Year : 2015

MOLECULAR BASIS AND REGULATION OF INSECT PATHOGENICITY IN PLANT-BENEFICIAL PSEUDOMONADS

Peter KUPFERSCHMIED

Peter Kupferschmied, 2015, Molecular Basis and Regulation of Insect Pathogenicity in Plant-Beneficial Pseudomonads

Originally published at : Thesis, University of Lausanne

Posted at the University of Lausanne Open Archive http://serval.unil.ch
Document URN : urn:nbn:ch:serval-BIB_FB118BB071585

UNIL | Université de Lausanne
Faculté de biologie
et de médecine

Département de microbiologie fondamentale

MOLECULAR BASIS AND REGULATION OF INSECT PATHOGENICITY IN PLANT-BENEFICIAL PSEUDOMONADS

Thèse de doctorat ès sciences de la vie (PhD)

présentée à la

Faculté de biologie et de médecine
de l'Université de Lausanne

par

Peter KUPFERSCHMIED

Master of Science in Biology de l'ETH Zurich

Jury

Prof. Edward E. Farmer, Président
Dr. Christoph Keel, Directeur de thèse
Prof. Julia A. Vorholt, Expert
Prof. Monica Höfte, Expert

Lausanne 2015

UNIL | Université de Lausanne
Faculté de biologie
et de médecine

Ecole Doctorale

Doctorat ès sciences de la vie

Imprimatur

Vu le rapport présenté par le jury d'examen, composé de

Président	Monsieur Prof. Edward E. **Farmer**
Directeur de thèse	Monsieur Dr Christoph **Keel**
Experts	Madame Prof. Monica **Höfte**
	Madame Prof. Julia A. **Vorholt**

le Conseil de Faculté autorise l'impression de la thèse de

Monsieur Peter Kupferschmied

Master in biology of ETH, Zürich

intitulée

MOLECULAR BASIS AND REGULATION OF INSECT PATHOGENICITY IN PLANT-BENEFICIAL PSEUDOMONADS

Lausanne, le 18 septembre 2015

pour le Doyen
de la Faculté de biologie et de médecine

Prof. Edward E. Farmer

Edward E. Farmer

« What is a scientist after all? It is a curious person looking through a keyhole, the keyhole of nature, trying to know what's going on. »

– Jacques-Yves Cousteau

For my family.

Table of Contents

Summary

Pseudomonas bacteria have the astonishing ability to survive within and adapt to different habitats, which has allowed them to conquer a wide range of ecological niches and to interact with different host organisms. Species of the *Pseudomonas fluorescens* group can readily be isolated from plant roots and are commonly known as plant-beneficial pseudomonads. They are capable of promoting plant growth, inducing systemic resistance in the plant host and antagonizing soil-borne phytopathogens. A defined subgroup of these pseudomonads evolved in addition the ability to infect and kill certain insect species. Profound knowledge about the interaction of these particular bacteria with insects could lead to the development of novel biopesticides for crop protection. This thesis thus aimed at a better understanding of the molecular basis, evolution and regulation of insect pathogenicity in plant-beneficial pseudomonads. More specifically, it was outlined to investigate the production of an insecticidal toxin termed Fit and to identify additional factors contributing to the entomopathogenicity of the bacteria.

In the first part of this work, the regulation of Fit toxin production was probed by epifluorescence microscopy using reporter strains of *Pseudomonas protegens* CHA0 that express a fusion between the insecticidal toxin and a red fluorescent protein in place of the native toxin gene. The bacterium was found to express its insecticidal toxin only in insect hemolymph but not on plant roots or in common laboratory media. The host-dependent activation of Fit toxin production is controlled by three local regulatory proteins. The histidine kinase of this regulatory system, FitF, is essential for the tight control of toxin expression and shares a sensing domain with DctB, a sensor kinase regulating carbon uptake in Proteobacteria. It is therefore likely that shuffling of a ubiquitous sensor domain during the evolution of FitF contributed to host-specific production of the Fit toxin. Findings of this study additionally suggest that host-specific expression of the Fit toxin is mainly achieved by repression in the presence of plant-derived compounds rather than by induction upon perceiving an insect-specific signal molecule.

In the second part of this thesis, mutant strains were generated that lack factors previously shown to be important for virulence in prominent pathogens. A screening for attenuation in insect virulence suggested that lipopolysaccharide (LPS) O-antigen and the PhoP-PhoQ two-component regulatory system significantly contribute to virulence of *P. protegens* CHA0. The genetic basis of O-antigen biosynthesis in plant-beneficial pseudomonads displaying insect pathogenicity was elucidated and revealed extensive differences between lineages due to reduction and horizontal acquisition of gene clusters during the evolution of several strains. Specific O side chains of LPS were found to be vital for strain CHA0 to successfully infect insects by ingestion or upon injection. Insecticidal pseudomonads with plant-beneficial properties were observed to be naturally resistant to polymyxin B, a model antimicrobial peptide. Protection against this particular antimicrobial compound was dependent on the presence of O-antigen and modification of the lipid A portion of LPS with 4-aminoarabinose. Since cationic antimicrobial peptides play a major role in the immune system of insects,

O-antigenic polysaccharides could be important for insecticidal pseudomonads to overcome host defense mechanisms. The PhoP-PhoQ system, which is well-known to control lipid A modifications in several pathogenic bacteria, was identified in *Pseudomonas chlororaphis* PCL1391 and *P. protegens* CHA0. No evidence was found so far that lipid A modifications contribute to insect pathogenicity in this bacterium. However, the sensor kinase PhoQ was required for full virulence of strain CHA0 suggesting that it additionally regulates the expression of virulence factors in this bacterium.

The findings of this thesis demonstrate that certain plant-associated pseudomonads are true insect pathogens and give some insights into how these microbes evolved to survive within and eventually kill the insect host. Results however also point out that more in-depth research is needed to know how exactly these fascinating bacteria manage to bypass or overcome host immune responses and to breach physical barriers to invade insects upon oral infection. To achieve this, future studies should not only focus on the bacterial side of the microbe-host interactions but also investigate the infection from a host-oriented view. The knowledge gained about the entomopathogenicity of plant-beneficial pseudomonads gives hope for their future application in agriculture to protect plants not only against plant diseases but also against insect pests.

Résumé

Les bactéries du genre *Pseudomonas* ont la capacité étonnante de s'adapter à différents habitats et d'y survivre, ce qui leur a permis de conquérir un large éventail de niches écologiques et d'interagir avec différents organismes hôte. Les espèces du groupe *Pseudomonas fluorescens* peuvent être facilement isolées de la rhizosphère et sont communément connues comme des *Pseudomonas* bénéfiques pour les plantes. Elles sont capables d'induire la résistance systémique des plantes, d'induire leur croissance et de contrer des phytopathogènes du sol. Un sous-groupe de ces *Pseudomonas* a de plus développé la capacité d'infecter et de tuer certaines espèces d'insectes. Approfondir les connaissances sur l'interaction de ces bactéries avec les insectes pourraient conduire au développement de nouveaux biopesticides pour la protection des cultures. Le but de cette thèse est donc de mieux comprendre la base moléculaire, l'évolution et la régulation de la pathogénicité des *Pseudomonas* plante-bénéfiques envers les insectes. Plus spécifiquement, ce travail a été orienté sur l'étude de la production de la toxine insecticide appelée Fit et sur l'indentification d'autres facteurs de virulence participant à la toxicité de la bactérie envers les insectes.

Dans la première partie de ce travail, la régulation de la production de la toxine Fit a été évaluée par microscopie à épifluorescence en utilisant des souches rapportrices de *Pseudomonas protegens* CHA0 qui expriment la toxine insecticide fusionnée à une protéine fluorescente rouge, au site natif du gène de la toxine. Celle-ci a été détectée uniquement dans l'hémolymphe des insectes et pas sur les racines des plantes, ni dans les milieux de laboratoire standards, indiquant une production dépendante de l'hôte. L'activation de la production de la toxine est contrôlée par trois protéines régulatrices dont l'histidine kinase FitF, essentielle pour un contrôle précis de l'expression et possédant un domaine "senseur" similaire à celui de la kinase DctB qui régule l'absorption de carbone chez les Protéobactéries. Il est donc probable que, durant l'évolution de FitF, un réarrangement de ce domaine "senseur" largement répandu ait contribué à une production hôte-spécifique de la toxine. Les résultats de cette étude suggèrent aussi que l'expression de la toxine Fit est plutôt réprimée en présence de composés dérivés des plantes qu'induite par la perception d'un signal d'insecte spécifique.

Dans la deuxième partie de ce travail, des souches mutantes ciblant des facteurs de virulence importants identifiés dans des pathogènes connus ont été générées, dans le but d'identifier ceux avec une virulence envers les insectes atténuée. Les résultats ont suggéré que l'antigène O du lipopolysaccharide (LPS) et le système régulateur à deux composantes PhoP/PhoQ contribuent significativement à la virulence de *P. protegens* CHA0. La base génétique de la biosynthèse de l'antigène O dans les *Pseudomonas* plante-bénéfiques et avec une activité insecticide a été élucidée et a révélé des différences considérables entre les lignées suite à des pertes de gènes ou des acquisitions de gènes par transfert horizontal durant l'évolution de certaines souches. Les chaînes latérales du LPS ont été montrées comme vitales pour une infection des insectes réussie par la souche CHA0, après ingestion ou injection. Les *Pseudomonas* plante-bénéfiques, avec

une activité insecticide sont naturellement résistants à la polymyxine B, un peptide antimicrobien modèle. La protection contre ce composé antimicrobien particulier dépend de la présence de l'antigène O et de la modification du lipide A, une partie du LPS, avec du 4-aminoarabinose. Comme les peptides antimicrobiens cationiques jouent un rôle important dans le système immunitaire des insectes, l'antigène O pourrait être important chez les *Pseudomonas* insecticides pour surmonter les mécanismes de défense de l'hôte. Le système PhoP/PhoQ, connu pour contrôler les modifications du lipide A chez plusieurs bactéries pathogènes, a été identifié chez *Pseudomonas chlororaphis* PCL1391 et *P. protegens* CHA0. Pour l'instant, il n'y a pas d'évidence que des modifications du lipide A contribuent à la pathogénicité de cette bactérie envers les insectes. Cependant, le senseur-kinase PhoQ est requis pour une virulence optimale de la souche CHA0, ce qui suggère qu'il régule aussi l'expression des facteurs de virulence de cette bactérie.

Les découvertes de cette thèse démontrent que certains *Pseudomonas* associés aux plantes sont de véritables pathogènes d'insectes et donnent quelques indices sur l'évolution de ces microbes pour survivre dans l'insecte-hôte et éventuellement le tuer. Les résultats suggèrent également qu'une recherche plus approfondie est nécessaire pour comprendre comment ces bactéries sont capables de contourner ou surmonter la réponse immunitaire de l'hôte et de briser les barrières physiques pour envahir l'insecte lors d'une infection orale. Pour cela, les futures études ne devraient pas uniquement se concentrer sur le côté bactérien de l'interaction hôte-microbe, mais aussi étudier l'infection du point de vue de l'hôte. Les connaissances gagnées sur la pathogénicité envers les insectes des *Pseudomonas* plante-bénéfiques donnent un espoir pour une future application en agriculture, pour protéger les plantes, non seulement contre les maladies, mais aussi contre les insectes ravageurs.

Acknowledgements

There are many people I would like to thank for contributing to this thesis in some way.
I first and foremost would like to thank my supervisor Christoph Keel for letting me do my PhD in his lab, for giving me the freedom to carry out research the way I wanted to and listening to all my ideas and ever-changing interpretations of results. I would like to thank all past and present members of the Keel lab - especially Nicola Imperiali, Céline Terrettaz, Maria Péchy-Tarr, Carmen Menzi, and Tiancong Chai - for all their contributions, support and time for discussions. Thanks to them, it was never boring in the lab.
Furthermore, I am deeply grateful to all the people outside of the research group who contributed to my thesis by sharing their knowledge, discussing results, taking their time for administrative issues or helping with experiments: Monika Maurhofer, Pascale Flury, Beat Ruffner, Nicolas Wenner, Karine Lapouge, Justine Collier, Po-Chi Lin, Claire Prigent-Combaret, Guy Condemine, Artur Reimer, Carmen Fernandez Fernandez, Silvia Moreno, Vladimir Sentchilo, Shawna McCallin, Paul Majcherczyk, Nazife Beqa, Maria Diniz, Nadine Thomas, Jan van der Meer, Aline Roth, Teresa Teixeira, and everyone I forgot to mention.
I could not have done my PhD without the support from my family – thanks mom, dad, Doris, and Karent! Thank you Dave, Giovanni, Kim, Dalmo, and Jen for being there as friends and all the fantastic journeys. And last but not least I would like to thank Sandrine, Jean-Louis, Yvonne, and José for giving me a roof over my head during my time in Lausanne.

CHAPTER 1

General Introduction and Thesis Outline

Most parts of this chapter were previously published in Kupferschmied P, Maurhofer M, and Keel C (2013) Promise for plant pest control: root-associated pseudomonads with insecticidal activities. *Front Plant Sci* 4:287. doi: 10.3389/fpls.2013.00287. Author contributions: Peter Kupferschmied, Christoph Keel and Monika Maurhofer wrote the article.

Insect pests and available control measures

With the world population still experiencing continuous growth, an immediate priority of agriculture is to increase crop production to assure food security while becoming more sustainable (Gatehouse et al., 2011). One way to do so is by improving the management of pests. Weeds, plant pathogens and certain animal species are considered to be the major pests of economic significance and together they are estimated to reduce the world's annual crop yield by approximately 30-40 per cent (Oerke, 2006). Due to their incredible diversity and adaptability, insects are probably the single most challenging pest to control in agriculture worldwide. Insects do not only cause major damage to agricultural crops as pests, but are also vectors of diseases. Since the introduction of synthetic insecticides, their application has made a major contribution to improve food production, but it was also soon discovered to be problematic in many ways. The rapid appearance of resistance to insecticides is a major concern in pest management. Today insect pest species of economic importance as pests that are resistant to more than 30 different chemical insecticides are no longer a rarity (Arthropod Pesticide Resistance Database, Michigan State University). Moreover, chemical insecticides are troublesome because of their potentially nocuous effects on the environment and public health (Heckel, 2012).

After decades of intensive pesticide application, it has become evident that there is no silver bullet solution to the control of pests in sustainable agriculture. The integration of many different, complementary approaches of chemical and biological control methods to solve the diverse and challenging problems with pests is the basic idea behind integrated pest management (IPM) programs (van den Bosch and Stern, 1962). Since its inception, IPM has become an increasingly important and popular toolbox-like approach to protect plants in agriculture against weeds, pathogens and animal pests (Oerke, 2006). Its tactics are designed to decrease the amount of chemical pesticides applied through careful forecasting or even to replace them by biological alternatives. The two main alternatives to synthetic insecticides are the exploitation of semiochemicals (like pheromones) to change the behavior of insects or the use of biological control agents (parasites, predators, and pathogens) to reduce the pest population size (Bale et al., 2008). IPM-based systems are becoming progressively more popular due to the increased public awareness of the above mentioned problematic effects of synthetic pesticides and interest in the development of alternative approaches for plant pest control. Microbial products that are based on insecticidal microorganisms for biological pest management strategies are receiving particular attention.

Bacillus thuringiensis (Bt) is a Gram-positive, spore-forming soil bacterium and the insecticidal organism which is dominating the market for products for microbial control of insects (Bravo et al., 2011; Sanahuja et al., 2011). The reason for its success is the production of pore-forming δ-endotoxins, namely Cry and Cyt proteins (Crickmore et al., 1998; 2013). These so-called crystal proteins are produced during sporulation and show potent and specific insecticidal activity. Once proteolytically activated, the Cry and Cyt proteins act in

the midgut of insects as pore-forming toxins via binding to specific receptors or directly to membrane lipids, respectively (Bravo et al., 2007; Vachon et al., 2012). In addition to the well-known crystal toxins, Bt produces an array of additional virulence factors that contribute to the insecticidal activity of this bacterium (Nielsen-LeRoux et al., 2012). Bt is typically applied as topical sprays and has several advantages over conventional chemical insecticides. The bacterium's pathogenic activity is specific towards a narrow range of insect species and its application is considered to be environmentally sound and harmless to humans and other mammals. However, the use of Bt as a biological control agent has some limitations. The bacterium shows low environmental persistence after topical application, mainly because it is sensitive to solar irradiation as well as to the chemical environment on plant leaves, and is not a competitive plant colonizer (Bizzarri and Bishop, 2008; Raymond et al., 2010). Therefore, and because the susceptible stages of the pest insects are during the early instar larvae, Bt provides only short-term crop protection in the field and requires precise application practices (Bravo et al., 2011). The recent discovery that at least some Bt strains are capable of colonizing crop plants as endophytes and as such translocate throughout the plant (Monnerat et al., 2009) may open up an avenue for new Bt application strategies.

To overcome the problem of the low persistence of Bt on plants, genetically modified (GM) crops that express variants of the Cry toxins have been developed and successfully commercialized. Planting of GM crops reduced the amount of pesticides applied by 8.9% in the period from 1996 to 2011 (James, 2012) and is a component of IPM strategies due to its compatibility with biological control methods (Bale et al., 2008). However, the major drawback of this new biotechnology has been the development of resistance against the Cry toxins by pests (Bravo et al., 2011). Due to the relatively simple mode of action of Cry toxins and the absence of complementary virulence factors normally found in the complete microorganism, resistance is much more probable to develop towards the insect toxin in the GM plants than to the entire microorganism (Cory and Franklin, 2012). Infections by microbial pathogens are complex and likely to require more diverse polygenic resistance mechanisms in pest insects. In addition, there are public concerns about transgenic crops regarding their impact on biodiversity and the consumer's health and the possible dependency of farmers on seed companies. Especially in Europe, the public acceptance for GM crops is currently fairly low for these reasons.

Belowground pest insects are especially difficult to control, because they are hidden in the soil and therefore hard to detect and to get access to. Although root herbivory can cause significant damage to crops, even leading to a sudden collapse of the plant population, there is still a considerable lack of research data about root feeders and their impact on plants (Hunter, 2001; Blossey and Hunt-Joshi, 2003). For instance, the Western corn rootworm *Diabrotica virgifera virgifera* is a significant economic pest insect of maize in the United States and in Europe and acquired the nickname "billion dollar bug", not without reason (Gray et al., 2009). Even if this troublesome insect species has been the subject of many scientific studies, this root feeder remains challenging to control because of its cryptic lifestyle, the adaptation to crop rotation and the

development of resistance to certain insecticides. While the use of chemical pesticides for pest management in soils is extremely restricted, microbial control is a promising approach to address problems with soil-dwelling insects due to the more favorable environmental conditions for microbes in contrast to aboveground habitats (e.g., absence of ultraviolet radiation and lower risk of desiccation in the soil). Species of *Photorhabdus* and *Xenorhabdus*, bacteria which are living in symbiosis with entomopathogenic nematodes, are used in agriculture as soil-applied insecticides (Lacey and Georgis, 2012). However, contrarily to Bt, they currently only play a minor role on the market for microbial insecticides. Commercial products for pest control are based on formulations of entomopathogenic nematodes of the genera *Heterorhabditis* and *Steinernema* with select strains of *Photorhabdus* and *Xenorhabdus* (Ehlers, 2001). Preparations of *Heterorhabditis* and *Steinernema* vectoring the entomopathogenic bacteria have been applied with varying success to control larval forms of some of the most notorious soil pest insects, including the black cutworm *Agrotis ipsilon* of the order Lepidoptera, *Diabrotica* spp. and *Diaprepes* sp. and *Otiorhynchus* sp. root weevils of the Coleoptera, and the cabbage root fly *Delia radicum* and fungus gnats (*Sciaridae*) of the Dipterae (Denno et al., 2008; Lacey and Shapiro-Ilan, 2008; Degenhardt et al., 2009; Toepfer et al., 2010; Campos-Herrera et al., 2012; Shapiro-Ilan and Gaugler, 2013). The two nematodes have also been used in combination with the entomopathogenic fungus *Metarhizium* and Bt maize to improve root protection from damage caused by *Diabrotica* spp. (Petzold-Maxwell et al., 2013).

Photorhabdus and *Xenorhabdus* are fascinating entomopathogenic bacteria and they have been studied extensively for their insect pathogenicity and mutualistic interaction with nematodes, as well as for their production of an array of protein toxins and toxic secondary metabolites with insecticidal potential (ffrench-Constant et al., 2007; Herbert and Goodrich-Blair, 2007; Bode, 2009; Waterfield et al., 2009; Nielsen-LeRoux et al., 2012). They provide a rich source of novel insecticidal toxins for crop protection, as it will be exemplified later in this chapter. There have been efforts to isolate new strains of these entomopathogens to mine for novel antimicrobial and insecticidal compounds (Thanwisai et al., 2012), and to create insect-resistant plants using toxins from *Photorhabdus luminescens* (Liu et al., 2003). In contrast to Bt, which relies on the oral route of infection in order to kill the insect host, *Photorhabdus* and *Xenorhabdus* species are "delivered" directly into the insect hemocoel by their nematode vectors, which then invade the insects either via penetration of the cuticle or through natural openings. As a result, this infection strategy makes the bacteria dependent on their nematode symbiont, which in turn makes applications of these microorganisms for insect pest management in the soil much more complex than it would be in the case of a free-living, entomopathogenic rhizobacterium.

Certain root-associated bacteria of the genus *Pseudomonas* could constitute a promising alternative to the above-mentioned two groups of commercialized entomopathogens, in particular when addressing the notorious problem of soil-dwelling pests. As described in more detail below, these well-known rhizobacteria are capable of protecting plant roots against fungal and oomycete pathogens and simultaneously show

potent oral insecticidal activity (Fig. 1A). Some of these bacterial strains are already successfully used as antifungal biocontrol agents in agriculture (Berg, 2009). Therefore, these root-associated bacteria could be exploited for the development of novel microbial products which would protect plant roots simultaneously against phytopathogens and herbivorous insects and could become an important element of IPM.

Interaction of beneficial pseudomonads with plants and phytopathogens: cooperation, competition, and antagonism

The genus *Pseudomonas* makes up a remarkably ubiquitous and diverse group of microorganisms. These Gram-negative bacteria are highly adaptive and can use a wide variety of compounds as an energy source, and as a result, there is practically no place on earth where they cannot be found (Wu et al., 2010; Silby et al., 2011). The environmental niches that they colonize range from oil-spilled seawater (Viggor et al., 2013) to soil (Weller et al., 2002), plant surfaces (Hirano and Upper, 2000; Loper et al., 2012) and insect guts (Vodovar et al., 2005). Some of them live a life as saprophytes, while some are plant pathogens or opportunistic human pathogens, and yet others entertain commensal or almost mutualistic relationships with plants. The latter are in most cases root-colonizing members of the *Pseudomonas fluorescens* group according to Mulet and colleagues (Mulet et al., 2010; 2012b), and include amongst others the species *P. fluorescens*, *P. protegens* and *P. chlororaphis*. Among them, plant-beneficial pseudomonads are well known for their multiple skills that enable them to not only survive and compete in the rhizosphere, which is an ecological hot spot attracting many different kinds of organisms, but also to undergo intimate interactions with the plant itself (Fig. 1A; Lugtenberg and Kamilova, 2009; Hol et al., 2013). To this effect, the root-colonizing pseudomonads first became renowned thanks to the ability of some strains to protect plants against the attack by some of the most notorious soil-borne fungal and oomycete pathogens, including *Gaeumannomyces*, *Thielaviopsis*, *Rhizoctonia, Fusarium oxysporum,* and *Pythium* sp. (Table 1; Cook et al., 1995; Haas and Défago, 2005; Mercado-Blanco and Bakker, 2007).

The mechanisms by which pseudomonads suppress plant diseases have been studied for many years. These bacteria are excellent root colonizers and compete effectively with pathogens for rhizosphere niches and macro- and micronutrients (Mercado-Blanco and Bakker, 2007; Lugtenberg and Kamilova, 2009). Notably, pseudomonads produce high affinity iron-chelators (so-called siderophores such as pyoverdines and pyochelins) by which they sequester iron, which is in limited supply in soil, and render it unavailable for the pathogens (Table 1; Keel et al., 1989; Loper and Buyer, 1991; Cornelis, 2010; Youard et al., 2011). Probably the most potent mechanism by which pseudomonads can suppress soilborne pathogens is antibiosis (Haas and Keel, 2003). Many disease-suppressive strains produce one, two or even an entire cocktail of secondary metabolites with potent antifungal activity by which they can ward off plant pathogens. Phenazines, 2,4-diacetylphloroglucinol (DAPG), pyoluteorin, pyrrolnitrin, hydrogen cyanide (HCN), and cyclic lipopeptides are

metabolites with a documented role in disease suppression (Table 1; Haas and Keel, 2003; de Werra et al., 2008; Gross and Loper, 2009; Mentel et al., 2009; Raaijmakers et al., 2010; Rochat et al., 2010; Jousset et al., 2011). The pseudomonads use several of these compounds also for self-defense against predatory protozoa and nematodes (Bjørnlund et al., 2009; Jousset et al., 2009; Raaijmakers and Mazzola, 2012). Most remarkably, root-inhabiting pseudomonads producing DAPG, phenazines or cyclic lipopeptides are key components of soils that are naturally suppressive to specific soil-borne diseases such as take-all of wheat, black root of tobacco, and *Rhizoctonia* root rot of sugar beet (Weller et al., 2002; Défago and Haas, 2005; Mazurier et al., 2009; Mendes et al., 2011; Almario et al., 2013).

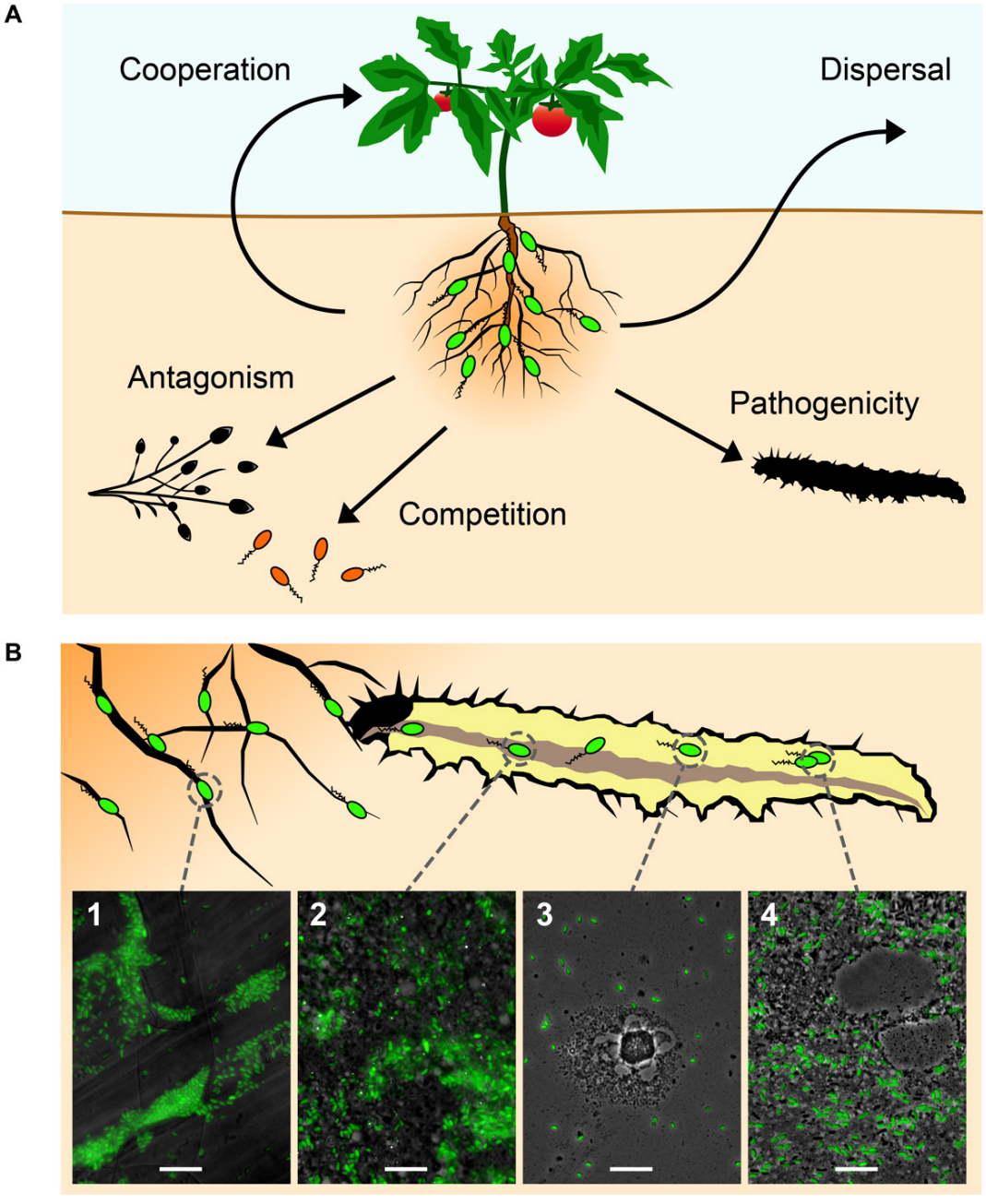

Figure 1: Certain plant root-associated *Pseudomonas* bacteria exhibit insect pathogenicity as an additional trait to the well-studied biocontrol activity against phytopathogens (see text for more details). **(A)** The most important interactions of these plant-beneficial pseudomonads (in green) include cooperation with the plant host (growth promotion and induction of systemic resistance) and competition with and antagonism of soil-borne phytopathogens. In addition, they show insecticidal activity and can use insects as vectors for dispersal. **(B)** Certain strains of *P. protegens* and *P. chlororaphis* are capable of infecting and efficiently killing insect larvae after oral uptake. *P. protegens* strain CHA0 (here tagged with GFP for microscopical visualization) typically forms microcolonies on roots (1) of various plant species (here tomato). Following ingestion by herbivorous insects, the entomopathogenic *P. protegens* strain is able to colonize the midgut (2) of pest insect larvae (here the large cabbage white *Pieris brassicae*), possibly by competing with the intestinal microbiota. By a so far unknown mechanism CHA0 cells then cross the intestinal epithelial barrier and invade the hemocoel within less than one day after oral infection (3). Once in this body compartment, the bacteria proliferate, resist uptake and elimination by hemocytes and cause disease (4). Bars represent 10 µm.

Several root-associated *Pseudomonas* strains are able to reduce plant diseases not only by directly antagonizing pathogens but also indirectly by activating plant defenses (Table 1). The beneficial effects of induced systemic resistance (ISR) triggered by root-colonizing pseudomonads in mono- and dicotyledonous plants against plant pests caused by fungal, oomycete, bacterial and viral pathogens, and also by herbivorous insects are extensively documented (Maurhofer et al., 1994; Bakker et al., 2007; De Vleesschauer and Höfte, 2009; van de Mortel et al., 2012; Zamioudis and Pieterse, 2012; Balmer et al., 2013). Nevertheless, pseudomonads sometimes can also negatively interfere with plant defenses against insects or with the attraction of parasitoids of leaf-feeding insects (Pineda et al., 2012; 2013). A number of bacterial determinants eliciting ISR have been identified, including iron-chelators such as pyoverdines and pyochelins, and antimicrobials such as DAPG, phenazines and lipopeptides (Table 1; Bakker et al., 2007; De Vleesschauer and Höfte, 2009). Most pseudomonads that are capable of inducing systemic resistance do this by priming plants in a way which leads to an accelerated, mostly jasmonate-signaling dependent response upon pathogen or insect attack (Prime-A-Plant Group, 2006; Bakker et al., 2007; De Vleesschauer and Höfte, 2009).

There are two main strategies by which we can exploit these pseudomonads with their astonishing repertoire of plant-beneficial activities for improving crop performance and crop health. The first is to adapt cropping systems in a way that attracts the beneficial rhizobacteria, fosters their populations and stimulates their activity (Janvier et al., 2007; Berendsen et al., 2012). This may be achieved in numerous ways, e.g. by adapting tillage or crop rotation practices, by soil amendments such as quality composts or by the use of inter- or covercrops (Mazzola, 2004; Janvier et al., 2007). The second strategy is to apply *Pseudomonas*-based biopesticides either as a seed treatment, soil drench or foliar spray. Several products based on plant-beneficial pseudomonads for use in integrated biological control have been commercialized mainly for the US market, including AtEze (*P. chlororaphis*) with activity against *Pythium*, *Rhizoctonia*, and *Fusarium* root diseases of vegetables and ornamentals in greenhouses, BlightBan A506 (*P. fluorescens*) used against fire blight on apple and pear, and Bio-Save 10 LP/11 LP (*Pseudomonas syringae*) used for the control of post-

harvest diseases of fruits and potato (Fravel, 2005). In several European countries, two formulations based on *P. chlororaphis*, i.e. Cedomon and Cerall, are sold as a seed treatment against seed-borne diseases of cereals (Mark et al., 2006) and the *Pseudomonas*-based product Proradix (Buddrus-Schiemann et al., 2010) was recently placed on the market for use as a potato tuber treatment against diseases caused by *Rhizoctonia*, *Phytophthora, Streptomyces* and *Erwinia*. Considerations for the selection, production, delivery, field testing, and registration of *Pseudomonas* and other biocontrol agents for commercial purposes have been reviewed elsewhere (Walsh et al., 2001; Fravel, 2005; Mark et al., 2006; Berg, 2009; Höfte and Altier, 2010).

Table 1: Prominent root-associated *Pseudomonas fluorescens* group strains with biocontrol activity against plant diseases and effectors contributing to pathogen suppression.

Strain[a]	Target soil-borne // leaf pathogens[b]	Pathogen suppression mechanisms[c]	Effectors (antibiotics / biosurfactants // siderophores)[d]	References[e]
Pseudomonas protegens				
CHA0	*Thielaviopsis, Pythium, Gaeumannomyces, Rhizoctonia, Fusarium // Hyaloperonospora*, TNV	Antibiosis, ISR	DAPG, pyrrolnitrin, pyoluteorin, HCN / orfamide // pyoverdine, enantiopyochelin	Haas and Keel (2003); Défago and Haas (2005); Youard et al. (2011)
Pf-5	*Pythium, Rhizoctonia, Drechslera, Sclerotinia // Pst*	Antibiosis, ISR	DAPG, pyrrolnitrin, pyoluteorin, HCN, rhizoxins / orfamide // pyoverdine, enantiopyochelin	Gross and Loper (2009); Loper et al. (2012); Weller et al. (2012)
Pseudomonas chlororaphis				
30-84	*Gaeumannomyces*	Antibiosis	Phenazines, pyrrolnitrin, HCN // pyoverdine	Pierson and Pierson (2010); Loper et al. (2012)
O6	*// Phytophthora, Corynespora, Pectobacterium*	Antibiosis, ISR	Phenazines, pyrrolnitrin, HCN // pyoverdine	De Vleesschauer and Höfte (2009); Park et al. (2011); Loper et al. (2012)
PCL1391	*Fusarium*	Antibiosis	Phenazines, HCN // pyoverdine	Chin-A-Woeng et al. (2001); Ruffner (2013)
Pseudomonas fluorescens				
2-79	*Gaeumannomyces*	Antibiosis	Phenazine // pyoverdine	Weller (2007); Mavrodi et al. (2010)
DR54	*Pythium, Rhizoctonia*	Antibiosis	/ Viscosinamide // pyoverdine	Nielsen and Sørensen (2003)
F113	*Pythium, Fusarium, Pectobacterium*	Antibiosis	DAPG, HCN // pyoverdine	Redondo-Nieto et al. (2013)
Pf29A	*Gaeumannomyces*	Alteration of fungal pathogenesis	ND	Daval et al. (2011); Marchi et al. (2013)
Q2-87	*Gaeumannomyces // Pst*	Antibiosis, ISR	DAPG, HCN // pyoverdine	Loper et al. (2012); Weller et al. (2012)
SBW25	*Pythium*	ND	/ Viscosin // pyoverdine	Loper et al. (2012); Trippe et al. (2013)
SS101	*Pythium // Phytophthora, Pst*	ISR	/ Massetolide // pyoverdine	Loper et al. (2012); van de Mortel et al. (2012)
WCS374	*Fusarium // Magnaporthe, Pst*	ISR	// Pyoverdine, pseudomonine	Bakker et al. (2007); De Vleesschauer and Höfte (2009)
WCS417	*Fusarium // Alternaria, Hyaloperonospora, Botrytis, Pst*	ISR	ND	Bakker et al. (2007); Van der Ent et al. (2008)

[a] *Strains belonging to the P. fluorescens group according to Mulet et al. (2010; 2012b).*
[b] *Pst, Pseudomonas syringae pv. tomato; TNV, tobacco necrosis virus.*
[c] *ISR, induced systemic resistance. ND, not determined.*

[d]*Major effectors with antimicrobial, biosurfactant, metal-chelating and/or plant defense-inducing properties produced by the respective strain. DAPG, 2,4-diacetylphloroglucinol; HCN, hydrogen cyanide.*
[e]*References from which further information on the strains can be accessed.*

Insecticidal activity in plant-beneficial *P. fluorescens* group bacteria: occurrence and molecular basis

Until very recently, insecticidal activities in the *P. fluorescens* group had only been sparsely documented (Table 2). Notably, strains of *P. fluorescens* were reported to exhibit insecticidal activity towards agricultural pest insects such as aphids (Hashimoto, 2002), phytophagous ladybird beetles (Otsu et al., 2004), and termites (Devi and Kothamasi, 2009). In the same vein, a bioformulation of a combination of two *P. fluorescens* strains was demonstrated to simultaneously reduce the incidence of a herbivorous insect (the rice leafroller *Cnaphalocrocis medinalis*) and a phytopathogenic fungus (*Rhizoctonia solani*) in rice under greenhouse and field conditions (Commare et al., 2002; Karthiba et al., 2010). Furthermore, a number of *P. fluorescens* strains were found to be capable of either killing the common fruit fly *Drosophila melanogaster* or of causing morphological defects to the widely used laboratory insect (de Lima Pimenta et al., 2003; Olcott et al., 2010). Although in some cases protein extracts (Prabakaran et al., 2002) or metabolites of *P. fluorescens* group strains, such as HCN (Devi and Kothamasi, 2009) and the lipopeptides viscosin (Hashimoto, 2002) and orfamide (Yang et al., 2013), were shown to have insecticidal properties, the molecular basis and regulation of the insecticidal activity in these bacteria remains obscure.

Table 2: Insecticidal activity in *Pseudomonas* species and currently known effectors and regulatory mechanisms involved in insect virulence.

Bacterial strain[a]	Target insect	Application of bacteria / bacterial product[b]	Effector / regulatory mechanism involved in insect virulence[c]	Reference
Pseudomonas protegens				
CHA0	*Galleria mellonella, Manduca sexta*	Injection	Fit toxin (similar to Mcf toxin of *Photorhabdus*)	Péchy-Tarr et al. (2008; 2013)
	Spodoptera littoralis	Feeding (D, L)	Fit toxin, GacA (global regulator of virulence and biocontrol)	Ruffner et al. (2013)
	Heliothis virescens, Plutella xylostella	Feeding (L)	ND	Ruffner et al. (2013)
	Odontotermes obesus	Contact (live cells)	HCN (biocide)	Devi and Kothamasi (2009)
Pf-5	*G. mellonella, M. sexta*	Injection	Fit toxin	Péchy-Tarr et al. (2008)
	Drosophila melanogaster	Feeding (D)	GacA	Olcott et al. (2010)
F6	*Myzus persicae*	Contact (purified metabolite)	Orfamide (biosurfactant)	Jang et al. (2013)
Pseudomonas chlororaphis				
30-84	*G. mellonella*	Injection	ND	Ruffner (2013)
PCL1391	*S. littoralis*	Feeding (D, L)	Fit toxin	Ruffner et al. (2013)
	H. virescens, P. xylostella	Feeding (L)	ND	Ruffner et al. (2013)
ST-1	*Bombyx mori*	Injection	ND	Tao et al. (2011)

Pseudomonas fluorescens				
AH1, FP7 and Pf1	*Cnaphalocrocis medinalis*	Feeding (L)	ND	Commare et al. (2002); Karthiba et al. (2010)
HS870031	*Myzus persicae, Aphis gossypii, Aulacorthum solani*	Contact (purified metabolite)	Viscosin (biosurfactant)	Hashimoto (2002)
KPM-018P	*Epilachna vigintioctopunctata*	Feeding (oral injection, L)	ND	Otsu et al. (2004)
MF37	*D. melanogaster*	Pricking	Adherence factors (LPS, OMP)	de Lima Pimenta et al. (2003)
NN, Biotype C	*Apis mellifera*	Feeding (D)	ND	Horn and Eberspächer (1976)
NN	*Formica paralugubris*	Contact (live cells)	ND	Chapuisat et al. (2007)
SBW25	*D. melanogaster*	Feeding (D)	ND	Olcott et al. (2010)
Pseudomonas taiwanensis				
TKU015	*D. melanogaster*	Feeding (purified toxin)	TccC-like toxin (similar to *Photorhabdus* toxin complex component TccC)	Liu et al. (2010)
Pseudomonas sp.				
EP-3	*M. persicae*	Contact (purified metabolite)	Rhamnolipid (biosurfactant)	Kim et al. (2011)
ICTB-745	*Rhyzopertha dominica*	Contact (purified metabolites)	Rhamnolipids, PCA (antibiotic)	Kamal et al. (2012)
Pseudomonas entomophila				
L48	*D. melanogaster*	Feeding (D)	Monalysin (pore-forming toxin), AprA (metallo-protease), GacA, Pvf (signalling system), AlgR (regulator)	Vodovar et al. (2005; 2006); Liehl et al. (2006); Vallet-Gely et al. (2010b); Opota et al. (2011)
	G. mellonella	Force feeding of live cells	GacA	Fedhila et al. (2010)
Pseudomonas syringae				
B728a	*Acyrthosiphon pisum*	Feeding (D, L)	FliL (flagellum formation and motility)	Stavrinides et al. (2009)
Pseudomonas aeruginosa				
CHA	*D. melanogaster*	Pricking	T3SS and effectors (ExoS)	Fauvarque et al. (2002); Avet-Rochex et al. (2005)
PA14	*G. mellonella*	Injection	T3SS and effectors (ExoT, ExoU)	Miyata et al. (2003)
	D. melanogaster	Feeding (D)	Quorum sensing (RhlR)	Limmer et al. (2011)
PAO1	*D. melanogaster*	Injection	HCN	Broderick et al. (2008)
	B. mori	Injection	Superoxide dismutase (SodM, SodB), exotoxin A, GacA	Chieda et al. (2005; 2011); Iiyama et al. (2007)
	B. mori	Midgut injection	ExoS, pyoverdine (iron chelator)	Okuda et al. (2010)
	D. melanogaster	Feeding (D)	Quorum sensing (QscR), stringent response (ppGpp), control of biofilm formation	Chugani et al. (2001); Mulcahy et al. (2011); Vogt et al. (2011); de Bentzmann et al. (2012)
	Pieris rapae	Feeding (D)	Quorum sensing (LasI, RhlI)	Borlee et al. (2008)
NN	*Melanoplus bivittatus*	Injection, Feeding (L)	ND	Bucher and Stephens (1957); Stephens (1958)

[a] *NN, not named.*
[b] *Injection, bacterial cell suspension injected into the hemocoel if not mentioned otherwise. Feeding, oral administration of a bacterial cell suspension with artificial diet (D) or applied to plant leaves (L); Contact, bacterial cells or products sprayed on or put otherwise in contact with insect surface.*
[c] *ND, not determined; HCN, hydrogen cyanide; LPS, lipopolysaccharide; OMP, outer membrane protein; PCA, phenazine-1-carboxylic acid; T3SS, type III secretion system.*

The genome sequencing of the root-colonizing biocontrol agent *P. fluorescens* strain Pf-5 (now called *P. protegens* Pf-5; Ramette et al., 2011) published by Paulsen *et al.* (2005) and of the closely related *P.*

fluorescens strain CHA0 (recently renamed *P. protegens* CHA0) (NCBI Database Bioproject PRJNA78307) and their analysis revealed astonishing results which opened a new door to future studies on plant-associated pseudomonads. After more than twenty years of research on the biocontrol properties of *P. fluorescens* group strains it came as a surprise that some of these bacteria do not only harbor numerous genes for the biosynthesis of antifungal metabolites, including DAPG, pyoluteorin, HCN, and pyrrolnitrin (see Table 1), in their genomes, but also possess a gene which codes for a protein that is similar to the potent insect toxin Mcf1 of the entomopathogen *P. luminescens* (Péchy-Tarr et al., 2008).

Mcf1 was discovered in a screening of a *P. luminescens* W14 cosmid library aiming at the identification of new insecticidal proteins and metabolites in this entomopathogenic bacterium (Daborn et al., 2002). A single gene which was called *makes caterpillars floppy* (*mcf*) made the *E. coli* cells expressing it capable of surviving within and killing larvae of the tobacco hornworm *Manduca sexta* upon injection into the hemocoel. When expressed heterologously in *E. coli*, Mcf1 was shown to cause hemocytes and midgut epithelial cells to undergo programmed cell death. The disintegration of the midgut caused by Mcf1 was proposed to contribute to the "floppy" phenotype of insects infected with *P. luminescens*, thereby giving the name to the newly discovered toxin. The pro-apoptotic action of Mcf1 was attributed to the predicted Bcl2-homology 3-like (BH3-like) domain at the N-terminus of the protein. The BH3 domain is a well-studied and important peptide motif of proteins making up part of the pro-apoptotic signal-transduction cascades in animal cells (Cory and Adams, 2002). Mcf1 has been shown to also trigger apoptosis in mammalian cells and the N-terminal part of the toxin containing the BH3-like domain was sufficient for the observed toxicity (Dowling et al., 2004). The potent insect toxin seems to hijack the apoptosis cascades of the cells of the innate immune system and thereby to contribute to the immune suppressive activity of *P. luminescens*.

An exciting feature of the *mcf1*-related gene of *P. fluorescens* group strains Pf-5 and CHA0 is that, in contrast to *mcf1*, it is part of an eight-gene cluster (Péchy-Tarr et al., 2008). The cluster was termed *fit* for *P. fluorescens* insecticidal toxin. The gene *fitD,* which codes for the actual insect toxin with a molecular weight of 327 kDa, is flanked by four genes (*fitABC-E*) predicted to encode a type I secretion system and three genes (*fitFGH*) coding for regulatory proteins. The toxin gene is co-transcribed with the genes encoding the proteins for the putative secretion system, thereby suggesting that the toxin may be transported across the bacterial cell wall via this type I secretion system (Péchy-Tarr et al., 2013). While the transport of the Fit toxin still remains to be investigated, the roles and importance of the individual regulatory proteins of the Fit cluster have been elucidated and are described in more detail below. Because the putative BH3-like domain of Mcf1 is also conserved in the Fit toxin, it is imaginable that FitD induces apoptosis in insect cells as well.

So far the Fit toxin gene has been detected in the genomes of only a narrow group of plant-associated pseudomonads, namely in isolates of *P. protegens* and *P. chlororaphis* (Table 2; Loper et al., 2012; Ruffner et al., 2013; Shen et al., 2013). Strains of these two bacterial species generally showed a high toxicity towards larvae of lepidopteran insects. The *P. protegens* strains CHA0 and Pf-5 were lethal to larvae of *M. sexta* and

the greater wax moth *Galleria mellonella* upon injection of very low doses into the hemocoel of these insects (Péchy-Tarr et al., 2008). The Fit toxin thereby significantly contributed to the insecticidal activity of these microorganisms. Furthermore, as with Mcf1, heterologous expression of the Fit toxin in *E. coli* resulted in the capacity of the bacterium to kill the insect host upon injection.

P. protegens strain CHA0 and *P. chlororaphis* strain PCL1391 were later also shown to display potent oral insecticidal activity in feeding assays with artificial diet or leaves treated with the bacteria (Table 2; Ruffner et al., 2013). When bacterial suspensions containing low cell concentrations were sprayed on plant leaves, both strains efficiently killed larvae of several agriculturally important lepidopteran pest insects, notably the African cotton leafworm *Spodoptera littoralis*, the tobacco budworm *Heliothis virescens* and the diamondback moth *Plutella xylostella* that fed on the leaves. The Fit toxin was found to substantially contribute to the oral insecticidal activity of the two model strains. In contrast, a related but naturally Fit-deficient *P. fluorescens* group strain displayed almost no oral toxicity in the same assay (Ruffner et al., 2013). Thus the presence of the Fit toxin gene in plant-colonizing pseudomonads seems to correlate well with high toxicity of these strains towards insects. This and observations with additional strains suggest that the gene could potentially be used as a suitable molecular marker for insecticidal activity in fluorescent pseudomonads (Ruffner et al., 2009; Ruffner, 2013). In addition to the Fit toxin, traits regulated by the GacS/GacA two-component system, which is known to control pathogenic and beneficial activities in pseudomonads (Haas and Keel, 2003; Lapouge et al., 2008), contribute significantly to the oral insecticidal activity of *P. protegens* CHA0 (Ruffner et al., 2013). Additional toxicity assays suggest specificity in the insecticidal spectrum of *P. protegens* CHA0. In particular, during a quest for potential side effects of the pseudomonad towards beneficial insects, the Fit toxin producers were found to exhibit no oral toxicity towards an ecologically and economically important pollinator, the large earth bumblebee *Bombus terrestris* (Ruffner, 2013).

The potential of these plant root-associated pseudomonads as entomopathogenic microorganisms can be demonstrated impressively by feeding Chinese cabbage leaves containing drops of a suspension of GFP-tagged *P. protegens* CHA0 to larvae of the large cabbage white *Pieris brassicae*. The bacteria seem to be capable of colonizing the insect gut and subsequently translocating into the hemocoel by so far unknown means, where they replicate and cause disease (Fig. 1B). The invasion of the insect blood system within a short time period of less than one day after oral uptake of the microorganisms strongly suggests that these bacteria should be considered as true insect pathogens.

The deletion of the Fit toxin gene in the chromosomes of *P. protegens* or *P. chlororaphis* strains is not sufficient to render them non-toxic to insects (Péchy-Tarr et al., 2008; 2013; Ruffner et al., 2013). This suggests that additional virulence factors are waiting to be discovered in these insecticidal pseudomonads. Candidate virulence factors that could play a role in insect pathogenicity in some of these strains are the so-called toxin complexes (Tc). Tc, which were first identified in *P. luminescens*, are large multimeric insecticidal

protein complexes displayed on the surface of these bacteria (Bowen et al., 1998; ffrench-Constant et al., 2007). Although the exact mode of action of these orally active toxins is still not fully resolved, recent studies provide evidence that some Tc subunits function as a molecular syringe allowing membrane translocation of functional Tc components that induce actin clustering and death in target cells (Lang et al., 2010; Gatsogiannis et al., 2013). Tc components have also been investigated as alternatives to the Bt toxins for the development of transgenic crops (Liu et al., 2003). Tc-related gene clusters occur in many other bacteria that interact with insects, including *Xenorhabdus nematophila*, *Yersinia pestis*, *Yersinia entomophaga*, *Serratia entomophila*, and Bt (Hurst et al., 2000; Waterfield et al., 2001; Blackburn et al., 2011; Landesberg et al., 2011; Spinner et al., 2012). Remarkably, Tc-related genes can also be found in certain strains of *P. chlororaphis* and *P. fluorescens* (Loper et al., 2012) and their role in insect pathogenicity should thus be investigated in future studies. In pseudomonads, a role for a Tc-related gene so far has only been demonstrated for *tccC* from *Pseudomonas taiwanensis* of which the purified product caused substantial mortality when fed to larvae of *Drosophila* (Liu et al., 2010).

Molecular basis of insect interaction in prominent pathogenic pseudomonads

Several observations suggest that natural interactions of pseudomonads with insects are most likely more widespread than recognized so far. First, members of the genus *Pseudomonas* make commonly part of microbial communities of various insect species. Indeed, using culture-dependent and -independent approaches, pseudomonads were identified as common inhabitants of the intestinal tract or otherwise associated with field-collected or laboratory-raised larvae, pupae and adults of representatives of the major insect orders. Examples include *Anopheles*, *Aedes* and *Culex* mosquitos, the *Drosophila* fruit fly, and the Hessian fly *Mayetiola destructor* in the order Diptera (Corby-Harris et al., 2007; Bansal et al., 2011; Osei-Poku et al., 2012), *S. littoralis*, the cotton bollworm *Helicoverpa armigera*, and the gypsy moth *Lymantria dispar* in the Lepidoptera (Broderick et al., 2004; Tang et al., 2012), the wireworm *Limonius canus*, the forest cockchafer *Melolontha hippocastani*, and *Periplaneta* and *Blattella* cockroaches in the Coleoptera (Lacey et al., 2007; Saitou et al., 2009; Arias-Cordero et al., 2012), *Camponotus* ants and several bee species in the Hymenoptera (Mohr and Tebbe, 2006; Li et al. 2012), and the leafhopper *Homalodisca vitripennis* and several aphids in the Hemiptera (Hashimoto et al., 2002; Lacava et al., 2007). Many of these insects feed on roots or aboveground parts of plants or spend a part of their life cycle in aquatic habitats, i.e. in environments that are typically colonized by pseudomonads. It is therefore likely that pseudomonads are commonly acquired by insects via ingestion or contact. These highly versatile bacteria then may be very well adapted to live inside or otherwise associated with their arthropod host, exploiting it as a shelter, vector or food source.

Second, the genomes of many *Pseudomonas* strains contain genetic loci with predicted function in insect interaction and insect toxicity. These loci are related to genes encoding known insect virulence determinants in the entomopathogens *Photorhabdu*s and *Xenorhabdus*, namely the Mcf toxins, the Tc toxin complexes, the XaxAB cytolysin, and several lytic enzymes (ffrench-Constant et al., 2007; Vigneux et al., 2007; Lindeberg et al., 2008; Stavrinides et al., 2009; Silby et al., 2011; Loper et al., 2012). To date, the function of most of these loci in pseudomonads remains nebulous. A clear role in insect toxicity so far has only been established for the Mcf homologue Fit (see above).

Third, following oral infection several *Pseudomonas* species are capable not only of colonizing insects but also of exhibiting significant pathogenicity towards insects. Besides the above-described plant-beneficial *P. protegens* and *P. chlororaphis* of the *P. fluorescens* group (Mulet et al., 2012b), currently only three pathogenic species are known to be capable of efficiently killing insects, (i) the entomopathogen *Pseudomonas entomophila*, (ii) the opportunistic human pathogen *Pseudomonas aeruginosa*, and (iii) the plant pathogen *Pseudomonas syringae*. Studies of the interactions of the three pathogens with insect hosts have significantly advanced our understanding of the molecular mechanisms involved in bacterial invasion of insects, escape from the insect immune response, gut and hemocoel colonization, and insect toxicity. They have also provided first insights into the ecology of vectoring of pseudomonads by insects. Studies on these pathogens can thus provide a valuable source of inspiration for future work on interactions of plant-beneficial pseudomonads with insects.

The entomopathogen *P. entomophila* is a bacterium that naturally infects *Drosophila* and originally was isolated from a fruit fly in Guadeloupe. The species which affiliates with the *Pseudomonas putida* phylogenetic group (Loper et al., 2012; Mulet et al., 2012a; 2012b) is also pathogenic towards lepidopteran insects (Vallet-Gely et al., 2008; Fedhila et al., 2010). Following oral infection, this bacterium is capable of persisting in the gut of *Drosophila*, inducing local and systemic immune responses and, at high doses, of killing the insect, and thus constitutes an exciting model for studies into virulence and host immune defense mechanisms (Vodovar et al., 2005; Vallet-Gely et al., 2008; 2010b). *P. entomophila* virulence is multi-factorial and depends on the GacS/GacA two-component system (Vodovar et al., 2005; Liehl et al., 2006). A second global regulatory system involving a yet unidentified signal molecule synthesized by the Pvf proteins contributes to control of *P. entomophila* virulence and immune response induction independently of GacS/GacA (Vallet-Gely et al., 2010b). Two important virulence factors have been identified in the entomopathogen. One is the Gac controlled metalloprotease AprA which counteracts the local immune response in the *Drosophila* gut via degradation of antimicrobial peptides (AMP) produced by the insect (Liehl et al., 2006). The other is a Gac and Pvf controlled pore-forming protein toxin termed Monalysin which contributes to the massive damage to the fly gut caused by *P. entomophila* in a mechanism involving suppression of immune and repair programs in the intestinal tract (Opota et al., 2011; Chakrabarti et al., 2012). However, both AprA and Monalysin deficient mutants (but not *gacA* mutants) retain some degree of

insect toxicity pointing to the existence of additional virulence factors. The genomic sequence of *P. entomophila* reveals a number of loci that encode potential candidate virulence factors, e.g., Tc-related toxins, HCN, hemolysins, and lipopetides (Vodovar et al., 2006), which await to be explored. One of these factors, a lipopeptide with a role in hemolytic activity, was recently determined not to be required for virulence in *Drosophila* (Vallet-Gely et al., 2010a).

P. aeruginosa is an opportunistic human pathogen (Gellatly and Hancock, 2013) and several strains are capable of infecting mammalian, invertebrate (nematodes and insects) and plant hosts, and these multihost interactions can be used to unravel conserved and variable virulence strategies of the bacterium (Mahajan-Miklos et al., 2000; Hendrickson et al., 2001; Kim et al., 2008). In general, the capability of *P. aeruginosa* to infect and kill insects was not used to investigate insect pathogenicity of the bacterium *per se* but rather to profit of convenient infection models for exploring the molecular basis of virulence of the human pathogen, even more as insects rely on innate defense mechanisms resembling those in mammalian hosts to fight microbial infections (Vallet-Gely et al., 2008). The entomopathogenic potential of the species was recognized already in reports dating back to the early last century (Bacot, 1911; Cameron, 1934; Bucher and Stephens, 1957; Angus, 1965). For instance, a *P. aeruginosa* isolate was reported to be responsible for a disease in laboratory rearings of grasshoppers (Bucher and Stephens, 1957). The authors demonstrated that the disease can be produced artificially by injecting the isolate into the hemocoel (LD_{50} of 10-20 cells per insect) or by feeding the insects with the bacterium (LD_{50} of about 10^4 cells per insect). A follow-up study then provided evidence for the passage of small numbers of the *P. aeruginosa* isolate from the gut into the hemocoel (Stephens, 1958). A field experiment with the isolate to control grasshoppers was not successful (Baird, 1958; Angus, 1965).

A majority of recent studies on *P. aeruginosa* insect virulence rely on variations of two *Drosophila* infection models, i.e. the fly nicking and fly feeding models thought to reflect acute or chronic infections, respectively (Sibley et al., 2008; Apidianakis and Rahme, 2009). In the nicking model rapid killing within 1 to 2 days after pricking flies with a needle dipped into a bacterial culture is observed, whereas the feeding model allows to monitor an extended infection process of 1 to 2 weeks after ingestion of a high concentration of bacteria by the flies. Using these models, considerable strain variation in virulence of *P. aeruginosa* to *Drosophila* was observed (Lutter et al. 2012) coinciding with similar observations for *P. fluorescens* group bacteria (Olcott et al., 2010). The variations in the pathogenicity are likely to mirror differences in the genomic equipage with relevant virulence genes and in the regulation of these genes in the different strains. Virulence gene expression by *P. aeruginosa* in the *Drosophila* intestinal tract and as a consequence insect pathogenicity is also influenced by other microorganisms present in the gut (Sibley et al., 2008).

As for *P. entomophila*, *P. aeruginosa* virulence towards *Drosophila* is multifactorial. Following ingestion, *P. aeruginosa* is able to colonize various parts of the *Drosophila* intestinal tract, counteract the insect immune defense, cross the intestinal barrier, and proliferate in the hemolymph (Sibley et al., 2008; Limmer et al.,

2011; Mulcahy et al., 2011). Global regulatory mechanisms involved in virulence control such as quorum sensing (QS) and the ppGpp-mediated stringent response are essential for the infection process (Chugani et al., 2001; Limmer et al., 2011; Vogt et al., 2011). The importance of QS signaling in the insect gut is highlighted in another feeding model involving the small cabbage white *Pieris rapae* in which interruption of QS signaling by mutation or by a chemical inhibitor reduced the virulence of *P. aeruginosa* (Borlee et al., 2008). In a recent study, *P. aeruginosa* was found to be capable of establishing a biofilm infection in the *Drosophila* crop following ingestion, thereby inducing an AMP immune response in the fly (Mulcahy et al., 2011). Remarkably, a mutant defective in biofilm formation had an improved capacity to cross the intestinal barrier and to disseminate into the hemolymph and was more virulent than the wild-type parent (Mulcahy et al., 2011). By contrast, hyperbiofilm strains were markedly less virulent to flies, an observation that was confirmed by another study (de Bentzmann et al., 2012) and is in accordance with the common association of biofilm formation with chronic infection in *P. aeruginosa* (Gellatly and Hancock, 2013) and other bacterial pathogens.

Multiple virulence traits of *P. aeruginosa* have a role in the acute infection model of *Drosophila* (Kim et al., 2008), including the capacity to suppress the insect's AMP defense response (Apidianakis et al., 2005), HCN production (Broderick et al., 2008) and delivery of type III secretion system (T3SS) effectors (Fauvarque et al., 2002; Avet-Rochex et al., 2005). The variety of virulence factors contributing to acute infection is further highlighted by studies involving the silkworm *Bombyx mori* and the *Galleria* waxmoth, two widely used lepidopteran model insects. The global regulator GacA (Chieda et al., 2005), the ADP-ribosylating exotoxin A (Chieda et al., 2011), and superoxide dismutases (Iiyama et al., 2007), but not pyocyanin (Chieda et al., 2008) contribute to injectable activity of *P. aeruginosa* in the silkworm model. Several T3SS effectors including ExoT are important for virulence in the *Galleria* injection model (Miyata et al., 2003). A T3SS effector (ExoS) is also required for virulence and translocation of *P. aeruginosa* from the midgut to the hemolymph in the *Bombyx* model (Okuda et al., 2010).

P. syringae is an important member of the phyllosphere bacterial community and well known for its plant pathogenic, ice-nucleating and epiphytic activities (Hirano and Upper, 2000). However, possible activities of *P. syringae* in interactions with insects so far have attracted only little attention. Interestingly, a recent study suggests that at least some *P. syringae* strains may exhibit significant insecticidal activity (Stavrinides et al., 2009). In the study, the bean pathogen *P. syringae* pv. *syringae* B728a was found to kill the pea aphid *Acyrthosiphon pisum* within less than two days when fed to the insect in artificial diet. By contrast, the tomato pathogen *P. syringae* pv. *tomato* DC3000 did not harm the aphid even though cell densities of the strain in infected insects raised to higher levels than those of strain B728a. In another study, *P. syringae* pv. *mori* did not survive in the intestinal tract of *Bombyx mori* larvae fed an artificial diet containing the phytopathogen (Watanabe et al., 1998). This may suggest that, as with strains of the *P. fluorescens* and *P. aeruginosa* groups, the capacity for potent insect pathogenicity is associated only with certain *P. syringae*

pathovars or strains and as such depends on the genomic background of the respective strain. The molecular basis of aphid toxicity of *P. syringae* pv. *syringae* B728a is unclear. Similarly to many other *P. syringae* strains, the genome of B728a harbors sequences related to those encoding the *Photorhabdus* Tc toxin complexes (Lindeberg et al., 2008). However, these were not required for virulence of *P. syringae* B728a in the aphid model (Stavrinides et al., 2009).

The work of Stavrinides and colleagues puts forward another interesting aspect of *Pseudomonas*-insect associations. They show that following natural infection of pea aphids by *P. syringae* present on leaves, the bacteria multiply inside the insect host and then can be spread at high cell concentrations onto fresh leaf surfaces in the honeydew deposited by the aphids (Stavrinides et al., 2009; Nadarasah and Stavrinides, 2011). Only very few other reports provide experimental evidence for insect vectoring of pseudomonads. For instance, the root-associated bacterium *P. chlororaphis* was demonstrated to be transmitted between corn plants by the Southern corn rootworm *Diabrotica undecimpunctata howardi* feeding on roots colonized by the bacterium (Snyder et al., 1998). In other reports, *P. fluorescens* strains were found to persist in the gut of the Colorado potato beetle *Leptinotarsa decemlineata* fed with the bacteria in laboratory experiments or prior to overwintering in the field (Castrillo et al., 2000a; 2000b). The ice-nucleation active bacteria markedly increased the supercooling point of the insects, leading the authors to speculate on a possibility for the biological control of the freeze-intolerant pest insects by reducing the survival of overwintering populations with a *Pseudomonas* treatment. Finally, insects may also be considered as potential vectors for the dispersal of biocontrol pseudomonads. This is documented by field experiments in which honeybees were successfully used to disseminate *P. fluorescens* strain A506, a biocontrol agent of fire blight and the active ingredient of the commercial product BlightBan A506, to pear and apple blossoms (Johnson et al., 1993). Together, all these studies illustrate that insects may not only constitute alternatives hosts for pseudomonads but also may serve as vectors and shelters for their survival and multiplication.

Potential of pseudomonads for the control of root-feeding pest insects

As it was illustrated earlier in this chapter, natural isolates of *P. protegens* and *P. chlororaphis* possess multiple activities that are beneficial to the plant in terms of growth and protection against various pests. These include antagonism of soil-borne phytopathogens, plant growth promotion, induction of systemic resistance, and insect pathogenicity (Fig. 1). It is therefore that these bacteria have a high potential as plant protection products. Because they can promote the growth of plants and protect plant roots against several pests simultaneously, *Pseudomonas*-based formulations may become products of high profit potential (Chandler et al., 2011). While plant root-associated pseudomonads have been successfully used for the formulation of commercial fungicides (Fravel, 2005; Berg, 2009), no insecticidal products with *Pseudomonas* strains as active ingredient currently exist on the market for biopesticides.

The way to a product based on root-associated pseudomonads for efficient plant protection against insects and phytopathogenic fungi obviously is not free of obstacles. Pseudomonads are known to be challenging microorganisms when it comes to formulation (Walsh et al., 2001). The survival of the bacteria during the manufacturing process and long-term storage is a critical issue. Furthermore, *Pseudomonas*-based products were reported to exhibit inconsistency under field conditions and they have raised some concerns of the general public about biosafety because this bacterial genus includes opportunistic human pathogens such as *P. aeruginosa*. As with every new biopesticide, the expensive and time-consuming registration procedure is a major hurdle for the successful application of a biocontrol agent (Bale et al., 2008). Nevertheless, the few strains of the *P. fluorescens* group which are approved in many countries for their use as fungicides in agriculture already went through the evaluation of environmental risks and the registration procedure. The products passed all tests on biosafety and efficacy, and these bacterial strains should therefore be studied for their effects on insects to possibly extend their application range in the future by modifying their formulations.

Novel *Pseudomonas* strains can also readily be isolated from various insect species. An obvious approach to discover strains with entomopathogenic potential could therefore be the isolation of pseudomonads from the respective target organism. During the selection of strains for a new plant protection product the efficacy of the bacterium as an insecticidal organism, the persistence and competition on plant roots, and the resistance during the formulation process should be considered (Walsh et al., 2001). Moreover, a detailed risk analysis needs to be performed to ensure that the bacterial strains have no deleterious effects on human health and on the environment. This requires amongst others more research on the molecular basis and regulation of insecticidal activity in these root-associated pseudomonads. The importance of such investigations is impressively illustrated by the above described discovery of the sophisticated regulatory switch allowing *P. protegens* to launch Fit toxin expression specifically in an insect host while arresting production of the insecticidal factor on roots (Péchy-Tarr et al., 2013), thus procuring a natural containment mechanism for biocontrol. The collaboration of the scientific community with commercial companies may then be the key to the development and commercialization of new biopesticides based on entomopathogenic, root-associated *Pseudomonas* strains, just like the development of products such as Proradix, Cedomon and Cerall already has demonstrated (Johnsson et al., 1998; Buddrus-Schiemann et al., 2010).

Microbial control agents are considered environmentally friendly and harmless to mammals, making them ideal components of IPM systems. Commercial insecticides based on entomopathogenic bacteria are mostly applied as inundative releases for short-term pest control when insect populations have already reached a certain threshold (Lacey et al., 2001). Many strains of the *P. fluorescens* group are well adapted to the life on plant roots and show environmental persistence. These microbes are very competitive and aggressive root colonizers (Lugtenberg and Kamilova, 2009), and would thus ideally be applied as inoculations for long-term

control before pest insects pose a problem to the particular plant population. As for the commercially available *Pseudomonas*-based biofungicides, it could be possible to apply entomopathogenic strains of *P. protegens* and *P. chlororaphis* as seed coatings for inoculative releases and thereby use these microorganisms in a preventative manner. Because plant-associated pseudomonads are already successfully used as biological fungicides in agriculture, insecticidal products for crop protection with entomopathogenic *Pseudomonas* bacteria as active ingredient could fit well into integrated systems. They would extend the existing toolbox for IPM and help to optimize the protection of plants against pest insects that feed on roots during at least a part of their life cycle and remain a challenging problem in many agricultural systems. As mentioned before, formulations with plant-beneficial pseudomonads possessing insecticidal activity could potentially be developed to provide products to the farmers that may permit long-term control of root-feeding insects and soil-borne phytopathogens simultaneously in an IPM framework. Future research should investigate the interaction of these pseudomonads with other IPM components. Combinations with other biocontrol agents such as entomopathogenic fungi or nematodes or further IPM tactics could show a synergistic effect on the suppression of plant pests (Lacey et al., 2001; Lacey and Shapiro, 2008; Karthiba et al., 2010; Hol et al., 2013).

The analysis and comparison of whole genome sequences in order to find candidate genes or gene clusters contributing to the insecticidal activity is a powerful approach to discover novel virulence factors and to extend the knowledge about these bacteria. It is further important to learn from existing data on other entomopathogenic bacteria to get a better understanding of the relevant virulence factors and their regulation, the mechanisms of colonization and invasion, and other functions required for insect pathogenicity of the plant-beneficial *Pseudomonas* strains. This includes in particular research on *Photorhabdus/Xenorhabdus* and pathogenic *Pseudomonas* species, but also studies about less known bacteria capable of killing insects, e.g. the aphid-infecting plant pathogen *Dickeya dadantii* (Costechareyre et al., 2013), could be inspiring for future investigations. Moreover, it is fundamental to carry out future research related to the control of soil-dwelling pest insects by beneficial root-associated pseudomonads under (near) natural conditions. This implies investigations into the interactions of these biocontrol bacteria with the natural microbiota of the insect gut just as the assessment of the efficacy of killing of insects under field conditions. Such approaches may help lessen known problems of inconsistency of *Pseudomonas*-based products in the field from the beginning.

We think that the current knowledge about the insect pathogenicity of certain root-associated pseudomonads and the powerful tools that are available for further investigations into this exciting feature are promising and a motivation for the development and application of microbial pesticides based on well-selected strains of these bacteria for a better management of root-feeding pest insects in the near future.

Research objectives and thesis outline

Even though previous investigations could demonstrate that strains of *P. protegens* and *P. chlororaphis* display insecticidal activity and identified the Fit toxin as a potent effector (Péchy-Tarr et al., 2008; Ruffner et al., 2013), there was only little knowledge about the evolution, molecular basis and regulation of their entomopathogenicity at the time this thesis project was started.

While the Fit toxin was shown to play an important role in CHA0 and PCL1391 during insect infection, it was unclear under which conditions the insecticidal protein is produced by *P. protegens* and by which regulators and signals its expression is controlled. Although there was evidence that the LysR-type regulator FitG and the response regulator FitH are important for induction and repression of toxin production (Péchy-Tarr et al., 2013), respectively, it remained unclear which factors and signals control the expression of the Fit toxin upstream of the two known regulatory proteins.

The first part of this thesis was thus focused on answering remaining questions concerning the regulation of Fit toxin expression *in vitro* and *in vivo* using *P. protegens* CHA0 as a model.

- In **chapter 2**, a fluorescence microscopy-based approach using reporter strains expressing a FitD-mCherry fusion protein was chosen to investigate under which conditions the Fit toxin is actually produced in *P. protegens* CHA0. This led to the discovery that the bacterium produces the insecticidal toxin only when infecting insects but not when colonizing roots or growing in standard growth media. Studies on the host range of strain CHA0 in addition suggest that this microbe is able to infect mainly larvae of lepidopteran insects.
- In **chapter 3**, the question about the role of the sensor kinase FitF, which is encoded at the *fit* locus right next to *fitH* and *fitG*, was addressed using similar techniques as in the previous chapter. The sensor protein was discovered to be required for the observed host-specific activation of the expression of the Fit toxin via FitG and FitH and it evolved most likely by recruiting a sensory domain from a ubiquitous regulatory protein, which controls the uptake of C_4-dicarboxylates in Proteobacteria, via domain shuffling.

~ ~ ~

The Fit toxin seems to be a contributory and not a requisite virulence factor, because a Δ*fitD* mutant is still able to kill insect larvae, although with a certain delay compared to the wild type (Péchy-Tarr et al., 2008; Ruffner et al., 2013). Therefore, there must be additional traits that are essential for or contribute to the capability of *P. protegens* and *P. chlororaphis* strains to colonize insects and cause disease. As suggested before, reports about the molecular basis of pathogenicity in well-characterized pathogenic bacteria could

provide a valuable source of inspiration for unraveling the underlying mechanisms of interactions of plant-beneficial pseudomonads with insects.

The second part of this thesis therefore aimed at identifying additional virulence factors and regulators in *P. protegens* CHA0 and *P. chlororaphis* PCL1391 by screening and characterizing determinants known to be important in pathogenic bacteria for infection of their hosts.

- The goal of **chapter 4** was to create a list of candidate virulence factors and regulators, specifically knock out relevant genes in *P. protegens* CHA0 or *P. chlororaphis* PCL1391, and screen the generated mutant strains in a *G. mellonella* injection assay for reduced virulence. Genes whose inactivation caused a delayed insect mortality in the screening encoded an O-antigen glycosyltransferase, the sensor kinase of the two-component regulatory system PhoP-PhoQ and the transcriptional regulator FleQ.

- **Chapter 5** investigated the genetic basis of O-antigen biosynthesis and its biological role by using a combined bioinformatic, genetic and molecular approach and aimed at confirming that O-polysaccharides are important for insect pathogenicity. The experiments that were carried out uncovered for the first time the genetics of O-antigen biosynthesis in plant-beneficial pseudomonads and showed that a particular type of O-antigen is indeed vital for these bacteria to infect and kill insects. Additional results further indicate that this surface constituent is important for the bacteria to survive the defense responses of the innate immune system, in particular as a protection against host antimicrobial peptides.

- In **chapter 6**, the main interest was to identify and characterize the role of the PhoP-PhoQ system. The presented data demonstrate that the two-component system is conserved in insect-pathogenic pseudomonads and that the sensor protein PhoQ is important for full virulence. Modification of lipid A, which is probably directly controlled by the response regulator PhoP, however does not seem to play an important role during systemic infection of insects. The lack of knowledge on how these pseudomonads deal with the insect immune system to successfully infect the host and cause disease makes it difficult to interpret some of the results from chapter 5 and 6.

- The general discussion in **chapter 7** thus gives some ideas and suggestions on how we can get a better understanding of how plant-beneficial pseudomonads with entomopathogenic properties interact with the insect immune system.

References

Almario, J., Prigent-Combaret, C., Muller, D., and Moënne-Loccoz, Y. (2013). Effect of clay mineralogy on iron bioavailability and rhizosphere transcription of 2,4-diacetylphloroglucinol biosynthetic genes in biocontrol *Pseudomonas protegens*. *Mol. Plant-Microbe Interact.* 26, 566–574.

Angus, T. A. (1965). Symposium on microbial insecticides. I. Bacterial pathogens of insects as microbial insecticides. *Bacteriol. Rev.* 29, 364–372.

Apidianakis, Y., and Rahme, L. G. (2009). *Drosophila melanogaster* as a model host for studying *Pseudomonas aeruginosa* infection. *Nat. Protoc.* 4, 1285–1294.

Apidianakis, Y., Mindrinos, M. N., Xiao, W., Lau, G. W., Baldini, R. L., Davis, R. W., and Rahme, L. G. (2005). Profiling early infection responses: *Pseudomonas aeruginosa* eludes host defenses by suppressing antimicrobial peptide gene expression. *Proc. Natl. Acad. Sci. U.S.A.* 102, 2573–2578.

Arias-Cordero, E., Ping, L., Reichwald, K., Delb, H., Platzer, M., and Boland, W. (2012). Comparative evaluation of the gut microbiota associated with the below- and above-ground life stages (larvae and beetles) of the forest cockchafer, *Melolontha hippocastani*. *PLoS One* 7, e51557.

Avet-Rochex, A., Bergeret, E., Attree, I., Meister, M., and Fauvarque, M. O. (2005). Suppression of *Drosophila* cellular immunity by directed expression of the ExoS toxin GAP domain of *Pseudomonas aeruginosa*. *Cell. Microbiol.* 7, 799–810.

Bacot, A. W. (1911). The persistence of *Bacillus pyocyaneus* in pupae and imagines of *Musca domestica* raised from larvae experimentally infected with the bacillus. *Parasitology* 4, 68–73.

Baird, R. B. (1958). Field experiments with *Pseudomonas aeruginosa* (Schroeter) Migula to control grasshoppers. *Can. Entomol.* 90, 89–91.

Bakker, P. A. H. M., Pieterse, C. M. J., and Van Loon, L. C. (2007). Induced systemic resistance by fluorescent *Pseudomonas* spp. *Phytopathology* 97, 239–243.

Bale, J. S., van Lenteren, J. C., and Bigler, F. (2008). Biological control and sustainable food production. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 363, 761–776.

Balmer, D., Planchamp, C., and Mauch-Mani, B. (2013). On the move: induced resistance in monocots. *J. Exp. Bot.* 64, 1249–1261.

Bansal, R., Hulbert, S., Schemerhorn, B., Reese, J. C., Whitworth, R. J., Stuart, J. J., and Chen, M. S. (2011). Hessian fly-associated bacteria: transmission, essentiality, and composition. *PLoS One* 6, e23170.

Berendsen, R. L., Pieterse, C. M. J., and Bakker, P. A. H. M. (2012). The rhizosphere microbiome and plant health. *Trends Plant Sci*. 17, 478–486.

Berg, G. (2009). Plant-microbe interactions promoting plant growth and health: perspectives for controlled use of microorganisms in agriculture. *Appl. Microbiol. Biotechnol.* 84, 11–18.

Bizzarri, M. F., and Bishop, A. H. (2008). The ecology of *Bacillus thuringiensis* on the phylloplane: colonization from soil, plasmid transfer, and interaction with larvae of *Pieris brassicae*. *Microb. Ecol.* 56, 133–139.

Bjørnlund, L., Rønn, R., Péchy-Tarr, M., Maurhofer, M., Keel, C., and Nybroe, O. (2009). Functional GacS in *Pseudomonas* DSS73 prevents digestion by *Caenorhabditis elegans* and protects the nematode from killer flagellates. *ISME J*. 3, 770–779.

Blackburn, M. B., Martin, P. A. W., Kuhar, D., Farrar, R. R. Jr, and Gundersen-Rindal, D. E. (2011). The occurrence of *Photorhabdus*-like toxin complexes in *Bacillus thuringiensis*. *PLoS One* 6, e18122.

Blossey, B., and Hunt-Joshi, T. R. (2003). Belowground herbivory by insects: influence on plants and aboveground herbivores. *Annu. Rev. Entomol.* 48, 521–547.

Bode, H. B. (2009). Entomopathogenic bacteria as a source of secondary metabolites. *Curr. Opin. Chem. Biol.* 13, 224–230.

Borlee, B. R., Geske, G. D., Robinson, C. J., Blackwell, H. E., and Handelsman, J. (2008). Quorum-sensing signals in the microbial community of the cabbage white butterfly larval midgut. *ISME J.* 2, 1101–1111.

Bowen, D., Rocheleau, T. A., Blackburn, M., Andreev, O., Golubeva, E., Bhartia, R., and ffrench-Constant, R. H. (1998). Insecticidal toxins from the bacterium *Photorhabdus luminescens*. *Science* 280, 2129–2132.

Bravo, A., Gill, S. S., and Soberón, M. (2007). Mode of action of *Bacillus thuringiensis* Cry and Cyt toxins and their potential for insect control. *Toxicon* 49, 423–435.

Bravo, A., Likitvivatanavong, S., Gill, S. S., and Soberón, M. (2011). *Bacillus thuringiensis*: A story of a successful bioinsecticide. *Insect Biochem. Mol. Biol.* 41, 423–231.

Broderick, K. E., Chan, A., Balasubramanian, M., Feala, J., Reed, S. L., Panda, M., et al. (2008). Cyanide produced by human isolates of *Pseudomonas aeruginosa* contributes to lethality in *Drosophila melanogaster*. *J. Infect. Dis.* 197, 457–464.

Broderick, N. A., Raffa, K. F., Goodman, R. M., and Handelsman, J. (2004). Census of the bacterial community of the gypsy moth larval midgut by using culturing and culture-independent methods. *Appl. Environ. Microbiol.* 70, 293–300.

Bucher, G. E., and Stephens, J. M. (1957). A disease of grasshoppers caused by the bacterium *Pseudomonas aeruginosa* (Schroeter) Migula. *Can. J. Microbiol.* 3, 611–625.

Buddrus-Schiemann, K., Schmid, M., Schreiner, K., Welzl, G., and Hartmann, A. (2010). Root colonization by *Pseudomonas* sp. DSMZ 13134 and impact on the indigenous rhizosphere bacterial community of barley. *Microb. Ecol.* 60, 381–393.

Cameron, G. R. (1934). Inflammation in the caterpillars of *Lepidoptera*. *J. Pathol. Bacteriol.* 38, 441–466.

Campos-Herrera, R., Barbercheck, M., Hoy, C. W., and Stock, S. P. (2012). Entomopathogenic nematodes as a model system for advancing the frontiers of ecology. *J. Nematol.* 44, 162–176.

Castrillo, L. A., Lee, R. E. Jr., Lee, M. R., and Rutherford, S. T. (2000a). Identification of ice-nucleating active *Pseudomonas fluorescens* strains for biological control of overwintering Colorado potato beetles (*Coleoptera: Chrysomelidae*). *J. Econ. Entomol.* 93, 226–233.

Castrillo, L. A., Lee, R. E. Jr., Lee, M. R., and Wyman, J. A. (2000b). Long-term retention of ice-nucleating active *Pseudomonas fluorescens* by overwintering Colorado potato beetles. *Cryo Letters* 21, 5–12.

Chakrabarti, S., Liehl, P., Buchon, N., and Lemaitre, B. (2012). Infection-induced host translational blockage inhibits immune responses and epithelial renewal in the *Drosophila* gut. *Cell Host Microbe* 12, 60–70.

Chandler, D., Bailey, A. S., Tatchell, G. M., Davidson, G., Greaves, J., and Grant, W. P. (2011). The development, regulation and use of biopesticides for integrated pest management. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 366, 1987–1998.

Chapuisat, M., Oppliger, A., Magliano, P., and Christe, P. (2007). Wood ants use resin to protect themselves against pathogens. *Proc. Biol. Sci.* 274, 2013–2017.

Chieda, Y., Iiyama, K., Lee, J. M., Kusakabe, T., Yasunaga-Aoki, C., and Shimizu, S. (2008). Inactivation of pyocyanin synthesis genes has no effect on the virulence of *Pseudomonas aeruginosa* PAO1 toward the silkworm, *Bombyx mori. FEMS Microbiol. Lett.* 278, 101–107.

Chieda, Y., Iiyama, K., Lee, J. M., Kusakabe, T., Yasunaga-Aoki, C., and Shimizu, S. (2011). Virulence of an exotoxin A-deficient strain of *Pseudomonas aeruginosa* toward the silkworm, *Bombyx mori*. *Microb. Pathog.* 51, 407–414.

Chieda, Y., Iiyama, K., Yasunaga-Aoki, C., Lee, J. M., Kusakabe, T., and Shimizu, S. (2005). Pathogenicity of *gacA* mutant of *Pseudomonas aeruginosa* PA01 in the silkworm, *Bombyx mori*. *FEMS Microbiol. Lett.* 244, 181–186.

Chin-A-Woeng, T. F., Thomas-Oates, J. E., Lugtenberg, B. J., and Bloemberg, G. V. (2001). Introduction of the *phzH* gene of *Pseudomonas chlororaphis* PCL1391 extends the range of biocontrol ability of phenazine-1-carboxylic acid-producing *Pseudomonas* spp. strains. *Mol. Plant Microbe Interact.* 14, 1006–1015.

Chugani, S. A., Whiteley, M., Lee, K. M., D'Argenio, D., Manoil, C., and Greenberg, E. P. (2001). QscR, a modulator of quorum-sensing signal synthesis and virulence in *Pseudomonas aeruginosa*. *Proc. Natl. Acad. Sci. U.S.A.* 98, 2752–2757.

Commare, R. R., Nandakumar, R., Kandan, A., Suresh, S., Bharathi, M., Raguchander, T., and Samiyappan, R. (2002). *Pseudomonas fluorescens* based bio-formulation for the management of sheath blight disease and leaffolder insect in rice. *Crop Prot.* 21, 671–677.

Cook, R. J., Thomashow, L. S., Weller, D. M., Fujimoto, D., Mazzola, M., Bangera, G., and Kim, D.-S. (1995). Molecular mechanisms of defense by rhizobacteria against root disease. *Proc. Natl. Acad. Sci. U.S.A.* 92, 4197–4201.

Corby-Harris, V., Pontaroli, A. C., Shimkets, L. J., Bennetzen, J. L., Habel, K. E., and Promislow, D. E. (2007). Geographical distribution and diversity of bacteria associated with natural populations of *Drosophila melanogaster*. *Appl. Environ. Microbiol.* 73, 3470–3479.

Cornelis, P. (2010). Iron uptake and metabolism in pseudomonads. *Appl. Microbiol. Biotechnol.* 86, 1637–1645.

Cory, J. S., and Franklin, M. T. (2012). Evolution and the microbial control of insects. *Evol. Appl.* 5, 455–469.

Cory, S., and Adams, J. M. (2002). The Bcl2 family: regulators of the cellular life-or-death switch. *Nat. Rev. Cancer* 2, 647–656.

Costechareyre, D., Chich, J. F., Strub, J. M., Rahbé, Y., and Condemine, G. (2013). Transcriptome of *Dickeya dadantii* infecting *Acyrthosiphon pisum* reveals a strong defense against antimicrobial peptides. *PLoS One* 8, e54118.

Crickmore, N., Baum, J., Bravo, A., Lereclus, D., Narva, K., Sampson, K., et al. (2013). *Bacillus thuringiensis* toxin nomenclature http://www.btnomenclature.info/.

Crickmore, N., Zeigler, D. R., Feitelson, J., Schnepf, E., Van Rie, J., Lereclus, D., et al. (1998). Revision of the nomenclature for the *Bacillus thuringiensis* pesticidal crystal proteins. *Microbiol. Mol. Biol. Rev.* 62, 807–813.

Daborn, P. J., Waterfield, N., Silva, C. P., Au, C. P., Sharma, S., and ffrench-Constant, R. H. (2002). A single *Photorhabdus* gene, makes caterpillars floppy (*mcf*), allows *Escherichia coli* to persist within and kill insects. *Proc. Natl. Acad. Sci. U.S.A.* 99, 10742–10747.

Daval, S., Lebreton, L., Gazengel, K., Boutin, M., Guillerm-Erckelboudt, A. Y., and Sarniguet, A. (2011). The biocontrol bacterium *Pseudomonas fluorescens* Pf29Arp strain affects the pathogenesis-related gene expression of the take-all fungus *Gaeumannomyces graminis* var. *tritici* on wheat roots. *Mol. Plant Pathol.* 12, 839–854.

de Bentzmann, S., Giraud, C., Bernard, C. S., Calderon, V., Ewald, F., Plésiat, P., et al. (2012). Unique biofilm signature, drug susceptibility and decreased virulence in *Drosophila* through the *Pseudomonas aeruginosa* two-component system PprAB. *PLoS Pathog.* 8, e1003052.

de Lima Pimenta, A., Di Martino, P., Le Bouder, E., Hulen, C., and Blight, M. A. (2003). In vitro identification of two adherence factors required for in vivo virulence of *Pseudomonas fluorescens*. *Microb. Infect*. 5, 1177–1187.

De Vleesschauer, D., and Höfte, M. (2009). Rhizobacteria-induced systemic resistance. *Adv. Bot. Res.* 51, 223–281.

de Werra, P., Baehler, E., Huser, A., Keel, C., and Maurhofer, M. (2008). Detection of plant-modulated alterations in antifungal gene expression in *Pseudomonas fluorescens* CHA0 on roots by flow cytometry. *Appl. Environ. Microbiol.* 74, 1139–1349.

Degenhardt, J., Hiltpold, I., Köllner, T. G., Frey, M., Gierl, A., Gershenzon, J., et al. (2009). Restoring a maize root signal that attracts insect-killing nematodes to control a major pest. *Proc. Natl. Acad. Sci. U.S.A.* 106, 13213–13218.

Denno, R. F., Gruner, D. S., and Kaplan, I. (2008). Potential for entomopathogenic nematodes in biological control: a meta-analytical synthesis and insights from trophic cascade theory. *J. Nematol.* 40, 61–72.

Devi, K. K., and Kothamasi, D. (2009). *Pseudomonas fluorescens* CHA0 can kill subterranean termite *Odontotermes obesus* by inhibiting cytochromec oxidase of the termite respiratory chain. *FEMS Microbiol. Lett*. 300, 195–200.

Dowling, A. J., Daborn, P. J., Waterfield, N. R., Wang, P., Streuli, C. H., and ffrench-Constant, R. H. (2004). The insecticidal toxin Makes caterpillars floppy (Mcf) promotes apoptosis in mammalian cells. *Cell. Microbiol*. 6, 345–353.

Ehlers, R. U. (2001). Mass production of entomopathogenic nematodes for plant protection. *Appl. Microbiol. Biotechnol.* 56, 623–633.

Fauvarque, M. O., Bergeret, E., Chabert, J., Dacheux, D., Satre, M., and Attree, I. (2002). Role and activation of type III secretion system genes in *Pseudomonas aeruginosa*-induced *Drosophila* killing. *Microb. Pathog.* 32, 287–295.

Fedhila, S., Buisson, C., Dussurget, O., Serror, P., Glomski, I. J., Liehl, P., et al. (2010). Comparative analysis of the virulence of invertebrate and mammalian pathogenic bacteria in the oral insect infection model *Galleria mellonella*. *J. Invertebr. Pathol.* 103, 24–29.

ffrench-Constant, R. H., Dowling, A., and Waterfield, N. R. (2007). Insecticidal toxins from *Photorhabdus* bacteria and their potential use in agriculture. *Toxicon* 49, 436–451.

Fravel, D. R. (2005). Commercialization and implementation of biocontrol. *Annu. Rev. Phytopathol.* 43, 337–359.

Gatehouse, A. M., Ferry, N., Edwards, M. G., and Bell, H. A. (2011). Insect-resistant biotech crops and their impacts on beneficial arthropods. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 366, 1438–1452.

Gatsogiannis, C., Lang, A. E., Meusch, D., Pfaumann, V., Hofnagel, O., Benz, R., et al. (2013). Syringe-like injection mechanism in *Photorhabdus luminescens* toxins. *Nature* 495, 520–523.

Gellatly, S. L., and Hancock, R. E. (2013). *Pseudomonas aeruginosa*: new insights into pathogenesis and host defenses. *Pathogens Dis.* 67, 159–173.

Gray, M. E., Sappington, T. W., Miller, N. J., Moeser, J., and Bohn, M. O. (2009). Adaptation and invasiveness of Western corn rootworm: intensifying research on a worsening pest. *Annu. Rev. Entomol.* 54, 303–321.

Gross, H., and Loper, J. E. (2009). Genomics of secondary metabolite production by *Pseudomonas* spp. *Nat. Prod. Rep*. 26, 1408–1446.

Haas, D., and Défago, G. (2005). Biological control of soil-borne pathogens by fluorescent pseudomonads. *Nat. Rev. Microbiol.* 3, 307–319.

Haas, D., and Keel, C. (2003). Regulation of antibiotic production in root-colonizing *Pseudomonas* spp. and relevance for biological control of plant disease. *Annu. Rev. Phytopathol.* 41, 117–153.

Hashimoto, Y. (2002). Study of the bacteria pathogenic for aphids, isolation of bacteria and identification of insecticidal compound. *Rep. Hokkaido Prefect. Agric. Exp. Sta.* 102, 1–48.

Heckel, D. G. (2012). Insecticide resistance after Silent spring. *Science* 337, 1612–1614.

Hendrickson, E. L., Plotnikova, J., Mahajan-Miklos, S., Rahme, L. G., and Ausubel, F. M. (2001). Differential roles of the *Pseudomonas aeruginosa* PA14 *rpoN* gene in pathogenicity in plants, nematodes, insects, and mice. *J. Bacteriol.* 183, 7126–7134.

Herbert, E. E., and Goodrich-Blair, H. (2007). Friend and foe: the two faces of *Xenorhabdus nematophila*. *Nat. Rev. Microbiol.* 5, 634–646.

Hirano, S. S., and Upper, C. D. (2000). Bacteria in the leaf ecosystem with emphasis on *Pseudomonas syringae* - a pathogen, ice nucleus, and epiphyte. *Microbiol. Mol. Biol. Rev.* 64, 624–653.

Höfte, M., and Altier, N. (2010). Fluorescent pseudomonads as biocontrol agents for sustainable agricultural systems. *Res. Microbiol.* 161, 464–471.

Hol, W. H., Bezemer, T. M., and Biere, A. (2013). Getting the ecology into interactions between plants and the plant growth-promoting bacterium *Pseudomonas fluorescens*. *Front. Plant Sci.* 4, 81.

Horn, H., and Eberspächer, J. (1976). Die Waldtrachtkrankheit der Honigbiene ii. Nachweis von Bakterien in der Hämolymphe waldtrachtkranker Bienen und der zusätzliche Einfluss der Fütterung auf die Waldtrachtkrankheit. *Apidologie* 17, 307–324.

Hunter, M. D. (2001). Out of sight, out of mind: the impacts of root-feeding insects in natural and managed systems. *Agric. Forest Entomol.* 3, 3–9.

Hurst, M. R., Glare, T. R., Jackson, T. A., and Ronson, C. W. (2000). Plasmid-located pathogenicity determinants of *Serratia entomophila*, the causal agent of amber disease of grass grub, show similarity to the insecticidal toxins of *Photorhabdus luminescens*. *J. Bacteriol.* 182, 5127–5138.

Iiyama, K., Chieda, Y., Lee, J. M., Kusakabe, T., Yasunaga-Aoki, C., and Shimizu, S. (2007). Effect of superoxide dismutase gene inactivation on virulence of *Pseudomonas aeruginosa* PAO1 toward the silkworm, *Bombyx mori*. *Appl. Environ. Microbiol.* 73, 1569–1575.

James, C. (2012). Global status of commercialized biotech/GM crops: 2012. *ISAAA Brief No. 44*. Ithaca, NY: ISAAA.

Jang, J. Y., Yang, S. Y., Kim, Y. C., Lee, C. W., Park, M. S., Kim, J. C., and Kim, I. S. (2013). Identification of orfamide A as an insecticidal metabolite produced by *Pseudomonas protegens* F6. *J. Agric. Food Chem.* 61, 6786–91.

Janvier, C., Villeneuve, F., Alabouvette, C., Edel-Hermann, V., Mateille, T., and Steinberg, C. (2007). Soil health through soil disease suppression: Which strategy from descriptors to indicators? *Soil Biol. Biochem.* 39, 1–23.

Johnson, K. B., Stockwell, V. O., Burgett, D. M., Sugar, D., and Loper, J. E. (1993). Dispersal of *Erwinia amylovora* and *Pseudomonas fluorescens* by honey bees from hives to apple and pear blossoms. *Phytopathology* 83, 478–484.

Johnsson, L., Hökeberg, M., and Gerhardson, B. (1998). Performance of the *Pseudomonas chlororaphis* biocontrol agent MA 342 against cereal seed-borne diseases in field experiments. *Eur. J. Plant Pathol.* 104, 701–711.

Jousset, A., Rochat, L., Lanoue, A., Bonkowski, M., Keel, C., and Scheu, S. (2011). Plants respond to pathogen infection by enhancing the antifungal gene expression of root-associated bacteria. *Mol. Plant-Microbe Interact.* 24, 352–358.

Jousset, A., Rochat, L., Péchy-Tarr, M., Keel, C., Scheu, S., and Bonkowski, M. (2009). Predators promote defence of rhizosphere bacterial populations by selective feeding on non-toxic cheaters. *ISME J*. 3, 666–674.

Kamal, A., Shaik, A. B., Kumar, C. G., Mongolla, P., Rani, P. U., Krishna, K. V., et al. (2012). Metabolic profiling and biological activities of bioactive compounds produced by *Pseudomonas* sp. strain ICTB-745 isolated from Ladakh, India. *J. Microbiol. Biotechnol.* 22, 69–79.

Karthiba, L., Saveetha, K., Suresh, S., Raguchander, T., Saravanakumar, D., and Samiyappan, R. (2010). PGPR and entomopathogenic fungus bioformulation for the synchronous management of leaffolder pest and sheath blight disease of rice. *Pest Manag. Sci*. 66, 555–564.

Keel, C., Voisard, C., Berling, C. H., Kahr, G., and Défago, G. (1989). Iron sufficiency is a prerequisite for suppression of tobacco black root rot by *Pseudomonas fluorescens* strain CHA0 under gnotobiotic conditions. *Phytopathology* 79, 584–589.

Kim, S. K., Kim, Y. C., Lee, S., Kim, J.C., Yun, M. Y., and Kim, I. S. (2011). Insecticidal activity of rhamnolipid isolated from *Pseudomonas* sp. EP-3 against green peach aphid (*Myzus persicae*). *J. Agric. Food Chem.* 59, 934–938.

Kim, S. H., Park, S. Y., Heo, Y. J., and Cho, Y. H. (2008). *Drosophila melanogaster*-based screening for multihost virulence factors of *Pseudomonas aeruginosa* PA14 and identification of a virulence-attenuating factor, HudA. *Infect. Immun.* 76, 4152–4162.

Lacava, P. T., Parker, J., Andreote, F. D., Dini-Andreote, F., Ramirez, J. L., and Miller, T. A. (2007). Analysis of the bacterial community in glassy-winged sharpshooter heads. *Entomol. Res.* 37, 261–266.

Lacey, L. A., and Georgis, R. (2012). Entomopathogenic nematodes for control of insect pests above and below ground with comments on commercial production. *J. Nematol.* 44, 218–225.

Lacey, L. A., Frutos, R., Kaya, H. K., and Vail, P. (2001). Insect pathogens as biological control agents: do they have a future? *Biol. Control* 21, 230–248.

Lacey, L. A., and Shapiro-Ilan, D. I. (2008). Microbial control of insect pests in temperate orchard systems: potential for incorporation into IPM. *Annu. Rev. Entomol.* 53, 121–144.

Lacey, L. A., Unruh, T. R., Simkins, H., and Thomsen-Archer, K. (2007). Gut bacteria associated with the Pacific Coast wireworm, *Limonius canus*, inferred from 16s rDNA sequences and their implications for control. *Phytoparasitica* 35, 479–489.

Landsberg, M. J., Jones, S. A., Rothnagel, R., Busby, J. N., Marshall, S. D., Simpson, R. M., et al. (2011). 3D structure of the *Yersinia entomophaga* toxin complex and implications for insecticidal activity. *Proc. Natl. Acad. Sci. U.S.A.* 108, 20544–20549.

Lang, A. E., Schmidt, G., Schlosser, A., Hey, T. D., Larrinua, I. M., Sheets, J. J., et al. (2010). *Photorhabdus luminescens* toxins ADP-ribosylate actin and RhoA to force actin clustering. *Science* 327, 1139–1142.

Lapouge, K., Schubert, M., Allain, F. H., and Haas, D. (2008). Gac/Rsm signal transduction pathway of gamma-proteobacteria: from RNA recognition to regulation of social behaviour. *Mol. Microbiol*. 67, 241–253.

Li, X., Nan, X., Wei, C., and He, H. (2012). The gut bacteria associated with *Camponotus japonicus* Mayr with culture-dependent and DGGE methods. *Curr. Microbiol.* 65, 610–616.

Liehl, P., Blight, M., Vodovar, N., Boccard, F., and Lemaitre, B. (2006). Prevalence of local immune response against oral infection in a *Drosophila/Pseudomonas* infection model. *PLoS Pathog.* 2, e56.

Limmer, S., Haller, S., Drenkard, E., Lee, J., Yu, S., Kocks, C., et al. (2011). *Pseudomonas aeruginosa* RhlR is required to neutralize the cellular immune response in a *Drosophila melanogaster* oral infection model. *Proc. Natl. Acad. Sci. U.S.A.* 108, 17378–17383.

Lindeberg, M., Myers, C. R., Collmer, A., and Schneider, D. J. (2008). Roadmap to new virulence determinants in *Pseudomonas syringae*: insights from comparative genomics and genome organization. *Mol. Plant-Microbe Interact.* 21, 685–700.

Liu, D., Burton, S., Glancy, T., Li, Z. S., Hampton, R., Meade, T., and Merlo, D. (2003). Insect resistance conferred by 283-kDa *Photorhabdus luminescens* protein TcdA in *Arabidopsis thaliana*. *Nat. Biotechnol.* 21, 1222–1228.

Liu, J.-R., Lin, Y.-D., Chang, S.-T., Zeng, Y.-F., and Wang, S.-L. (2010). Molecular cloning and characterization of an insecticidal toxin from *Pseudomonas taiwanensis*. *J. Agric. Food Chem.* 58, 12343–12349.

Loper, J. E., and Buyer, J. S. (1991). Siderophores in microbial interactions on plant surfaces. *Mol. Plant-Microbe Interact.* 4, 5–13.

Loper, J. E., Hassan, K. A., Mavrodi, D. V., Davis, E. W. 2nd, Lim, C. K., Shaffer, B. T., et al. (2012). Comparative genomics of plant-associated *Pseudomonas* spp.: insights into diversity and inheritance of traits involved in multitrophic interactions. *PLoS Genet.* 8, e1002784.

Lugtenberg, B., and Kamilova, F. (2009). Plant-growth-promoting rhizobacteria. *Annu. Rev. Microbiol.* 63, 541–556.

Lutter, E. I., Purighalla, S., Duong, J., and Storey, D. G. (2012). Lethality and cooperation of *Pseudomonas aeruginosa* quorum-sensing mutants in *Drosophila melanogaster* infection models. *Microbiology* 158, 2125–2132.

Mahajan-Miklos, S., Rahme, L. G., and Ausubel, F. M. (2000). Elucidating the molecular mechanisms of bacterial virulence using non-mammalian hosts. *Mol. Microbiol.* 37, 981–988.

Marchi, M., Boutin, M., Gazengel, K., Rispe, C., Gauthier, J. P., Guillerm-Erckelboudt, A. Y., et al. (2013). Genomic analysis of the biocontrol strain *Pseudomonas fluorescens* Pf29Arp with evidence of T3SS and T6SS gene expression on plant roots. *Environ. Microbiol. Rep.* 5, 393–403.

Mark, G. L., Morrissey, J. P., Higgins, P., and O'Gara, F. (2006). Molecular-based strategies to exploit *Pseudomonas* biocontrol strains for environmental biotechnology applications. *FEMS Microbiol. Ecol.* 56, 167–177.

Maurhofer, M., Hase, C., Meuwly, P., Métraux, J.-P., and Défago, G. (1994). Induction of systemic resistance of tobacco to tobacco necrosis virus by the root-colonizing *Pseudomonas fluorescens* strain CHA0: Influence of the *gacA* gene and of pyoverdine production. *Phytopathology* 84, 139–146.

Mavrodi, D. V., Peever, T. L., Mavrodi, O. V., Parejko, J. A., Raaijmakers, J. M., Lemanceau, P., et al. (2010). Diversity and evolution of the phenazine biosynthesis pathway. *Appl. Environ. Microbiol.* 76, 866–879.

Mazurier, S., Corberand, T., Lemanceau, P., and Raaijmakers, J. M. (2009). Phenazine antibiotics produced by fluorescent pseudomonads contribute to natural soil suppressiveness to Fusarium wilt. *ISME J.* 3, 977–991.

Mazzola, M. (2004). Assessment and management of soil microbial community structure for disease suppression. *Annu. Rev. Phytopathol.* 42, 35–59.

Mendes, R., Kruijt, M., de Bruijn, I., Dekkers, E., van der Voort, M., Schneider, J. H. M., et al. (2011). Deciphering the rhizosphere microbiome for disease-suppressive bacteria. *Science* 332, 1097–1100.

Mentel, M., Ahuja, E. G., Mavrodi, D. V., Breinbauer, R., Thomashow, L. S., and Blankenfeldt, W. (2009). Of two make one: the biosynthesis of phenazines. *ChemBioChem* 10, 2295–2304.

Mercado-Blanco, J., and Bakker, P. A. (2007). Interactions between plants and beneficial *Pseudomonas* spp.: exploiting bacterial traits for crop protection. *Antonie Leeuwenhoek J. Microbiol*. 92, 367–389.

Miyata, S., Casey, M., Frank, D. W., Ausubel, F. M., and Drenkard, E. (2003). Use of the *Galleria mellonella* caterpillar as a model host to study the role of the type III secretion system in *Pseudomonas aeruginosa* pathogenesis. *Infect. Immun.* 71, 2404–2413.

Mohr, K. I., and Tebbe, C. C. (2006). Diversity and phylotype consistency of bacteria in the guts of three bee species (*Apoidea*) at an oilseed rape field. *Environ. Microbiol.* 8, 258–272.

Monnerat, R. G., Soares, C. M., Capdeville, G., Jones, G., Martins, E. S., Praça, L., et al. (2009). Translocation and insecticidal activity of *Bacillus thuringiensis* living inside of plants. *Microb. Biotechnol.* 2, 512–520.

Mulcahy, H., Sibley, C. D., Surette, M. G., and Lewenza, S. (2011). *Drosophila melanogaster* as an animal model for the study of *Pseudomonas aeruginosa* biofilm infections in vivo. *PLoS Pathog.* 7, e1002299.

Mulet, M., Gomila, M., Lemaitre, B., Lalucat, J., and García-Valdés, E. (2012a). Taxonomic characterisation of *Pseudomonas* strain L48 and formal proposal of *Pseudomonas entomophila* sp. nov. *Syst. Appl. Microbiol.* 35, 145–149.

Mulet, M., Gomila, M., Scotta, C., Sánchez, D., Lalucat, J., and García-Valdés, E. (2012b). Concordance between whole-cell matrix-assisted laser-desorption/ionization time-of-flight mass spectrometry and multilocus sequence analysis approaches in species discrimination within the genus *Pseudomonas*. *Syst. Appl. Microbiol.* 35, 455–464.

Mulet, M., Lalucat, J., and García-Valdés, E. (2010). DNA sequence-based analysis of the *Pseudomonas* species. *Environ. Microbiol.* 12, 1513–1530.

Nadarasah, G., and Stavrinides, J. (2011). Insects as alternative hosts for phytopathogenic bacteria. *FEMS Microbiol. Rev.* 35, 555–575.

Nielsen-LeRoux, C., Gaudriault, S., Ramarao, N., Lereclus, D., and Givaudan, A. (2012). How the insect pathogen bacteria *Bacillus thuringiensis* and *Xenorhabdus/Photorhabdus* occupy their hosts. *Curr. Opin. Microbiol.* 15, 220–231.

Nielsen, T. H., and Sørensen, J. (2003). Production of cyclic lipopeptides by *Pseudomonas fluorescens* strains in bulk soil and in the sugar beet rhizosphere. *Appl. Environ. Microbiol.* 69, 861–868.

Oerke, E.-C. (2006). Crop losses to pests. *J. Agric. Sci.* 144, 31–43.

Okuda, J., Hayashi, N., Okamoto, M., Sawada, S., Minagawa, S., Yano, Y., and Gotoh, N. (2010). Translocation of *Pseudomonas aeruginosa* from the intestinal tract is mediated by the binding of ExoS to an Na,K-ATPase regulator, FXYD3. *Infect. Immun.* 78, 4511–4522.

Olcott, M. H., Henkels, M. D., Rosen, K. L., Walker, F. L., Sneh, B., Loper, J. E., and Taylor, B. J. (2010). Lethality and developmental delay in *Drosophila melanogaster* larvae after ingestion of selected *Pseudomonas fluorescens* strains. *PLoS One* 5, e12504.

Opota, O., Vallet-Gély, I., Vincentelli, R., Kellenberger, C., Iacovache, I., Gonzalez, M. R., et al. (2011). Monalysin, a novel ß-pore-forming toxin from the *Drosophila* pathogen *Pseudomonas entomophila*, contributes to host intestinal damage and lethality. *PLoS Pathog.* 7, e1002259.

Osei-Poku, J., Mbogo, C. M., Palmer, W. J., and Jiggins, F. M. (2012). Deep sequencing reveals extensive variation in the gut microbiota of wild mosquitoes from Kenya. *Mol. Ecol.* 21, 5138–5150.

Otsu, Y., Matsuda, Y., Mori, H., Ueki, H., Nakajima, T., Fujiwara, K., et al. (2004). Stable phylloplane colonization by entomopathogenic bacterium *Pseudomonas fluorescens* KPM-018P and biological control of phytophagous ladybird beetles *Epilachna vigintioctopunctata* (Coleoptera: Coccinellidae). *Biocontrol Sci. Technol.* 14, 427–439.

Park, J. Y., Oh, S. A., Anderson, A. J., Neiswender, J., Kim, J. C., and Kim, Y. C. (2011). Production of the antifungal compounds phenazine and pyrrolnitrin from *Pseudomonas chlororaphis* O6 is differentially regulated by glucose. *Lett. Appl. Microbiol.* 52, 532–537.

Paulsen, I. T., Press, C. M., Ravel, J., Kobayashi, D. Y., Myers, G. S., Mavrodi, D. V., et al. (2005). Complete genome sequence of the plant commensal *Pseudomonas fluorescens* Pf-5. *Nat. Biotechnol.* 23, 873–878.

Péchy-Tarr, M., Borel, N., Kupferschmied, P., Turner, V., Binggeli, O., Radovanovic, D., et al. (2013). Control and host-dependent activation of insect toxin expression in a root-associated biocontrol pseudomonad. *Environ. Microbiol*. 15, 736–750.

Péchy-Tarr, M., Bruck, D. J., Maurhofer, M., Fischer, E., Vogne, C., Henkels, M. D., et al. (2008). Molecular analysis of a novel gene cluster encoding an insect toxin in plant-associated strains of *Pseudomonas fluorescens*. *Environ. Microbiol.* 10, 2368–2386.

Petzold-Maxwell, J. L., Jaronski, S. T., Clifton, E. H., Dunbar, M. W., Jackson, M. A., and Gassmann, A. J. (2013). Interactions among Bt maize, entomopathogens, and rootworm species (*Coleoptera: Chrysomelidae*) in the field: effects on survival, yield, and root injury. *J. Econ. Entomol.* 106, 622–632.

Pierson, L.S. 3rd, and Pierson, E.A. (2010). Metabolism and function of phenazines in bacteria: impacts on the behavior of bacteria in the environment and biotechnological processes. *Appl. Microbiol. Biotechnol.* 86, 659–670.

Pineda, A., Soler, R., Weldegergis, B. T., Shimwela, M. M., van Loon, J. J., and Dicke, M. (2013). Non-pathogenic rhizobacteria interfere with the attraction of parasitoids to aphid-induced plant volatiles via jasmonic acid signalling. *Plant Cell Environ.* 36, 393–404.

Pineda, A., Zheng, S. J., van Loon, J. J., and Dicke, M. (2012). Rhizobacteria modify plant-aphid interactions: a case of induced systemic susceptibility. *Plant Biol. (Stuttg).* 14 Suppl. 1, 83–90.

Prabakaran, G., Paily, K. P., Padmanabhan, V., Hoti, S. L., and Balaraman, K. (2002). Isolation of a *Pseudomonas fluorescens* metabolite/exotoxin active against both larvae and pupae of vector mosquitoes. *Pest Manag. Sci.* 59, 21–24.

Prime-A-Plant Group, Conrath, U., Beckers, G. J., Flors, V., García-Agustín, P., Jakab, G., et al. (2006). Priming: getting ready for battle. *Mol. Plant-Microbe Interact.* 19, 1062–1071.

Raaijmakers, J. M., and Mazzola, M. (2012). Diversity and natural functions of antibiotics produced by beneficial and plant pathogenic bacteria. *Annu. Rev. Phytopathol.* 50, 403–424.

Raaijmakers, J. M., De Bruijn, I., Nybroe, O., and Ongena, M. (2010). Natural functions of lipopeptides from *Bacillus* and *Pseudomonas*: more than surfactants and antibiotics. *FEMS Microbiol. Rev.* 34, 1037–1062.

Ramette, A., Frapolli, M., Fischer-Le Saux, M., Gruffaz, C., Meyer, J. M., Défago, G., et al. (2011). *Pseudomonas protegen*s sp. nov., widespread plant-protecting bacteria producing the biocontrol compounds 2,4-diacetylphloroglucinol and pyoluteorin. *Syst. Appl. Microbiol.* 34, 180–188.

Raymond, B., Johnston, P. R., Nielsen-LeRoux, C., Lereclus, D., and Crickmore, N. (2010). *Bacillus thuringiensis*: an impotent pathogen? *Trends Microbiol.* 18, 189–194.

Redondo-Nieto, M., Barret, M., Morrissey, J., Germaine, K., Martínez-Granero, F., Barahona, E., et al. (2013). Genome sequence reveals that *Pseudomonas fluorescens* F113 possesses a large and diverse array of systems for rhizosphere function and host interaction. *BMC Genomics* 14, 54.

Rochat, L., Péchy-Tarr, M., Baehler, E., Maurhofer, M., and Keel, C. (2010). Combination of fluorescent reporters for simultaneous monitoring of root colonization and antifungal gene expression by a biocontrol pseudomonad on cereals with flow cytometry. *Mol. Plant-Microbe Interact.* 23, 949–961.

Ruffner, B. (2013). Insecticidal activity in plant-beneficial pseudomonads: molecular basis and ecological relevance. Ph.D. thesis No. 21005, ETH Zurich.

Ruffner, B., Péchy-Tarr, M., Ryffel, F., Hoegger, P., Obrist, C., Rindlisbacher, A., et al. (2013). Oral insecticidal activity of plant-associated pseudomonads. *Environ. Microbiol.* 15, 751–763.

Ruffner, B., Péchy-Tarr, M., Keel, C., and Maurhofer, M. (2009). Occurrence and molecular diversity of the Fit insect toxin locus in plant-beneficial pseudomonads. *IOBC/WPRS Bulletin* 45, 251–254.

Saitou, K., Furuhata, K., Kawakami, Y., and Fukuyama, M. (2009). Isolation of *Pseudomonas aeruginosa* from cockroaches captured in hospitals in Japan, and their antibiotic susceptibility. *Biocontrol Sci.* 14, 155–159.

Sanahuja, G., Banakar, R., Twyman, R. M., Capell, T., and Christou, P. (2011). *Bacillus thuringiensis*: a century of research, development and commercial applications. *Plant Biotechnol. J.* 9, 283–300.

Shapiro-Ilan, D. I., and Gaugler, R. (2013). Biological control. A guide to natural enemies in North America. Nematodes (*Rhabditida: Steinernematidae* & *Heterorhabditidae*) [Online]. http://www.biocontrol.entomology.cornell.edu/pathogens/nematodes.html.

Shen, X., Hu, H., Peng, H., Wang, W., and Zhang, X. (2013). Comparative genomic analysis of four representative plant growth-promoting rhizobacteria in *Pseudomonas*. *BMC Genomics* 14, 271.

Sibley, C. D., Duan, K., Fischer, C., Parkins, M. D., Storey, D. G., Rabin, H. R., and Surette, M. G. (2008). Discerning the complexity of community interactions using a *Drosophila* model of polymicrobial infections. *PLoS Pathog.* 4, e1000184.

Silby, M. W., Winstanley, C., Godfrey, S. A. C., Levy, S. B., and Jackson, R. W. (2011). *Pseudomonas* genomes: diverse and adaptable. *FEMS Microbiol. Rev.* 35, 652–680.

Snyder, W. E., Tonkyn, D. W., and Kluepfel, D. A. (1998). Insect-mediated dispersal of the rhizobacterium *Pseudomonas chlororaphis*. *Phytopathology* 88, 1248–1254.

Spinner, J. L., Jarrett, C. O., LaRock, D. L., Miller, S. I., Collins, C. M., and Hinnebusch, B. J. (2012). *Yersinia pestis* insecticidal-like toxin complex (Tc) family proteins: characterization of expression, subcellular localization, and potential role in infection of the flea vector. *BMC Microbiol.* 12, 296.

Stavrinides, J., McCloskey, J. K., and Ochman, H. (2009). Pea aphid as both host and vector for the phytopathogenic bacterium *Pseudomonas syringae*. *Appl. Environ. Microbiol.* 75, 2230–2235.

Stephens, J. M. (1958). Occurrence of *Pseudomonas aeruginosa* (Schroeter) Migula in haemolymph of grasshoppers after infection by feeding. *Can. J. Microbiol.* 4, 191–193.

Tao, H. P., Shen, Z. Y., Zhu, F., Xu, X. F., Tang, X. D., and Xu, L. (2011). Isolation and identification of a pathogen of silkworm *Bombyx mori*. *Curr. Microbiol.* 62, 876–883.

Tang, X., Freitak, D., Vogel, H., Ping, L., Shao, Y., Cordero, E. A., et al. (2012). Complexity and variability of gut commensal microbiota in polyphagous lepidopteran larvae. *PLoS One* 7, e36978.

Thanwisai, A, Tandhavanant, S., Saiprom, N., Waterfield, N. R., Ke Long, P., Bode, H. B, et al. (2012). Diversity of *Xenorhabdus* and *Photorhabdus* spp. and their symbiotic entomopathogenic nematodes from Thailand. *PLoS One* 7, e43835.

Toepfer, S., Kurtz, B., and Kuhlmann, U. (2010). Influence of soil on the efficacy of entomopathogenic nematodes in reducing *Diabrotica virgifera virgifera* in maize. *J. Pest Sci.* 83, 257–264.

Trippe, K., McPhail, K., Armstrong, D., Azevedo, M., and Banowetz, G. (2013). *Pseudomonas fluorescens* SBW25 produces furanomycin, a non-proteinogenic amino acid with selective antimicrobial properties. *BMC Microbiol.* 13, 111.

Vachon, V., Laprade, R., and Schwartz, J. L. (2012). Current models of the mode of action of *Bacillus thuringiensis* insecticidal crystal proteins: a critical review. *J. Invertebr. Pathol.* 111, 1–12.

Vallet-Gely, I., Lemaitre, B., and Boccard, F. (2008). Bacterial strategies to overcome insect defences. *Nat. Rev. Microbiol.* 6, 302–313.

Vallet-Gely, I., Novikov, A., Augusto, L., Liehl, P., Bolbach, G., Péchy-Tarr, M., et al. (2010a). Association of hemolytic activity of *Pseudomonas entomophila*, a versatile soil bacterium, with cyclic lipopeptide production. *Appl. Environ. Microbiol.* 76, 910–921.

Vallet-Gely, I., Opota, O., Boniface, A., Novikov, A., and Lemaitre, B. (2010b). A secondary metabolite acting as a signalling molecule controls *Pseudomonas entomophila* virulence. *Cell. Microbiol.* 12, 1666–1679.

van de Mortel, J. E., de Vos, R. C. H., Dekkers, E., Pineda, A., Guillod, L., Bouwmeester, K., et al. (2012). Metabolic and transcriptomic changes induced in *Arabidopsis* by the rhizobacterium *Pseudomonas fluorescens* SS101. *Plant Physiol.* 160, 2173–2188.

van den Bosch, R., and Stern, V. M. (1962). The integration of chemical and biological control of arthropod pests. *Annu. Rev. Entomol.* 7, 367–386.

Van der Ent, S., Verhagen, B. W., Van Doorn, R., Bakker, D., Verlaan M. G., Pel, M. J., et al. (2008). MYB72 is required in early signaling steps of rhizobacteria-induced systemic resistance in *Arabidopsis*. *Plant Physiol.* 146, 1293–1304.

Viggor, S., Juhanson, J., Joesaar, M., Mitt, J., Truu, J., Vedler, E., et al. (2013). Dynamic changes in the structure of microbial communities in Baltic Sea coastal seawater microcosms modified by crude oil, shale oil or diesel fuel. *Microbiol. Res.* 168, 415–27.

Vigneux, F., Zumbihl, R., Jubelin, G., Ribeiro, C., Poncet, J., Baghdiguian, S., et al. (2007). The *xaxAB* genes encoding a new apoptotic toxin from the insect pathogen *Xenorhabdus nematophila* are present in plant and human pathogens. *J. Biol. Chem.* 282, 9571–9580.

Vodovar, N., Vallenet, D., Cruveiller, S., Rouy, Z., Barbe, V., Acosta, C., et al. (2006). Complete genome sequence of the entomopathogenic and metabolically versatile soil bacterium *Pseudomonas entomophila*. *Nat. Biotechnol.* 24, 673–679.

Vodovar, N., Vinals, M., Liehl, P., Basset, A., Degrouard, J., Spellman, P., et al. (2005). *Drosophila* host defense after oral infection by an entomopathogenic *Pseudomona*s species. *Proc. Natl. Acad. Sci. U.S.A.* 102, 11414–11419.

Vogt, S. L., Green, C., Stevens, K. M., Day, B., Erickson, D. L., Woods, D. E., and Storey, D. G. (2011). The stringent response is essential for *Pseudomonas aeruginosa* virulence in the rat lung agar bead and *Drosophila melanogaster* feeding models of infection. *Infect. Immun.* 79, 4094–4104.

Walsh, U. F., Morrissey, J. P., and O'Gara, F. (2001). *Pseudomonas* for biocontrol of phytopathogens: from functional genomics to commercial exploitation. *Curr. Opin. Biotechnol.* 12, 289–295.

Watanabe, K., Hara, W., and Sato, M. (1998). Evidence for growth of strains of the plant epiphytic bacterium *Erwinia herbicola* and transconjugation among the bacterial strains in guts of the silkworm *Bombyx mori*. *J. Invertebr. Pathol.* 72, 104–111.

Waterfield, N. R., Bowen, D. J., Fetherston, J. D., Perry, R. D., and ffrench-Constant, R. H. (2001). The *tc* genes of *Photorhabdus*: a growing family. *Trends Microbiol.* 9, 185–191.

Waterfield, N. R, Ciche, T., and Clarke, D. (2009). *Photorhabdus* and a host of hosts. *Annu. Rev. Microbiol.* 63, 557–574.

Weller, D. M. (2007). *Pseudomonas* biocontrol agents of soilborne pathogens: Looking back over 30 years. *Phytopathology* 97, 250–256.

Weller, D. M., Mavrodi, D. V., van Pelt, J. A., Pieterse, C. M., van Loon, L. C., and Bakker, P. A. (2012). Induced systemic resistance in *Arabidopsis thaliana* against *Pseudomonas syringae* pv. *tomato* by 2,4-diacetylphloroglucinol-producing *Pseudomonas fluorescens*. *Phytopathology* 102, 403–412.

Weller, D. M., Raaijmakers, J. M., Gardener, B. B. M., and Thomashow, L. S. (2002). Microbial populations responsible for specific soil suppressiveness to plant pathogens. *Annu. Rev. Phytopathol.* 40, 309–348.

Wu, X., Monchy, S., Taghavi, S., Zhu, W., and Ramos, J. (2010). Comparative genomics and functional analysis of niche-specific adaptation in *Pseudomonas putida*. *FEMS Microbiol. Rev.* 35, 299–323.

Youard, Z. A., Wenner, N., and Reimmann, C. (2011). Iron acquisition with the natural siderophore enantiomers pyochelin and enantio-pyochelin in *Pseudomonas* species. *Biometals* 24, 513–522.

Zamioudis, C., and Pieterse, C. M. (2012). Modulation of host immunity by beneficial microbes. *Mol. Plant-Microbe Interact.* 25, 139–150.

CHAPTER 2

Host-dependent activation of insect toxin expression in *Pseudomonas protegens*

Parts of this chapter were previously published in Péchy-Tarr M, Borel N, Kupferschmied P, Turner V, Binggeli O, Radovanovic D, Maurhofer M, and Keel C (2013) Control and host-dependent activation of insect toxin expression in a root-associated biocontrol pseudomonad. *Environ Microbiol* 15:3. doi: 10.1111/1462-2920.12050. Author contributions: Christoph Keel, Peter Kupferschmied, Maria Péchy-Tarr, and Monika Maurhofer wrote the article; Maria Péchy-Tarr, Peter Kupferschmied and Christoph Keel conceived and designed the experiments; Maria Péchy-Tarr, Naomi Borel, Peter Kupferschmied, Olivier Binggeli, Vincent Turner, and Dragica Radovanovic performed the experiments.

Abstract

Pseudomonas protegens is a plant-associated bacterium that is not only able to promote plant growth and efficiently protect roots from attack by fungal phytopathogens but also to turn into an insect pathogen. The microorganism is capable of killing certain pest insects upon oral infection. The bacterium is therefore a promising candidate for the formulation of novel biopesticides which are on the rise as viable alternatives to harmful chemical insecticides. The major goal of our work is to understand the molecular mechanisms that allow *P. protegens* and related bacteria to detect, to survive within and to kill the insect host. The entomopathogenic activity of *P. protegens* relies in part on the production of an insecticidal toxin termed Fit. In this study, we discovered that the pseudomonad produces the Fit toxin in the insect host, but not on plant roots and in standard batch cultures, indicating that its expression is host-dependent. Fit toxin production does not seem to be induced by specific insect-derived signal molecules as hypothesized previously but to depend on multiple factors. Amongst others, magnesium seems to play a role in the activation of the toxin in *P. protegens* during insect interaction. In contrast, the two-component regulatory system GacS-GacA, which positively regulates the production of most antifungal secondary metabolites necessary for the bacterium's biocontrol properties, most likely represses Fit toxin expression during insect interaction. Our results suggest that *P. protegens* is killing mainly larvae of lepidopteran insect species and that the expression of the Fit toxin has been optimized for infection of those insects. This study sets out a rationale for considering and investigating the use of plant-beneficial pseudomonads with insecticidal properties as biopesticides to protect not only roots but also aboveground plant parts against herbivorous pest insects.

Introduction

Insects are a class of animals which are highly diverse and represent more than half of all living species described today. While we depend on some of them, like, for example, insect species serving as pollinators of plants, we encounter challenging situations when insect outbreaks endanger our crops as pests or vectors of diseases. Insects are especially difficult to control when they are feeding on belowground plant parts. In addition, the application of synthetic insecticides is notoriously problematic because of potentially nocuous effects on the environment and public health. Namely, these chemicals cause environmental pollution, their production and application as sprays require fossil fuels, and they undermine sustainable pest management by showing nontarget effects on natural enemies of herbivores and increasing pesticide use due to the reduced effectiveness on resistant pests [1]. It has further been reported that children show unique susceptibilities to the potential toxicity of pesticides that they encounter daily [2]. As a result, there is an increasing interest worldwide to replace chemical pesticides by microbial products that are based on entomopathogenic microorganisms for biological pest management strategies. In recent years it has become evident that certain plant root-associated bacteria of the genus *Pseudomonas* display insect pathogenicity in addition to their already well-known biocontrol properties that help them to efficiently protect plants against fungal and oomycete phytopathogens [3-5]. These microorganisms have been studied for decades because of their ability to promote plant growth and to suppress plant diseases [6]. They contribute to the natural disease suppressiveness of certain soils by producing and secreting a cocktail of antifungal secondary metabolites, such as acylphloroglucinols, hydrogen cyanide, phenazines, and lipopeptides [7]. Several *Pseudomonas* strains are registered and available for the protection of plants from phytopathogenic fungi in the field [8]. *Pseudomonas protegens* strain CHA0, used as a model strain in this study, is one of the best investigated representatives of plant growth-promoting rhizobacteria and was shown to be capable of killing certain insect larvae upon oral administration or injection of low doses of bacterial cells [4-6,9]. The observed insecticidal activity of *P. protegens* CHA0 can partially be attributed to the presence of a gene (*fitD*) which codes for a large insecticidal protein toxin (termed Fit) and is located on a genomic island on the chromosome (Figure 1). The *fitD* gene is flanked by seven genes encoding a putative type I secretion system and three regulatory proteins. FitD is similar to Mcf1, an insect toxin produced by the entomopathogenic nematode symbiont *Photorhabdus luminescens* [10]. Mcf1 was reported to induce programmed cell death (apoptosis) in insect cells of the midgut epithelium and to paralyze and destroy insect hemocytes [10,11]. Since FitD is very similar to Mcf1 in terms of sequence identity, the Fit toxin might demonstrate a similar mode of action. The Fit toxin gene does not only occur in *P. protegens* but also in *Pseudomonas chlororaphis* and strains that phylogenetically cluster between these two bacterial species [4]. All strains that possess the Fit toxin also seem to be able to kill larvae of certain insect species when taken up orally ([4], P. Flury, in preparation).

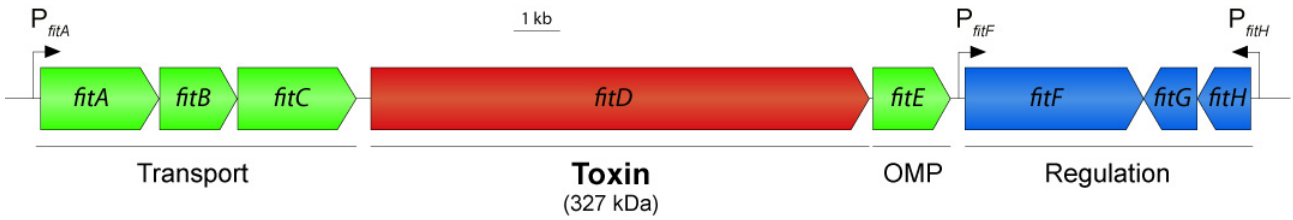

Figure 1: **The Fit insect toxin cluster of *Pseudomonas protegens* CHA0.** Genes encoding a putative type I secretion system are highlighted in green and the three regulatory genes in blue. The actual protein toxin is encoded by *fitD* (in red) which is transcribed as a part of the *fitABCDE* operon under the control of the P_{fitA} promoter. OMP, outer membrane protein.

Studies on the transcription of the eight genes in the *fit* gene cluster using quantitative reverse-transcription PCR (qRT-PCR) and Northern blot analysis suggested that the toxin gene and the genes coding for the putative type I secretion system form an operon (*fitABCDE*) [9]. The genes *fitG* and *fitH* are co-transcribed as well, while *fitF* is transcribed separately. Both, overexpression of *fitG* (a LysR-type regulator expressing gene) and the deletion of *fitH* (coding for a putative response regulator) resulted in a significant increase in the expression of the Fit toxin in CHA0 [9]. This strongly indicated that FitG is the activator and FitH the repressor in this local regulatory system. Although the third regulatory protein FitF (a hybrid histidine kinase-response regulator) was predicted to be the sensory protein of the Fit regulation system, no phenotype regarding toxin expression was observed when *fitF* was overexpressed or deleted in the chromosome of strain CHA0.

Application of these insect-pathogenic pseudomonads as biological pesticides in the future requires a good understanding of the underlying molecular mechanisms and regulation of insecticidal activity in these bacteria. It further needs knowledge about the host range of these entomopathogenic microorganisms. This study hence aimed at assessing the host range of *P. protegens* CHA0 and investigating the conditions under which the Fit toxin is produced in this model strain. We discovered that the Fit toxin is only expressed in *P. protegens* CHA0 during interaction with insects, but not when colonizing plant roots and growing in standard batch cultures. Additional results suggest that the *P. protegens* strain is capable of killing mainly larvae of leaf-feeding lepidopteran insects, while most other insect species tested seem to be immune to infection by this bacterial strain.

Results

Assessing the host range of *Pseudomonas protegens* CHA0

The capacity of *P. protegens* CHA0 to kill different insect species was investigated by performing a variety of biotoxicity assays (Table 1). Oral insecticidal activity in this bacterial strain was mostly restricted to lepidopteran species. Outside of the Lepidoptera, CHA0 has so far only been observed to be able to

efficiently kill pea aphids when taken up orally. Interestingly, the *P. protegens* strain does not seem to be lethal to all lepidopteran species upon oral administration. In this particular insect order, mortality after oral infection by CHA0 was only observed in the leaf-feeding species tested. The soil-dwelling pest insects *Agrotis ipsilon* and *Agrotis segetum* were completely resistant to CHA0 when fed with bacteria-containing artificial diet or feeding on bean plants inoculated with the entomopathogen. Young larvae of *Galleria mellonella*, an insect species which is a pest of beehives and a popular model for systemic infection of insects in laboratories, were resistant to CHA0 upon ingestion.

Table 1: Assessment of the host range of *Pseudomonas protegens* CHA0. If not otherwise stated, larvae of the respective insect species were used for the toxicity assays.

Insect order and species	Systemic toxicity	Oral toxicity
Lepidoptera		
Spodoptera littoralis (African cotton leafworm)	Yes	Yes (artificial diet, leaves) [4]
Pieris brassicae (cabbage white)	ND	Yes (artificial diet, leaves)
Plutella xylostella (diamondback moth)	ND	Yes (artificial diet, leaves) [4]
Heliothis virescens (tobacco budworm)	ND	Yes (leaves) [4]
Manduca sexta (tobacco hornworm)	Yes [3]	NA
Agrotis ipsilon (black cutworm)	ND	No (artificial diet, bean plants)
Agrotis segetum (turnip moth)	ND	No (artificial diet)
Galleria mellonella (greater wax moth)	Yes [3]	No (artificial diet)
Hemiptera		
Acyrthosiphon pisum (pea aphid)	ND	Yes (artificial diet)
Coleoptera		
Tenebrio molitor (mealworm)	Yes	No (on oat bran)
Melolontha melolontha (European cockchafer)	ND	No (contact assay) [12]
Hymenoptera		
Linepithema humile (Argentine ant) [A]	ND	No (in sugar water)
Bombus terrestris (bumblebee) [A]	ND	No (artificial diet) [12]
Diptera		
Aedes aegypti (yellow fever mosquito)	ND	No (contact assay) [13]
Drosophila melanogaster Oregon	ND	No (artificial diet) [14]
Orthoptera		
Schistocerca gregaria (desert locust) [A]	ND	No (on oat bran)

ND, not determined; A, adults.

Quantification of single cell fluorescence intensities by epifluorescence microscopy

The Fit toxin contributes to the capability of *P. protegens* CHA0 to kill lepidopteran insects [3,4]. In order to study the expression and regulation of the insecticidal toxin in live bacteria at the single cell level by epifluorescence microscopy, we previously constructed reporter strains by replacing the native chromosomal *fitD* gene with a *fitD-mcherry* fusion gene [9]. This has allowed monitoring the expression of mCherry-tagged Fit toxin under the control of the natural promoter(s) in different genetic backgrounds. To obtain quantitative data using these reporter bacteria, protocols for the semi-automatic quantification of fluorescence intensities of *Pseudomonas* bacteria in different *in vitro* and *in vivo* systems were developed in this study. Briefly, in this method, samples of bacterial cultures were taken, the bacteria were immobilized on agarose patches and phase contrast and fluorescence images were taken with an epifluorescence microscope. Single cells on micrographs were automatically identified in ImageJ using either the phase contrast (for *in vitro* assays) or the fluorescence images (for *in vivo* assays). The fluorescence intensity of each identified cell in the respective fluorescence channel was then recorded automatically, which can result in data about thousands of bacterial cells per population.

This newly developed method was used to quantify the expression of the FitD-mCherry reporter in LB medium in different genetic backgrounds of *P. protegens* CHA0 (Figure 2). It confirmed that FitG is an activator and FitH a repressor of Fit toxin expression in this bacterium. Additionally, it gave a quantitative impression of how the expression levels varied within a given population and between genotypes, which is harder to appreciate in the corresponding micrographs (Figure 2A), and showed that, statistically, CHA0 did not express its insecticidal toxin in the common laboratory growth medium (Figure 2B).

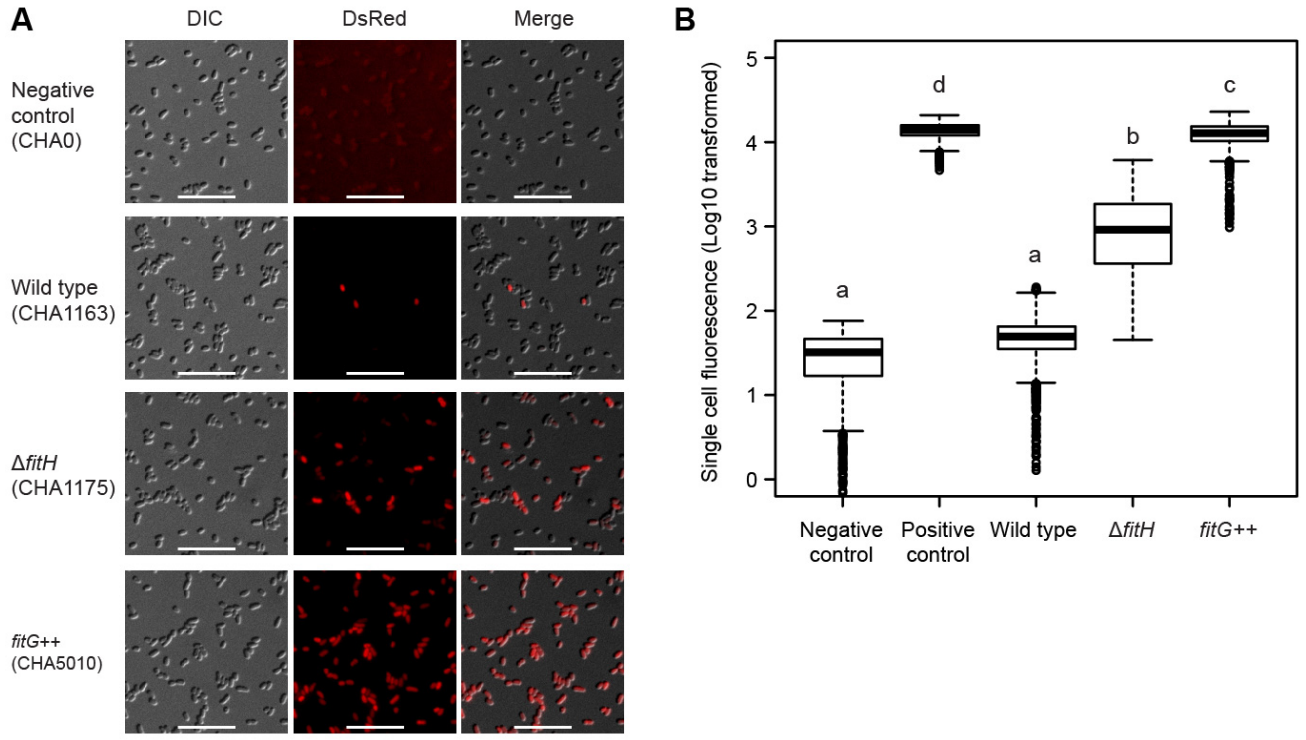

Figure 2: Visualization and quantification of Fit toxin production in single cells of *Pseudomonas protegens* CHA0 by fluorescence microscopy. (A) Visualization of Fit insect toxin expression in single cells of *P. protegens* CHA0 and isogenic mutants grown in batch culture. Cells of the wild type (CHA1163), the Δ*fitH* deletion mutant (CHA1175) and the *fitG* overexpressing (*fitG*++) mutant (CHA5010; $P_{tac/laclq}$-*fitG*; IPTG-induced), all equipped for expression of a chromosomally encoded FitD-mCherry fusion protein, were harvested from late exponential growth-phase cultures in LB medium and analyzed by fluorescence microscopy. Left panels correspond to DIC micrographs, middle panels to DsRed micrographs, and right panels to merge pictures of DIC and DsRed micrographs. Scale bars, 10 mm. (B) Red fluorescence intensities (arbitrary units) of individual cells of the respective strains measured in the DsRed channel (n = 1535–2015 cells per strain). Negative control, CHA0. Positive control, CHA0-*mche* (wild type expressing a constitutive mCherry tag). Exposure time: 2 s, except for the positive control (0.5 s). Treatments labelled with a different letter are significantly different (p-value < 0.05) according to Wilcoxon-Mann-Whitney's test with Bonferroni correction for multiple testing. The experiment was repeated and similar results were obtained.

The Fit toxin is expressed in insect hemolymph but not on plant roots

The FitD-mCherry reporter strains, equipped with a constitutive green fluorescent protein (GFP) tag, and the fluorescence microscopy-based quantification method were used to investigate whether the Fit toxin was expressed during insect infection. When injected into last-instar larvae of *G. mellonella* and re-isolated from hemolymph about 20 h later, most of the wild-type FitD-mCherry reporter bacteria (CHA1176) were red fluorescent (Figure 3). The Δ*fitH* reporter strain CHA1178, which was used as a positive control, was similarly red fluorescent while the negative control (CHA0-*gfp2*) did not display any red fluorescence. Quantification of FitD-mCherry expression in these bacterial cells indicated that the wild type expressed *fitD* at a similar level as the Δ*fitH* mutant strain (Figure 3B). These findings strongly suggest that, in contrast to LB, *P. protegens* CHA0 expresses its Fit toxin in insect hemolymph.

The same reporter strains were grown on roots of cucumber plants to study whether the Fit toxin is also produced during plant colonization. In contrast to some cells of the Δ*fitH* reporter strain, none of the FitD-mCherry reporter bacteria with the wild-type genetic background showed any detectable red fluorescence (Figure 4). Quantification did not reveal any significant difference in red fluorescence intensities between the wild-type reporter bacteria and the negative control strain (Figure 4B). This indicates that the Fit toxin is not produced in *P. protegens* CHA0 when the microorganism grows on cucumber roots. Altogether, the results suggest that Fit toxin production is only activated in strain CHA0 in insect hemolymph and is therefore host-dependent.

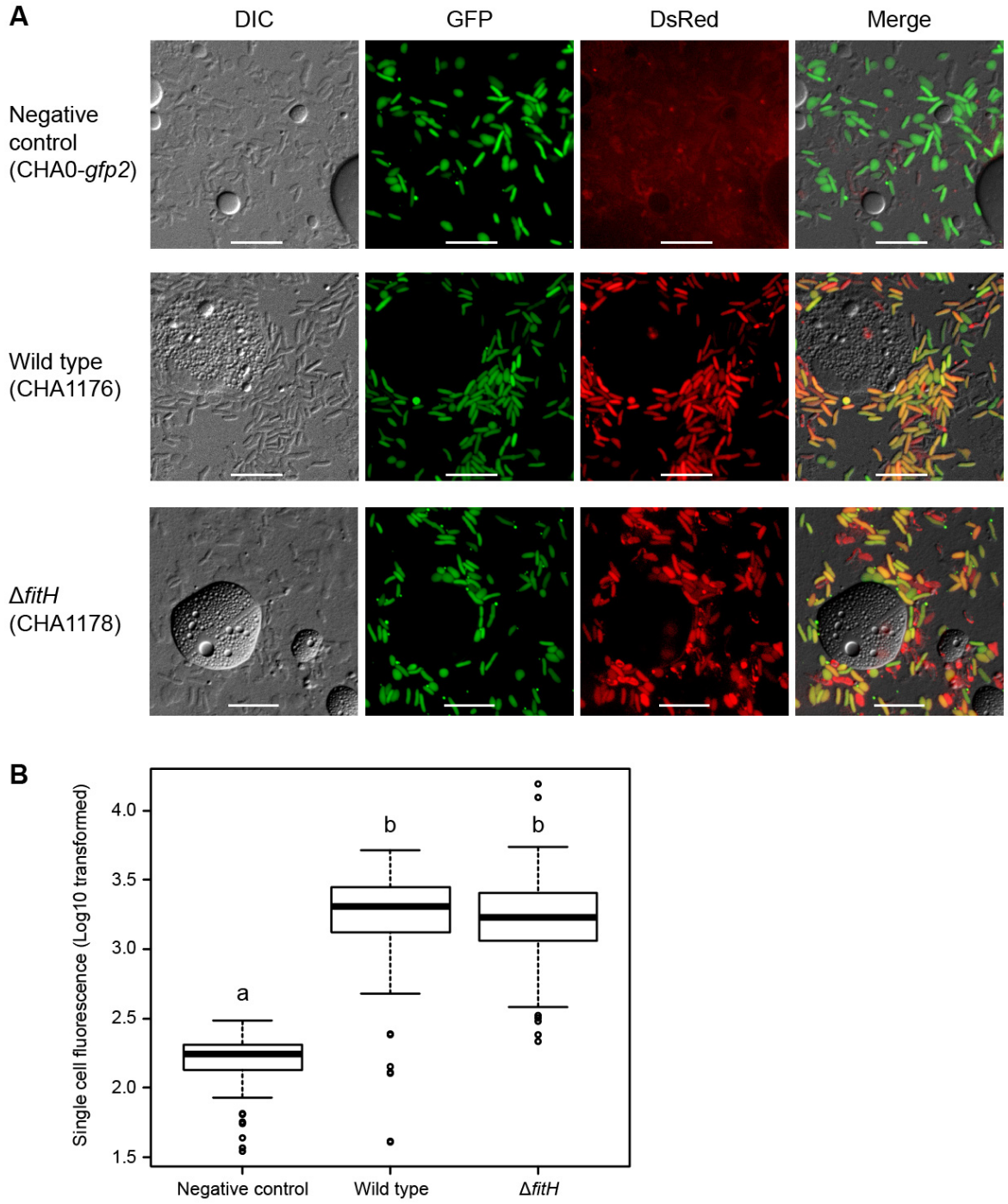

Figure 3: Fit toxin expression in *Pseudomonas protegens* CHA0 is induced in insect hemolymph of *Galleria mellonella*. (A) Visualization of Fit insect toxin expression by *Pseudomonas protegens* wild type CHA0 and the Δ*fitH* mutant in the hemolymph of larvae of *Galleria mellonella*. Toxin expression was monitored by fluorescence microscopy analysis of single cells of GFP-tagged derivatives of the strains expressing a chromosomally encoded FitD-mCherry fusion protein, i.e. strains CHA1176 and CHA1178, respectively. Strain CHA0-*gfp2* expressing only the GFP tag served as a negative control. Scale bar, 10 µm. (B) Red fluorescence intensities quantified for individual cells of the respective strains in hemolymph recovered 24 h post bacterial injection (n = 71 to 158 cells per strain). Cells were selected using the GFP channel images. Treatments labelled with a different letter are significantly different (p-value ≤ 0.01) according to Wilcoxon-Mann-Whitney's test with Bonferroni correction for multiple testing. The experiment was repeated with similar results.

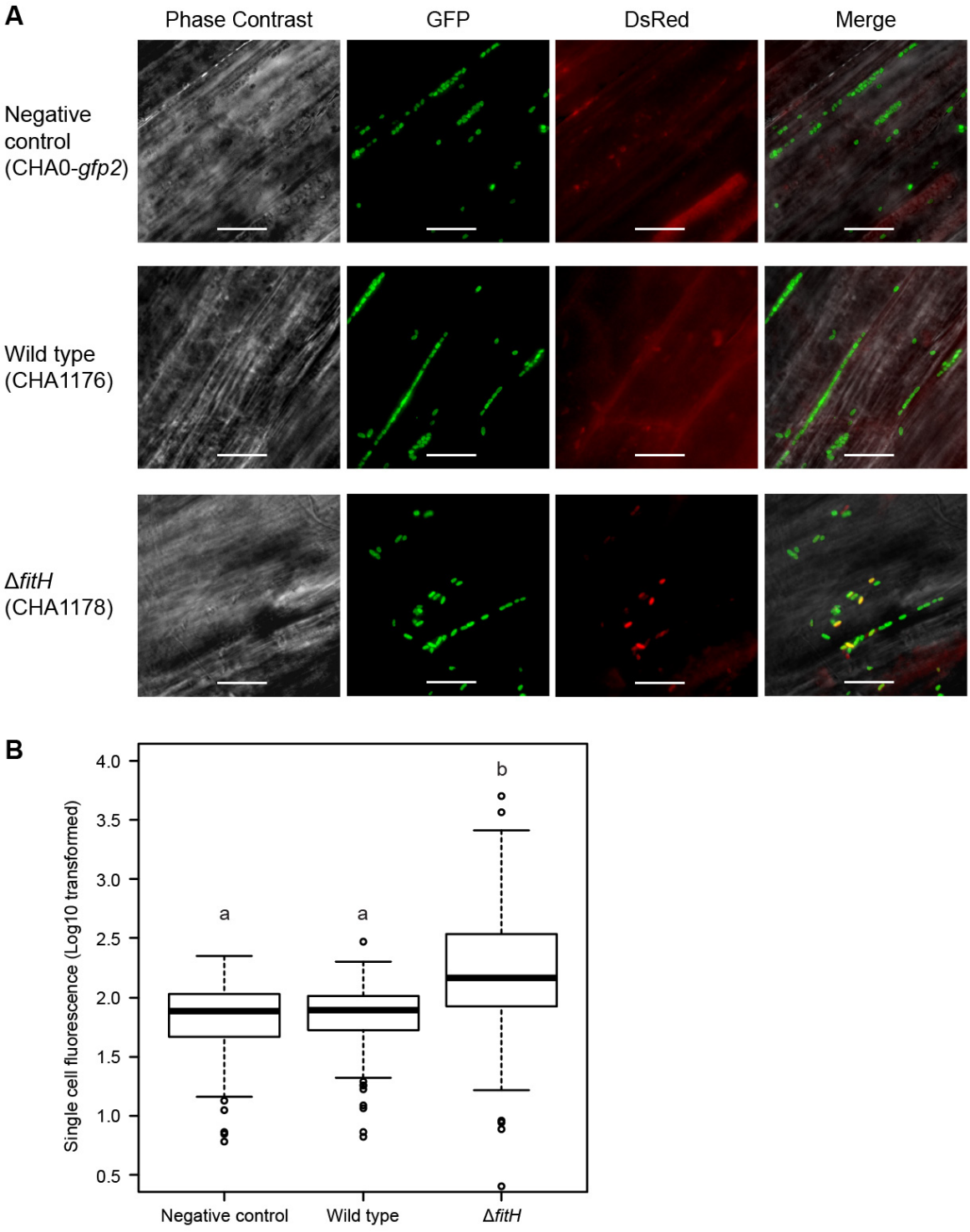

Figure 4: Expression of *fitD* in *Pseudomonas protegens* CHA0 is repressed during root colonization. (A) Monitoring of Fit insect toxin expression by *P. protegens* wild type CHA0 and the Δ*fitH* mutant on roots of cucumber plants. Toxin expression was studied by fluorescence microscopy analysis of single cells of GFP-tagged derivatives of the strains equipped for expression of a chromosomally encoded FitD-mCherry fusion protein, i.e. strains CHA1176 and CHA1178, respectively. Strain CHA0-*gfp2* expressing only the GFP tag served as a negative control. Scale bar, 10 µm. (B) Red fluorescence intensities quantified for individual cells of the respective strains in root washes recovered four days post bacterial inoculation (n = 98 to 279 cells per strain). Cells were selected using the GFP channel images. Treatments labelled with a different letter are significantly different (p-value < 0.05) according to Wilcoxon-Mann-Whitney's test with Bonferroni correction for multiple testing. The experiment was repeated with similar results.

Regulation of Fit toxin expression by environmental conditions and a global regulatory system

As it will be discussed in more detail in Chapter 3, the Fit toxin was also discovered to be highly expressed in Grace's Insect Medium (GIM), a lepidopteran insect hemolymph-mimicking medium [15], and to a lower extent in M9 minimal medium. This made it possible to study the regulation of the insecticidal toxin *in vitro* under different growth conditions, which has proven to be more convenient and reproducible than performing similar experiments *in vivo* in insect larvae.

The initial model which was used to describe the host-dependent activation of Fit toxin production in *P. protegens* CHA0 included insect-derived signal molecules that induce the expression of *fitD* during insect infection via the regulatory system composed of FitF, FitH and FitG [9]. It was thus hypothesized that GIM contains such signal molecules and that they could be identified by removing or replacing components of the medium. M9 minimal medium was used as a base medium to test different components of GIM in order to identify the proposed signal molecule(s). Although *P. protegens* CHA0 did not grow as well as in GIM or LB, the strain expressed FitD-mCherry relatively well in a defined medium termed GM9, which contained high concentrations of magnesium chloride and sucrose in addition to the classical components of the M9 medium and a low phosphate content to avoid precipitation of the salts (Figure 5A). In addition, removing or exchanging components of the GM9 medium did not abrogate the expression of FitD-mCherry in the wild-type reporter strain (Figure 5 and data not shown). These observations suggest that no particular molecule is essential for the expression of *fitD* in CHA0 and that a signal in the sense of a specific insect-derived molecule does not exist.

Varying the concentration of salt in the medium, in particular that of magnesium, seemed to significantly change the expression level of *fitD* in the wild-type FitD-mCherry reporter strain of CHA0 (Figure 5B). The expression of the Fit toxin increased with increasing concentrations of magnesium and was optimal at concentrations above 40 mM (Figure 5C). Importantly, the effect was not caused by increased osmolarity, since changing the concentration of sucrose in the medium did not affect the expression of FitD-mCherry (Figure 5D). This indicated that high salt concentrations might be an important factor for optimal Fit toxin production in *P. protegens* CHA0. However, these results have to be interpreted carefully since modification of the media in some cases also affected the growth rate of the bacteria which might have changed the expression of *fitD* indirectly as well.

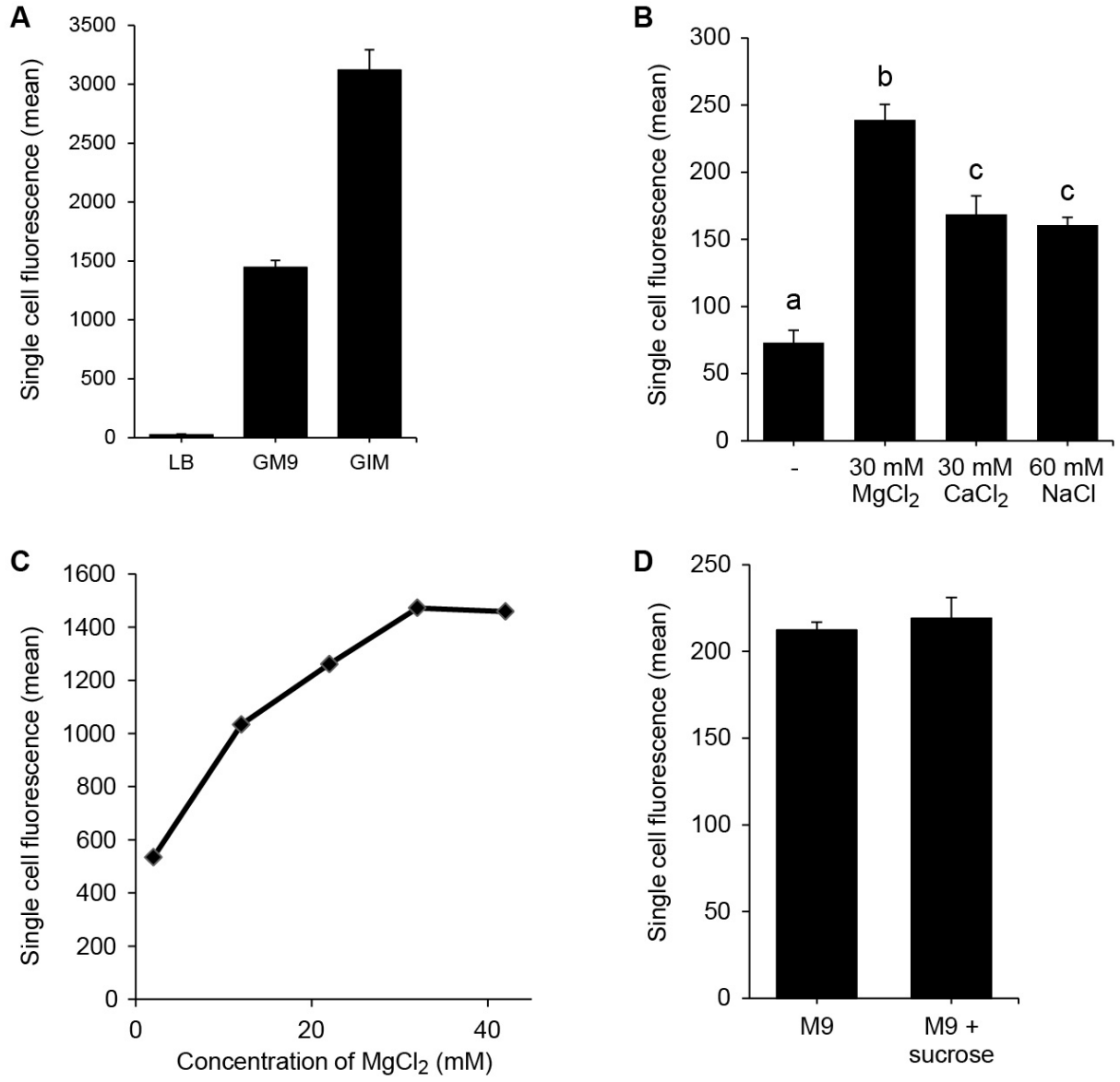

Figure 5: Fit toxin expression in *Pseudomonas protegens* CHA0 is modulated by the surrounding salt concentration. The FitD-mCherry reporter strain CHA1163 was grown in different media for 24 h at 25°C and 180 rpm and single cell fluorescence intensities were quantified by fluorescence microscopy. Shown are means and standard deviations of averages of single cell fluorescence intensities (arbitrary units) from three independent cultures (except for C, which shows means of single cell fluorescence from one single culture). (A) Expression of FitD-mCherry by CHA0 in LB, GM9 and GIM. (B) Production of FitD-mCherry in GM9 or GM9 plus 30 mM $MgCl_2$, 30 mM $CaCl_2$ or 60 mM NaCl. Treatments labelled with different letters are significantly different (p-value < 0.05; one-way ANOVA with a Tukey's HSD post hoc test). (C) Expression of FitD-mCherry in GM9 supplemented with different concentrations of $MgCl_2$. (D) Expression of FitD-mCherry by CHA0 in M9 minimal medium with or without 27 g/L sucrose. All experiments were repeated with similar results.

While other factors tested, such as osmolarity, iron availability, carbon source, nitrogen availability, and pH, did not seem to significantly affect the production of the Fit toxin (data not shown), results indicated that the expression of *fitD* is highly temperature dependent (Figure 6A). Toxin expression was optimal at a temperature between 20 and 25°C and sharply decreased at temperatures above 25°C.

Global regulatory systems could also influence the expression of *fitD*. The best-characterized two-component regulatory system in plant-beneficial pseudomonads, GacS-GacA [6,16], repressed the production of the insecticidal toxin in CHA0 (Figure 6B). In a *gacA* mutant of CHA0, the level of FitD-mCherry expression in single cells was highly increased in the stationary phase compared to the wild type when the bacteria were grown in GIM.

In summary, all the above mentioned findings indicate that there are no specific insect-derived signal molecules necessary to induce expression of the Fit toxin in *P. protegens* CHA0. On the contrary, several factors seem to modulate the level of toxin production in this bacterium without being essential for *fitD* expression.

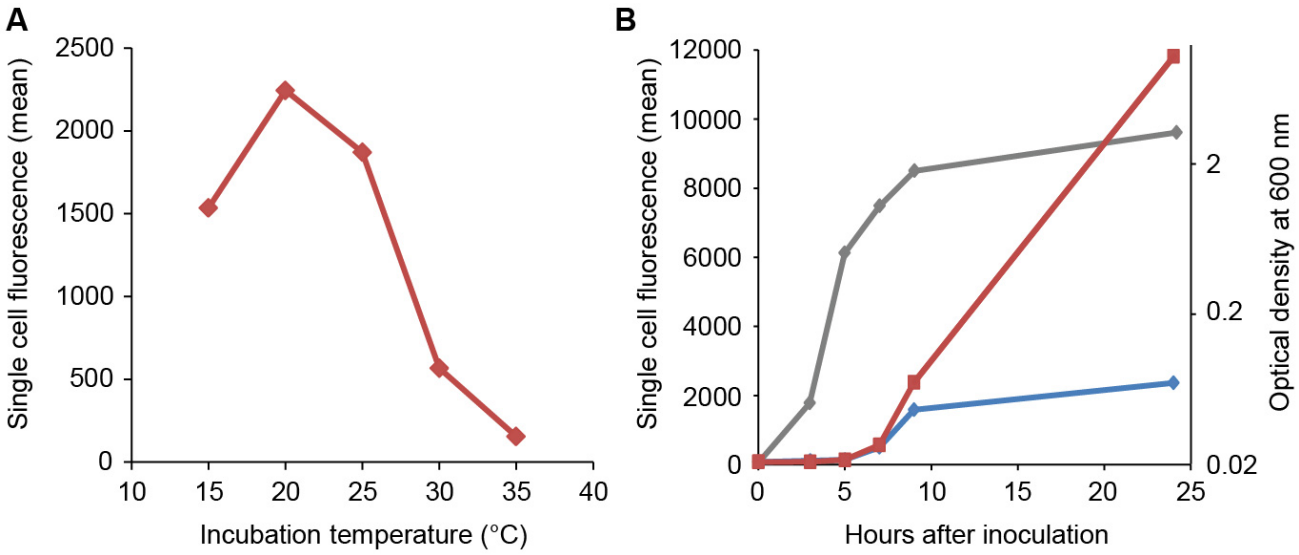

Figure 6: High temperatures and the two-component regulatory system GacS-GacA repress the expression of the Fit toxin in *Pseudomonas protegens* CHA0. (A) The FitD-mCherry reporter strain CHA1163 was grown for 24 h to stationary phase in GIM at five different temperatures. Shown are means of single cell fluorescence intensities (arbitrary units) from one single bacterial culture determined by fluorescence microscopy. (B) The CHA0 wild type (in blue) and *gacA* mutant strain (in red) expressing the FitD-mCherry reporter (CHA1163 and CHA1169, respectively) were grown in GIM at 25°C and 180 rpm. Samples were taken at different time points and red fluorescence intensities of single cells were quantified by fluorescence microscopy. Shown are means from a single culture per strain. The optical density at 600 nm of the wild-type culture is shown in gray (the growth of the mutant was similar). Both experiments were repeated with similar results.

Discussion

In this study, we found evidence that *P. protegens* CHA0 is mainly lethal to insect species of the order Lepidoptera, while most other species tested were resistant to this insect pathogen upon oral administration. While all lepidopteran species feeding on above-ground parts of plants were susceptible, two soil-dwelling species of the genus *Agrotis* were resistant to this rhizobacterium. This result asks for

considering the use of entomopathogenic pseudomonads for the control of above-ground insect pests. Saprophytic fluorescent *Pseudomonas* strains were isolated from the phyllosphere [17-19] and others successfully applied as foliar sprays to protect plants against fungal infection [20,21]. Meena *et al.* even claimed to have observed phyllosphere colonization after inoculation of groundnut seeds with *P. fluorescens* Pf1 and reported that this bacterium is a good leaf colonizer [20]. Additionally, the insect-pathogenic *P. protegens* Pf-5, a close relative of strain CHA0, was shown to survive on apple and pear leaves for over one week in the field [22]. Interestingly, leaf colonizing pseudomonads (*Pseudomonas syringae*) were previously suggested to have adapted to insects to use them as secondary hosts for bacterial proliferation and vectoring [23]. In the future, it should therefore be investigated whether and to which extent plants can be protected against leaf-feeding insects under lab conditions and on the field by foliar application of *P. protegens* or *P. chlororaphis* strains. In the same vein, it could further make sense to screen leaf isolates for plant-beneficial pseudomonads with insecticidal properties to obtain bacterial strains that are well adapted to the life on plant leaves.

We discovered that production of the Fit toxin in *P. protegens* CHA0 is specifically induced upon infection of the insect host, whereas it is not detectable on plant roots or in standard batch culture. Tight regulation may indicate that insect toxin production is very costly to the bacterium and is only activated when needed, i.e. to contribute to insect killing. The observed specific activation of the toxin in insects may provide a certain containment for future biocontrol applications.

We chose a highly sensitive approach to visualize and quantify Fit insect toxin expression in single live *P. protegens* cells, using fluorescence microscopy in combination with reporter strains expressing a full-length FitD-mCherry fusion protein. The Fit toxin is one of only a few bacterial toxins that have been visualized in this way; another example is the cholera toxin labelled with GFP or mRFP [24]. Furthermore, only a limited number of insect toxins have been visualized during interaction with the insect host so far, i.e. besides the Bt Cry and Cyt toxins [25], in particular the *P. luminescens* Mcf and Tc toxins and a Cyt toxin homologue produced by *Dickeya dadantii* which were detected with specific antibodies or following labelling with fluorescent dyes [11,26,27].

To our best knowledge, the present study is the very first to quantify expression of a bacterial insect toxin directly in individual live cells during host interaction. Other studies have quantified *in vivo* insect toxin expression (i.e., Tc and Mcf toxins in *Photorhabdus*) indirectly by RT-qPCR and Western blotting [28], or by using fluorescent reporters fused to promoters of the toxin genes [29]. Besides its usefulness for direct *in vivo* monitoring, our fluorescence microscopy approach has the advantage of being very sensitive, allowing to detect and quantify even very low fluorescence levels emitted by the fusion protein, which is a clear plus over the FACS-based single cell monitoring approach that our group used previously for following expression of *P. protegens* antifungal genes on roots [30]. Moreover, by fluorescence tagging the Fit toxin at its native genomic locus, control by the natural promoter(s) and regulators can be studied without bias [31].

We first hypothesized that particular insect-derived signal molecules exist that lead to the observed activation of Fit toxin expression in insect hemolymph. The search for these molecules however did not reveal any specific compound whose presence in GIM would solely explain the activation of Fit production in this growth medium. In contrast, the level of toxin expression seems to depend on multiple factors. Based on the obtained results, it can alternatively be hypothesized that expression of *fitD* is activated in insect hemolymph and GIM because of the lack of inhibiting molecules and that the level of induction depends on certain environmental conditions, such as high ionic strength. It can be speculated that certain plant-derived molecules act as inhibitors of Fit toxin expression. Alternatively, the signal molecules that were proposed to be recognized by the sensor kinase FitF and lead to the activation of Fit toxin production could also be produced by the bacteria themselves under hemolymph-like growth conditions.

Interestingly, results of this study point to an optimization of Fit toxin production in *P. protegens* for specific expression in herbivorous insects. Toxin production seemed to be optimal when the magnesium concentration was similar to those found in hemolymph of phytophagous insect species [32-34]. Additionally, the level of Fit production in GIM, which mimics the conditions of *Spodoptera* hemolymph, could not be further increased by modifying the insect medium (data not shown). Virulence determinants of human pathogenic bacteria are often fully expressed at body temperature [35], i.e. close to the temperature of optimal growth of these bacteria. In contrast, *P. protegens* CHA0 expresses its insecticidal toxin optimally at temperatures around 20 to 25°C, which is closer to temperatures found in the natural habitat of these microbes and probably ideal for insect infection.

Our group has previously shown that FitG and FitH tightly control the expression of *fitD* in *P. protegens* CHA0 [9]. Besides these local regulatory proteins, global regulatory systems might also influence the production of Fit toxin in this bacterium. Here, we show that the well-studied two-component regulatory system GacS-GacA, which positively regulates the production of secondary metabolites with antifungal activities during root colonization [36,37], most likely represses Fit toxin production in *P. protegens* in hemolymph. Repression of *fitD* expression by GacS-GacA can however not explain why *P. protegens* CHA0 does not produce FitD in standard batch cultures, since a *gacA* mutant expressed FitD-mCherry at only very low levels in LB (data not shown).

Our novel reporter approach opens possibilities for studying the regulation of toxin expression during insect infection in real time and for answering remaining questions. The developed tools and protocols were for example employed to address the role of the sensor histidine kinase FitF in Fit toxin regulation. As it will be described in details in chapter 3, upon activation, FitF most likely phosphorylates FitH which subsequently leads to an activation of toxin production via FitG. In the future, the FitD-mCherry reporter fusion could be helpful to study which global regulatory proteins and signals, besides GacS-GacA, influence the host-dependent control of Fit toxin expression in *P. protegens* CHA0. Good candidates are known global regulators like PhoP-PhoQ, FleQ, the H-NS-like proteins MvaT and MvaV, sigma factors, and second

messenger molecules such as cyclic di-GMP [38-41]. In *P. chlororaphis*, it would further be interesting to investigate whether quorum sensing systems influence the expression of *fitD*.

Now that we know under which conditions *P. protegens* CHA0 activates the production of its insecticidal toxin, it should be studied how the Fit toxin is transported and whether it is post-translationally modified. Although we found indications that genes coding for a type I secretion system are co-transcribed with *fitD* [9], we have no experimental evidence that the transport system is actually involved in the export of the Fit toxin. It further remains unclear what the exact mode of action of the Fit toxin is and how specific it is.

Our research demonstrates that plant-beneficial pseudomonads with insecticidal activities are promising candidates for the development of novel biopesticides. Efficiency in killing and resistance to stress are however critical points for a successful application of entomopathogenic bacteria. For plant protection, it will thus be important to study how efficient these insect-pathogenic pseudomonads can kill pest insects in the field - in the rhizosphere and in the phyllosphere - and to develop appropriate formulations based on well-selected isolates.

Material and Methods

Bacterial strains, media and culture conditions

All strains used in this study are listed in Table 2. Bacteria were routinely cultured at 25°C in LB (LB Broth Miller, BD Difco), or in nutrient yeast broth (NYB) or on nutrient agar (NA) [42]. When appropriate, growth media were supplemented with ampicillin (100 µg/ml), chloramphenicol (10 µg/ml), kanamycin (25 µg/ml), gentamicin (10 µg/ml), or tetracycline (125 µg/ml).

For Fit toxin expression studies, the following media were used. LB; sterile-filtered Grace's Insect Medium (GIM) (G9771, with L-glutamine, without sodium bicarbonate, adjusted to pH 5.5 with sodium bicarbonate) (Sigma-Aldrich); M9 minimal medium [43] with 10 mM L-malate; and GM9 (23 mM $MgCl_2$ x 6 H_2O, 12 mM KH_2PO_4, 9 mM NaCl, 80 mM NH_4Cl, 2 mM $MgSO_4$, 0.1 mM $CaCl_2$, 134 µM EDTA, 31 µM $FeCl_3$ × 6 H_2O, 6.2 µM $ZnCl_2$, 760 nM $CuCl_2$ × 2 H_2O, 420 nM $CoCl_2$ × 2 H_2O, 1.62 µM H_3BO_3, 81 nM $MnCl_2$ × 4 H_2O, 5 mM L-malate, 0.5 mM fumarate, 0.5 mM succinate, 3.9 mM D-glucose, 2.2 mM D-fructose, 1 mM glycine, 1 mM alanine, 79 mM sucrose, pH 5.5 - 6).

Table 2: Bacterial strains used in this study

Strain	Genotype, phenotype or relevant characteristics	Reference or source
Pseudomonas protegens		
CHA0	Wild type	[44]
CHA0-*gfp2*	CHA0::attTn*7*-*gfp2*; Gm^r	[9]
CHA0-*mche*	CHA0::attTn*7*-*mcherry*; Gm^r	[30]
CHA1158-*gfp2*	*ΔfitH*::attTn*7*-*gfp2*; *ΔfitH* mutant of CHA0 with a constitutive GFP cell tag; Gm^r	[9]
CHA1163	CHA0::*fitD-mcherry*; CHA0 derivative expressing a C-terminal FitD-mCherry fusion protein	[9]
CHA1169	CHA1163::*gacA*; *gacA* mutant expressing a C-terminal FitD-mCherry fusion protein; Km^r	M. Péchy-Tarr, unpublished
CHA1175	CHA1158::*fitD-mcherry*; *ΔfitH* mutant expressing a C-terminal FitD-mCherry fusion protein	[9]
CHA1176	CHA1163::attTn*7*-*gfp2*; Gm^r	[9]
CHA1178	CHA1175::attTn*7*-*gfp2*; Gm^r	[9]
CHA5010	CHA0::attTn*7*-$P_{tac/laclq}$-*fitG*::*fitD-mcherry*; $P_{tac/laclq}$-*fitG* derivative of CHA0 expressing a C-terminal FitD-mCherry fusion protein; Gm^r	[9]

Biotoxicity assays

For all assays, bacterial suspensions of *P. protegens* CHA0 were prepared by growing the strain overnight in 10 ml LB at 25°C and 180 rpm, washing the cells once in 0.9% NaCl solution and adjusting the optical density at 600 nm (OD_{600}) to 2 with saline solution, if not otherwise stated.

For the assay with *L. humile*, equal volumes of the bacterial suspension and a saturated household sugar solution were mixed and aliquots of 200 µl were added as drops to boxes containing 15 ants each. After an incubation of 24 h another 50 µl of the same suspension was added to each group of animals. Survival of the ants was monitored over 3 days at room temperature. The experiment was performed in triplicates and water was used as a negative control.

The ability of CHA0 to kill larvae of *S. littoralis* was assessed on artificial diet as described before [4]. For *P. brassicae*, aliquots of 40 µl and 10 µl of a bacterial suspension were spotted in six-well plates on pieces of leaves of *Brassica napa* (Chinese cabbage) or artificial diet, which was used before for *Spodoptera*, respectively. Individual L3 larvae were added to the wells and incubated for several days at room temperature. Saline solution served as a negative control.

For feeding assay with *T. molitor*, aliquots of 100 µl of a suspension of CHA0 cells were mixed in small Petri dishes with approximately 20 oat brans. Ten larvae of *T. molitor* were added per dish and incubated for several days at room temperature. The assay with adults of *S. gregaria* was performed analogously. For injection assays with *T. molitor* larvae, a bacterial suspension at an OD_{600} of 1 was diluted 1,000-times in saline solution and aliquots of 2.5 µl were injected into the forth last segment of the larvae. The experiment was performed with 18 larvae per treatment and with NaCl solution as a negative control.

For *G. mellonella* feeding assays, aliquots of 20 µl of a bacterial suspension were pipetted onto artificial diet (for approx. 100 g: 13 g/L flour, 10 g/L maize flour (polenta), 13 g/L wheat bran, 10 g/L milk powder, 5 g/L

Bacto™ Yeast Extract, 25 g/L honey, 20 ml glycerol, 7 ml distilled H_2O; sterilized by incubating twice for 1 h at 80°C), which was modified after [45], in Petri dishes. Twenty young larvae of *G. mellonella* were added per dish and incubated at room temperature for 7 days.

For *A. ipsilon*, aliquots of 10 µl of a bacterial suspension at an OD_{600} of 3 were pipetted onto artificial bean diet (for approx. 500 ml: 2 ml olive oil, 56 g finely grounded Borlotti beans, 13 g Bacto™ Yeast Extract, 20 g oat bran, 1 tab of Sanatogen® GOLD Vitamins, 8 g Bacto™ Agar, 400 ml distilled H_2O; incubated twice 1h at 80°C for sterilization), modified after [46], in Petri dishes. Saline solution was used as a negative control. Eight L3 larvae were added to each dish (2 Petri dishes per treatment) and incubated for 5 days at room temperature. Additionally, 15 *A. ipsilon* larvae were put into pots with Borlotti bean plants which were inoculated with 2 ml of bacterial suspensions 5 days before. Survival of the larvae was monitored for 5 days at room temperature. For *A. segetum*, the assay was performed in triplicates as described for *A. ipsilon* with the bean diet, but with 5 larvae per treatment and higher doses of CHA0 (20 µl of bacterial suspension at an OD_{600} of 10).

In order to assess the toxicity of CHA0 to *A. pisum*, a bacterial suspension at an OD_{600} of 1 was diluted 10^4-times in AP3 medium, which is a standard diet used for aphid bioassays [47]. Nymphs of *A. pisum* reared on *Vicia faba* (broad bean) were transferred to small Petri dishes (10 individuals per dish), which were then sealed with Parafilm® (stretched at least twice for a very thin layer). Aliquots of 50 µl of the diluted bacterial suspension were pipetted on top of the paraffin film and a second Parafilm® layer was placed on top of it. The nymphs were feeding on the artificial diet enclosed in these Parafilm® sachets through the thin membrane. The dishes were incubated 3 days in a growth chamber set to 80% relative humidity for 16 h with light (160 µE/m^2/s) at 22°C, followed by an 8-h dark period at 18°C. Survival was monitored every day.

Visualization and quantification of Fit toxin expression in batch cultures

Expression of the FitD-mCherry fusion by derivatives of *P. protegens* CHA0 (i.e., CHA1163, CHA1175, and CHA5010) in batch culture was monitored by fluorescence microscopy. Strains CHA0 and CHA0-*mche* were included as negative and positive controls, respectively. Bacteria were grown for 24 h at 30°C in 10 ml LB contained in 50-ml Erlenmeyer flasks with agitation at 180 rpm. Immediately prior to microscopy, cells were harvested by centrifugation, washed twice in 0.9% NaCl solution and immobilized on pads composed of 1% agarose. Bacterial cells were visualized with an Axio Imager.M1 microscope (Carl Zeiss MicroImaging GmbH, Jena, Germany) equipped with a Plan-Apochromat 100X/1.40 oil differential interference contrast (DIC) objective and a Cascade:1K EMCCD camera (Photometrics, Tucson, AZ, USA). Image processing (and false-coloring) was performed using Metamorph 7.5 (Universal Imaging, Downingtown, PA) and Adobe Photoshop CS2 (Adobe Systems). Cells were observed with the DIC and DsRed channels set at an exposure time of 80 ms and 2 s, respectively. For quantification of FitD-mCherry expression in individual cells, phase contrast

pictures taken with a Plan-Apochromat 100X/1.45 oil Ph3 objective were processed with ImageJ (Rasband, W.S., ImageJ, U.S. National Institutes of Health, Bethesda, Maryland, USA; http://imagej.nih.gov/ij/, 1997-2011). A macro was used to delimit cells on the Ph3 image and to measure the mean fluorescence intensity (grey value) of each selected cell on the corresponding DsRed image. Images of at least 10 separate views were analyzed for each strain. Cells were monitored at an exposure time of 2 s and 70 ms for the DsRed and Ph3 channels, respectively.

For the comparison of Fit toxin expression under different growth conditions, CHA1163 was grown for 24 h in 10 ml of the respective medium in 50-ml Erlenmeyer flasks at 25°C and 180 rpm. Quantification of red fluorescence intensities was performed as described above. Wild type CHA0 grown in the same media served as a negative control. A similar assay was performed to study the expression of the Fit toxin in GIM at different incubation temperatures.

For monitoring the expression of FitD-mCherry in the wild-type and *gacA* mutant background of CHA0, 20 ml of GIM contained in 100-ml Erlenmeyer flasks were inoculated with 200 µl of a bacterial suspension of CHA1163 or CHA1169 at an OD_{600} of 2. The bacterial cultures were incubated at 25°C and 180 rpm and samples were taken at different time points for quantification of red fluorescence intensities by fluorescence microscopy as described above.

Visualization and quantification of Fit toxin expression in insects and on plant roots

For microscopic observations of Fit toxin expression in insects, *Galleria* larvae were injected with 2×10^3 cells of reporter strains expressing the FitD-mCherry protein fusion and a GFP tag (i.e., CHA1176, CHA1178) or the GFP tag alone (i.e., CHA0-*gfp2*, CHA1158-*gfp2*). After incubation for 24 h, larvae were shock frozen in liquid nitrogen. For observation of bacteria in hemolymph, frozen larvae were broken apart. Each fracture surface was gently pressed for 2 min against a 1% agarose pad placed on a microscope slide to extract and fix the hemolymph. To visualize FitD-mCherry and GFP tag expression by bacteria, the same fluorescence microscopy equipment as described above was used, with exposure times set at 80 ms for DIC, 8 s for DsRed, and 300 ms for GFP.

For quantification of FitD-mCherry expression in individual bacterial cells, hemolymph collected from frozen larvae was diluted 20 times with ice-cold, sterile 0.9% NaCl solution and filtered through a 5-µm filter (Sartorius Minisart, Göttingen, Germany). Cells recovered in the subsequent centrifugation step were washed three times and fixed on 1% agarose pads. Bacteria were monitored with the Ph3 objective at following exposure times: Ph3, 70 ms; DsRed, 4 s; and GFP, 300 ms. Using an ImageJ macro, *P. protegens* cells were automatically identified and delimited on the GFP image based on the fluorescence of their GFP tag. Individual selected cells were then analyzed for mean fluorescence intensity (grey value) on the corresponding DsRed image to calculate FitD-mCherry expression levels. The average background DsRed

fluorescence of images was used to correct the measured single cell fluorescence values. At least 10 images were analyzed per strain.

To investigate Fit toxin expression by *P. protegens* on roots, GFP-tagged CHA0 derivatives expressing the FitD-mCherry protein fusion (CHA1176, CHA1178) were monitored in a hydroponic plant assay system. Variants expressing either the FitD-mCherry fusion alone (CHA1163, CHA1175) or the GFP tag alone (CHA0-*gfp2*, CHA1158-*gfp2*) were included as controls. For the assay, three sterile-grown, 60-h-old cucumber seedlings (*Cucumis sativus* cv. Chinese Snake) [48] were transferred to a cyg seed germination pouch (18 cm high by 16.5 cm wide; Mega International, West St. Paul, MN, U.S.A.) containing a paper wick moistened with 15 ml of sterile distilled water [30]. Growth pouches were wrapped in aluminium foil to protect roots from light and were placed in a growth chamber set to 80% relative humidity for 16 h with light (160 µE/m^2/s) at 22°C, followed by an 8-h dark period at 18°C. After incubation for three days, each seedling was inoculated with 1 ml of a suspension containing 10^8 washed cells prepared from exponential-growth-phase LB cultures of the *P. protegens* reporter strains. After incubation for another three to five days, fractions of the upper, middle and lower parts of the roots from each growth pouch were carefully harvested, cut into small pieces, placed on 1% agarose pads and fixed with 1% (vol/vol) formaldehyde solution. Visualization by fluorescence microscopy was performed with Ph3 (70 ms), GFP (300 ms) and DsRed (4 s) channels. For quantification of average FitD-mCherry expression levels in individual bacterial cells, the remaining roots from the same growth pouch were placed into a 50-ml Falcon tube containing 10 ml of autoclaved water with 1% of formaldehyde solution. Tubes were vigorously agitated at 300 rpm for 20 min to remove adhering bacteria from the roots. Cells were harvested by centrifugation, fixed on 1% agarose pads and monitored by fluorescence microscopy. Images recorded on the Ph3, GFP and DsRed channels were processed with ImageJ using the same approach and settings as described above for quantification of bacterial fluorescence in insects.

Statistical analysis

Statistical analysis of experiments was performed with R version 2.13.1 (http://www.r-project.org) by Wilcoxon-Mann-Whitney's test with Bonferroni correction for multiple testing or one-way analysis of variance (ANOVA) with Tukey's HSD test for post-hoc comparisons.

Acknowledgments

We would like to thank Romain Libbrecht for providing Argentine ants and the necessary equipment to run the experiment with them, Guy Condamine for helpful inputs on aphid bioassays and providing AP3 medium, and Justine Collier Close and Katharina Eich for advice with fluorescence microscopy.

References

1. Heckel DG (2012) Ecology. Insecticide resistance after Silent spring. Science 337: 1612-1614.
2. Roberts JR, Karr CJ (2012) Pesticide exposure in children. Pediatrics 130: e1765-1788.
3. Péchy-Tarr M, Bruck DJ, Maurhofer M, Fischer E, Keel C (2008) Molecular analysis of a novel gene cluster encoding an insect toxin in plant-associated strains of *Pseudomonas fluorescens*. Environ Microbiol 10: 2368-2386.
4. Ruffner B, Péchy-Tarr M, Ryffel F, Hoegger P, Obrist C, et al. (2013) Oral insecticidal activity of plant-associated pseudomonads. Environ Microbiol 15: 751-763.
5. Kupferschmied P, Maurhofer M, Keel C (2013) Promise for plant pest control: root-associated pseudomonads with insecticidal activities. Front Plant Sci 4: 287.
6. Haas D, Défago G (2005) Biological control of soil-borne pathogens by fluorescent pseudomonads. Nat Rev Microbiol 3: 307-319.
7. Mendes R, Kruijt M, de Bruijn I, Dekkers E, van der Voort M, et al. (2011) Deciphering the rhizosphere microbiome for disease-suppressive bacteria. Science 332: 1097-1100.
8. Berg G (2009) Plant-microbe interactions promoting plant growth and health: perspectives for controlled use of microorganisms in agriculture. Appl Microbiol Biotechnol 84: 11-18.
9. Péchy-Tarr M, Borel N, Kupferschmied P, Turner V, Binggeli O, et al. (2013) Control and host-dependent activation of insect toxin expression in a root-associated biocontrol pseudomonad. Environ Microbiol 15: 736-750.
10. Daborn PJ, Waterfield N, Silva CP, Au CP, Sharma S, et al. (2002) A single *Photorhabdus* gene, makes caterpillars floppy (*mcf*), allows *Escherichia coli* to persist within and kill insects. Proc Natl Acad Sci U S A 99: 10742-10747.
11. Vlisidou I, Dowling AJ, Evans IR, Waterfield N, ffrench-Constant RH, et al. (2009) *Drosophila* embryos as model systems for monitoring bacterial infection in real time. PLoS Pathog 5: e1000518.
12. Ruffner B (2013) Insecticidal activity in plant-beneficial pseudomonads: Molecular basis and ecological relevance. PhD thesis; ETH Zurich, Zurich, Switzerland.
13. Kupferschmied P (2010) Biotoxicity and interference competition of plant-associated beneficial pseudomonads. Master's research project; ETH Zurich, Zurich, Switzerland.
14. Binggeli O (2009) Molecular analysis of insecticidal activity in the plant-beneficial bacterium *Pseudomonas fluorescens*. Master's thesis; University of Lausanne, Lausanne, Switzerland.
15. Grace TD (1962) Establishment of four strains of cells from insect tissues grown in vitro. Nature 195: 788-789.
16. Lapouge K, Schubert M, Allain FHT, Haas D (2008) Gac/Rsm signal transduction pathway of gamma-proteobacteria: from RNA recognition to regulation of social behaviour. Mol Microbiol 67: 241-253.
17. Behrendt U, Ulrich A, Schumann P (2003) Fluorescent pseudomonads associated with the phyllosphere of grasses; *Pseudomonas trivialis* sp. nov., *Pseudomonas poae* sp. nov. and *Pseudomonas congelans* sp. nov. Int J Syst Evol Microbiol 53: 1461-1469.
18. Someya N, Morohoshi T, Ikeda T, Tsuchiya K, Ikeda S (2012) Genetic diversity and ecological evaluation of fluorescent pseudomonads isolated from the leaves and roots of potato plants. Microbes Environ 27: 122-126.
19. De Leij F, Sutton EJ, Whipps JM, Fenlon JS, Lynch JM (1995) Impact of field release of genetically modified *Pseudomonas fluorescens* on indigenous microbial populations of wheat. Appl Environ Microbiol 61: 3443-3453.
20. Meena B, Radhajeyalakshmi R, Marimuthu T, Vidhyasekaran P, Velazhahan R (2002) Biological control of groundnut late leaf spot and rust by seed and foliar applications of a powder formulation of *Pseudomonas fluorescens*. Biocontrol Sci Technol 12: 195-204.
21. Meena B (2012) PGPR-mediated systemic resistance for sustainable agriculture. In: Maheshwari DK, editor. Bacteria in Agrobiology: Plant Probiotics. Berlin: Springer.
22. Stockwell VO, Hockett K, Loper JE (2009) Role of RpoS in stress tolerance and environmental fitness of the phyllosphere bacterium *Pseudomonas fluorescens* strain 122. Phytopathology 99: 689-695.

23. Stavrinides J, McCloskey JK, Ochman H (2009) Pea aphid as both host and vector for the phytopathogenic bacterium *Pseudomonas syringae*. Appl Environ Microbiol 75: 2230-2235.
24. Tinker JK, Erbe JL, Holmes RK (2005) Characterization of fluorescent chimeras of cholera toxin and *Escherichia coli* heat-labile enterotoxins produced by use of the twin arginine translocation system. Infect Immun 73: 3627-3635.
25. Soberón M, Gill SS, Bravo A (2009) Signaling versus punching hole: How do *Bacillus thuringiensis* toxins kill insect midgut cells? Cell Mol Life Sci 66: 1337-1349.
26. Silva CP, Waterfield NR, Daborn PJ, Dean P, Chilver T, et al. (2002) Bacterial infection of a model insect: *Photorhabdus luminescens* and *Manduca sexta*. Cell Microbiol 4: 329-339.
27. Costechareyre D, Balmand S, Condemine G, Rahbe Y (2012) *Dickeya dadantii*, a plant pathogenic bacterium producing Cyt-like entomotoxins, causes septicemia in the pea aphid *Acyrthosiphon pisum*. PLoS ONE 7: e30702.
28. Daborn PJ, Waterfield N, Blight MA, ffrench-Constant RH (2001) Measuring virulence factor expression by the pathogenic bacterium *Photorhabdus luminescens* in culture and during insect infection. J Bacteriol 183: 5834-5839.
29. Münch A, Stingl L, Jung K, Heermann R (2008) *Photorhabdus luminescens* genes induced upon insect infection. BMC Genomics 9: 229.
30. Rochat L, Péchy-Tarr M, Baehler E, Maurhofer M, Keel C (2010) Combination of fluorescent reporters for simultaneous monitoring of root colonization and antifungal gene expression by a biocontrol pseudomonad on cereals with flow cytometry. Mol Plant Microbe Interact 23: 949-961.
31. Crivat G, Taraska JW (2012) Imaging proteins inside cells with fluorescent tags. Trends Biotechnol 30: 8-16.
32. Chapman RF (1998) Circulatory systems, blood and immune systems. In: Chapman RF, editor. The Insects: Structure and Function. Cambridge: Cambridge University Press.
33. Pannabecker TL, Andrews F, Beyenbach KW (1992) A quantitative analysis of the osmolytes in the hemolymph of the larval gypsy moth, *Lymantria dispar*. J Insect Physiol 38: 823-830.
34. Wyatt GR (1961) Biochemistry of insect hemolymph. Annu Rev Entomol 6: 75-102.
35. Konkel ME, Tilly K (2000) Temperature-regulated expression of bacterial virulence genes. Microbes Infect 2: 157-166.
36. Haas D, Keel C (2003) Regulation of antibiotic production in root-colonizing *Peudomonas* spp. and relevance for biological control of plant disease. Annu Rev Phytopathol 41: 117-153.
37. Laville J, Voisard C, Keel C, Maurhofer M, Défago G, et al. (1992) Global control in *Pseudomonas fluorescens* mediating antibiotic synthesis and suppression of black root rot of tobacco. Proc Natl Acad Sci U S A 89: 1562-1566.
38. Schnider U, Keel C, Blumer C, Troxler J, Défago G, et al. (1995) Amplification of the housekeeping sigma-factor in *Pseudomonas fluorescens* CHA0 enhances antibiotic production and improves biocontrol abilities. J Bacteriol 177: 5387-5392.
39. Schnider-Keel U, Lejbølle KB, Baehler E, Haas D, Keel C (2001) The sigma factor AlgU (AlgT) controls exopolysaccharide production and tolerance towards desiccation and osmotic stress in the biocontrol agent *Pseudomonas fluorescens* CHA0. Appl Environ Microbiol 67: 5683-5693.
40. Péchy-Tarr M, Bottiglieri M, Mathys S, Lejbølle KB, Schnider-Keel U, et al. (2005) RpoN (σ^{54}) controls production of antifungal compounds and biocontrol activity in *Pseudomonas fluorescens* CHA0. Mol Plant Microbe Interact 18: 260-272.
41. Baehler E, de Werra P, Wick LY, Péchy-Tarr M, Mathys S, et al. (2006) Two novel MvaT-like global regulators control exoproduct formation and biocontrol activity in root-associated *Pseudomonas fluorescens* CHA0. Mol Plant Microbe Interact 19: 313-329.
42. Sambrook J, Russel DW (2001) Molecular Cloning: A Laboratory Manual. Cold Spring Harbor (New York): Cold Spring Harbor Laboratory Press.
43. Kupferschmied P, Péchy-Tarr M, Imperiali N, Maurhofer M, Keel C (2014) Domain shuffling in a sensor protein contributed to the evolution of insect pathogenicity in plant-beneficial *Pseudomonas protegens*. PLoS Pathog 10: e1003964.

44. Stutz E, Défago G, Kern H (1986) Naturally occurring fluorescent pseudomonads involved in suppression of black root rot of tobacco. Phytopathology 76: 181-185.
45. Birah A, Chilana P, Shukla UK, Gupta GP (2008) Mass rearing of greater wax moth (*Galleria mellonella* L.) on artificial diet. Indian J Entomol 70: 389-392.
46. Reese JC, English LM, Yonke TR, Fairchild ML (1972) A method for rearing black cutworms. J Econ Entomol 65: 1047-1050.
47. Rahbé Y, Febvay G (1993) Protein toxicity to aphids - an in vitro test on *Acyrthosiphon pisum*. Entomol Exp Appl 67: 149-160.
48. Keel C, Ucurum Z, Michaux P, Adrian M, Haas D (2002) Deleterious impact of a virulent bacteriophage on survival and biocontrol activity of *Pseudomonas fluorescens* strain CHAO in natural soil. Mol Plant Microbe Interact 15: 567-576.

CHAPTER 3

Domain shuffling in a sensor protein contributed to the evolution of insect pathogenicity in plant-beneficial *Pseudomonas protegens*

This chapter was previously published in Kupferschmied P, Péchy-Tarr M, Imperiali N, Maurhofer M, and Keel C (2014) Domain shuffling in a sensor protein contributed to the evolution of insect pathogenicity in plant-beneficial *Pseudomonas protegens*. *PLoS Pathog* 10:2. doi: 10.1371/journal.ppat.1003964. Author contributions: Peter Kupferschmied, Christoph Keel and Monika Maurhofer wrote the article; Peter Kupferschmied and Christoph Keel conceived and designed the experiments; Peter Kupferschmied, Nicola Imperiali and Maria Péchy-Tarr performed the experiments.

Abstract

Pseudomonas protegens is a biocontrol rhizobacterium with a plant-beneficial and an insect pathogenic lifestyle, but it is not understood how the organism switches between the two states. Here we focus on understanding the function and possible evolution of a molecular sensor that enables *P. protegens* to detect the insect environment and produce a potent insecticidal toxin specifically during insect infection but not on roots. By using quantitative single cell microscopy and mutant analysis we provide evidence that the sensor histidine kinase FitF is a key regulator of insecticidal toxin production. Our experimental data and bioinformatic analyses indicate that FitF shares a sensing domain with DctB, a histidine kinase regulating carbon uptake in Proteobacteria. This suggested that FitF has acquired its specificity through domain shuffling from a common ancestor. We constructed a chimeric DctB-FitF protein and showed that indeed it is functional in regulating toxin expression in *P. protegens*. The shuffling event and subsequent adaptive modifications of the recruited sensor domain were critical for the microorganism to express its potent insect toxin in the observed host-specific manner. Inhibition of the FitF sensor during root colonization could explain the mechanism by which *P. protegens* differentiates between the plant and insect host. Our study establishes FitF of *P. protegens* as a prime model for molecular evolution of sensor proteins and bacterial pathogenicity.

Author Summary

Pseudomonas bacteria are well-known for their capability of adapting to different environments which enables them to interact with various host organisms. *Pseudomonas protegens* is a plant-associated biocontrol bacterium with lifestyles that are of interest for agricultural applications, among them one as a competitive root colonizer protecting plants against pathogenic fungi and the other as an insect pathogen invading and killing insect species of importance as pests in agriculture. We recently discovered that *P. protegens* produces a potent insecticidal toxin only during infection of insects but not when growing on plant roots. Since sensor proteins enable bacteria to sense and respond to changing environments and are important for pathogen-host interactions, we investigated whether a specific sensory protein could explain our observation. We found that this particular protein tightly controls toxin production and during its evolution has recruited a common sensor domain from a regulatory protein involved in control of nutrient uptake. This so-called domain shuffling event was important for the ability of *P. protegens* to produce its insecticidal toxin only when it infects insects. Our study provides a prime example of how a sensory system can evolve and contribute to the evolution of bacterial pathogenicity.

Introduction

Pseudomonas protegens is a beneficial root-associated bacterium of the *Pseudomonas fluorescens* group that is able to promote the growth of crop plants and to efficiently protect their roots against fungal and oomycete phytopathogens [1,2]. *P. protegens* can also turn into an insect pathogen [3-5]. The bacterium produces a potent insecticidal toxin termed Fit (for *P. fluorescens* insecticidal toxin) which is required for its capacity to efficiently kill larvae of important agricultural pest insects upon oral or systemic infection [5,6]. The gene encoding the Fit protein toxin is part of an eight-gene cluster which comprises also genes coding for a type I secretion system and three regulatory proteins (Figure S1 and [6,7]). Expression of the insecticidal toxin is activated during infection of the insect host, but not on plant roots or in standard laboratory media [7]. We recently demonstrated that toxin expression is tightly controlled by two regulators, named FitG (an activator) and FitH (a repressor) [7]. The third regulatory protein encoded in the Fit cluster is named FitF and codes for a putative sensor histidine kinase-response regulator hybrid protein. We hypothesize that FitF is responsible for the detection of the host environment and for activating insecticidal toxin production via FitH and FitG specifically upon infection of the insect host (Figure S1).

Sensor proteins enable bacteria to sense the environment they live in and to adapt their behavior accordingly, which is particularly relevant for pathogen-host interactions [8-10]. The number of sensor protein types is particularly high in bacteria such as pseudomonads that inhabit diverse and changing environments [11,12]. An important category of sensor proteins is that of the two-component regulatory systems, which couple extracellular stimuli to adaptive responses. A typical two-component system consists of a membrane-bound sensor histidine kinase, which perceives a stimulus, and a cytosolic response regulator, which transduces the signal into an output, such as altering specific gene expression. Signal transduction is achieved by phosphotransfer reactions between the sensor kinase and the response regulator. In some cases, like in the so-called phosphorelay system, the sensor histidine kinase is a hybrid response regulator protein undergoing multiple intramolecular phosphotransfer reactions, before finally activating a separate response regulator protein [13,14].

Sensor and signal transduction proteins usually show a modular organization of conserved domains [14], which can be highly variable in their order and topological organization [8]. Not surprisingly, therefore, it has been proposed that the modularity of two-component systems enables rapid evolution and generation of new functional properties. Gene duplication and domain shuffling are considered to be driving mechanisms for the formation of new two-component systems in bacteria [10,12]. More than 70% of estimated recently duplicated histidine kinases have input domains different from those of their closest paralogs, suggesting frequent domain shuffling events [10]. It was proposed that by shuffling of the sensor domain recently duplicated histidine kinases gained new sensing specificity and thus might have enabled the bacteria to respond to a broader range of environmental changes [12].

The major goal of our work is to understand the molecular mechanisms that allow *P. protegens* and related bacteria to survive within and to kill the insect host. Of particular interest for the underlying work was the question as to how insect pathogenicity may have evolved and has been selected for. Because sensory systems are essential for niche adaptation, we felt that an evolutionary analysis of the chemosensory systems enabling insect recognition in *P. protegens* and in particular of the Fit system would be fundamental to the understanding of host adaptation.

Here we thus report the detailed regulation of Fit toxin expression and in particular describe the role of the hybrid sensor kinase protein FitF. We noticed that the periplasmic region of FitF is strikingly similar to the sensor domain of the histidine kinase DctB, which regulates the uptake of C_4-dicarboxylates in Proteobacteria [15]. The crystal structures of DctB of *Vibrio cholerae* and *Sinorhizobium meliloti* have been solved [16,17] and show an inserted repeat of a Per-Arnt-Sim (PAS)-like fold (PASp) in the periplasmic sensory domain, which was later termed the PhoQ/DcuS/CitA (PDC) domain [18]. PAS domains are universally distributed among all kingdoms of life, are the most frequent type of signal sensors in bacteria, can fulfill several functions and can bind chemically diverse small-molecule ligands [9,19-21]. The membrane distal PASp domain of DctB binds C_4-dicarboxylates such as malate, fumarate and succinate [15].

We present several lines of evidence illustrating that the periplasmic sensory domain of FitF evolved from a common ancestor with DctB, enabling *P. protegens* to survive and switch on toxin expression only in the insect host. By expressing a chimeric DctB-FitF protein in *P. protegens* and thereby testing the proposed domain shuffling event, we show that the DctB sensor domain is effectively suitable to drive the expression of the insecticidal toxin in a similar way as wild-type FitF. We found that the periplasmic sensor region of FitF possesses an important and conserved peptide motif and demonstrate by site-directed mutagenesis that, as for DctB, it is essential for the function of the histidine kinase. Bioinformatic analyses further support that the specific tandem PASp domain probably served as a sensory module for numerous proteins in *P. protegens* and other bacterial species, highlighting its importance, mobility and evolutionary plasticity. Our work reveals how the FitF sensor kinase could have evolved into a crucial virulence gene expression regulator, and has contributed to the ability of *P. protegens* to exploit a new ecological niche by recruiting a functional domain from an ancestor of sensor proteins involved in the regulation of the primary metabolism. In addition, our evolutionary analysis of the Fit regulatory system could provide a unique model system to study the hypothesis of domain shuffling in sensor protein evolution, which so far had been postulated mainly on the basis of bioinformatic analysis of proteins [10,22] and construction of artificial chimeric proteins [23-25].

Results

FitF is essential for Fit toxin expression in the insect host

The *fit* locus (EU400157) of *P. protegens* comprises three genes (*fitF*, *fitG*, and *fitH*) that code for regulatory proteins (Figure S1). We previously demonstrated that expression of the insecticidal Fit toxin can be activated in strain CHA0 in Lysogeny Broth (LB) by overexpression of *fitG* or deletion of *fitH*, thus identifying the encoded proteins as an activator and repressor of insect toxin expression, respectively [7]. The third gene *fitF*, which was predicted to code for a sensor histidine kinase-response regulator hybrid protein (Figure 1A), was hypothesized to function as a detector of the insect environment and a regulator of Fit toxin production [7].

To demonstrate that FitF is necessary for Fit toxin production, we used reporter strains of *P. protegens* CHA0 in which the full-length *fitD* gene was translationally fused at its native locus to *mcherry* by markerless gene replacement [7]. Epifluorescence microscopy confirmed that FitD-mCherry was visibly expressed in *P. protegens* CHA0 cells during infection of larvae of the greater wax moth *Galleria mellonella*, but was absent when *fitF* was inactivated by an in-frame deletion (Figure 1B). Also, the virulence of the CHA0 *fitF* deletion mutant in a *Galleria* injection assay was statistically significantly decreased compared to the wild type and was similar to a *fitD* deletion mutant (Fit toxin-deficient) (Figure 1C). These results demonstrate that FitF is essential for the activation of Fit toxin expression by *P. protegens* CHA0 in the insect host.

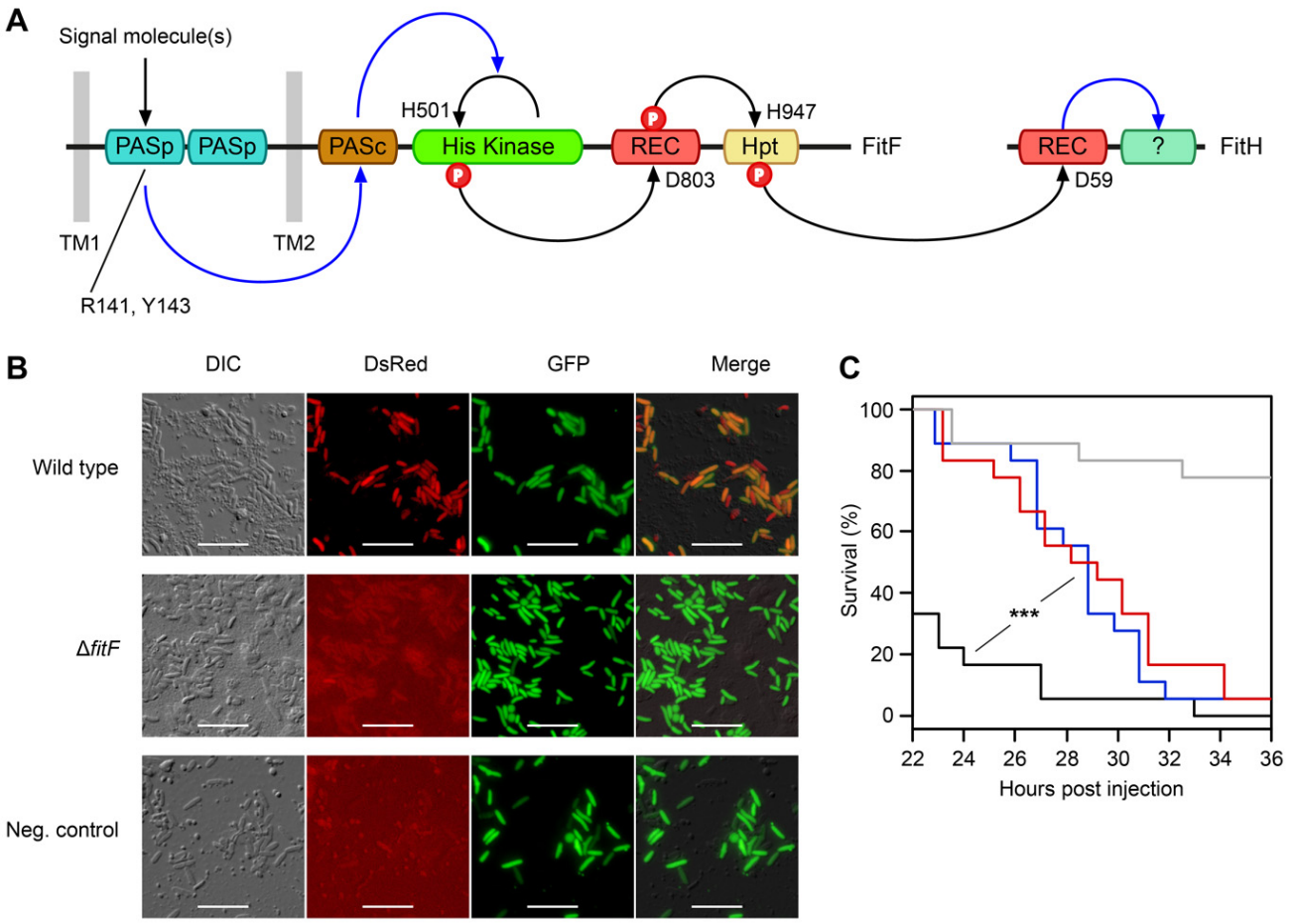

Figure 1. The hybrid sensor kinase FitF is essential for Fit toxin expression. (A) Domain topology of FitF and FitH and putative signal transduction pathways (blue arrays) and phosphotransfer reactions (black arrows) between domains and proteins predicted by NCBI Conserved Domain Search [42] and SMART [43]. The conserved amino acid residues predicted by NCBI Conserved Domain Search to be phosphorylated or to be important for signal recognition are indicated with their respective amino acid positions. Hpt, phosphotransfer domain; PASc, cytoplasmic Per-Arnt-Sim (PAS) domain; PASp, periplasmic PAS domain; REC, receiver domain; TM, transmembrane region. (B) Epifluorescence microscopy of hemolymph extracts from larvae of *G. mellonella* infected with FitD-mCherry reporter strains with the wild-type (CHA1176) and Δ*fitF* mutant (CHA1174-*gfp2*) background for 24 h. The injected strains harbor a constitutive GFP cell tag for identification, expression of FitD-mCherry can be seen in the DsRed channel. Strain CHA0-*gfp2* was used as a negative control. Bars represent 10 µm, micrographs are false-colored. The experiment was repeated twice with similar results. (C) Systemic virulence assay with injection of wild-type (in black, CHA0) and isogenic mutants (Δ*fitF* in red, CHA1154; Δ*fitD* in blue, CHA1151) of *P. protegens* CHA0 into last instar larvae of *G. mellonella*. Saline solution served as a negative control (in gray). Significant differences between the different treatments are indicated with *** (p-value < 0.0001; Log-rank test). The experiment was repeated twice with similar results.

Activation of Fit toxin expression in an insect-mimicking medium

Although FitD-mCherry was readily expressed during infection of larvae, it was hardly detectable when *P. protegens* CHA0 was growing in standard bacterial culture media such as LB or Brain Heart Infusion (BHI) (Figure 2A). Fit toxin production was strongly induced when the bacteria were grown in Grace's Insect Medium (GIM), with on average 60-fold higher red fluorescence levels of individual cells than in LB. GIM,

which is a defined medium rich in amino acids and C_4-dicarboxylates, is widely used for insect cell cultures and reflects closely the composition of Lepidopteran hemolymph [26]. In GIM, wild-type bacteria expressed the Fit toxin mostly at the end of exponential growth but no longer produced it in stationary phase (Figure S2A). Compared to LB, FitD-mCherry expression was also significantly higher in M9 minimal medium supplemented with L-malate as sole carbon source, but not in fetal bovine serum or in marine broth, although both media provide conditions similar to insect hemolymph (Figure 2A). Interestingly, FitD-mCherry production was significantly lower in M9 or GIM supplemented with plant root extracts (Figure 2B). Also more than 20% (v/v) of LB mixed in with GIM abolished FitD-mCherry expression (data not shown). Altering pH in M9 medium did not impede FitD-mCherry expression (data not shown).

Expression levels of the FitD-mCherry fusion protein in GIM were similar in the *P. protegens* wild type and in a *fitH* deletion mutant, which constitutively expresses the toxin (Figure S3). Furthermore, deletion of *fitF* abolished the expression of FitD-mCherry in GIM (Figure 2C), but could be fully rescued by complementation of the mutant strain by insertion of a single copy of the *fitF* gene into the chromosome (Figure 2C). Interestingly, the *fitF* deletion mutant of strain CHA0 could also be fully complemented with the homologue *fitF* from *P. chlororaphis* strain PCL1391 (Figure 2C), even though *P. chlororaphis* FitF is predicted to harbor two cytoplasmic PAS domains instead of one for FitF from *P. protegens* [4,5,27]. Results of FitD-mCherry expression were confirmed by assaying the activity of the P_{fitA} promoter, which drives the expression of toxin and type I transporter genes [7], using a GFP-based transcriptional reporter fusion (Figure S2B).

Using a hemolymph-mimicking medium, we were thus able to confirm the essential role of FitF in regulation of insect toxin production in a controlled and reproducible manner in an *ex vivo* environment.

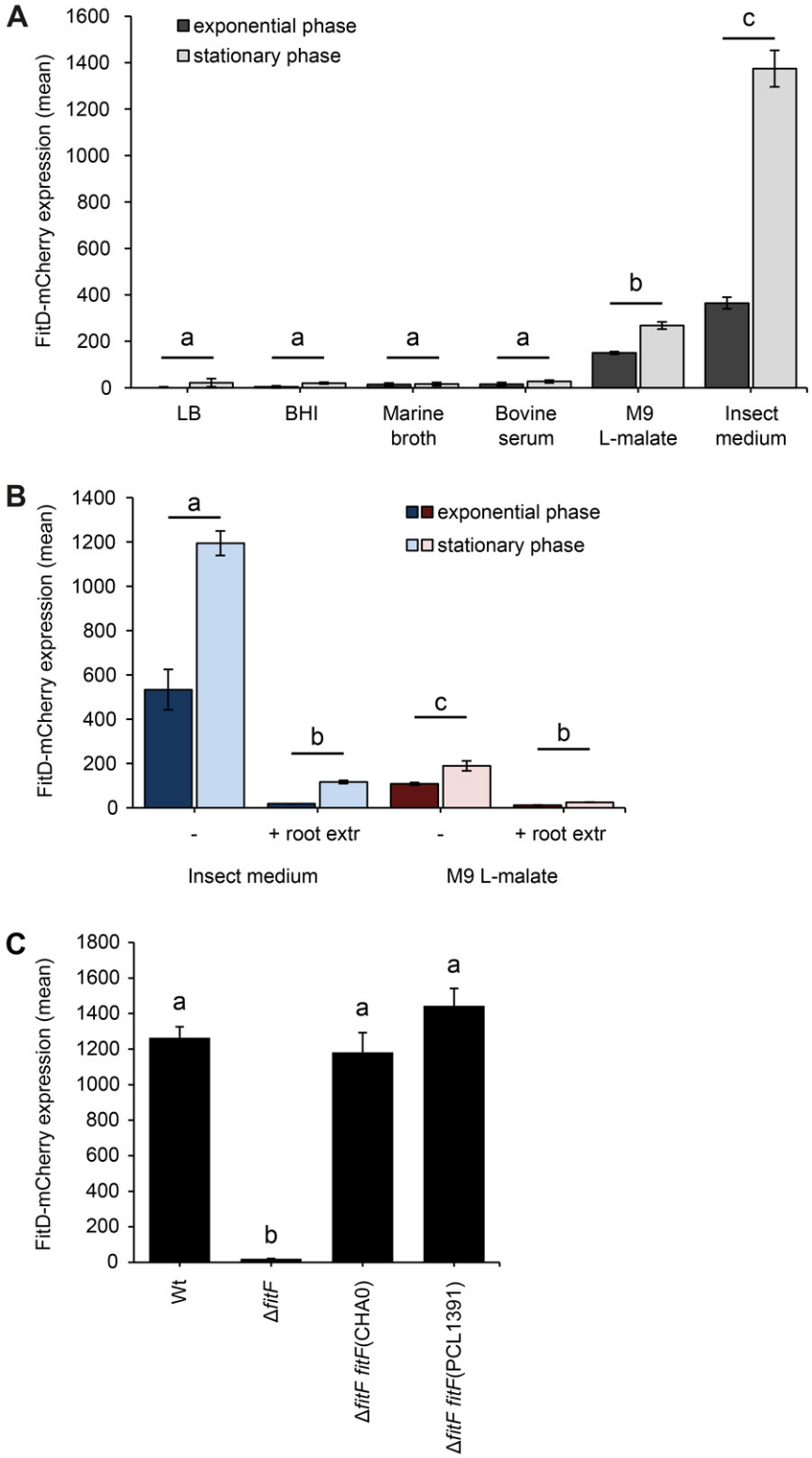

Figure 2. Expression of the Fit insect toxin can be induced in an insect hemolymph-mimicking medium (GIM). (A) The FitD-mCherry reporter strain of *P. protegens* CHA0 (CHA1163) was grown in different media and red fluorescence intensities of single cells were quantified by epifluorescence microscopy in the exponential (8 h post inoculation) and stationary (24 h post inoculation) growth phase. Results are the mean and standard deviation of population averages of single cell fluorescence intensities from three independent cultures (n = on average approx. 3200 cells per treatment and time point). Treatments labeled with a different letter are significantly different (p-values < 0.0001; two-way ANOVA with Tukey's HSD test for post-hoc comparisons). The experiment was performed three times with similar results. (B) Quantification of the expression of FitD-mCherry in the wild-type background of CHA0

(CHA1163) in GIM and M9 L-malate with or without root extracts from field-grown wheat (n = on average approx. 2600 cells per treatment and time point). Characters indicate significant differences between the treatments (p-values < 0.05; two-way ANOVA with Tukey's HSD test for post-hoc comparisons). The experiment was repeated twice with similar results. (C) Quantification of the expression of FitD-mCherry in the wild-type (CHA1163) and Δ*fitF* deletion mutant (CHA1174) background of strain CHA0 grown in GIM for 24 h at 25°C (n = 2768−3239 cells per strain). Re-introducing a single copy of *fitF* from CHA0 (CHA5066) or PCL1391 (CHA5073) in the bacterial chromosome rescued the expression of FitD-mCherry. Means labeled with a different letter are significantly different (p-value < 0.05; one-way ANOVA with Tukey's HSD test for post-hoc comparisons). The experiment was performed three times with similar results.

FitF has a periplasmic region homologous to the C_4-dicarboxylate-sensing PASp domains of DctB

FitF is predicted to possess two transmembrane domains, a periplasmic sensor domain, a cytoplasmic PAS domain, a histidine kinase domain (comprising a conserved phosphoacceptor domain and an ATPase domain), a CheY-homologous receiver domain, and a phosphotransfer domain (Figure 1A). BLAST comparisons with the amino acid sequence of the periplasmic region of FitF (FitFp) of *P. protegens* CHA0 indicated 54% amino acid sequence similarity (27% sequence identity) across the whole length to the double PASp domain of the C_4-dicarboxylate sensor DctB (DctBp) of *V. cholerae* (Figure 3A). Phylogenetic analysis further indicated that FitFp homologues from various strains of *P. protegens* and *P. chlororaphis* group with DctBp homologues of different proteobacterial species, while the periplasmic regions of DctB-related CitA and DcuS proteins appear to be phylogenetically more distant (Figure 3B and Table S1). CLANS cluster analysis revealed similar results with FitFp clustering in close proximity to homologs of DctBp and CitA and DcuS clustering further away (Figure S4 and Table S1). We found a conserved “FRPYF” motif among the FitFp homologues (Figure 3A), which is similar to the previously reported signal molecule-binding "RXYF" motif in DctB homologues and other proteins with double-PASp domains [28,29]. Protein threading and modeling approaches predicted a similar secondary and tertiary structure for FitFp as DctBp (Figure 3C). This suggests that the FitFp and DctBp domains share a common ancestor. Concurrently, FitF and DctB display different domain topologies in their cytoplasmic portions, which is in contrast to the similarity in the periplasmic region of the proteins.

By using *in vivo* site-directed mutagenesis, we replaced a number of residues in *fitF* and *fitH* and studied the effect on FitD-mCherry expression in *P. protegens*. Change of Arg141 and of Tyr143 in the RXYF motif of FitF to Ala following the mutagenesis of *dctB* described by Nan *et al.* [28], resulted in almost completely abolished FitD-mCherry production (Figure 3D). In contrast, change of Asp149 to Ala (used as an internal negative control) did not alter the expression of the insecticidal toxin. Changing Tyr143 to Phe reduced expression of FitD-mCherry by approximately 45%. Replacement of predicted conserved phosphorylation residues of the histidine kinase and receiver domains in FitF (H501 and D803) and FitH (D59) (Figure 1A) by alanine diminished the expression of FitD-mCherry (Figure 4). Together, these data demonstrate

conspicuous structural and functional relatedness between the periplasmic domain of FitF and the sensor domain of DctB, with a conserved peptide motif being crucial for activation of Fit toxin expression.

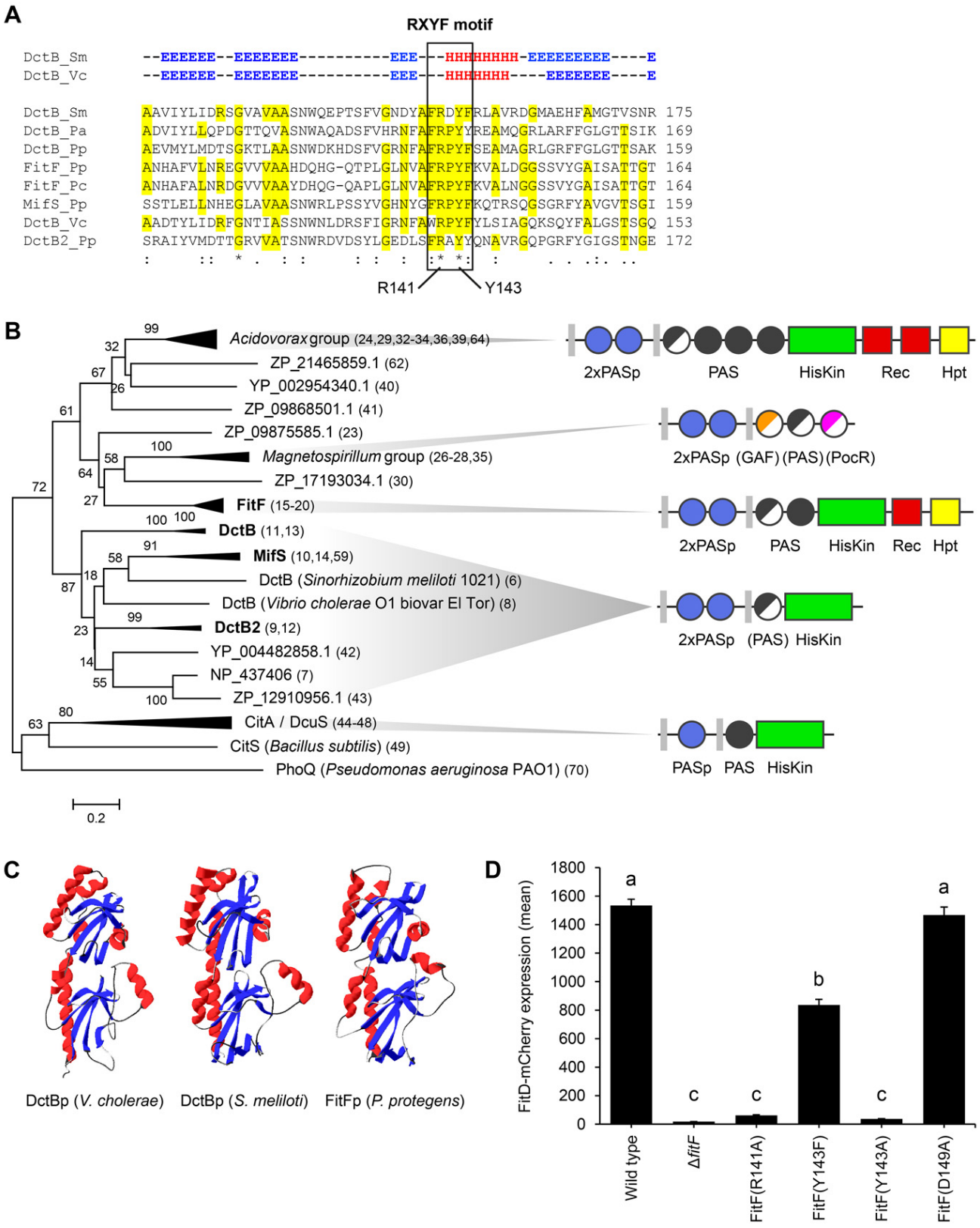

Figure 3. FitFp is homologous to the periplasmic DctB-like sensor domain. (A) Multiple sequence alignment of the periplasmic region of FitF and DctB homologs (selection). Amino acid residues that are identical to FitF are highlighted in yellow. Secondary structures of DctB were deduced from the corresponding crystal structures and are displayed on top (H, alpha helix; E, beta sheet; -, coil). Pa, *P. aeruginosa* PAO1; Pp, *P. protegens* CHA0; Pc, *P. chlororaphis* PCL1391; Sm, *S. meliloti*; Vc, *V. cholerae*. (B) Phylogenetic tree with sequences obtained from BLASTp searches using the periplasmic sequence of FitF of *P. protegens* CHA0 and of homologs of DctBp. MAFFT was used for sequence alignment and the Minimum Evolution method in MEGA [44] for inferring the evolutionary history of the proteins. The percentage of replicate trees in which the associated proteins clustered together in the bootstrap test (500 replicates) is shown next to the branches. Evolutionary distances, which were computed using the Poisson correction method, are drawn to scale and are in the units of the number of amino acid substitutions per site. The corresponding protein sequences can be found in File S1. The predicted domain topology of the entire proteins is depicted for groups of interest. Domains that are displayed in half do not exist in all proteins of the respective group. PhoQ was used as out group. (C) Tertiary structure prediction for *P. protegens* FitFp by Phyre2 in comparison with crystal structures of DctBp of *V. cholerae* (PDB code 3BY9) and *S. meliloti* (PDB code 3E4O). Other modeling programs predicted highly similar structures (data not shown). (D) Site-directed mutagenesis of the native *fitF* gene in the FitD-mCherry reporter strain CHA1163. The sites of the mutated residues are depicted in panel A and Figure 1C. Microscopic quantification of the expression of FitD-mCherry in the wild-type and individual mutant backgrounds of CHA0 grown for 24 h in GIM. Results are the mean and standard deviation of population averages of single cell fluorescence intensities from three independent cultures (n = on average approx. 2900 cells per strain). Characters indicate significant differences between the means (p-values < 0.01; one-way ANOVA with Tukey's HSD test for post-hoc comparisons). The experiment was performed three times with similar results.

An artificial chimera of DctB and FitF is functional

Because of the conspicuous similarity between the FitFp and DctBp domains, we hypothesized that perhaps the actual FitF protein might have been the result of a fusion of an ancestor DctBp domain into a FitF precursor. To simulate the proposed domain shuffling event and to test experimentally whether the sensor module of DctB is effectively suitable to regulate the expression of the Fit toxin, we created an artificial DctBp-FitFc chimera in which the periplasmic domain of DctB of *P. protegens* CHA0 was fused to the cytoplasmic portion of FitF (FitFc) (Figure 5A).

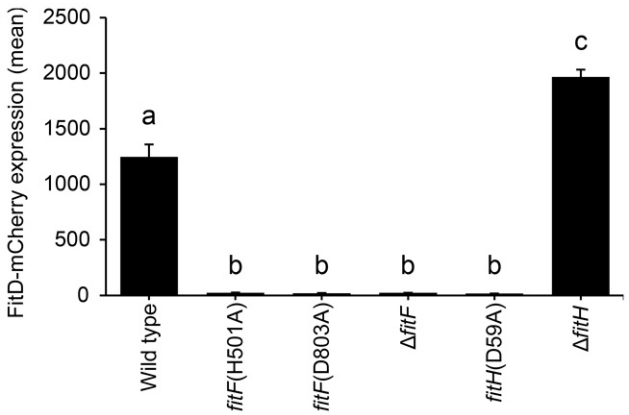

Figure 4. Site-directed mutagenesis of *fitF* and *fitH*. Site-directed mutagenesis of the native *fitF* and *fitH* genes in the FitD-mCherry reporter strain CHA1163. Quantification of the expression of FitD-mCherry in the wild-type (CHA1163) and individual mutant

backgrounds of CHA0 (CHA5056, CHA5075, CHA1174, CHA5084, and CHA1175) grown for 24 h in GIM. Results are the mean and standard deviation of population averages of single cell fluorescence intensities from three independent cultures (n = on average approx. 2900 cells per strain). Characters indicate significant differences between the means (p-values < 0.001; one-way ANOVA with Tukey's HSD test for post-hoc comparisons). The experiment was repeated twice with similar results.

Indeed, expression of the DctBp-FitFc chimeric protein in a Δ*fitF* mutant background of strain CHA0 led to FitD-mCherry production in GIM, but not in LB (Figure 5B). Still, FitD-mCherry expression was significantly higher in GIM in the Δ*fitF* mutant complemented with wild-type *fitF* than with the *dctB'-'fitF* chimeric gene. Remarkably, however, FitD-mCherry production was activated in CHA0 expressing the DctBp-FitFc chimeric protein when the bacteria were growing on plant roots, while toxin production was completely off in bacteria expressing wild-type FitF (Figure 5C). Furthermore, bacteria with the DctBp-FitFc background produced FitD-mCherry at significantly higher levels in minimal medium with L-malate as sole carbon source than bacteria expressing wild-type FitF (Figure 5B). In a *Galleria* injection assay the DctBp-FitFc chimera fully complemented the *fitF* mutant (Figure 5D). These results thus indicate that the DctB sensor domain can replace the FitFp domain of FitF. Yet, this causes a shift in sensor protein sensitivity resulting in a loss of responsiveness in an insect environment and a gain of responsiveness in a root environment.

A chimera of the more distantly related PASp sensor domain of CitA and FitFc was functional and even less responsive to the insect mimicking medium than the DctBp-FitFc chimera (Figure 5).

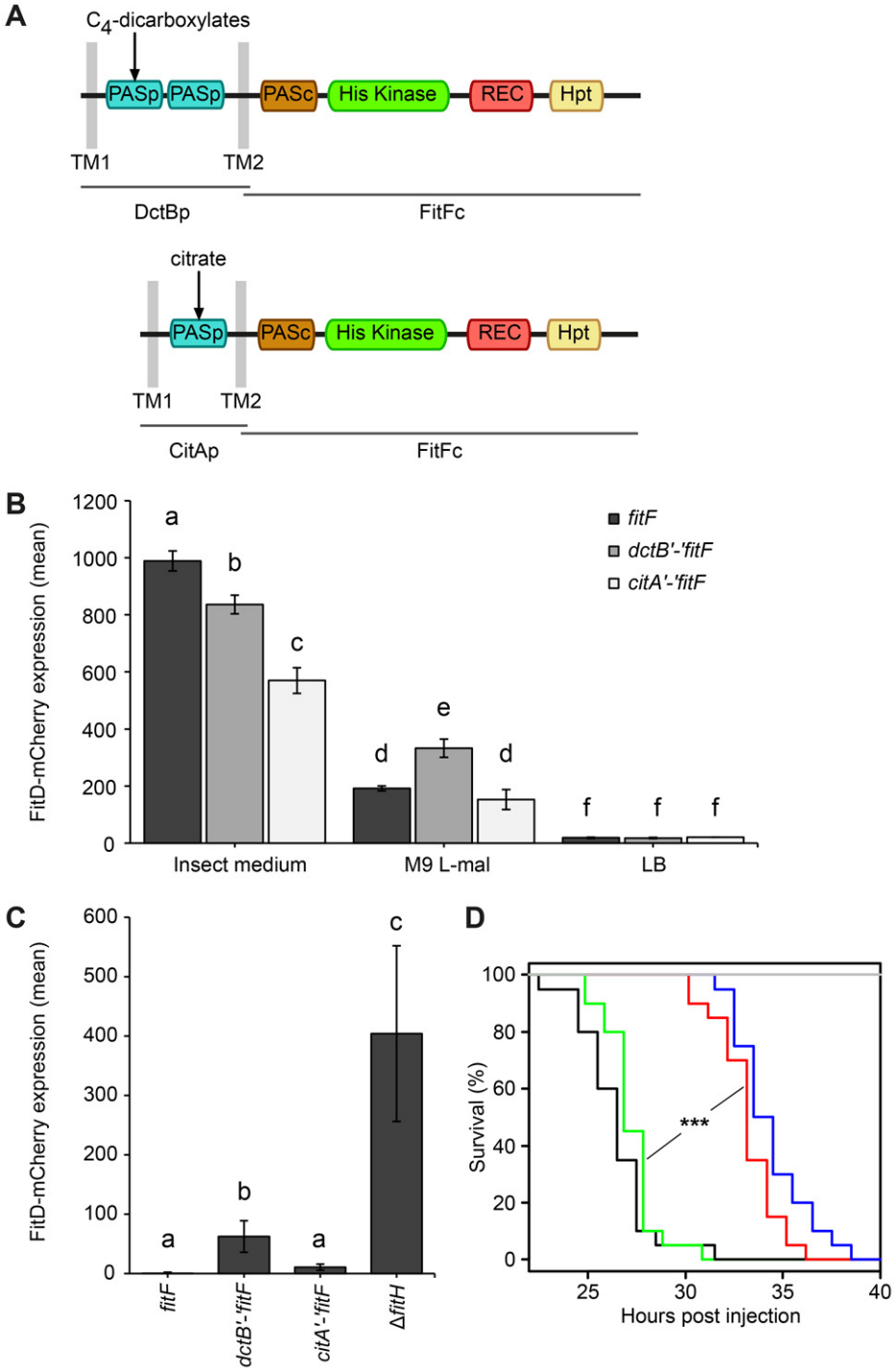

Figure 5. A DctBp-FitFc chimera regulates toxin expression similarly to wild-type FitF. (A) A chimeric protein of the cytoplasmic portion of FitF and the N-terminal part of DctB including its double-PASp sensor domain and the transmembrane regions was constructed by fusing the respective *P. protegens* CHA0 genes using the conserved DNA sequence coding for the second transmembrane region as a linker. A CitAp-FitFc chimera was constructed analogously using *E. coli citA*. (B) Expression of FitD-mCherry in the Δ*fitF* reporter strain CHA1174 complemented with either wild-type *fitF* (CHA5066), the *dctB'-'fitF* chimeric gene (CHA5093) or the *citA'-'fitF* chimeric gene (CHA5151) in different media for 24 h. Results are the mean and standard deviation of population averages of single cell fluorescence intensities from three independent cultures (n = on average approx. 3590 cells per treatment). Characters indicate significant differences between the means (p-values < 0.05; one-way ANOVA with Tukey's HSD test for post-hoc comparisons). The experiment was performed three times with similar results. (C) Quantification by epifluorescence

microscopy of FitD-mCherry expression in reporter strains CHA5066, CHA5093, CHA5151, and CHA1175 (Δ*fitH*, positive control), all harboring the plasmid pPROBE-TT for GFP-tagging of the cells, grown for five days on roots of cucumber. Shown are means and standard deviations of population averages of single cell fluorescence intensities of bacteria isolated from six independent plants (n = on average approx. 1170 cells per strain). Characters indicate significant differences between the means (p-values < 0.05; one-way ANOVA with Tukey's HSD test for post-hoc comparisons). The experiment was repeated twice with similar results. (D) *Galleria* injection assay with wild-type (in black, CHA0) and isogenic mutants (Δ*fitF* in red, CHA1154; Δ*fitD* in blue, CHA1151; Δ*fitF dctB'-'fitF* in green, CHA5150) of *P. protegens* CHA0 into last instar larvae of *G. mellonella*. Saline solution served as a negative control (in gray). Significant differences between the different treatments are indicated with *** (p-value < 0.0001; Log-rank test). The experiment was repeated twice with similar results.

Activation of Fit toxin production is host-specific

In order to investigate whether toxin production is not only host-dependent but also specific toward certain insect orders, the expression of FitD-mCherry by *P. protegens* CHA0 was studied in additional insect species. The expression of the Fit toxin was activated in the hemocoel of the African cotton leafworm *Spodoptera littoralis* (Lepidoptera) and the mealworm *Tenebrio molitor* (Coleoptera) (Figure 6A). In contrast to the Δ*fitH* mutant of strain CHA0, however, the insecticidal toxin was hardly produced in the phylogenetically distant pea aphid *Acyrthosiphon pisum* (Hemiptera) (Figure 6A). In addition, as already shown for cucumber [7], no toxin expression was detectable on roots of wheat and tomato (Figure 6B). Moreover, the presence of a phytopathogenic fungus (*Fusarium oxysporum*) on tomato roots did not activate Fit toxin production in the bacteria (Figure 6B). These results suggest that *P. protegens* CHA0 is capable of expressing its insecticidal toxin in a host-specific manner.

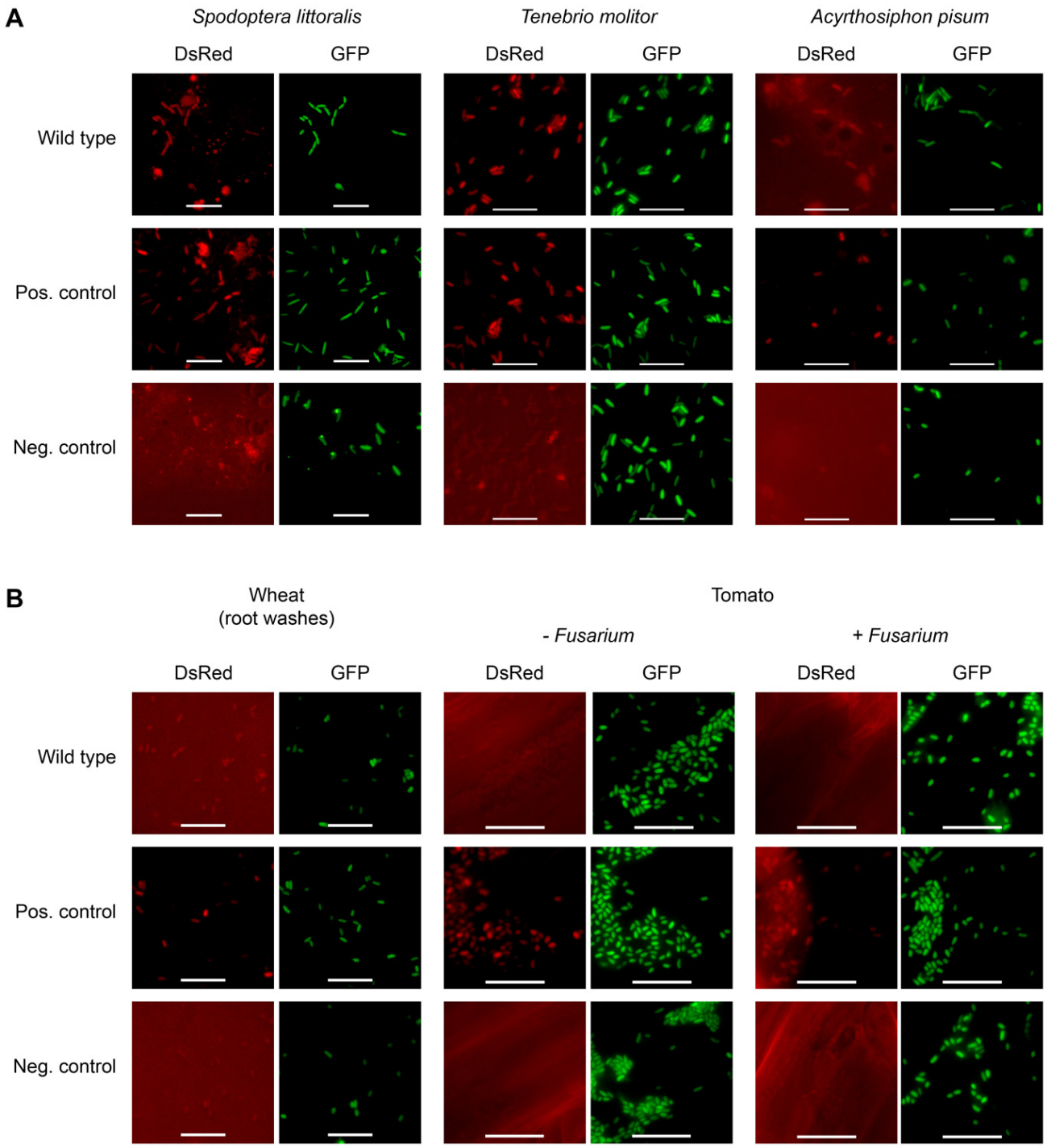

Figure 6. Fit toxin expression is controlled in a host-specific manner. The insectidical toxin is expressed by *P. protegens* CHA0 only in certain insect species and not on plant roots. (A) Epifluorescence microscopy of hemolymph isolated from *S. littoralis*, *T. molitor* and *A. pisum* infected with FitD-mCherry reporter strains with the wild-type (CHA1176) and Δ*fitH* mutant (CHA1178, positive control) background. The bacteria harbor a constitutive GFP cell tag for identification, expression of FitD-mCherry can be seen in the DsRed channel. Strain CHA0-*gfp2* was used as a negative control. Bars represent 10 µm, micrographs are false-colored. The experiments were performed at least twice with similar results. (B) Epifluorescence microscopy of plant roots (or root washes) three to five days after the inoculation with the same reporter strains as in panel A, with or without co-inoculation with the phytopathogen *Fusarium oxysporum* f. sp. *radicis-lycopersici*. The experiments were performed twice with similar results.

Discussion

Fit toxin production is dependent on the sensor kinase FitF

Here we show that the histidine kinase FitF is responsible for activation of Fit toxin expression in *P. protegens* CHA0. We deleted *fitF* in the CHA0 genome and our results show unambiguously that FitF is essential for the induction of Fit toxin expression and for full virulence of the bacterial strain in the insect host (Figure 1 and Figure 2C). We assume that FitF is the primary sensor to signal *P. protegens* the appropriate conditions to start toxin expression, activating a phosphorelay from the histidine kinase to the receiver and phosphotransfer domain of FitF (Figure 1A). FitF then most likely inactivates FitH via phosphorylation of a conserved aspartate residue, since the substitution of this residue by alanine locked the protein in its repressing state (Figure 4). Inactivation of FitH might derepress FitG, which subsequently activates transcription of the *fitABCDE* operon (Figure S1).

FitF acquired the mobile DctB-like sensor domain by domain shuffling

The periplasmic region of FitF showed remarkable structural and functional similarity to the sensor domain of DctB (Figure 3 and Figure 5). In particular, a RXYF motif was found in FitFp and we could show by site-directed mutation analysis that this conserved and known peptide motif is crucial for the activation of Fit toxin expression in *P. protegens* (Figure 3D). However, these two proteins differ substantially in their domain topologies in the cytoplasmic portion (Figure 3D). This suggested that an ancestor DctBp domain was acquired through shuffling in a precursor FitF. We present experimental and bioinformatic evidence that FitF most likely evolved via a fusion of two genes coding for a histidine kinase-response regulator hybrid protein and a duplicated DctB homolog (Figure 7). We noticed that DctB and FitF share a high degree of primary sequence identity in the second transmembrane region. It may therefore be possible that the fusion occurred via homologous recombination within the DNA sequence coding for the second transmembrane alpha helix.

Despite limited primary sequence conservation between DctBp and FitFp, a constructed DctBp-FitFc chimera was functional and, most interestingly, induced Fit toxin production in *P. protegens* in the insect medium, although to significantly lower expression levels than wild-type FitF. This strongly suggests that the tandem PASp sensor of DctB is functionally analogous to that of FitF and may have been at the basis of sensor specificity acquisition by FitF. This experiment is limited by the fact that the chimeric protein was constructed using sequences of extant proteins as it is not possible to reconstruct the sensor protein as it was shortly after the proposed domain shuffling event.

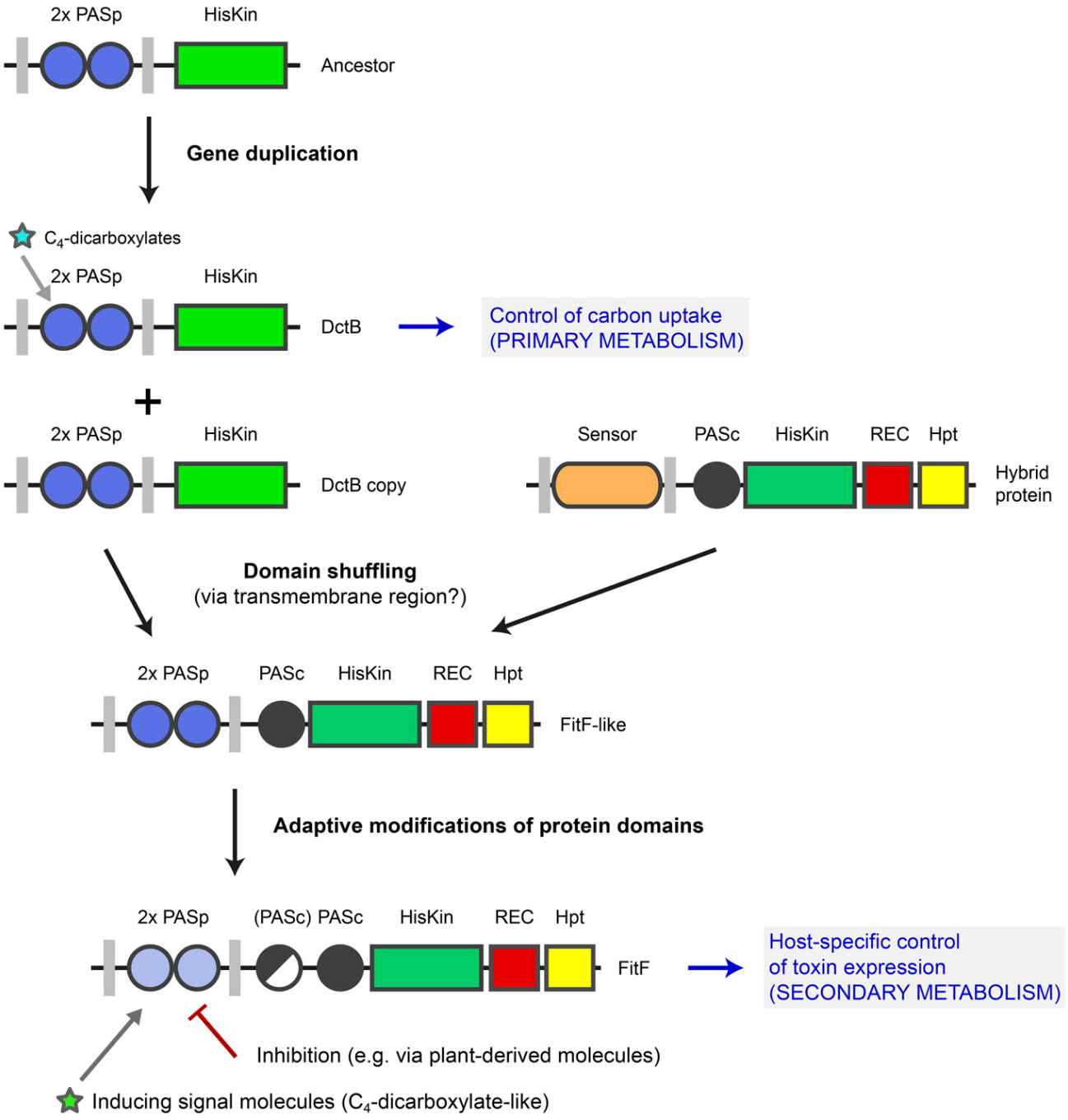

Figure 7. Model for evolution of FitF via a domain shuffling event involving a DctB ancestor. The ancestor of the gene coding for the sensor kinase DctB was duplicated several times in various proteobacterial species. One *dctB* gene copy underwent a fusion with a gene encoding a histidine kinase-response regulator hybrid protein, possibly by homologous recombination via a conserved region coding for the second transmembrane region of the sensor proteins. This domain shuffling event resulted in the expression of a hybrid histidine kinase with a dual PASp domain architecture in the periplasmic portion. Selective pressure then led to adaptive modifications in the protein sequence and domain topology (i.e. insertion of a second PASc domain in *P. chlororaphis*). Domain shuffling and subsequent modifications during the evolution of FitF significantly contributed to the ability of *P. protegens* CHA0 to produce its insecticidal toxin in a host-specific manner and as a result to the evolution of insect pathogenicity in this biocontrol bacterium. Inhibition of FitF by plant-derived molecules may be a mechanism helping the bacterium to distinguish between the plant and insect host. The evolution of FitF may have taken place in bacterial species other than *P. protegens*, implying horizontal gene transfer.

Protein comparisons further suggested that similar double-PASp domains occur widely among prokaryotes and in a variety of modular proteins (Figure 3B and Figure S4). Domains homologous to DctBp cannot only be found in histidine kinases but also in cyclic di-GMP modulating proteins (Figure 3B and Figure S4). PAS domains are known to be the most frequent type of sensor domains in bacteria [9,20]. It is thus imaginable that such domains have been frequently interchanged and that such shuffling has been fundamental to evolution of FitF specificity.

In contrast to DctB, FitF possesses a cytoplasmic PAS domain as a linker between the sensor and kinase domain (Figure 1). We noticed that DctB proteins with an inserted PASc domain also occur in certain *Acidovorax* species. Furthermore, the C_4-dicarboxylate sensing DcuS and CitA proteins of *Escherichia coli* possess a DctB-like PASp sensor domain in the periplasmic portion and a PASc domain as a linker between the sensor and the histidine kinase domain [15,30]. These observations further support the notion that an ancestral DctB-like sensor domain served as an adaptable and mobile module for the evolution of diverse proteins, since it can be fused to a variety of other protein domains. This is further supported by our observation that a fusion of the periplasmic sensor domain of CitA to FitFc was functional (Figure 5).

Domain shuffling may require gene duplication and recombination [12]. In this respect, it is interesting to note that like many *Pseudomonas* species, *P. protegens* encodes three paralogs of the *dctB* gene (Figure 3). The *dctB* paralogs are functionally different. One of them (DctB) is involved in regulation of the uptake of C_4-dicarboxylates (Figure S5 and [31]), whereas another (named MifS) was reported to be a regulator of biofilm formation in *P. aeruginosa* [32]. *Pseudomonas fulva* strain 12-X encodes four *dctB* paralogs (GeneBank CP002727), suggesting that duplications of *dctB* must have occurred frequently and could have been the basis for domain shuffling events in these bacteria.

The molecular mechanism of domain shuffling in the bacterial kingdom is still unknown. However, it has been reported that hybrid sensor kinases as is FitF show particularly high levels of DNA polymorphism and fast evolutionary rates [33]. Moreover, they are thought to have mostly evolved by lateral recruitment of individual protein domains [19]. Therefore, not only lineage-specific expansion but also recombination with horizontally acquired sequences could have played a role in the evolution of FitF. The sensor protein could have evolved by shuffling of functional domains that originated from different bacterial species.

Adaptive modifications to ensure host-specific expression of the insecticidal toxin

We discovered that Fit toxin expression in *P. protegens* CHA0 can be highly induced independently of the host organism in an insect hemolymph-mimicking medium (Figure 2A). The physicochemical conditions given by the insect medium are thus sufficient for the observed activation of toxin production during infection of the insect host. Despite extensive testing (not shown), however, we currently do not know the precise chemical structure of the signaling compound(s) that trigger FitF activation. The fact that the DctBp-FitFc

chimera controlled Fit toxin production similarly to wild-type FitF, suggests that the signal molecule may be similar to C_4-dicarboxylates. However, the chimera seemed to respond differentially to changing environmental conditions (Figure 5B and C). In addition and in contrast to DctB [28], the conservative replacement of the important tyrosine residue Y143 by phenylalanine did not diminish Fit toxin expression in the insect medium (Figure 3D). Moreover, certain cells within the population of bacteria with the DctBp-FitFc chimera expressed the insect toxin on plant roots, which was not the case with bacteria expressing wild-type FitF (Figure 5C). These results indicate that the signal molecules recognized by FitFp are no longer (only) C_4-dicarboxylates. Molecules that bind to the sensor domain of FitF could be detected when solving the crystal structure of its periplasmic sensor domain in future studies, as it was demonstrated for several proteins with double-PASp sensor domains in the work of Zhang and Hendrickson [29].

Our findings suggest that even though a DctBp domain may have been at the basis of acquisition of FitF sensory capacity, further adaptive mutations occurred after the domain shuffling event, shifting the spectrum of recognized signals to ensure specificity of toxin production toward the insect environment. Indeed, we found indications that the Fit toxin is produced by wild type *P. protegens* CHA0 in a host-specific manner (Figure 6).

Competitive inhibition by plant molecules as a mechanism for host recognition?

Interestingly, FitD-mCherry expression by *P. protegens* diminished when induction media were supplemented with plant root extract (Figure 2B). We speculate that this may be the result of a competitive inhibition rather than of absence of inducer compounds, because the rest of the induction medium was kept the same. If FitF could be directly or indirectly inhibited by plant molecules, this would explain the observed loss in toxin expression on roots, and could form a mechanism for host (plant or insect) differentiation. Activation of toxin expression in the insect host via FitF would then be the result of absence of inhibiting plant-derived molecules and the simultaneous presence of specific activating signal molecules in insect hemolymph (Figure 7). Competitive interactions are known from studies on DctB, where it was reported that molecules structurally resembling C_4-dicarboxylates (e.g. malonate) can bind to the membrane distal PASp domain of DctB but do not lead to an activation of the kinase by conformational change [17]. The possibility of competition between activating and inhibitory molecules for the signal binding pocket of DctB was not discussed so far, but would be an interesting aspect for future research on PAS sensor domains. Alternatively, the observed inhibition of toxin production on roots could be due to repression of FitF by another protein. In the case of DctB it was suggested that the activity of the sensor kinase can be controlled by the transporter DctA directly by protein-protein interaction [15]. The proposed inhibition of FitF could also be mediated indirectly through changes in the metabolism of the bacterium when growing on roots.

In summary, the present study provides evidence that a virulence-associated sensor histidine kinase, contributing to control the switch of the pseudomonad between a plant-beneficial and an insect pathogenic lifestyle, evolved by acquisition of a prominent sensory domain from a common ancestor of a protein, which regulates carbon uptake and primary carbon metabolism. This event was crucial for the ability of the microorganism to activate toxin expression in insects in a host-specific manner and thus to the adaptation of this bacterium to the insect environment.

To our best knowledge, *P. protegens* at first is well adapted to the life on plant roots. The microorganism acquired and evolved virulence determinants, such as the *fit* cluster, and adapted to the insect environment, allowing it to survive within and to kill larvae of certain insect species. Since two-component signal transduction pathways are often involved in sensing and responding to changing environments, they have played a fundamental role in the adaptation of bacteria to a range of ecological niches [12]. *P. protegens* has the ability to tightly control Fit toxin production in a way that the toxin is only expressed during infection of certain insects but not on plant roots (Figure 6 and [7]). As we show here, FitF thereby plays an important role as a regulatory protein. We recently demonstrated that the Fit toxin is required for full virulence upon oral or systemic infection of insect larvae [5-7]. Therefore, the proposed domain shuffling event during the evolution of FitF has significantly contributed to the adaptation of this bacterium to a new niche and thus to the evolution of insect pathogenicity.

With the existing molecular techniques, the provided reporter constructs, the possibility to induce the expression of the Fit toxin *in vitro* in an insect medium, and the current knowledge about the regulation of Fit toxin expression, the Fit regulatory system could serve as a prime example for future studies on domain shuffling and related molecular mechanisms driving the evolution of sensory systems involved in the regulation of bacterial virulence and on the evolution of pathogenesis in general.

Material and Methods

Bacterial strains, plasmids, media, and culture conditions

All strains and plasmids used in this study are listed in Table S2. Bacteria were routinely cultured in LB (LB Broth Miller, BD Difco), or in nutrient yeast broth (NYB) or on nutrient agar (NA) [34]. *E. coli* cells were grown at 37°C while *P. protegens* was cultured at 25°C. When appropriate, growth media were supplemented with ampicillin (100 µg/ml), chloramphenicol (10 µg/ml), kanamycin (25 µg/ml), gentamicin (10 µg/ml), tetracycline (25 µg/ml or 125 µg/ml for *E. coli* and *P. protegens*, respectively), or isopropyl β-D-1-thiogalactopyranoside (IPTG) (0.1 mM).

For Fit toxin expression studies, the following media were used. LB; Brain Heart Infusion (BHI) (BD Bacto); sterile-filtered Grace's Insect Medium (GIM) (G9771, with L-glutamine, without sodium bicarbonate, adjusted to pH 5.5 with sodium bicarbonate) (Sigma-Aldrich); M9 minimal medium (50 mM $Na_2HPO_4 \times 2\ H_2O$, 22 mM KH_2PO_4, 9 mM NaCl, 19 mM NH_4Cl, 2 mM $MgSO_4$, 0.1 mM $CaCl_2$, 134 µM EDTA, 31 µM $FeCl_3 \times 6\ H_2O$, 6.2 µM $ZnCl_2$, 760 nM $CuCl_2 \times 2\ H_2O$, 420 nM $CoCl_2 \times 2\ H_2O$, 1.62 µM H_3BO_3, 81 nM $MnCl_2 \times 4\ H_2O$, pH 7) with 10 mM L-malate, except for growth curve assays which were performed with 20 mM L-malate; sterile-filtered Fetal Bovine Serum (Invitrogen Gibco); and Marine Broth 2216 (BD Difco). Cold root extracts were prepared by adding 4 g/L of washed and cut roots of field-grown wheat to M9 L-malate or GIM. The mixture was aggitated for 30 min at 300 rpm and room temperature and sterilized by using 5 µm and 0.45 µm filters. Dose-response assays were performed with LB, GIM and different ratios of LB and GIM.

Recombinant DNA techniques

DNA manipulations and PCRs were conducted according to standard protocols [34]. Genomic DNA was extracted using the Promega Wizard Genomic DNA Purification Kit. Plasmid DNA was routinely extracted and purified using the QIAprep Spin Miniprep Kit (Qiagen). Larger scale plasmid preparations were performed with the Genomed JETStar Plasmid Purification Midi Kit. DNA gel extractions were conducted using the MinElute Gel Extraction Kit and the QIAquick Gel Extraction Kit (Qiagen). DNA restriction and modification enzymes were from Promega and were used according to the manufacturer's recommendations. DNA enzyme reaction cleanups were performed using the QIAquick PCR Purification Kit (Qiagen). PCR was routinely conducted using the PrimeSTAR HS high-fidelity DNA polymerase kit (Takara Bio Inc.) for molecular cloning and the GoTaq DNA Polymerase kit (Promega) for analytic purposes according to the recommendations of the manufacturer. Primers used for this study were obtained from Microsynth AG (Balgach, Switzerland) and are listed in Table S3. DNA sequencing was conducted at GATC Biotech (Konstanz, Germany). Sequences were analyzed using the DNASTAR Lasergene software suite.

In-frame deletion of *fitF* and integration of reporter constructs

For the construction of the Δ*fitF* mutant CHA1154, a 2982-bp fragment was deleted in-frame in the *fitF* gene as follows. Using CHA0 DNA as a template, a 722-bp KpnI-EcoRI fragment encompassing the first 42 codons of *fitF* and the adjacent upstream region was amplified by PCR with primers PfitF1 and PfitF2 (Table S3). An 884-bp EcoRI-XbaI fragment comprising the last 41 codons of *fitF* plus downstream region was amplified by PCR using primers PfitF3 and PfitF4. The fragments obtained were digested with KpnI and EcoRI and with EcoRI and XbaI, respectively, and cloned by triple ligation into pUK21 opened with KpnI and XbaI. The 1.6-kb KpnI-XbaI insert in the resulting plasmid was checked by sequencing, excised and cloned into the suicide plasmid pME3087 digested with the same enzymes, giving pME8256 (Table S2). The constructed

replacement vector was then used to delete *fitF* in *P. protegens* CHA0 by D-cycloserine counterselection as described before [35,36], resulting in strain CHA1154 (Table S2). The suicide plasmid pME8217 was used to replace the native *fitD* with the *fitD-mcherry* fusion in strain CHA1154 by homologous recombination, generating strain CHA1174 (Table S2). For insect assays, the strain CHA1174 additionally was marked with a constitutively expressed GFP tag using the Tn7 delivery vector pBKminiTn*7-gfp2*, producing CHA1174-*gfp2* (Table S2).

In vivo site-directed mutagenesis of *fitF* and *fitH*

For the mutagenesis of the periplasmic region of FitF, a region of *fitF* of 979 bp length encompassing the site of interest in the centre was amplified by PCR with CHA0 DNA using the primers fitF-mut1-hr-F and fitF-mut1-hr-R (Table S3). The resulting fragment was digested with EcoRI and BamHI and ligated into the suicide vector pEMG [37] opened with the same enzymes. The insert of the resulting plasmid pME8271 was checked by DNA sequencing. To introduce mutations into the insert sequence of pME8271 to subsequently replace the single amino acid residues R141, Y143, and D149 of FitF, primer pairs fitF-R141A-F/ fitF-R141A-R, fitF-Y143A-F/ fitF-Y143A-R, fitF-Y143F-F/ fitF-Y143F-R, and fitF-D149-F/fitF-D149-R (Table S3), respectively, were used to amplify the vector pME8271 by PCR. The template plasmids used for the PCR were degraded by DpnI for 1 h at 37°C and PCR-amplified vectors were obtained by electroporation of *E. coli* DH5α λpir cells with purified PCR reaction and selection for kanamycin resistance. The insert sequences of the resulting plasmids were controlled by DNA sequencing.

For the replacement of H501 of FitF by alanine, a 489-bp fragment of the upstream region was amplified by PCR with primers fitF-mut2-hr-F and fitF-mut2-R using CHA0 DNA (Table S3). A 524-bp fragment of the downstream region was amplified by PCR using primers fitF-mut2-F and fitF-mut2-hr-R using CHA0 DNA as template. The two fragments were combined by overlap extension PCR using the primers fitF-mut2-hr-F and fitF-mut2-hr-R, creating a 984-bp KpnI-HindIII fragment. The PCR product was digested by KpnI and HindIII and ligated into the plasmid pUK21. The insert was checked by sequencing, excised by digestion with KpnI and BamHI and cloned into the suicide plasmid pEMG by ligation. The resulting plasmid pME8265 was then used to create strain CHA5056 (Table S2).

An analogous approach (leaving out the cloning of the PCR fragment into the plasmid pUK21) was used to create the suicide vector for the replacement of D803 of FitF and D59 of FitH by alanine. For FitF(D803A) the primers fitF-REC-hr-F, fitF-REC-hr-R, fitF-D803A-F, and fitF-D803A-R were used to construct the suicide plasmid pME8302 and create strain CHA5075. For FitH(D59A) the primers fitH-REC-hr-F, fitH-REC-hr-R, fitH-D59A-F, and fitH-D59A-R were used to construct the suicide plasmid pME8303 and generate strain CHA5084. Isogenic mutants of *P. protegens* strain CHA0 were constructed by allelic replacement using the I-SceI system with pEMG. The I-SceI system protocol described by Martinez-Garcia and de Lorenzo [37] was modified for *P.*

protegens for this study. Briefly, the pEMG suicide vector bearing sequences homologous to genomic counterparts was integrated into the chromosome of *P. protegens* via homologous recombination after delivery by electroporation of competent cells. Bacteria were selected for kanamycin resistance on agar plates and competent cells were transformed with the expression plasmid pSW-2 by electroporation. Bacterial cells were selected for gentamicin resistance on agar plates and grown overnight at 30°C in LB supplemented with 10 µg/ml gentamicin. Ten milliliter of fresh LB was inoculated with 2 ml of overnight culture, supplemented with 2 mM m-toluate and 10 µg/ml gentamicin and incubated for 7 h at 30°C to allow second homologous recombinations to occur. Bacterial cultures were diluted and plated on nutrient agar plates without antibiotics. Isolated colonies were screened for kanamycin sensitivity and mutants were identified by specific PCR and sequencing of the respective genomic region.

In-frame deletion of *dctB* homologs

Deletions of the three *dctB* homologs in *P. protegens* CHA0 were performed based on homologous recombinations using the suicide vector pEMG and the I-SceI system.

For the construction of suicide vectors for in-frame gene deletions of CHA0 *dctB* (PFLCHA0_c03070), *dctB2* (PFLCHA0_c48560) and *mifS* (PFLCHA0_c47820), upstream and downstream regions of 500-600 bp length flanking the region to be deleted, encompassing the first five codons and the last 7-18 codons of the open reading frames, were amplified by PCR using the primers listed in Table S3. The resulting BamHI-HindIII fragments were digested with BamHI and HindIII and cloned by triple ligation into pEMG opened with BamHI. Correct insert sequences of the obtained plasmids pME8307, pME8308 and pME8309 for Δ*dctB1*, Δ*dctB2* and Δ*mifS*, respectively, were confirmed by DNA sequencing (Table S2). The constructed suicide plasmids then served to construct strains CHA5085, CHA5090 and CHA5089, respectively, using the I-SceI system (Table S2).

Inducible expression of *fitF*

For complementation of the Δ*fitF* mutant of CHA0, the *fitF* genes of strains *P. protegens* CHA0 and *P. chlororaphis* PCL1391 were cloned under the control of the $P_{tac/laclq}$ promoter and introduced into the unique chromosomal Tn*7* attachment site of strain CHA1174 using the mini-Tn*7* delivery vector pME9411 as follows. Primers fitF-F-SD-new and fitF-R-HindIII were used to amplify the *fitF* gene of strain CHA0 by PCR. The 3.2-kb EcoRI-HindIII fragment was digested with EcoRI and HindIII and ligated into plasmid pME4510 opened with the same restriction enzymes. After blunt-ending the EcoRI restriction site, the fragment was ligated into pME9411 opened with SmaI and HindIII, to obtain pME8288, and the correct insertion was confirmed by sequencing. The pME9411 derivative and the Tn*7* transposition helper plasmid pUX-BF13 were co-electroporated into competent cells of the recipient strain CHA1174 to create strain CHA5066 (Table S2).

An analogous approach was taken to complement the Δ*fitF* mutant of CHA0 *in trans* with *fitF* of strain PCL1391 [5]. A 1188-bp EcoRI–BamHI fragment (primers PCL-fitF-F-SD and PCL-fitF-br-R), a 1704-bp BamHI–StuI fragment (primers PCL-fitF-br-F and PCL-fitF-StuI-R), and a 957-bp StuI–HindIII fragment (primers PCL-fitF-StuI-F and PCL-fitF-R) were amplified by PCR with the indicated primer pairs using chromosomal DNA from strain PCL1391. The individual fragments were digested with the respective restriction enzymes and ligated individually into plasmid pUK21 opened with the same enzymes. The inserts in the resulting plasmids were checked by sequencing. The insert fragments were excised from the plasmids with the respective enzymes and cloned by quadruple ligation into plasmid pME4510 opened with EcoRI and HindIII. After blunt-ending the EcoRI restriction site, the fragment was ligated into pME9411 opened with SmaI and HindIII, and the correct insertion was confirmed by sequencing. The resulting mini-Tn*7*-$P_{tac/laclq}$-*fitF*(PCL1391) delivery plasmid pME8295 then served to generate strain CHA5073 (Table S2).

Construction of the *dctB'-'fitF* and *citA'-'fitF* chimeras

Primers ME8300-F and ME8300-SpeI-R were used to amplify the *lacIq* gene and the IPTG-inducible promoter region of the plasmid pME6032 by PCR. The PCR product was purified, digested with NcoI and HindIII, and ligated into the vector pME6182 opened with the same enzymes. The insert in the resulting plasmid pME8300 was checked by DNA sequencing.

Primers dctB-F-SpeI and dctB-R-overlap were used to amplify an 879-bp fragment of *dctB* using genomic DNA from strain CHA0. Primers fitFc-F and fitF-R-HindIII were used to amplify a 2271-bp fragment of *fitF* by PCR with CHA0 DNA. The two fragments were combined by overlap extension PCR using the primers dctB-F-SpeI and fitF-R-HindIII, creating a 3.3-kb SpeI-HindIII fragment. The PCR product was digested by SpeI and HindIII and ligated into the plasmid pME8300. The insert of the resulting plasmid pME8317 was checked by DNA sequencing. The $P_{tac/laclq}$-*dctB*'-'*fitF* construct was then integrated into the chromosome of the Δ*fitF* mutant of CHA1163 (CHA1174) using the mini-Tn7 delivery system, yielding strain CHA5093 (Table S2).

Analogously, the *citA'-'fitF* chimera was constructed with primer pairs citA-F-SpeI / citA-R-overlap and fitFc-F2 / fitF-R-HindIII using genomic DNA from *E. coli* K-12 and *P. protegens* CHA0, respectively, as a template. The resulting plasmid pME8354 was used to create strain CHA5151 (Table S2).

Quantification of Fit toxin expression in batch cultures using GFP reporters

For assays with transcriptional reporter strains, GFP fluorescence was measured with a BMG FLUOstar Galaxy multidetection microplate reader as detailed previously [7,38].

Quantification of Fit toxin expression in batch cultures by epifluorescence microscopy

Bacterial strains were grown overnight in 10 ml of LB at 25°C and 180 rpm. Bacterial cells were washed once in 0.9% NaCl solution and the optical density at 600 nm was adjusted to 1, if not otherwise specified. Ten milliliters of the respective medium (LB, BHI, marine broth, FBS, M9 L-malate, or GIM) in 50-ml Erlenmeyer flasks was inoculated 1:100 with the bacterial suspension and incubated for 8 h (exponential growth phase) and 24 h (stationary growth phase) at 25°C and 180 rpm. Quantification of red fluorescence intensities of single cells by epifluorescence microscopy was performed as described previously [7]. Exposure times were 2 sec for the DsRed channel and 80 msec for the Ph3 channel. The CHA0 wild-type strain was used to correct for autofluorescence of the bacterial cells.

Bacterial infection of insects and monitoring of Fit toxin expression by epifluorescence microscopy

Injection assays for virulence determination using last-instar larvae of *G. mellonella* (Reptile-food.ch GmbH, Dübendorf, Switzerland) were performed as described before [7]. For complementation assays, IPTG was added to the inoculi to a final concentration of 1 mM. Reporter strains of *P. protegens* CHA0 were in injected in and extracted from forth instar larvae of *S. littoralis* (Syngenta Crop Protection, Stein, Switzerland) and last instar larvae of *T. molitor* (The Animal House, Zuzwil, Switzerland) as described before for *G. mellonella* [7]. *A. pisum* (The Animal House) was infected with reporter strains of *P. protegens* CHA0 by placing 20 adult individuals in a small Petri dish on leaves of white beans (*Phaseolus vulgaris*) that contained drops of bacterial suspensions (at a concentration of 10^8 cfu per ml, 100 µl per dish). After three days of incubation at room temperature, adult aphids were shock frozen in liquid nitrogen, surface-sterilized with 70% ethanol for 2 min and hemolymph was extracted by crushing them on microscope slides. Extracted hemolymph was fixed on 1% agarose pads placed on microscope slides and observed by epifluorescence microscopy as described previously [7].

Monitoring of Fit toxin expression on roots by epifluorescence microscopy

Visualization of Fit toxin expression on tomato (*Solanum lycopersicum* cv. Marmande) and wheat (*Triticum aestivum* cv. Arina) roots was performed as described previously for cucumber [7]. Infection of tomato roots with the crown and root rot pathogen *Fusarium oxysporum* f. sp. *radicis-lycopersici* isolate Forl22 was done as detailed elsewhere [39]. Fit toxin expression on cucumber (*Cucumis sativus* cv. Chinese Snake) roots with the DctBp-FitFc chimera was studied as follows. Cucumber seedlings were grown axenically for three days at room temperature in the dark and inoculated with different reporter strains of *P. protegens* CHA0 by placing them for 30 min in bacterial suspension, which was prepared from an overnight culture in LB by washing

them once in saline solution and adjusting the optical density at 600 nm to 1. The seedlings were then placed into 50-ml tubes (three plants per tube) containing 35-ml of 0.35% (w/v) water agar supplemented with 0.1 mM IPTG, 125 µg/ml tetracycline and 10 µg/ml gentamicin if necessary. The tubes were wrapped in aluminum foil for the lower part to protect roots from light and incubated in a growth chamber set to 80% relative humidity for 16 h with light (160 µE/m^2/s) at 22°C, followed by an 8-h dark period at 18°C. After incubation for five days, roots were individually removed, cut into smaller pieces and placed into Eppendorf tubes containing 100 µl of saline solution supplemented with 0.1% Silwet L-77 for the isolation of the bacteria (GE Bayer Silicones Sàrl, Switzerland). The mixture was vigorously agitated for 2 min and 5 µl were used for epifluorescence microscopy as described above. Quantification of single cell fluorescence was performed by using the GFP (2 sec exposure time) and DsRed (2 sec exposure time) channels.

Bioinformatics

Homologs of the periplasmic domains of *P. protegens* FitF were identified from the NCBI nonredundant protein sequence database using PSI-BLAST and an E-value cutoff of 1e-12 [40]. Periplasmic regions of membrane-bound proteins were determined by predicting transmembrane regions using DAS [41] and PRED-TMR (http://athina.biol.uoa.gr/PRED-TMR/input.html). Functional domains of proteins were predicted using the NCBI Conserved Domain Search [42] and SMART [43] with default parameters. Multiple sequence alignments including sequences from reference proteins with known functions were performed with MAFFT version 7 (http://mafft.cbrc.jp/alignment/server) and phylogenetic analyses were conducted in MEGA5 using the Minimum Evolution method for inferring the evolutionary history [44]. Cluster analyses were performed with CLANS [45] as described earlier [46] using 2D clustering with default parameters.

Secondary and tertiary structure predictions of the periplasmic region of FitF were performed using ESyPred3D [47], I-TASSER [48], LOMETS [49], Phyre2 [50], SABLE (http://sable.cchmc.org), and SWISS-MODEL [51] using default parameters and the crystal structure of the *V. cholerae* DctB sensor domain (3BY9) as template if required. Structure models were visualized using the Swiss-PdbViewer version 4.0.3 (http://spdbv.vital-it.ch).

Statistical analysis

Significant differences between treatments or strains were calculated in R version 2.13.1 (http://www.r-project.org) by one-way or two-way analysis of variance (ANOVA) with Tukey's HSD test for post-hoc comparisons. The Log-Rank test of the Survival package of R was used to calculate significant differences in insect toxicity between *P. protegens* CHA0 and isogenic mutant strains in the *Galleria* injection assay.

Acknowledgments

We would like to thank Jan Roelof van der Meer and Karine Lapouge for fruitful discussions and carefully reading the manuscript, and Sophie Martin for critically reading the manuscript. We gratefully acknowledge the help of Christian Ahrens and Stefan Zoller with the phylogenetic analysis of FitF. We thank Jérôme Wassef and Karent Paola Bermudez Valdes for technical assistance.

References

1. Haas D, Défago G (2005) Biological control of soil-borne pathogens by fluorescent pseudomonads. Nat Rev Microbiol 3: 307-319.
2. Ramette A, Frapolli M, Fischer-Le Saux M, Gruffaz C, Meyer JM, et al. (2011) *Pseudomonas protegens* sp nov., widespread plant-protecting bacteria producing the biocontrol compounds 2,4-diacetylphloroglucinol and pyoluteorin. Syst Appl Microbiol 34: 180-188.
3. Kupferschmied P, Maurhofer M, Keel C (2013) Promise for plant pest control: root-associated pseudomonads with insecticidal activities. Front Plant Sci 4: 287.
4. Loper JE, Hassan KA, Mavrodi DV, Davis EW, Lim CK, et al. (2012) Comparative genomics of plant-associated *Pseudomonas* spp.: insights into diversity and inheritance of traits involved in multitrophic nteractions. PLoS Genet 8: e1002784.
5. Ruffner B, Péchy-Tarr M, Ryffel F, Hoegger P, Obrist C, et al. (2013) Oral insecticidal activity of plant-associated pseudomonads. Environ Microbiol 15: 751-763.
6. Péchy-Tarr M, Bruck DJ, Maurhofer M, Fischer E, Keel C (2008) Molecular analysis of a novel gene cluster encoding an insect toxin in plant-associated strains of *Pseudomonas fluorescens*. Environ Microbiol 10: 2368-2386.
7. Péchy-Tarr M, Borel N, Kupferschmied P, Turner V, Binggeli O, et al. (2013) Control and host-dependent activation of insect toxin expression in a root-associated biocontrol pseudomonad. Environ Microbiol 15: 736-750.
8. Gao R, Stock AM (2009) Biological insights from structures of two-component proteins. Annu Rev Microbiol 63: 133-154.
9. Krell T, Lacal J, Busch A, Silva-Jimenez H, Guazzaroni ME, et al. (2010) Bacterial sensor kinases: diversity in the recognition of environmental signals. Annu Rev Microbiol 64: 539-559.
10. Alm E, Huang K, Arkin A (2006) The evolution of two-component systems in bacteria reveals different strategies for niche adaptation. PLoS Comput Biol 2: e143.
11. Galperin MY (2005) A census of membrane-bound and intracellular signal transduction proteins in bacteria: bacterial IQ, extroverts and introverts. BMC Microbiol 5: 35.
12. Capra EJ, Laub MT (2012) Evolution of two-component signal transduction systems. Annu Rev Microbiol 66: 325-347.
13. Raghavan V, Groisman EA (2010) Orphan and hybrid two-component system proteins in health and disease. Curr Opin Microbiol 13: 226-231.
14. Jung K, Fried L, Behr S, Heermann R (2012) Histidine kinases and response regulators in networks. Curr Opin Microbiol 15: 118-124.
15. Scheu PD, Kim OB, Griesinger C, Unden G (2010) Sensing by the membrane-bound sensor kinase DcuS: exogenous versus endogenous sensing of C(4)-dicarboxylates in bacteria. Future Microbiol 5: 1383-1402.
16. Cheung J, Hendrickson WA (2008) Crystal structures of C-4-dicarboxylate ligand complexes with sensor domains of histidine kinases DcuS and DctB. J Biol Chem 283: 30256-30265.
17. Zhou YF, Nan BY, Nan J, Ma QJ, Panjikar S, et al. (2008) C(4)-dicarboxylates sensing mechanism revealed by the crystal structures of DctB sensor domain. J Mol Biol 383: 49-61.
18. Cheung J, Hendrickson WA (2010) Sensor domains of two-component regulatory systems. Curr Opin Microbiol 13: 116-123.
19. Zhang W, Shi L (2005) Distribution and evolution of multiple-step phosphorelay in prokaryotes: lateral domain recruitment involved in the formation of hybrid-type histidine kinases. Microbiology 151: 2159-2173.
20. Chang C, Tesar C, Gu M, Babnigg G, Joachimiak A, et al. (2010) Extracytoplasmic PAS-like domains are common in signal transduction proteins. J Bacteriol 192: 1156-1159.
21. Henry JT, Crosson S (2011) Ligand-binding PAS domains in a genomic, cellular, and structural context. Annu Rev Microbiol 65: 261-286.

22. Stephenson K, Hoch JA (2002) Evolution of signalling in the sporulation phosphorelay. Mol Microbiol 46: 297-304.
23. Riechmann L, Winter G (2000) Novel folded protein domains generated by combinatorial shuffling of polypeptide segments. Proc Natl Acad Sci U S A 97: 10068-10073.
24. Möglich A, Ayers RA, Moffat K (2010) Addition at the molecular level: signal integration in designed Per-ARNT-Sim receptor proteins. J Mol Biol 400: 477-486.
25. Checa SK, Zurbriggen MD, Soncini FC (2012) Bacterial signaling systems as platforms for rational design of new generations of biosensors. Curr Opin Biotechnol 23: 766-772.
26. Grace TD (1962) Establishment of four strains of cells from insect tissues grown in vitro. Nature 195: 788-789.
27. Shen X, Hu H, Peng H, Wang W, Zhang X (2013) Comparative genomic analysis of four representative plant growth-promoting rhizobacteria in *Pseudomonas*. BMC Genomics 14: 271.
28. Nan BY, Liu X, Zhou YF, Liu JW, Zhang L, et al. (2010) From signal perception to signal transduction: ligand-induced dimeric switch of DctB sensory domain in solution. Mol Microbiol 75: 1484-1494.
29. Zhang Z, Hendrickson WA (2010) Structural characterization of the predominant family of histidine kinase sensor domains. J Mol Biol 400: 335-353.
30. Etzkorn M, Kneuper H, Dunnwald P, Vijayan V, Kramer J, et al. (2008) Plasticity of the PAS domain and a potential role for signal transduction in the histidine kinase DcuS. Nat Struct Mol Biol 15: 1031-1039.
31. Valentini M, Storelli N, Lapouge K (2011) Identification of C(4)-dicarboxylate transport systems in *Pseudomonas aeruginosa* PAO1. J Bacteriol 193: 4307-4316.
32. Petrova OE, Sauer K (2009) A novel signaling network essential for regulating *Pseudomonas aeruginosa* biofilm development. PLoS Pathog 5: e1000668.
33. Qian W, Han ZJ, He C (2008) Two-component signal transduction systems of *Xanthomonas* spp.: a lesson from genomics. Mol Plant Microbe Interact 21: 151-161.
34. Sambrook J, Russel DW (2001) Molecular Cloning: A Laboratory Manual. Cold Spring Harbor (New York): Cold Spring Harbor Laboratory Press.
35. Schnider-Keel U, Seematter A, Maurhofer M, Blumer C, Duffy B, et al. (2000) Autoinduction of 2,4-diacetylphloroglucinol biosynthesis in the biocontrol agent *Pseudomonas fluorescens* CHA0 and repression by the bacterial metabolites salicylate and pyoluteorin. J Bacteriol 182: 1215-1225.
36. Défago G, Haas D (1990) Pseudomonads as antagonists of soilborne plant pathogens: modes of action and genetic analysis. In: Bollag JM, Stotzky G, editors. Soil Biochemistry. New York, USA: Marcel Dekker. pp. 249–291.
37. Martinez-Garcia E, de Lorenzo V (2011) Engineering multiple genomic deletions in Gram-negative bacteria: analysis of the multi-resistant antibiotic profile of *Pseudomonas putida* KT2440. Environ Microbiol 13: 2702-2716.
38. Baehler E, Bottiglieri M, Pechy-Tarr M, Maurhofer M, Keel C (2005) Use of green fluorescent protein-based reporters to monitor balanced production of antifungal compounds in the biocontrol agent *Pseudomonas fluorescens* CHA0. J Appl Microbiol 99: 24-38.
39. Sharifi-Tehrani A, Zala M, Natsch A, Moenne-Loccoz Y, Defago G (1998) Biocontrol of soil-borne fungal plant diseases by 2,4-diacetylphloroglucinol-producing fluorescent pseudomonads with different restriction profiles of amplified 16S rDNA. Eur J Plant Pathol 104: 631-643.
40. Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, et al. (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res 25: 3389-3402.
41. Cserzo M, Wallin E, Simon I, von Heijne G, Elofsson A (1997) Prediction of transmembrane alpha-helices in prokaryotic membrane proteins: the dense alignment surface method. Protein Eng 10: 673-676.
42. Marchler-Bauer A, Lu S, Anderson JB, Chitsaz F, Derbyshire MK, et al. (2011) CDD: a Conserved Domain Database for the functional annotation of proteins. Nucleic Acids Res 39: D225-229.
43. Schultz J, Milpetz F, Bork P, Ponting CP (1998) SMART, a simple modular architecture research tool: identification of signaling domains. Proc Natl Acad Sci U S A 95: 5857-5864.
44. Tamura K, Peterson D, Peterson N, Stecher G, Nei M, et al. (2011) MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol 28: 2731-2739.

45. Frickey T, Lupas A (2004) CLANS: a Java application for visualizing protein families based on pairwise similarity. Bioinformatics 20: 3702-3704.
46. Krämer J, Fischer JD, Zientz E, Vijayan V, Griesinger C, et al. (2007) Citrate sensing by the C-4-dicarboxylate/citrate sensor kinase DcuS of *Escherichia coli*: Binding site and conversion of DcuS to a C-4-dicarboxylate- or citrate-specific sensor. J Bacteriol 189: 4290-4298.
47. Lambert C, Leonard N, De Bolle X, Depiereux E (2002) ESyPred3D: Prediction of proteins 3D structures. Bioinformatics 18: 1250-1256.
48. Roy A, Kucukural A, Zhang Y (2010) I-TASSER: a unified platform for automated protein structure and function prediction. Nat Protoc 5: 725-738.
49. Wu S, Zhang Y (2007) LOMETS: a local meta-threading-server for protein structure prediction. Nucleic Acids Res 35: 3375-3382.
50. Kelley LA, Sternberg MJ (2009) Protein structure prediction on the Web: a case study using the Phyre server. Nat Protoc 4: 363-371.
51. Arnold K, Bordoli L, Kopp J, Schwede T (2006) The SWISS-MODEL workspace: a web-based environment for protein structure homology modelling. Bioinformatics 22: 195-201.

Supporting Information

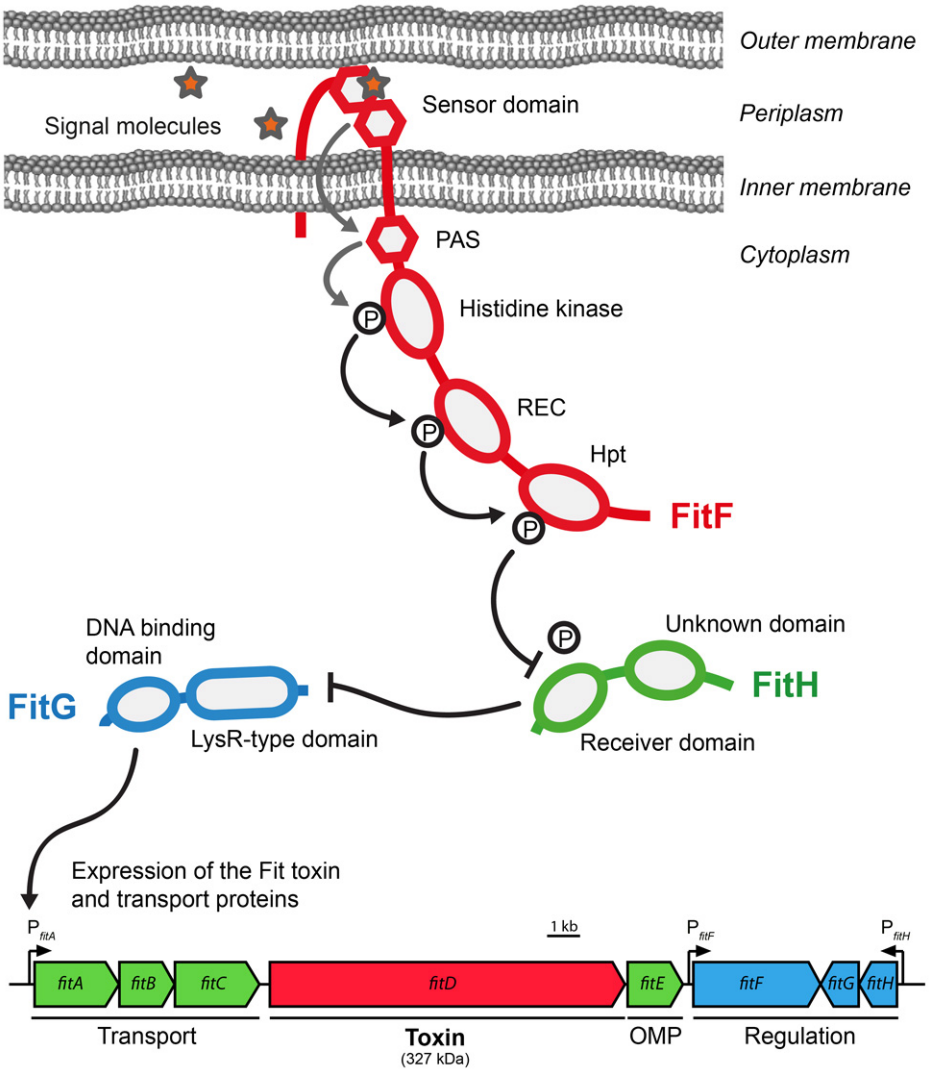

Figure S1. Model for the local regulation of Fit toxin expression in *Pseudomonas protegens*. The histidine kinase-response regulator hybrid FitF recognizes so far unknown signal molecules with its periplasmic sensor domain. During infection of the insect host, FitF undergoes a conformational change which autophosphorylates the histidine kinase. Subsequent phosphotransfer reactions lead to the inactivation of the repressor FitH by phosphorylation of a conserved aspartate residue by FitF. When FitH gets inactivated, the inducer FitG is released from repression and drives the transcription of the *fitABCDE* operon and thus activates the expression of the Fit toxin.

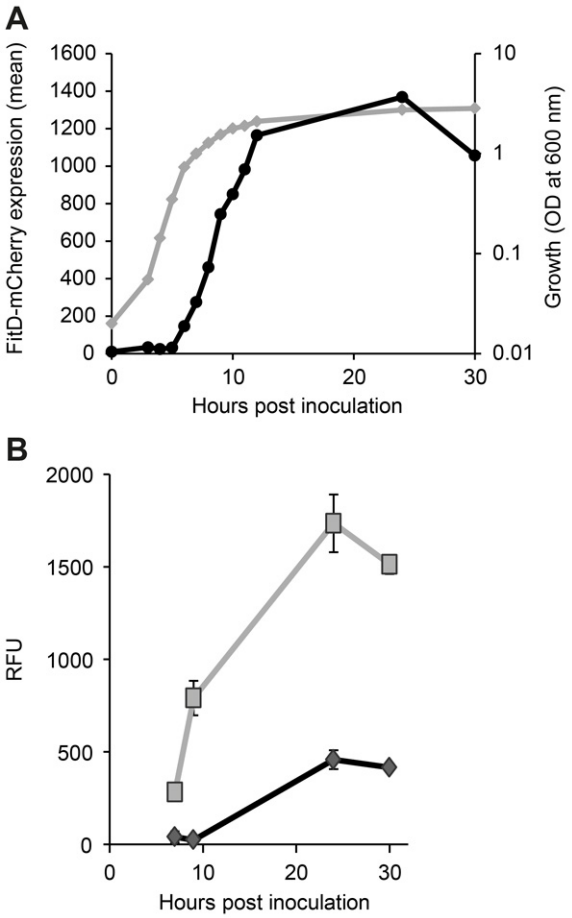

Figure S2. Fit toxin expression over time in the insect medium. (A) Expression of FitD-mCherry over time in the wild-type background of strain CHA0 (CHA1163) grown in GIM (black circles). Bacterial growth (recorded as the optical density of the culture at 600 nm) is displayed for the corresponding time points (gray diamonds). Shown are population averages from a single culture of CHA1163. (B) P_{fitA} promoter activity in the wild-type strain of CHA0 (CHA0 pME8203) grown in LB (black diamonds) and GIM (gray squares) over time. Results are the mean and standard deviation of population averages from three independent cultures. RFU, relative fluorescence units. Both experiments were repeated at least twice with similar results.

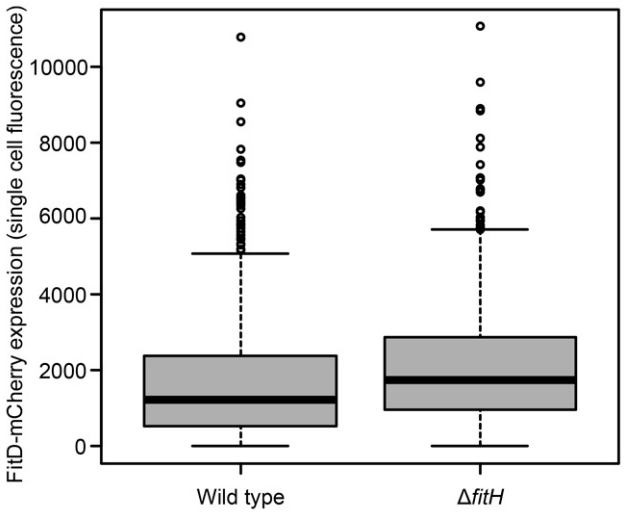

Figure S3. Highly induced toxin expression in the insect medium. Fit toxin expression in the insect medium in the wild-type and *fitH* deletion mutant background of *P. protegens* CHA0. Shown are single cell fluorescence intensities of one single bacterial culture incubated for 24 h at 25°C (n = on average 960 cells per strain).

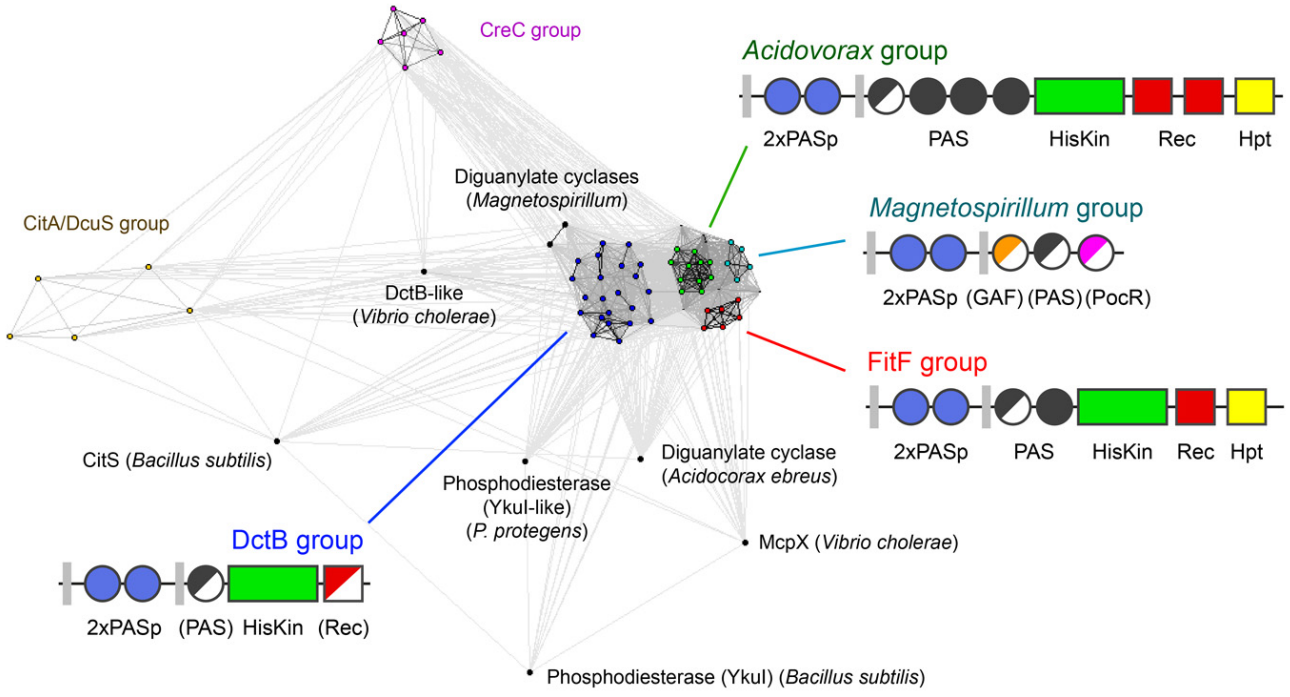

Figure S4. Cluster analysis of proteins with sensor domains homologous to DctBp. CLANS cluster analysis of periplasmic sensor domains of FitF, DctB, and of proteins with sequence or structural homology to DctBp. Predicted domain topologies are shown for groups of interest. Domains that are displayed in half do not exist in all proteins of the respective group. Protein identifications and the corresponding sequences can be found in Table S1 and File S1, respectively.

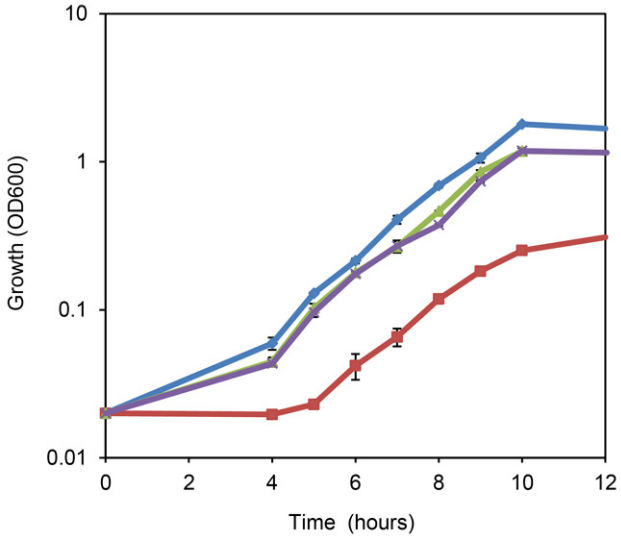

Figure S5. Growth curves of wild-type CHA0 and isogenic *dctB* mutants. The wild-type (blue) and isogenic mutant strains (CHA5085, Δ*dctB*, in red; CHA5089, Δ*mifS*, in green; CHA5090, Δ*dctB2*, in purple) of *P. protegens* CHA0 were grown in M9 minimal medium with L-malate as sole carbon source and growth (optical density at 600 nm) was recorded over time. Shown are means and standard deviations of three independent cultures. In some instances, the standard deviation bars are smaller than the symbols used. The experiment was repeated twice with similar results.

File S1. Protein sequences of periplasmic regions of proteins used in this study.

The file can be obtained from the PLoS Pathogen website:

http://journals.plos.org/plospathogens/article/asset?unique&id=info:doi/10.1371/journal.ppat.1003964.s006

Table S1. **Proteins used for phylogenetic and CLANS cluster analysis of FitFp.**

Group	Protein ID	Accession number (protein name, locus tag)	Bacterial strain
Acidovorax	24	ZP_09331456.1	*Acidovorax* sp. NO-1
Acidovorax	29	YP_006855860.1	*Acidovorax* sp. KKS102
Acidovorax	31	ZP_08946908.1	*Acidovorax radicis* N35
Acidovorax	32	YP_002552439.1	*Acidovorax ebreus* TPSY
Acidovorax	33	ZP_10391000.1	*Acidovorax* sp. CF316
Acidovorax	34	ZP_04761532.1	*Acidovorax delafieldii* 2AN
Acidovorax	36	ZP_08872608.1	*Verminephrobacter aporrectodeae* subsp. *tuberculatae* At4
Acidovorax	38	YP_004235565.1	*Acidovorax avenae* subsp. *avenae* ATCC 19860
Acidovorax	39	YP_970419.1	*Acidovorax citrulli* AAC00-1
CitA/DcuS	44	WP_001423264 (DcuS)	*Escherichia coli*
CitA/DcuS	45	NP_388326 (DctS)	*Bacillus subtilis* subsp. *subtilis* str. 168
CitA/DcuS	46	NP_391030 (MalK)	*Bacillus subtilis*
CitA/DcuS	47	YP_002240511 (CitA)	*Klebsiella pneumonia* 342
CitA/DcuS	48	WP_001498225 (CitA)	*Escherichia coli*
CreC	0	WP_020233602 (CreC)	*Escherichia coli*
CreC	1	EGK28744.1 (CreC)	*Shigella flexneri* K-218
CreC	2	ZP_06542774.1 (CreC)	*Salmonella enterica* subsp. *enterica* serovar Typhi str. AG3
CreC	3	YP_002917719.1 (CreC)	*Klebsiella pneumoniae* subsp. *pneumoniae* NTUH-K2044

CreC	4	NP_249155 (CreC)	*Pseudomonas aeruginosa* PAO1
CreC	5	EKM28643.1 (CreC)	*Vibrio cholerae* HENC-02
DctB	6	NP_438064 (DctB)	*Sinorhizobium meliloti* 1021
DctB	7	NP_437406	*Sinorhizobium meliloti* 1021
DctB	8	NP_231559 (DctB)	*Vibrio cholerae* O1 biovar El Tor str. N16961
DctB	9	NP_253852 (DctB, PA5165)	*Pseudomonas aeruginosa* PAO1
DctB	10	NP_254199 (MifS, PA5512)	*Pseudomonas aeruginosa* PAO1
DctB	11	NP_250027 (PA1336)	*Pseudomonas aeruginosa* PAO1
DctB	12	AGL82108 or YP_007997612 (DctB, PFLCHA0_c03070)	*Pseudomonas protegens* CHA0
DctB	13	AGL86606 or YP_008002110 (DctB2, PFLCHA0_c48560)	*Pseudomonas protegens* CHA0
DctB	14	AGL86532 or YP_008002036 (MifS-like, PFLCHA0_c47820)	*Pseudomonas protegens* CHA0
DctB	42	YP_004482858.1	*Marinomonas posidonica* IVIA-Po-181
DctB	43	ZP_12910956.1	*Agrobacterium tumefaciens* CCNWGS0286
DctB	50	WP_008902769.1	*Acidovorax* sp. NO-1
DctB	51	YP_002551517.1	Acidovorax ebreus TPSY
DctB	52	ZP_08950390.1	*Acidovorax radicis* N35
DctB	53	ZP_08946594.1	*Acidovorax radicis* N35
DctB	54	ZP_10388110.1	*Acidovorax* sp. CF316
DctB	55	ZP_10393609.1	*Acidovorax* sp. CF316
DctB	57	YP_421020.1	*Magnetospirillum magneticum* AMB-1
DctB	59	YP_001902255.1	*Xanthomonas campestris* pv. *campestris* str. B100
DctB	65	WP_010466438.1	*Acidovorax radicis*
DctB	66	WP_010459261.1	*Acidovorax radicis*
DctB	68	YP_004232774.1	*Acidovorax avenae* subsp. *avenae* ATCC 19860
DctB	69	YP_006856794.1	*Acidovorax* sp. KKS102
FitF	15	ABY91232 (FitF)	*Pseudomonas protegens* CHA0
FitF	16	EJM00432 (FitF)	*Pseudomonas* sp. GM17
FitF	17	(FitF)	*Pseudomonas chlororaphis* PCL1391
FitF	18	(FitF)	*Pseudomonas* sp. CMR12a
FitF	19	EIM16861 (FitF)	*Pseudomonas chlororaphis* O6
FitF	20	EJL06947 (FitF)	*Pseudomonas chlororaphis* subsp. *aureofaciens* 30-84
Magnetospirillum	25	ZP_00207923.1	*Magnetospirillum magnetotacticum* MS-1
Magnetospirillum	26	YP_422297.1	*Magnetospirillum magneticum* AMB-1
Magnetospirillum	27	ZP_23007253.1	*Magnetospirillum* sp. SO-1
Magnetospirillum	28	ZP_23002113.1	*Magnetospirillum* sp. SO-1
Magnetospirillum	35	YP_423510.1	*Magnetospirillum magneticum* AMB-1
PhoQ	70	NP_249871 (PA1180)	*Pseudomonas aeruginosa* PAO1

Table S2. **Bacterial strains and plasmids used in this study.**

Strain or plasmid	Genotype, phenotype or relevant characteristics	Reference or source
Pseudomonas protegens		
CHA0	Wild type	[1]
CHA0-*gfp2*	CHA0::attTn*7*-*gfp2*; Gm^r	[2]

CHA1151	Δ*fitD* in-frame deletion mutant of CHA0	[3]
CHA1154	Δ*fitF* in-frame deletion mutant of CHA0	This study
CHA1163	CHA0::*fitD-mcherry*; CHA0 derivative expressing a C-terminal FitD–mCherry fusion protein	[2]
CHA1174	CHA1154::*fitD-mcherry*	This study
CHA1174-*gfp2*	CHA1174::attTn*7*-*gfp2*; Gm^r	This study
CHA1175	Δ*fitH* in-frame deletion mutant of CHA1163	[2]
CHA1176	CHA1163::attTn*7*-*gfp2*; Gm^r	[2]
CHA1178	CHA1175::attTn*7*-*gfp2*; Gm^r	[2]
CHA1185	CHA0::attTn7-$P_{tac/laclq}$-*fitG*; inducible expression of *fitG* under the control of the $P_{tac/laclq}$ promoter in CHA0; Gm^r	[2]
CHA5056	CHA1163 *fitF*(H501A); reporter strain of CHA0 — expression of FitF(H501A) in a FitD-mCherry	This study
CHA5061	CHA1163 *fitF*(R141A); reporter strain of CHA0 — expression of FitF(R141A) in a FitD-mCherry	This study
CHA5062	CHA1163 *fitF*(Y143F); reporter strain of CHA0 — expression of FitF(Y143F) in a FitD-mCherry	This study
CHA5066	CHA1174::attTn*7*-$P_{tac/laclq}$-*fitF*; inducible expression of *fitF* from CHA0 under the control of the $P_{tac/laclq}$ promoter in CHA1174; Gm^r	This study
CHA5070	CHA1163 *fitF*(D149A); reporter strain of CHA0 — expression of FitF(D149A) in a FitD-mCherry	This study
CHA5071	CHA1163 *fitF*(Y143A); reporter strain of CHA0 — expression of FitF(Y143A) in a FitD-mCherry	This study
CHA5073	CHA1174::attTn*7*-$P_{tac/laclq}$-*fitF*; inducible expression of *fitF* from PCL1391 under the control of the $P_{tac/laclq}$ promoter in CHA1174; Gm^r	This study
CHA5075	CHA1163 *fitF*(D803A); reporter strain of CHA0 — expression of FitF(D803A) in a FitD-mCherry	This study
CHA5084	CHA1163 *fitH*(D59A); expression of FitH(D59A) in a FitD-mCherry reporter strain of CHA0	This study
CHA5085	Δ*dctB* (PFLCHA0_c03070) in-frame deletion mutant of CHA0	This study
CHA5089	Δ*mifS* (PFLCHA0_c47820) in-frame deletion mutant of CHA0	This study
CHA5090	Δ*dctB2* (PFLCHA0_c48560) in-frame deletion mutant of CHA0	This study
CHA5093	CHA1174::attTn7-$P_{tac/laclq}$-*dctB*'-'*fitF*; inducible expression of a DctBp-FitFc chimeric protein under the control of the $P_{tac/laclq}$ promoter in CHA1174; Gm^r	This study
CHA5150	attTn7-$P_{tac/laclq}$-*dctB*'-'*fitF*; inducible expression of a DctBp-FitFc chimeric protein under the control of the $P_{tac/laclq}$ promoter in CHA0 wild type; Gm^r	This study
CHA5151	CHA1174::attTn7-$P_{tac/laclq}$-*citA*'-'*fitF*; inducible expression of a CitAp-FitFc chimeric protein under the control of the $P_{tac/laclq}$ promoter in CHA1174; Gm^r	This study
Pseudomonas chlororaphis		
PCL1391	Wild type	[4]
Escherichia coli strains		
DH5α, DH5α λpir, HB101, K-12	Laboratory strains	[5]
Plasmids		
pBK-miniTn7-*gfp1*	pUC19-based delivery plasmid for miniTn7-*gfp1*; *mob*$^+$; Km^r, Cm^r, Ap^r	[6]
pBK-miniTn7-*gfp2*	pUC19-based delivery plasmid for miniTn7-*gfp2*; *mob*$^+$; Gm^r, Cm^r, Ap^r	[6]
pEMG	pSEVA212S; *ori*R6K, *lacZα* MCS flanked by two I-SceI sites; Km^r, Ap^r	[7]
pME497	Mobilizing plasmid; Ap^r	[8]
pME3087	Suicide vector; ColE1 replicon; RK2-mob; Tc^r	[8]
pME4510	Broad host range promoter-probe plasmid vector for Gram-negative bacteria; Gm^r	[9]
pME6182	Carrier plasmid for Tn*7* containing the mini Tn*7*-Gm transposon; Ap^r, Gm^r	[10]

pME8203	pPROBE-TT with a *fitA-gfp* transcriptional fusion; Tc^r	[2]
pME8217	Suicide vector for 3'-tagging of *fitD* with *mche* in strain CHA0; Tc^r	[2]
pME8256	pME3087-Δ*fitF*; suicide plasmid for the in-frame deletion of *fitF* in CHA0; Tc^r	This study
pME8265	pEMG-*fitF'*(H501A); fragment of *fitF* containing the mutation for H501A in FitF; Km^r	This study
pME8271	pEMG-*fitFp*-region; fragment of *fitF* for site-directed mutagenesis of the sensory domain of FitF; Km^r	This study
pME8275	pEMG-*fitF'*(R141A); fragment of *fitF* containing the mutation for R141A in FitF; Km^r	This study
pME8276	pEMG-*fitF'*(Y143F); fragment of *fitF* containing the mutation for Y143F in FitF; Km^r	This study
pME8288	pME9411-$P_{tac/laclq}$-*fitF*(CHA0); IPTG-inducible expression of *fitF* from CHA0; Gm^r, Ap^r	This study
pME8295	pME9411-$P_{tac/laclq}$-*fitF*(PCL1391); IPTG-inducible expression of *fitF* from PCL1391; Gm^r, Ap^r	This study
pME8298	pEMG-*fitF'*(D149A); fragment of *fitF* containing the mutation for D149A in FitF; Km^r	This study
pME8299	pEMG-*fitF'*(Y143A); fragment of *fitF* containing the mutation for Y143A in FitF; Km^r	This study
pME8300	pME9411 optimized for cloning; Gm^r, Ap^r	This study
pME8302	pEMG-*fitF'*(D803A); fragment of *fitF* containing the mutation for D803A in FitF; Km^r	This study
pME8303	pEMG-*fitH'*(D59A); fragment of *fitH* containing the mutation for D59A in FitH; Km^r	This study
pME8307	pEMG-Δ*dctB1*; suicide plasmid for the in-frame deletion of PFLCHA0_c03070 (putative *dctB*) in CHA0; Km^r	This study
pME8308	pEMG-Δ*dctB2*; suicide plasmid for the in-frame deletion of PFLCHA0_c48560 (putative *dctB2*) in CHA0; Km^r	This study
pME8309	pEMG-Δ*mifS*; suicide plasmid for the in-frame deletion of PFLCHA0_c47820 (putative *mifS*) in CHA0; Km^r	This study
pME8317	pME8300-$P_{tac/laclq}$-*dctB'-'fitF*; IPTG-inducible expression of a *dctB'-'fitF* hybrid; Gm^r, Ap^r	This study
pME8354	pME8300-$P_{tac/laclq}$-*citA'-'fitF*; IPTG-inducible expression of a *dctB'-'fitF* hybrid; Gm^r, Ap^r	This study
pME9411	Carrier plasmid for Tn*7* derived from pME6182 and pME6032 for $P_{tac/laclq}$ controlled target gene expression; Gm^r, Ap^r	[2]
pPROBE-TT	Promoter-probe vector based on eGFP; Tc^r	[11]
pSW-2	*ori*RK2, *xylS*, P_m::*I-sce*I; Gm^r	[7]
pUK21	Cloning vector; Km^r	[12]
pUX-BF13	Helper plasmid encoding Tn*7* transposition functions; R6K-replicon; Ap^r	[13]

Abbreviations: Ap^r, ampicillin; Cm^r, chloramphenicol; Gm^r, gentamicin; Km^r, kanamycin; and Tc^r, tetracycline resistance, respectively.

References:

1. Stutz E, Défago G, Kern H (1986) Naturally occurring fluorescent pseudomonads involved in suppression of black root rot of tobacco. Phytopathology 76: 181–185.
2. Péchy-Tarr M, Borel N, Kupferschmied P, Turner V, Binggeli O, et al. (2013) Control and host-dependent activation of insect toxin expression in a root-associated biocontrol pseudomonad. Environ Microbiol 15: 736-750.
3. Péchy-Tarr M, Bruck DJ, Maurhofer M, Fischer E, Keel C (2008) Molecular analysis of a novel gene cluster encoding an insect toxin in plant-associated strains of *Pseudomonas fluorescens*. Environ Microbiol 10: 2368-2386.
4. Chin-A-Woeng TFC, Bloemberg GV, van der Bij AJ, van der Drift KMGF, Schripsema J, et al. (1998) Biocontrol by phenazine-1-carboxamide-producing *Pseudomonas chlororaphis* PCL1391 of tomato

root rot caused by *Fusarium oxysporum* f. sp. *radicis-lycopersici*. Mol Plant Microbe Interact 11: 1069-1077.
5. Sambrook J, Russel DW (2001) Molecular Cloning: A Laboratory Manual. Cold Spring Harbor (New York): Cold Spring Harbor Laboratory Press.
6. Koch B, Jensen LE, Nybroe O (2001) A panel of Tn*7*-based vectors for insertion of the *gfp* marker gene or for delivery of cloned DNA into Gram-negative bacteria at a neutral chromosomal site. J Microbiol Methods 45: 187-195.
7. Martinez-Garcia E, de Lorenzo V (2011) Engineering multiple genomic deletions in Gram-negative bacteria: analysis of the multi-resistant antibiotic profile of *Pseudomonas putida* KT2440. Environ Microbiol 13: 2702-2716.
8. Voisard C, Bull C, Keel C, Laville J, Maurhofer M, et al. (1994) Biocontrol of root diseases by *Pseudomonas fluorescens* CHA0: current concepts and experimental approaches. In: O'Gara F, Dowling D, Boesten B, editors. Molecular ecology of rhizosphere microorganisms: biotechnology and the release of GMOs. Weinheim: VCH Publishers. pp. 67-89.
9. Rist M, Kertesz MA (1998) Construction of improved plasmid vectors for promoter characterization in *Pseudomonas aeruginosa* and other Gram-negative bacteria. FEMS Microbiol Lett 169: 179-183.
10. Péchy-Tarr M, Bottiglieri M, Mathys S, Lejbolle KB, Schnider-Keel U, et al. (2005) RpoN (sigma54) controls production of antifungal compounds and biocontrol activity in *Pseudomonas fluorescens* CHA0. Mol Plant Microbe Interact 18: 260-272.
11. Miller WG, Leveau JHJ, Lindow SE (2000) Improved *gfp* and *inaZ* broad-host-range promoter-probe vectors. Mol Plant Microbe Interact 13: 1243-1250.
12. Vieira J, Messing J (1991) New pUC-derived cloning vectors with different selectable markers and DNA replication origins. Gene 100: 189-194.
13. Bao Y, Lies DP, Fu H, Roberts GP (1991) An Improved Tn*7*-Based System for the Single-Copy Insertion of Cloned Genes into Chromosomes of Gram-Negative Bacteria. Gene 109: 167-168.

Table S3. **Primers used in this study.**

Name	Sequence 5' → 3', restriction enzyme(s)[1]	Usage
citA-F-SpeI	GACTAGTATGTTGCAGCTTAACGAGAATAAAC	*citA'-'fitF* chimera
citA-R-overlap	CCCACAGCAACAGTAAGAACACCAGAATCCCTAACAGCACGAC	*citA'-'fitF* chimera
dctB-F-SpeI	GACTAGTATGACCCCACCCCTTCCCC, SpeI	*dctB'-'fitF* chimera
dctB-R-overlap	CCGCCCCACAGCAACAGTAACAGCACCAGCAGCGCGG	*dctB'-'fitF* chimera
dctB1-1	CGGGATCCAACCCGCTGAGTGTCTAC, BamHI	Deletion of PFLCHA0_c03070 (*dctB*)
dctB1-2	CCCAAGCTTAAGGGGTGGGGTCATGGAAAAG, HindIII	Deletion of PFLCHA0_c03070 (*dctB*)
dctB1-3	CCCAAGCTTTCCGAGGACCGCGGGGT, HindIII	Deletion of PFLCHA0_c03070 (*dctB*)
dctB1-4	CGGGATCCAGTTCCTGACGGTCGCTGAG, BamHI	Deletion of PFLCHA0_c03070 (*dctB*)
dctB2-1	CGGGATCCGGAAGAGGCCACCAAGAAAGG, BamHI	Deletion of PFLCHA0_c48560 (*dctB2*)
dctB2-2	CCCAAGCTTGGGGTCGCATTTCATCACAGAT, HindIII	Deletion of PFLCHA0_c48560 (*dctB2*)
dctB2-3	CCCAAGCTTGCCTTTGTCCTCAGCTTGC, HindIII	Deletion of PFLCHA0_c48560 (*dctB2*)
dctB2-4	CGGGATCCGCACGTTGGCCGAGGTATC, BamHI	Deletion of PFLCHA0_c48560 (*dctB2*)
fitFc-F	TTACTGTTGCTGTGGGGCGG	*dctB'-'fitF* chimera
fitFc-F2	GTGTTCTTACTGTTGCTGTGGG	*citA'-'fitF* chimera
fitF-D149-F	CAAGGTGGCTCTCGCTGGCGGCAGCAG	Site-dir. mutagenesis of *fitF*
fitF-D149-R	CTGCTGCCGCCAGCGAGAGCCACCTTG	Site-dir. mutagenesis of *fitF*

fitF-D803A-F	CGCTGTGCTGATGGCTTGTCAGATGCCG	Site-dir. mutagenesis of *fitF*
fitF-D803A-R	CGGCATCTGACAAGCCATCAGCACAGCG	Site-dir. mutagenesis of *fitF*
fitF-F-SD-new	GGAATTCAGGAGATATACCCTTGTACGGGTTATTGGACTTC, EcoRI	Complementation of Δ*fitF*
fitF-mut1-F	GACTAGTGGCGCGGGCAAGGTGGCTCTCGATGGCGGC, SpeI	Site-dir. mutagenesis of *fitF*
fitF-mut1-hr-F	GGAATTCCAGGAAATGCCTCGGACAAG, EcoRI	Site-dir. mutagenesis of *fitF*
fitF-mut1-hr-R	CGGGATCCGGCAACAGCGTATCCAGGT, BamHI	Site-dir. mutagenesis of *fitF*
fitF-mut1-R	GACTAGTGGCCACGTTCAGCCCCAGG, SpeI	Site-dir. mutagenesis of *fitF*
fitF-mut2-F	CGACTGCATGTCCGCGGAGATCCGCACGC	Site-dir. mutagenesis of *fitF*
fitF-mut2-hr-F	GGGGTACCAGATCGAGCAGAGCGAACAGC, KpnI	Site-dir. mutagenesis of *fitF*
fitF-mut2-hr-R	CCCAAGCTTCGGCCTGGACAAAGACATTGA, HindIII	Site-dir. mutagenesis of *fitF*
fitF-mut2-R	GCGTGCGGATCTCCGCGGACATGCAGTCG	Site-dir. mutagenesis of *fitF*
fitF-R-HindIII	CCCAAGCTTCTAGCCCTGGAAGTCGACAAGCAGGT, HindIII	*dctB'-'fitF* and *citA'-'fitF* chimera; complementation of Δ*fitF*
fitF-R141A-F	CTGAACGTGGCCTTCGCCCCCTACTTCAAGG	Site-dir. mutagenesis of *fitF*
fitF-R141A-R	CCTTGAAGTAGGGGGCGAAGGCCACGTTCAG	Site-dir. mutagenesis of *fitF*
fitF-REC-hr-F	GGAATTCGTATCGGTGTGTGCCTGGTG, EcoRI	Site-dir. mutagenesis of *fitF*
fitF-REC-hr-R	CGGGATCCGCCTGTTCCTCCAGTTGTC, BamHI	Site-dir. mutagenesis of *fitF*
fitF-Y143A-F	GCCTTCCGCCCCGCCTTCAAGGTGGC	Site-dir. mutagenesis of *fitF*
fitF-Y143A-R	GCCACCTTGAAGGCGGGGCGGAAGGC	Site-dir. mutagenesis of *fitF*
fitF-Y143F-F	GCCTTCCGCCCCTTCTTCAAGGTGGC	Site-dir. mutagenesis of *fitF*
fitF-Y143F-R	GCCACCTTGAAGAAGGGGCGGAAGGC	Site-dir. mutagenesis of *fitF*
fitH-D59A-F	CGGCATCAGGATGGCGAGAATGATCAGATC	Site-dir. mutagenesis of *fitH*
fitH-D59A-R	GATCTGATCATTCTCGCCATCCTGATGCCG	Site-dir. mutagenesis of *fitH*
fitH-REC-hr-F	GGAATTCGTGATTTTCCAGGCAAGGTTGAGG, EcoRI	Site-dir. mutagenesis of *fitH*
fitH-REC-hr-R	CGGGATCCGACCCCGATGAGAATCTACCC, BamHI	Site-dir. mutagenesis of *fitH*
ME8300-F	CATGCCATGGACTGAATCCGGTGAGAATGG, NcoI	Cloning of pME8300
ME8300-SpeI-R	CCCAAGCTTGGGACTAGTTGTTTCCTGTGTGAAATTGTTATCC, HindIII, SpeI	Cloning of pME8300
mifS-1	CGGGATCCGACAAGTTCACCCGCCAGATC, BamHI	Deletion of PFLCHA0_c47820 (*mifS*)
mifS-2	CCCAAGCTTAGAGATCTGGGCCATGAGTCG, HindIII	Deletion of PFLCHA0_c47820 (*mifS*)
mifS-3	CCCAAGCTTAGCCTGCCCATCGACCTGG, HindIII	Deletion of PFLCHA0_c47820 (*mifS*)
mifS-4	CGGGATCCGGCTGCCAGTTCCAGTACCT, BamHI	Deletion of PFLCHA0_c47820 (*mifS*)
PCL-fitF-br-F	CTGGGGATCGTCATCAACAACG	Complementation of Δ*fitF*
PCL-fitF-br-R	GCCATTGCCAAAGGTCTGGTC	Complementation of Δ*fitF*
PCL-fitF-F-SD	GGAATTCAGGAGATATACCCTTGCACAGGTTAACCCACTTC, EcoRI	Complementation of Δ*fitF*
PCL-fitF-R	CCCAAGCTTCTAACCCTGGGCCGCGC, HindIII	Complementation of Δ*fitF*
PCL-fitF-StuI-F	CTGAACCAGGATGTGTTGCAG	Complementation of Δ*fitF*
PCL-fitF-StuI-R	CATCTGGCAATCCATCAGCAC	Complementation of Δ*fitF*
PfitF1	GGGGTACCAACAGCGATATCCGTGGCAATG, KpnI	Deletion of *fitF*
PfitF2	GGAATTCGTAGACGTCTTCGACTTGCACAC, EcoRI	Deletion of *fitF*
PfitF3	GGAATTCCTGGCGCTGATCGATGAACTGTA, EcoRI	Deletion of *fitF*
PfitF4	GCTCTAGATCCACCCGCACCATGAAGCTCA, XbaI	Deletion of *fitF*

[1] Restriction sites are underlined

CHAPTER 4

Screening for additional factors contributing to insect pathogenicity of *Pseudomonas protegens* and *Pseudomonas chlororaphis*

Abstract

The molecular and genetic basis of pathogenicity has been well-studied in the clinically important human pathogen *Pseudomonas aeruginosa* and the agriculturally important plant pathogen *Pseudomonas syringae*. Here, we used this knowledge to detect homologs of virulence-related genes in model strains of *Pseudomonas protegens* and *Pseudomonas chlororaphis*, which are plant-beneficial rhizobacteria displaying insect pathogenicity, to identify novel factors that contribute to their ability to invade and kill pest insects. The list of candidate virulence determinants was narrowed down to genes that encode proteins that influence the decoration of the cell surface, the site where microbe-host interactions take place. Mutant strains in which these conserved genes were inactivated were generated and used to screen for attenuation in virulence upon injection into the greater wax moth, *Galleria mellonella*. The screening identified O-antigen, the highly variable portion of lipopolysaccharide, as a prime candidate for a novel factor that contributes to virulence in these entomopathogenic pseudomonads. Mutation of *wbpL*, a gene that is most likely essential for the biosynthesis of O-antigen, rendered the *P. protegens* strain CHA0 virtually avirulent. The results of the screening additionally indicated that the two-component regulatory system PhoP-PhoQ and the transcriptional regulator FleQ are important for virulence in these bacteria as well. Since PhoP-PhoQ is generally known to regulate covalent modifications of lipid A, this study suggests that lipopolysaccharide plays a key role in the interaction of *P. protegens* and *P. chlororaphis* with their insect host and thus should be further characterized in these bacteria in future studies.

Introduction

Definitions of pathogenicity, virulence and virulence factors

Pathogenicity is a qualitative trait which is generally defined as the inherent capacity of a microorganism to cause disease or damage in a host [1]. Virulence, a quantitative trait, is difficult and complex to define but is generally accepted to represent the extent of the pathology caused by a particular microorganism. It can be described from a pathogen-centered or a host-centered perspective. From a pathogen-centered view, virulence factors can be defined as microbial products that permit a microorganism to cause disease, without being essential for viability [1]. Virulence, however, depends on the availability of a susceptible host, on the specific microorganism and the nature of its interaction with the host. Casadevall and Pirofski therefore suggested that host damage should be used to define the terms pathogenicity and virulence in a host-centered view, since damage is a central feature of infectious disease [1]. Thus, virulence factors can also be defined as components of a pathogen that damage the host and can include components essential for viability.

Our research aims at identifying factors that significantly contribute to the insect pathogenicity of *P. protegens* and *P. chlororaphis* in addition to an already well-characterized insecticidal toxin termed Fit [2-5]. Our goal is to get a better understanding of how these bacteria infect and kill insects of agricultural importance as pests. For this purpose, we define virulence factors here in the classical sense as features that in some way contribute to the ability of these bacteria to kill insect larvae without being required for normal growth of these microorganisms in common laboratory media.

Approaches to identification of pathogenicity factors

Today's genetic and bioinformatic tools allow the identification of virulence factors in a particular bacterial species in several different ways. Performing a whole-genome genetic screen can be a powerful approach to identify novel factors that significantly contribute to virulence in a chosen host organism or contribute to a specific property of the microorganism (such as biofilm formation or resistance to particular immune effectors) under *in vitro* conditions. Random transposon insertion mutagenesis can be used to conduct a loss-of-function screen [6-8]. To identify fitness determinants, the mutant library can be used as an inoculum to infect a model organism. Mutant cells that are less or no longer present at a defined time point after the infection can subsequently be identified for example via transposon sequencing [9]. Gain-of-function screens can be performed, for example, by heterologous expression of fragments of the microbe's genome in *Escherichia coli* and test the bacterium's increase in virulence, resistance, etc. [10,11]. Studying the differential expression of genes during host interaction can also be an approach to discover factors that might contribute to pathogenicity without necessarily increasing the fitness of the pathogen. This can be

done amongst others by performing RNA sequencing, microarray analysis, or promoter-trap studies [8,12-14].

An alternative approach to find new virulence factors in a given bacterium is to do classical forward genetics combined with bioinformatic tools. Candidate genes can be found by searching for homologs of genes that are known to contribute to virulence in a well-studied pathogen. Since their functions are mostly already well-known and generally well conserved, one can predict and characterize their roles in virulence more easily than for genes identified in a genetic screen. After the identification of such genes and a thorough literature search, defined mutants are generated and their virulence is assessed in an infection model. Similarly, novel virulence factors can theoretically also be identified by comparing genome sequences from pathogenic and related, non-pathogenic strains [15]. Genes that are only present in virulent strains might be required for them to persist within and cause damage to the host organism. This approach is, however, limited by the fact that virulence in bacteria is both multifactorial and combinatorial [16]. Genes that are important for virulence do not have to be present in all virulent strains and, on the other hand, can also be present in genomes of non-pathogenic strains.

In this study, we chose to identify novel virulence factors and regulators in *P. protegens* CHA0 and *P. chlororaphis* PCL1391 by searching for conserved genes known to be implicated in pathogenicity in other pseudomonads. At the current stage of our research it is the fastest, most cost-effective and probably the most promising way to find new key players in these entomopathogenic bacteria.

Results

Selection of candidate genes

Major virulence factors of the *Pseudomonas* genus can be found, including references, in the Virulence Factor Database (VFDB; http://www.mgc.ac.cn/VFs/) [17] and are listed in Table 1. Sequences of related genes and proteins were used to identify homologs in *P. chlororaphis* PCL1391 and *P. protegens* CHA0 by performing BLAST searches and homology searches as described in the Material and Methods section. For comparison, homologs were additionally searched for in the non-insecticidal *P. fluorescens* F113. About one third of the major *Pseudomonas* virulence factors also seem to be present in the entomopathogens *P. protegens* and *P. chlororaphis*. Aside from being required for virulence, most of them are also important for non-pathogenic lifestyles [15,18,19] and therefore conserved in strains such as *P. fluorescens* F113 as well.

Table 1: Major virulence factors in *Pseudomonas aeruginosa* listed in the Virulence Factor Database[1] and presence of homologs in selected species of the *Pseudomonas fluorescens* group.

Category (according to the VFDB[1])	Factor(s)	Presence of homologs of related genes		
		PCL1391	CHA0	F113
Adherence	Flagella	Yes	Yes	Yes
	LPS	Yes	Yes	Yes
	Type IV pili	Partly[2]	Partly[2]	Yes
Antiphagocytosis	Alginate	Yes	Yes	Yes
Biosurfactants	Rhamnolipid	No[3]	No[3]	No[3]
Iron uptake	Pyochelin	No	Yes	No
	Pyoverdine	Yes	Yes	Yes
Pigments	Pyocyanin	No[4]	No[4]	No[4]
Proteases	AprA	Yes	Yes	Yes
	LasA	No	No	No
	LasB	No	No	No
Regulation	Quorum sensing systems (*las* and *rhl*)	(Yes)[5]	No	(Yes)[5]
Secretion	HSI-1 (type VI secretion system)	Yes	Yes	Yes
	Type III secretion system	No	No	No
	xcp secretion system (type II secretion system)	Yes	No	Yes
Toxins	ExoA	No	No	No
	ExoS	No	No	No
	ExoT	No	No	No
	ExoU	No	No	No
	ExoY	No	No	No
	PLC	No	No	No

[1] From the Virulence Factor Database (VFDB; http://www.mgc.ac.cn/VFs/) [17], accessed on 23 March 2015.

[2] Several genes reported to be essential for type IV pili biogenesis in *P. aeruginosa* are absent in these strains (data not shown).

[3] Although these strains do not seem to synthesize rhamnolipid they might produce other types of biosurfactants. Many pseudomonads were reported to produce biosurfactants [20,21].

[4] *P. chlororaphis* produces other types of phenazines [5,22]. *P. fluorescens* and *P. protegens* synthesize other compounds with antimicrobial activities [5].

[5] Although no homologs of the *P. aeruginosa las* and *rhl* systems were detected, *P. chlororaphis* PCL1391 was previously reported to possess the quorum-sensing system PhzI/PhzR [23] and *P. fluorescens* F113 to make at least three different *N*-acylhomoserine lactones [24].

Feinbaum *et al.* screened a mutant library of *P. aeruginosa* PA14 for attenuation in a *Caenorhabditis elegans* model of infection and compared the discovered virulence factors with findings from similar studies using other animal models [6]. Only mutants that grew like the wild type in common laboratory medium were considered. Homology search conducted in this study revealed that many of the discovered genes are conserved in the selected *P. fluorescens* group strains (Table 2). The two-component system GacS-GacA has already been shown to also play an important role in virulence of *P. protegens* CHA0 in insects [3]. For the screening in this study, interesting candidates on the list include genes coding for the virulence regulator Vfr,

the alginate regulator KinB, the potassium sensor KdpD, the type VI secretion system, the type IV pili and the biosynthesis of O-antigen.

Table 2: Virulence factors and regulators discovered previously in a screen for attenuation of *P. aeruginosa* PA14 in *C. elegans*[1] and presence of homologs in *P. protegens* CHA0, *P. chlororaphis* PCL1391 and *P. fluorescens* F113.

Factor / Regulator	Protein name / function	Presence of homologs of related genes		
		PCL1391	CHA0	F113
GacA	Response regulator	Yes	Yes	Yes
Vfr	Cyclic AMP receptor-like protein	Yes	Yes	Yes
PchH	Putative ATP-binding component of ABC transporter (pyochelin cluster)	No	Yes	No
PA4005	Conserved hypothetical protein	Yes	Yes	Yes
PA14_27700	Putative transcriptional regulator	No	No	No
PepP	Aminopeptidase P	Yes	Yes	Yes
PA4664	Putative methyl transferase	Yes	Yes	Yes
LysC	Aspartate kinase alpha and beta chain	Yes	Yes	Yes
VqsR	Transcriptional regulator, LuxR family	No	No	No
KinB	Putative two-component sensor	Yes	Yes	Yes
PtsP	Phosphoenolpyruvate-protein phosphotransferase	Yes	Yes	Yes
PA0745	Probable enoyl-CoA hydratase/isomerase	No	No	No
RhlR	Acylhomoserine lactone dependent transcriptional regulator	No	No	No
PA2550	Putative acyl-CoA dehydrogenase	Yes	Yes	Yes
MinD	Cell division inhibitor	Yes	Yes	Yes
PA1592	Conserved hypothetical protein	No	No	Yes
GlnK	Nitrogen regulatory protein PII	Yes	Yes	Yes
AruD	Succinylglutamate 5-semialdehyde dehydrogenase	Yes	Yes	Yes
GshA	Glutamate-cysteine ligase	Yes	Yes	Yes
PA2015	Putative isovaleryl-CoA dehydrogenase	Yes	Yes	Yes
CspB	Putative major cold shock protein	No	No	No
PrpC	Citrate synthase 2	Yes	Yes	Yes
GacS	Sensor/response regulator hybrid	Yes	Yes	Yes
LasI	Autoinducer synthesis protein	No	No	No
PchI	Putative ATP-binding component of ABC transporter (pyochelin cluster)	No	No	No
AruG	Arginine/ornithine succinyltransferase AII subunit	Yes	Yes	Yes
FabF1	Beta-ketoacyl-acyl carrier protein synthase II	Yes	Yes	Yes
ClpA	ATP-dependent clp protease, ATP-binding subunit	Yes	Yes	Yes
AruB	Succinylarginine dihydrolase	Yes	Yes	Yes
PA1766	Conserved hypothetical protein	Yes	Yes	Yes
PA1216	Conserved hypothetical protein	No	No	No
PqsE	Quinolone signal response protein	No	No	No
KdpD	Two-component sensor	Yes	Yes	Yes
PA1767	Putative membrane protein	Yes	Yes	Yes
PrpB	Carboxyphosphonoenolpyruvate phosphonomutase	Yes	Yes	Yes
Fha2	Conserved hypothetical protein (type VI secretion system cluster)	Yes	No	Yes
GshB	Glutathione synthetase	Yes	Yes	Yes
AruC	N-succinylglutamate 5-semialdehyde dehydrogenase	Yes	Yes	Yes

WbpL	Putative glycosyltransferase L	Yes	Yes	Yes
PilF	Type 4 fimbrial biogenesis protein	Yes	Yes	Yes
ORF_11	O-antigen synthesis	No	No	Yes

[1] Table adapted from Feinbaum *et al.* (2012) [6]. The genes are listed in the order of decreasing importance for the virulence (based on their respective LT_{50} values) of PA14 in *C. elegans*.

In order to reduce the number of candidate genes for the screening in this study, the search for novel factors contributing to insect pathogenicity of *P. protegens* (or *P. chlororaphis*) was focused on selected genes that putatively influence the surface decoration in this bacterium (Table 3). These included genes required for the biosynthesis of exopolysaccharides (*alg* and *psl* operon), flagella (*fliC*), pili (*pil* cluster, *cupB* cluster, and *cupC* cluster), and O-antigen (*wbpL*). The *pgaABCD* operon which is putatively responsible for the synthesis of poly-*N*-acetylglucosamine (PNAG) in these bacteria was added to the list of candidate genes. PNAG biosynthesis has not been demonstrated in pseudomonads to date, but this particular polysaccharide plays an important role in biofilm formation and contributes to virulence in several non-pseudomonad pathogens [7,25-27]. Genes necessary for the biosynthesis of Pel polysaccharide were not included in the screen because this putative virulence factor is being investigated by other members of the research group. The transcriptional regulator FleQ is one of the main regulators controlling the transition from a planktonic to a sessile lifestyle and might therefore also influence the pathogenicity of entomopathogenic pseudomonads by changing their surface properties [28-31]. The cyclases WspR and TpbB were chosen because their overexpression might allow forcing the bacteria to induce the production of exopolysaccharides and to switch to biofilm mode of growth [32-34]. Genes contributing to (*arnA* and *pagL*) or regulating (*phoP-phoQ*) the modification of lipid A were added as well since lipopolysaccharide (LPS) is a generally recognized virulence factor [35,36]. Because the type II secretion system could be necessary to export surface proteins, it was put on the list, too. Vfr was added to the candidate list because it seems to be a major virulence regulator in *P. aeruginosa* and *P. syringae* [6,37,38].

Table 3: Candidate genes for the screening for attenuation in virulence in *P. protegens*.

Category	Gene (cluster)	Putative function of the encoded protein	References
Exopolysaccharides	*alg* cluster	Synthesis of alginate; contribution to biofilm formation	[39-42]
	psl cluster	Synthesis of Psl; contribution to biofilm formation	[40-42]
	pgaABCD	Synthesis of poly-N- acetylglucosamine (PNAG) ; contribution to biofilm formation and to resistance to phagocytosis	[25-27]
	wspR	Synthesis of c-di-GMP (cyclase); activation of exopolysaccharide synthesis	[32,33]
	tpbB	Synthesis of c-di-GMP (cyclase) ; activation of exopolysaccharide synthesis; also termed YfiN	[32,34]
Lipopolysaccharide	*wbpL*	Initial glycosyltransferase essential for the synthesis of O-antigen; contributes to resistance to host immune reactions and evasion of recognition by the innate immunity	[6,16,36]
	pagL	Lipid A 3-O-deacylase contributing to immune evasion	[43,44]
	arn cluster	Aminoarabinose modification of lipid A; contributing to resistance to antimicrobial peptides	[44]
Adhesion / motility	*fliC*	Flagellin; essential for swimming and contributing to attachment; recognized by innate immunity	[45]
	pil cluster	Type IV pili	[46]
	cupB cluster	Fimbrial pili	[47,48]
	cupC cluster	Fimbrial pili	[47,48]
Secretion	*hxc* cluster	Type II secretion system	[49-52]
Regulation	*fleQ*	c-di-GMP-dependent regulator controlling flagellar biogenesis and synthesis of exopolysaccharides	[28-31]
	phoP-phoQ	Two-component regulatory system controlling lipid A modifications and virulence	[53-55]
	vfr	cAMP-dependent virulence factor regulator	[37,38,56]

Screening for decreased biofilm formation and attenuation in virulence

Mutant strains that were suspected to produce less exopolysaccharides or to be affected in their ability to adhere where first tested for their capability to form biofilms in a simple *in vitro* assay. The goal of this approach was to narrow down the list of candidate genes to a few interesting genes in order to facilitate the subsequent virulence screening. The transcriptional regulator FleQ was, however, the only factor on the candidate list that seemed to be required for normal biofilm formation under the conditions tested (Figure 1A and B). Mutation of individual exopolysaccharide, pili and LPS biosynthesis genes (including *pgaABCD* and *wbpL*; data not shown) did not affect the ability of *P. protegens* CHA0 to form biofilms under *in vitro* conditions. The results indicate that CHA0 produces considerably more biofilm when grown in an insect-mimicking medium than in a common laboratory medium (Figure 1B). Moreover, it was possible to obtain hyper-biofilms by overexpression of selected diguanylate cyclases (WspR and TpbB) in *P. protegens* CHA0 or *P. chlororaphis* PCL1391 (Figure 1C). These enzymes synthesize c-di-GMP which is a second messenger that mediates the transition from the motile planktonic to the sedentary biofilm-associated bacterial “lifestyle” [32]. PCL1391 cells overexpressing WspR only grew in biofilms and no longer as motile planktonic cells (Figure 1C and D).

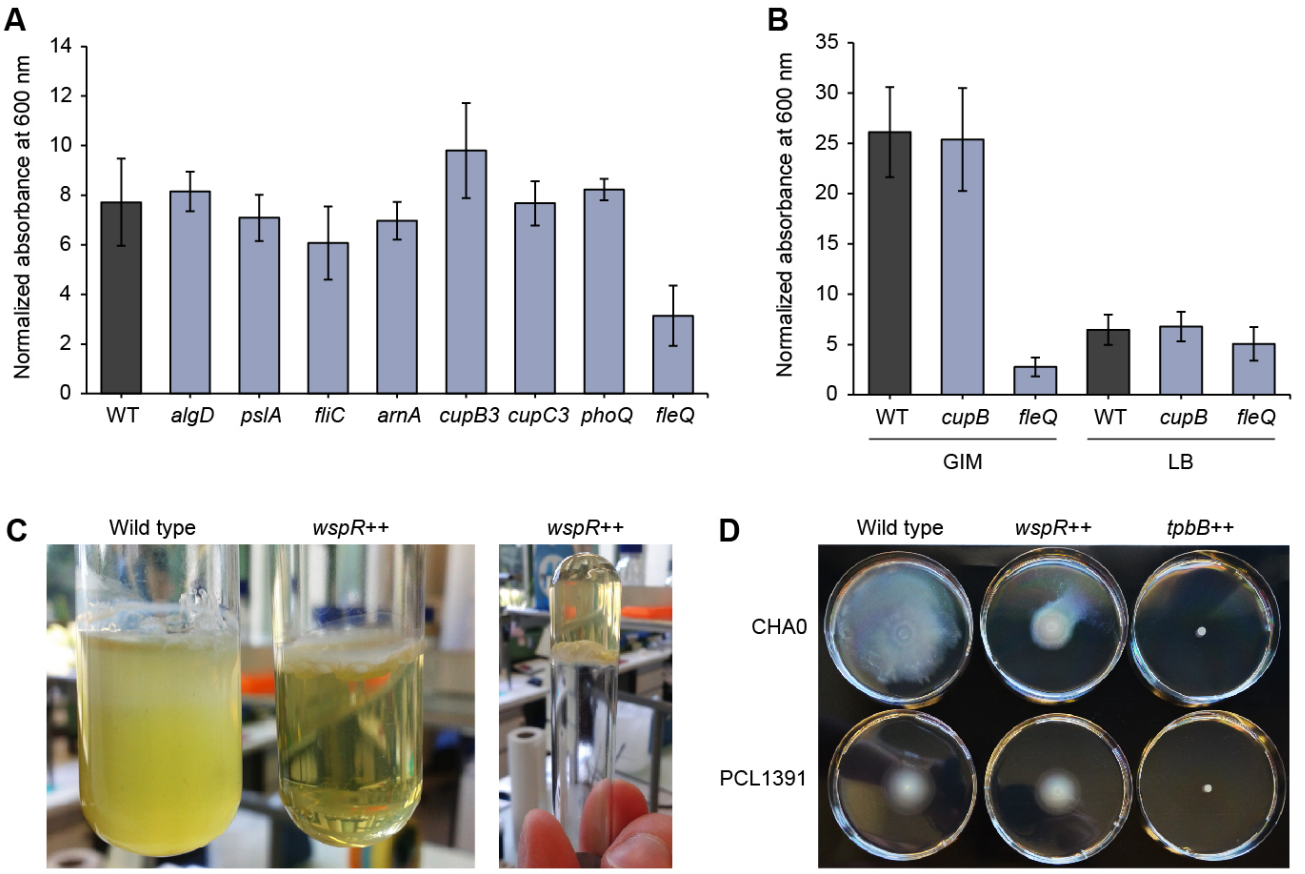

Figure 1: Influence of mutation or overexpression of selected genes on biofilm formation in *Pseudomonas protegens* CHA0 and *P. chlororaphis* PCL1391. (A) *P. protegens* CHA0 wild-type and mutant strains (CHA5105, Δ*fleQ*; CHA5126, *algD*; CHA5127, *pslA*; CHA5128, *fliC*; CHA5129, *arnA*; CHA5130, *cupB3*; CHA5131, *cupC3*; CHA5133, Δ*phoQ*) were grown in 96-well plates in Grace's Insect Medium for 24 hours without agitation. The biofilms were stained with crystal violet and the absorbance at 600 nm was measured and normalized with the optical density of the corresponding bacterial culture. Shown are means and standard deviations of eight independent cultures per strain. **(B)** The assay described in (A) was repeated with CHA0 and its isogenic Δ*cupB* (CHA5132) and Δ*fleQ* (CHA5105) mutant strains in GIM and Lysogeny Broth (LB). Only the *fleQ* deletion mutant was reduced in biofilm formation in GIM. **(C)** Overexpression of the diguanylate cyclase WspR (PCL-4) resulted in a strongly increased biofilm formation in *P. chlororaphis* PCL1391 in LB. The pellicle at the air-liquid interface was strong enough that the glass test tubes containing the bacterial culture could be turned upside-down. The corresponding CHA0 strain CHA5177 showed a similar phenotype. **(D)** Swimming assays were performed with wild-type and isogenic diguanylate-cyclase-overexpressing strains of *P. protegens* CHA0 (CHA5177 and CHA5178) and *P. chlororaphis* PCL1391 (PCL-4 and PCL-5) to assess whether these bacteria can be forced into sessile mode of growth (expression of *wspR* and *tpbB* was induced by adding IPTG to the soft agar). Strains overexpressing TpbB were unable to swim.

Larvae of the greater wax moth, *Galleria mellonella*, were used as a systemic infection model to screen for mutants that are reduced in virulence. Interestingly, mutation of *wbpL* (CHA5156) rendered *P. protegens* CHA0 almost avirulent in this infection model (Figure 2A and B). While larvae treated with wild-type cells are generally dead within 30 hours after injection, most insects infected with the *wbpL* mutant were still alive three days post injection. WbpL is the initial glycosyltransferase in the biosynthesis of O-antigen in *P. aeruginosa* and required for full virulence of this opportunistic pathogen without being necessary for normal growth (Table 2) [6,36]. The screening further revealed that the PhoP-PhoQ two-component system, which

regulates lipid A modifications in diverse bacteria [53-55], might be an important virulence regulator in CHA0 (Figure 2C). Deletion of *fleQ* also seemed to affect the bacterium's ability to kill insect larvae (data not shown). In contrast, mutation of *algD*, *pgaABCD*, *pilA*, *cupB*, *pagL*, *arnA*, or *hxcQ* did not significantly decrease the systemic virulence of CHA0 in the chosen insect model (Figure 2A and data not shown). Due to time constraints, the *fliC*, *cupC3*, *pslA*, *vfr*, *wspR*, and *tpbB* mutant strains have not been tested for virulence to date.

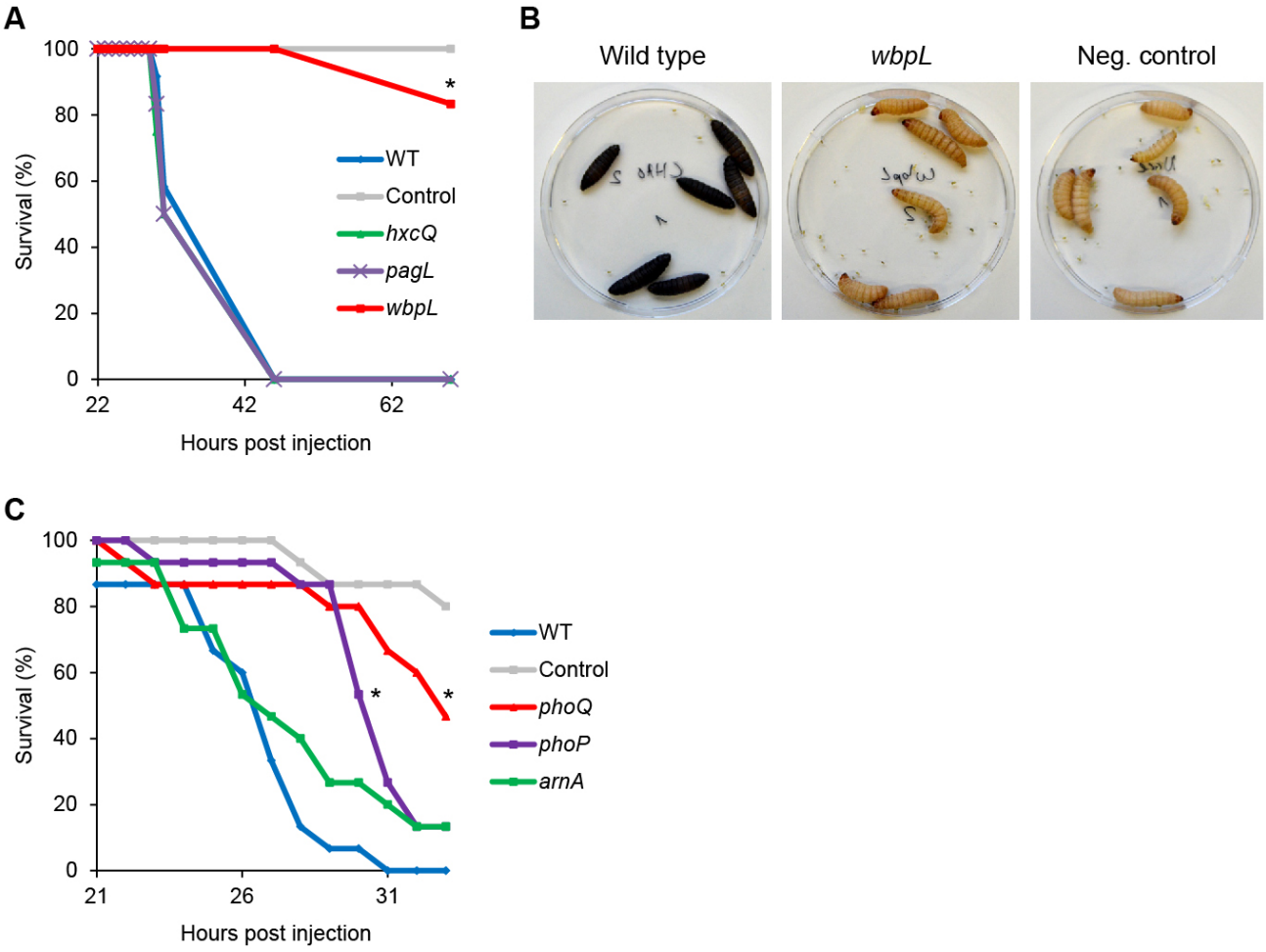

Figure 2: O-antigen and the PhoP-PhoQ regulatory system seem to be required for systemic virulence of *Pseudomonas protegens* CHA0. Low doses of *P. protegens* CHA0 or isogenic mutant strains (CHA5155, *hxcQ*; CHA5156, *wbpL*; CHA5157, Δ*pagL*) were injected into larvae of *Galleria mellonella* in order to assess whether selected genes play a role in virulence. Saline solution was used as a negative control. **(A)** The survival of the larvae was monitored over time. Larvae were considered dead when they did not respond to repeated stimulus. Strain CHA5156 was significantly less virulent than the wild type (*, p-value < 0.0001; Log-Rank test with Kaplan Meier estimations). **(B)** Pictures of larvae 48 hours after injection of either CHA0, CHA5156 (*wbpL*) or saline solution. While larvae infected with the wild type were dead and fully melanized (black color), the insects treated with the *wbpL* mutant were, as the negative control group, still alive and showed no signs of melanization. The assay was repeated with the *wbpL* mutant and gave similar results. **(C)** The same assay was performed with additional mutants of *P. protegens* CHA0, such as Δ*phoP* (CHA5134), Δ*phoQ* (CHA5133) and *arnA* (CHA5129). Strains marked with asterisks were significantly less virulent than the wild type (p-value < 0.001; Log-Rank test with Kaplan Meier estimations).

Discussion

In the present study we aimed at identifying new factors that significantly contribute to insect pathogenicity of plant-beneficial pseudomonads with insecticidal activities. To this end, we collected information about the genetic basis of pathogenicity in the well-studied opportunistic pathogens *P. aeruginosa* and *P. syringae* and made use of this knowledge to identify a set of genes encoding putative virulence factors and regulators in *P. protegens* and *P. chlororaphis*. The subsequent targeted mutagenesis and virulence screening identified *wbpL* as the gene whose mutagenesis in *P. protegens* CHA0 resulted in the strongest attenuation in virulence in the chosen insect model. *P. aeruginosa* produces two forms of O-antigen [36]. A *P. aeruginosa* strain lacking *wbpL* produces LPS without the variable side chains, which strongly suggests that the *wbpL*-encoded glycosyltransferase is essential for the synthesis of both types of O-antigen. It is thus likely that the *wbpL* mutant of *P. protegens* CHA0 was almost avirulent because it lacked the protective O-antigen layer. Since neither the genetic basis of O-antigen biosynthesis in plant-beneficial pseudomonads nor the function of this variable LPS portion in insect pathogenicity has been investigated to date, this apparently important virulence factor should be studied in details in the future.

The targeted screening performed in this study also suggested that FleQ and the PhoP-PhoQ regulatory system might be important for virulence of *P. protegens* and *P. chlororaphis*. PhoP-PhoQ is known to control modification of lipid A in response to limited extracellular magnesium and to contribute to virulence in several different pathogenic bacteria [53-55]. FleQ is regulating the biogenesis of flagella and the biosynthesis of exopolysaccharides in *P. aeruginosa*, and thereby contributes to the c-di-GMP-dependent switch between the sessile and the planktonic mode of growth [28-31]. Further experiments are necessary to confirm the roles of these regulators in virulence.

Because of time reasons not all generated mutants could be tested in a *Galleria* virulence assay. Thus, further virulence factors could be detected by testing the remaining mutant strains and also by assessing the virulence of all constructed mutants in an oral infection assay. The *pslA* and *vfr* mutants are of particular interest. The *psl* gene cluster seems to be only present in insecticidal strains of the *P. fluorescens* group, suggesting that Psl is of relevance for the virulence of these bacteria in insects (Flury *et al*., manuscript in preparation). In *P. aeruginosa*, the exopolysaccharide plays an important role in surface attachment, biofilm formation and differentiation, and protection from innate immune effectors [40]. Vfr seems to play a key role in virulence of *P. aeruginosa* and *P. syringae* [6,37,38]. In the plant-pathogen, the regulator controls the expression of a wide range of virulence factors, including flagella-, pili- and iron uptake-related proteins, in a cAMP-dependent manner [37]. Vfr could thus be a vital virulence regulator in plant-beneficial pseudomonads as well. It would further be interesting to see if flagella are essential for killing larvae via the oral route of infection. It is possible that flagellar motility and chemotaxis are necessary for crossing the epithelial barrier to gain access to the hemocoel. The role of iron scavenging in insect pathogenicity could

also be interesting to study in entomopathogenic pseudomonads. *P. protegens* CHA0 synthesizes two types of siderophores, pyochelin and pyoverdine, which were both shown to play a role in virulence of *P. aeruginosa* [57].

Results of this study suggest that biofilm formation in *P. protegens* CHA0 is not dependent on the synthesis of one single exopolysaccharide, but multifactorial. c-di-GMP is employed in bacteria as a second messenger to upregulate the production of exopolysaccharides, such as Pel and PNAG, and to stop the biogenesis of flagella in order to switch to the "biofilm lifestyle" [32]. Overexpression of diguanylate cyclases, which synthesize c-di-GMP [32], could be used as an approach to induce the production of the diverse exopolysaccharides in wild-type and mutant strains of *P. chlororaphis* and *P. protegens* in order to better understand their contributions to biofilm formation. Because deletion of one exopolysaccharide gene cluster usually increases the expression of the remaining ones [58], it may be necessary to generate double and triple mutants to decipher whether individual (or combinations of) polysaccharides play a role in virulence.

With the exception of *pgaABCD*, this study only considered genes from the *Pseudomonas* genus for the identification of new virulence factors in *P. protegens* and *P. chlororaphis*. The knowledge about factors contributing to virulence of other well-studied bacteria could, however, also aid extending the list of virulence factor candidates. For example, proteins that contribute to virulence in the entomopathogens *Photorhabdus luminescens*, *Xenorhabdus nematophila*, and *Serratia marcescens* might also be present and important in insect-pathogenic pseudomonads. The knowledge about the *P. luminescens mcf1* gene, which codes for a potent insecticidal toxin [11], for instance led to the discovery of the Fit toxin in *P. protegens* [5].

This study successfully identified O-antigen as a potential novel virulence factor in insecticidal pseudomonads with plant-beneficial properties. Its results additionally suggest that the PhoP-PhoQ system and FleQ play a role as virulence regulators in these microbes. Several additional mutant strains generated in this study remain to be tested for attenuation in virulence. The results presented here thus may open new doors for future research on the insect pathogenicity of plant-associated pseudomonads.

Material and Methods

Bioinformatics

Genes that are homologous (i.e., minimum 70% sequence identity over at least 70% of the coding sequence) to known virulence factors of the *Pseudomonas* genus were identified in *P. protegens* CHA0, *P. chlororaphis* PCL1391 and *P. fluorescens* F113 by performing nucleotide and protein BLAST searches locally or on the websites of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/) and the

Pseudomonas Genome Database (http://www.pseudomonas.com/), using sequences from *P. aeruginosa* PAO1 obtained from the *Pseudomonas* Genome Database.

Bacterial strains, plasmids, media, and culture conditions

All strains and plasmids used in this study are listed in Table 4. Bacterial strains were routinely maintained on nutrient agar (NA) plates or cultured in LB (LB Broth Miller, BD Difco) supplemented with appropriate antibiotics as needed. *Pseudomonas* strains were grown at 25°C while *E. coli* was cultured at 37°C. The following antibiotic concentrations were used: ampicillin, 100 µg/ml; chloramphenicol, 10 µg/ml; kanamycin, 25 µg/ml for *E. coli* and *P. protegens*, 50 µg/ml for *P. chlororaphis*; gentamicin, 10 µg/ml for *E. coli* and *P. protegens*, 20 µg/ml for *P. chlororaphis*; and tetracycline, 25 µg/ml or 125 µg/ml for *E. coli* and *Pseudomonas*, respectively. Isopropyl β-D-1-thiogalactopyranoside (IPTG) was added at a final concentration of 0.1 mM, if not otherwise stated.

Table 4: Bacterial strains and plasmids used in this study.

Strain	Genotype, phenotype or relevant characteristics	Reference or source
Pseudomonas protegens		
CHA0	Wild type	[59]
CHA5105	CHA0 Δ*fleQ* (PFLCHA0_c16720)	This study
CHA5119	CHA0 *pilA*::pEMG (PFLCHA0_c52590); Km^r	This study
CHA5126	CHA0 *algD*::pEMG (PFLCHA0_c10440); Km^r	This study
CHA5127	CHA0 *pslA*::pEMG (PFLCHA0_c42710); Km^r	This study
CHA5128	CHA0 *fliC*::pEMG (PFLCHA0_c16670); Km^r	This study
CHA5129	CHA0 *arnA*::pEMG (PFLCHA0_c30730); Km^r	This study
CHA5130	CHA0 *cupB3*::pEMG (PFLCHA0_c15000); Km^r	This study
CHA5131	CHA0 *cupC3*::pEMG (PFLCHA0_c39830); Km^r	This study
CHA5132	CHA0 Δ*cupB*; deletion of the *cupB* gene cluster (PFLCHA0_c14980-PFLCHA0_c15080)	This study
CHA5133	CHA0 Δ*phoQ* (PFLCHA0_c45400)	This study
CHA5134	CHA0 Δ*phoP* (PFLCHA0_c45410)	This study
CHA5155	CHA0 *hxcQ*::pEMG (PFLCHA0_c28140); Km^r	This study
CHA5156	CHA0 *wbpL*::pEMG (PFLCHA0_c43720); Km^r	This study
CHA5157	CHA0 Δ*pagL* (PFLCHA0_c51150)	This study
CHA5158	CHA0 Δ*pgaABCD* (PFLCHA0_c01630-01660)	This study
CHA5177	CHA0 attTn*7*-$P_{tac/laclq}$-*wspR*(PCL1391) (PCL1391_1037); Km^r, Gm^r	This study
CHA5178	CHA0 attTn*7*-$P_{tac/laclq}$-*tpbB*(PCL1391) (PCL1391_662); Km^r, Gm^r	This study
Pseudomonas chlororaphis		
PCL1391	Wild type	[60]
PCL-4	PCL1391 attTn*7*-$P_{tac/laclq}$-*wspR*(PCL1391) (PCL1391_1037); Gm^r	This study
PCL-5	PCL1391 attTn*7*-$P_{tac/laclq}$-*tpbB*(PCL1391) (PCL1391_662); Gm^r	This study
PCL-6	PCL1391 Δ*vfr* (PCL1391_5348)	This study
Escherichia coli		
DH5α, DH5α λpir	Laboratory strains	[61]

Plasmids		
pEMG	pSEVA212S; *ori*R6K, *lacZα* MCS flanked by two I-SceI sites; Km^r	[62]
pME8300	Carrier plasmid for Tn*7* for $P_{tac/laclq}$ controlled target gene expression; Gm^r, Ap^r	[63]
pME8323	pEMG-Δ*fleQ*; suicide plasmid for the in-frame deletion of *fleQ* (PFLCHA0_c16720); Km^r	This study
pME8332	pEMG-*pilA*; suicide plasmid for the disruption of *pilA* (PFLCHA0_c52590); Km^r	This study
pME8336	pEMG-Δ*cupB*; suicide plasmid for the deletion of the *cupB* gene cluster (PFLCHA0_c14980-PFLCHA0_c15080); Km^r	This study
pME8337	pEMG-*algD*; suicide plasmid for the disruption of *algD* (PFLCHA0_c10440); Km^r	This study
pME8338	pEMG-*pslA*; suicide plasmid for the disruption of *pslA* (PFLCHA0_c42710); Km^r	This study
pME8339	pEMG-*fliC*; suicide plasmid for the disruption of *fliC* (PFLCHA0_c16670); Km^r	This study
pME8340	pEMG-*arnA*; suicide plasmid for the disruption of *arnA* (PFLCHA0_c30730); Km^r	This study
pME8341	pEMG-*cupB3*; suicide plasmid for the disruption of *cupB3* (PFLCHA0_c15000); Km^r	This study
pME8342	pEMG-*cupC3*; suicide plasmid for the disruption of *cupC3* (PFLCHA0_c39830); Km^r	This study
pME8357	pEMG-*hxcQ*; suicide plasmid for the disruption of *hxcQ* (PFLCHA0_c28140); Km^r	This study
pME8358	pEMG-Δ*pagL*; suicide plasmid for the in-frame deletion of *pagL* (PFLCHA0_c51150); Km^r	This study
pME8359	pEMG-*wbpL*; suicide plasmid for the disruption of *wbpL* (PFLCHA0_c43720); Km^r	This study
pME8360	pEMG-Δ*pgaABCD*; suicide plasmid for the deletion of the *pga* operon (PFLCHA0_c01630-01660); Km^r	This study
pME8386	pEMG-Δ*vfr*(PCL1391); suicide plasmid for the in-frame deletion of PCL1391 *vfr* (PCL1391_5348); Km^r	This study
pME8390	pME8300-$P_{tac/laclq}$-*wspR*(PCL1391); IPTG-inducible expression of PCL1391 *wspR* (PCL1391_1037); Gm^r, Ap^r	This study
pME8391	pME8300-$P_{tac/laclq}$-*tpbB*(PCL1391); IPTG-inducible expression of PCL1391 *tpbB* (PCL1391_662); Gm^r, Ap^r	This study
pSW-2	*ori*RK2, *xylS*, P_m::*I-sce*I; Gm^r	[62]
pUX-BF13	Helper plasmid encoding Tn*7* transposition functions; R6K-replicon; Ap^r	[64]

Abbreviations: Ap^r, ampicillin; Cm^r, chloramphenicol; Gm^r, gentamicin; Km^r, kanamycin; and Tc^r, tetracycline resistance, respectively.

Recombinant DNA techniques

DNA manipulations and PCRs were performed according to standard protocols [61]. Genomic DNA was extracted with the Promega Wizard Genomic DNA Purification Kit. Plasmid DNA preparations were performed using the QIAprep Spin Miniprep Kit (Qiagen) and the Genomed JETStar Plasmid Purification Midi Kit for small and large scale purifications, respectively. DNA gel extractions were conducted using the MinElute Gel Extraction Kit and the QIAquick Gel Extraction Kit (Qiagen). DNA restriction and modification enzymes were purchased from Promega and were used according to the manufacturer's recommendations. DNA enzyme reaction cleanups were conducted using the QIAquick PCR Purification Kit (Qiagen). PCR for molecular cloning was conducted according to the recommendations of the manufacturer with the PrimeSTAR HS high-fidelity DNA polymerase kit (Takara Bio Inc.), while the DNA Polymerase kit (Promega) was used to perform PCRs for analytic purposes. The primers used in this study were synthesized by

Microsynth AG (Balgach, Switzerland) and are listed in Table 5. DNA sequencing was conducted at GATC Biotech (Konstanz, Germany) and obtained sequences were analyzed using the DNASTAR Lasergene software suite.

Table 5: Primers used in this study.

Name	Sequence 5' → 3', restriction enzyme(s)[1]	Purpose
algD-im-1	CGGGATCCGCGATCAAGGACTACGACT, BamHI	Disruption of *algD*
algD-im-2	GGAATTCTGCTGTCGTAGATGCTCAGG, EcoRI	Disruption of *algD*
arnA-im-1	CGGGATCCAACTGGGTGCTGGTCAAG, BamHI	Disruption of *arnA*
arnA-im-2	GGAATTCGTCCACCAGGCCCAGTTC, EcoRI	Disruption of *arnA*
cupB-del-1	CGGGATCCCCTTCTGCCGAACTGTTAGCG, BamHI	Deletion of *cupB*
cupB-del-2	CCCAAGCTTGGACAAGAGAGTCTTGCGTTTC, HindIII	Deletion of *cupB*
cupB-del-3	CCCAAGCTTGGTGTCAACCGGAAGAAGCTG, HindIII	Deletion of *cupB*
cupB-del-4	GGAATTCGAAAATCGAGCGTGACCATCTG, EcoRI	Deletion of *cupB*
cupB3-im-1	CGGGATCCAGCCGCTTTACCGTACCTTTTG, BamHI	Disruption of *cupB3*
cupB3-im-2	GGAATTCTGTAGCCCATGGCGAAGTTC, EcoRI	Disruption of *cupB3*
cupC3-im-1	CGGGATCCAGCGTTGACCAGAAGTCACC, BamHI	Disruption of *cupC3*
cupC3-im-2	GGAATTCGTAGTTGCCGACATCCTTGG, EcoRI	Disruption of *cupC3*
fleQ-1	CGGGATCCATTGAAGAAACCCGTGAGGC, BamHI	Deletion of *fleQ*
fleQ-2	CCCAAGCTTTAAAATCACCGCCAGGTCGCG, HindIII	Deletion of *fleQ*
fleQ-5	CCCAAGCTTTGACGCCGGTTTTTCAAGTCTTTG, HindIII	Deletion of *fleQ*
fleQ-6	GGAATTCATTTCATGGCCATCGTCTTCGCG, EcoRI	Deletion of *fleQ*
fliC-im-1	CGGGATCCTGAAGATCAACAGCGCAAAAG, BamHI	Disruption of *fliC*
fliC-im-2	GGAATTCAGCAGCGAAGTCGGTATCT, EcoRI	Disruption of *fliC*
hxcQ-im-1	GGAATTCGTGATCGAGAGCCTGATCGTC, EcoRI	Disruption of *hxcQ*
hxcQ-im-2	CGGGATCCGCTGCTGACCTCCTGGTAGA, BamHI	Disruption of *hxcQ*
pagL-del-1	CGGGATCCTTGCAGAGTGCCAAGTTC, BamHI	Deletion of *pagL*
pagL-del-2	CCCAAGCTTCAGTCGCTTCATCAGGAACATC, HindIII	Deletion of *pagL*
pagL-del-3	CCCAAGCTTATGCCGCTCTAAGGCCGCT, HindIII	Deletion of *pagL*
pagL-del-4	GGAATTCGGAGTGAGCTTTGCCTATG, EcoRI	Deletion of *pagL*
PCL.tpbB-expr-F	GACTAGTATGAGCTTCTTCAAGTCGCCTG, SpeI	Expression of PCL1391 *tpbB*
PCL.tpbB-expr-R	CCCAAGCTTGCAAGAAAGGTCGAGTCAGTG, HindIII	Expression of PCL1391 *tpbB*
PCL.vfr-del-1	GGAATTCGTGGATCTTGGTGAAGACCTTG, EcoRI	Deletion of PCL1391 *vfr*
PCL.vfr-del-2	GACTAGTGGTGACCGAGCCTTTGATGAT, SpeI	Deletion of PCL1391 *vfr*
PCL.vfr-del-3	GACTAGTGTATTCGGCACCCGTTAGAGC, SpeI	Deletion of PCL1391 *vfr*
PCL.vfr-del-4	CGGGATCCAGGATCTGCTCGCTCAGGTA, BamHI	Deletion of PCL1391 *vfr*
PCL.wspR-expr-F	GACTAGTATGAATGATTTACAGCTCGACG, SpeI	Expression of PCL1391 *wspR*
PCL.wspR-expr-R	CCCAAGCTTCACTCGATCCCCACCTGAT, HindIII	Expression of PCL1391 *wspR*
pga-del-1	CGGGATCCAAGCTGTCGCTGTCCAAG, BamHI	Deletion of *pgaABCD*
pga-del-2	CCCAAGCTTAGTGCGTTGCATTTCGTCAGC, HindIII	Deletion of *pgaABCD*
pga-del-3	CCCAAGCTTGGAGAAGATTGATTGCCCAGG, HindIII	Deletion of *pgaABCD*
pga-del-4	GGAATTCTCATCCCGAGCGTAGAGAG, EcoRI	Deletion of *pgaABCD*
phoP-del-1	GCGGATCCTGGCGAACTCAT, BamHI	Deletion of CHA0 *phoP*
phoP-del-2	GACTAGTAACGAGCGTTGCCGATGATTC, SpeI	Deletion of CHA0 *phoP*
phoP-del-3	GACTAGTCCAGGCAAAATTCGACATCTCC, SpeI	Deletion of CHA0 *phoP*
phoP-del-4	GGAATTCGATATGTTCCTGCCGGTGAC, EcoRI	Deletion of CHA0 *phoP*
phoQ-del-1	CGGGATCCTACGACCTGCTTTTCCCTTGC, BamHI	Deletion of CHA0 *phoQ*
phoQ-del-2	GACTAGTCACTTTCCGGCCCTGTAGG, SpeI	Deletion of CHA0 *phoQ*
phoQ-del-3	GACTAGTGCGCAGCGATCGAATCATCG, SpeI	Deletion of CHA0 *phoQ*

phoQ-del-4	GGAATTCAGACGTTCCCGATCCTCATC, EcoRI	Deletion of CHA0 *phoQ*
pilA-im-1	CGGGATCCAGGGTCAGGGTCTTGTTCAG, BamHI	Disruption of *pilA*
pilA-im-1	CGGGATCCAATCCGGTTTTACCCTGATCG, BamHI	Disruption of *pilA*
pslA-im-1	CGGGATCCACAACCGCATCACCGAAGTC, BamHI	Disruption of *pslA*
pslA-im-2	GGAATTCGTACTCCTTGACCGCCTCTTC, EcoRI	Disruption of *pslA*
wbpL-im-1	CGGGATCCGCTCGCTGGAGATTATTG, BamHI	Disruption of *wbpL*
wbpL-im-2	GGAATTCAGGGCAAGCAAACCAAGAAC, EcoRI	Disruption of *wbpL*

[1] Restriction sites are underlined

Construction of in-frame deletion mutants

In-frame deletions of selected genes and gene clusters in *P. protegens* CHA0 and *P. chlororaphis* PCL1391 were performed based on homologous recombinations using the suicide vector pEMG and the I-SceI system [62]. For the construction of the suicide vectors (Table 4), upstream and downstream regions of 500–600 bp length flanking the region to be deleted were amplified by PCR using the "del" primer pairs listed in Table 5 and chromosomal DNA from strains CHA0 or PCL1391 as DNA templates. Purified PCR products were digested using the corresponding restriction enzymes (indicated in Table 5) and subsequently cloned into the suicide vector pEMG via triple ligation. Correct insert sequences of the resulting plasmids were confirmed via DNA sequencing. The obtained suicide vectors then served to construct strains CHA5105, CHA5132, CHA5133, CHA5134, CHA5157, CHA5158, and PCL-6 (Table 4) using the I-SceI system as described previously [63].

Construction of gene disruption mutants

For a more rapid generation of mutant strains, selected genes of *P. protegens* CHA0 were mutated by sequence-specific insertion of pEMG-based suicide vectors via homologous recombination, which led to the disruption of the coding sequences (CDS). Suicide vectors were constructed by PCR amplification of approximately 500-bp regions in the middle of the genes of interest, using the "im" primer pairs listed in Table 5 and chromosomal DNA from strain CHA0. The purified PCR products were digested with BamHI and EcoRI (in case of *pilA* only with BamHI) and cloned by ligation into pEMG opened with the same restriction enzymes. The insert sequences of the constructed vectors were verified by DNA sequencing and the plasmids were integrated into the CHA0 chromosome by transformation of the bacterium via electroporation, resulting in strains CHA5119, CHA5126, CHA5127, CHA5128, CHA5129, CHA5130, CHA5131, CHA5155, and CHA5156 (Table 4).

Overexpression of diguanylate cyclases

To increase the levels of c-di-GMP within the bacterial cells, the diguanylate cyclase-encoding genes *wspR* and *tpbB* of *P. chlororaphis* PCL1391 were cloned into the vector pME8300 under the control of the IPTG-

inducible P_{tac} promoter and integrated into the chromosome at the Tn*7* attachment locus as described in the following. The CDS of the *wspR* and *tpbB* genes were amplified by PCR using the primer pairs PCL.wspR-expr-F/PCL.wspR-expr-R and PCL.tpbB-expr-F/PCL.tpbB-expr-R, respectively, and chromosomal DNA from strain PCL1391. The purified PCR products were digested with SpeI and HindIII and cloned into pME8300 opened with the same restriction enzymes. The correct insert sequences of the newly created vectors pME8390 and pME8391 (Table 4) were verified by DNA sequencing. The two plasmids were subsequently used for the transformation of competent cells by co-electroporation with the helper plasmid pUX-BF13 to generate strains CHA5177, CHA5178, PCL-4, and PCL-5 (Table 4).

Virulence, biofilm formation and motility assays

Injection assays for virulence determination using last-instar larvae of *G. mellonella* (Entomos AG, Grossdietwil, Switzerland) were performed as described before [4].

For quantitative studies of the biofilm formation of selected strains, 200 µl of Grace's Insect Medium (GIM, Sigma) or LB in a 96-well microplate (Greiner Bio-One, Kremsmünster, Austria) were inoculated 1:100 with bacterial suspension and incubated at 25°C for 24 h without agitation. The cell density at 600 nm was measured with a FLUOstar multidetection microplate reader (BMG Labtech GmbH, Offenburg, Germany). The supernatant was then removed and the plate was washed once with saline solution for 5 min at room temperature with agitation (500 rpm). The liquid was removed and 200 µl of a 0.1% crystal violet solution per well was added in order to stain the biofilm. The microplate was incubated for 15 min at room temperature without shaking and then washed three times with bi-distilled water. Two hundred microliters of ethanol were added into each well and the plate was incubated at room temperature for 15 min with shaking. The absorbance at 600 nm was determined using the FLUOstar multidetection microplate reader.

For qualitative studies of the biofilm formation, 3 ml of LB (supplemented with IPTG, if indicated) contained in glass test tubes were inoculated 1:100 with bacterial suspension. Pellicle formation at the air-liquid interface was observed after incubation without agitation at room temperature for at least 24 h.

For studying the motility of selected mutant strains, 5 µl of bacterial suspension at an OD_{600} of 1 was spotted in the middle of an LB swimming agar plate (25 g/L Bacto LB broth (Difco), 2 g/L agar agar (SERVA)). The plates were incubated at room temperature for 16 hours.

Statistical analysis

Statistical analysis of *Galleria* virulence assays was performed in RStudio version 0.98.1091 (http://www.rstudio.com/). The Log-Rank test of the *survival* package of R was used to calculate significant differences in survival between treatments with the wild-type and isogenic mutant strains.

Acknowledgements

I would like to thank Marion Müller and Carmen Menzi for their contributions to the work on FleQ and PhoP-PhoQ, respectively, and Pascale Flury and Theo Smits for providing me access to the genome data.

References

1. Casadevall A, Pirofski LA (1999) Host-pathogen interactions: redefining the basic concepts of virulence and pathogenicity. Infect Immun 67: 3703-3713.
2. Péchy-Tarr M, Bruck DJ, Maurhofer M, Fischer E, Keel C (2008) Molecular analysis of a novel gene cluster encoding an insect toxin in plant-associated strains of *Pseudomonas fluorescens*. Environ Microbiol 10: 2368-2386.
3. Ruffner B (2013) Insecticidal activity in plant-beneficial pseudomonads: Molecular basis and ecological relevance. PhD thesis; ETH Zurich, Zurich, Switzerland.
4. Péchy-Tarr M, Borel N, Kupferschmied P, Turner V, Binggeli O, et al. (2013) Control and host-dependent activation of insect toxin expression in a root-associated biocontrol pseudomonad. Environ Microbiol 15: 736-750.
5. Kupferschmied P, Maurhofer M, Keel C (2013) Promise for plant pest control: root-associated pseudomonads with insecticidal activities. Front Plant Sci 4: 287.
6. Feinbaum RL, Urbach JM, Liberati NT, Djonovic S, Adonizio A, et al. (2012) Genome-wide identification of *Pseudomonas aeruginosa* virulence-related genes using a *Caenorhabditis elegans* infection model. PLoS Pathog 8: e1002813.
7. Subashchandrabose S, Smith SN, Spurbeck RR, Kole MM, Mobley HL (2013) Genome-wide detection of fitness genes in uropathogenic *Escherichia coli* during systemic infection. PLoS Pathog 9: e1003788.
8. Turner KH, Everett J, Trivedi U, Rumbaugh KP, Whiteley M (2014) Requirements for *Pseudomonas aeruginosa* acute burn and chronic surgical wound infection. PLoS Genet 10: e1004518.
9. van Opijnen T, Camilli A (2013) Transposon insertion sequencing: a new tool for systems-level analysis of microorganisms. Nat Rev Microbiol 11: 435-442.
10. Dowling AJ, Wilkinson PA, Holden MT, Quail MA, Bentley SD, et al. (2010) Genome-wide analysis reveals loci encoding anti-macrophage factors in the human pathogen *Burkholderia pseudomallei* K96243. PLoS ONE 5: e15693.
11. Daborn PJ, Waterfield N, Silva CP, Au CP, Sharma S, et al. (2002) A single *Photorhabdus* gene, makes caterpillars floppy (*mcf*), allows *Escherichia coli* to persist within and kill insects. Proc Natl Acad Sci U S A 99: 10742-10747.
12. Boch J, Joardar V, Gao L, Robertson TL, Lim M, et al. (2002) Identification of *Pseudomonas syringae* pv. *tomato* genes induced during infection of *Arabidopsis thaliana*. Mol Microbiol 44: 73-88.
13. Münch A, Stingl L, Jung K, Heermann R (2008) *Photorhabdus luminescens* genes induced upon insect infection. BMC Genomics 9: 229.
14. Cattoir V, Narasimhan G, Skurnik D, Aschard H, Roux D, et al. (2013) Transcriptional response of mucoid *Pseudomonas aeruginosa* to human respiratory mucus. MBio 3: e00410-00412.
15. Loper JE, Hassan KA, Mavrodi DV, Davis EW, Lim CK, et al. (2012) Comparative genomics of plant-associated *Pseudomonas* spp.: insights into diversity and inheritance of traits involved in multitrophic nteractions. PLoS Genet 8: e1002784.
16. Lee DG, Urbach JM, Wu G, Liberati NT, Feinbaum RL, et al. (2006) Genomic analysis reveals that *Pseudomonas aeruginosa* virulence is combinatorial. Genome Biol 7: 10.1186/gb-2006-1187-1110-r1190.

17. Chen L, Yang J, Yu J, Yao Z, Sun L, et al. (2005) VFDB: a reference database for bacterial virulence factors. Nucleic Acids Res 33: D325-328.
18. Gellatly SL, Hancock RE (2013) *Pseudomonas aeruginosa*: new insights into pathogenesis and host defenses. Pathog Dis 67: 159-173.
19. Lugtenberg B, Kamilova F (2009) Plant-growth-promoting rhizobacteria. Annu Rev Microbiol 63: 541-556.
20. Huang CJ, Pauwelyn E, Ongena M, Dubois D, Leclère V, et al. (2015) Characterization of cichopeptins, new phytotoxic cyclic lipodepsipeptides produced by *Pseudomonas cichorii* SF1-54, and their role in bacterial midrib rot disease of lettuce. Mol Plant Microbe Interact: 10.1094/MPMI-1003-1015-0061-R.
21. Ben Belgacem Z, Bijttebier S, Verreth C, Voorspoels S, Van de Voorde I, et al. (2015) Biosurfactant production by *Pseudomonas* strains isolated from floral nectar. J Appl Microbiol 118: 1370-1384.
22. Chin-A-Woeng TF, Thomas-Oates JE, Lugtenberg BJ, Bloemberg GV (2001) Introduction of the *phzH* gene of *Pseudomonas chlororaphis* PCL1391 extends the range of biocontrol ability of phenazine-1-carboxylic acid-producing *Pseudomonas* spp. strains. Mol Plant Microbe Interact 14: 1006-1015.
23. Chin-A-Woeng TF, van den Broek D, de Voer G, van der Drift KM, Tuinman S, et al. (2001) Phenazine-1-carboxamide production in the biocontrol strain *Pseudomonas chlororaphis* PCL1391 is regulated by multiple factors secreted into the growth medium. Mol Plant Microbe Interact 14: 969-979.
24. Laue BE, Jiang Y, Chhabra SR, Jacob S, Stewart GS, et al. (2000) The biocontrol strain *Pseudomonas fluorescens* F113 produces the *Rhizobium small* bacteriocin, N-(3-hydroxy-7-cis-tetradecenoyl)homoserine lactone, via HdtS, a putative novel *N*-acylhomoserine lactone synthase. Microbiology 146 (Pt 10): 2469-2480.
25. Wang X, Preston JF, 3rd, Romeo T (2004) The *pgaABCD* locus of *Escherichia coli* promotes the synthesis of a polysaccharide adhesin required for biofilm formation. J Bacteriol 186: 2724-2734.
26. Arciola CR, Campoccia D, Ravaioli S, Montanaro L (2015) Polysaccharide intercellular adhesin in biofilm: structural and regulatory aspects. Front Cell Infect Microbiol 5: 7.
27. Kropec A, Maira-Litran T, Jefferson KK, Grout M, Cramton SE, et al. (2005) Poly-N-acetylglucosamine production in *Staphylococcus aureus* is essential for virulence in murine models of systemic infection. Infect Immun 73: 6868-6876.
28. Baraquet C, Murakami K, Parsek MR, Harwood CS (2012) The FleQ protein from *Pseudomonas aeruginosa* functions as both a repressor and an activator to control gene expression from the *pel* operon promoter in response to c-di-GMP. Nucleic Acids Res 40: 7207-7218.
29. Jain R, Kazmierczak BI (2014) A conservative amino acid mutation in the master regulator FleQ renders *Pseudomonas aeruginosa* aflagellate. PLoS ONE 9: e97439.
30. Hickman JW, Harwood CS (2008) Identification of FleQ from *Pseudomonas aeruginosa* as a c-di-GMP-responsive transcription factor. Mol Microbiol 69: 376-389.
31. Arora SK, Ritchings BW, Almira EC, Lory S, Ramphal R (1997) A transcriptional activator, FleQ, regulates mucin adhesion and flagellar gene expression in *Pseudomonas aeruginosa* in a cascade manner. J Bacteriol 179: 5574-5581.
32. Hengge R (2009) Principles of c-di-GMP signalling in bacteria. Nat Rev Microbiol 7: 263-273.
33. Hickman JW, Tifrea DF, Harwood CS (2005) A chemosensory system that regulates biofilm formation through modulation of cyclic diguanylate levels. Proc Natl Acad Sci U S A 102: 14422-14427.
34. Giardina G, Paiardini A, Fernicola S, Franceschini S, Rinaldo S, et al. (2013) Investigating the allosteric regulation of YfiN from *Pseudomonas aeruginosa*: clues from the structure of the catalytic domain. PLoS ONE 8: e81324.
35. Raetz CR, Whitfield C (2002) Lipopolysaccharide endotoxins. Annu Rev Biochem 71: 635-700.
36. Lam JS, Taylor VL, Islam ST, Hao Y, Kocincova D (2011) Genetic and functional diversity of *Pseudomonas aeruginosa* lipopolysaccharide. Front Microbiol 2: 118.
37. Taguchi F, Ichinose Y (2013) Virulence factor regulator (Vfr) controls virulence-associated phenotypes in *Pseudomonas syringae* pv. *tabaci* 6605 by a quorum sensing-independent mechanism. Mol Plant Pathol 14: 279-292.

38. West SE, Sample AK, Runyen-Janecky LJ (1994) The *vfr* gene product, required for *Pseudomonas aeruginosa* exotoxin A and protease production, belongs to the cyclic AMP receptor protein family. J Bacteriol 176: 7532-7542.
39. Hay ID, Wang Y, Moradali MF, Rehman ZU, Rehm BH (2014) Genetics and regulation of bacterial alginate production. Environ Microbiol 16: 2997-3011.
40. Mann EE, Wozniak DJ (2012) *Pseudomonas* biofilm matrix composition and niche biology. FEMS Microbiol Rev 36: 893-916.
41. Ryder C, Byrd M, Wozniak DJ (2007) Role of polysaccharides in *Pseudomonas aeruginosa* biofilm development. Curr Opin Microbiol 10: 644-648.
42. Franklin MJ, Nivens DE, Weadge JT, Howell PL (2011) Biosynthesis of the *Pseudomonas aeruginosa* extracellular polysaccharides, alginate, Pel, and Psl. Front Microbiol 2: 167.
43. Ernst RK, Adams KN, Moskowitz SM, Kraig GM, Kawasaki K, et al. (2006) The *Pseudomonas aeruginosa* lipid A deacylase: selection for expression and loss within the cystic fibrosis airway. J Bacteriol 188: 191-201.
44. Raetz CR, Reynolds CM, Trent MS, Bishop RE (2007) Lipid A modification systems in gram-negative bacteria. Annu Rev Biochem 76: 295-329.
45. Haiko J, Westerlund-Wikström B (2013) The role of the bacterial flagellum in adhesion and virulence. Biology (Basel) 2: 1242-1267.
46. Burrows LL (2012) *Pseudomonas aeruginosa* twitching motility: type IV pili in action. Annu Rev Microbiol 66: 493-520.
47. Vallet I, Olson JW, Lory S, Lazdunski A, Filloux A (2001) The chaperone/usher pathways of *Pseudomonas aeruginosa*: identification of fimbrial gene clusters (*cup*) and their involvement in biofilm formation. Proc Natl Acad Sci U S A 98: 6911-6916.
48. Ruer S, Stender S, Filloux A, de Bentzmann S (2007) Assembly of fimbrial structures in *Pseudomonas aeruginosa*: functionality and specificity of chaperone-usher machineries. J Bacteriol 189: 3547-3555.
49. Ball G, Durand E, Lazdunski A, Filloux A (2002) A novel type II secretion system in *Pseudomonas aeruginosa*. Mol Microbiol 43: 475-485.
50. Filloux A (2004) The underlying mechanisms of type II protein secretion. Biochim Biophys Acta 1694: 163-179.
51. Jyot J, Balloy V, Jouvion G, Verma A, Touqui L, et al. (2011) Type II secretion system of *Pseudomonas aeruginosa*: in vivo evidence of a significant role in death due to lung infection. J Infect Dis 203: 1369-1377.
52. Johnson TL, Abendroth J, Hol WG, Sandkvist M (2006) Type II secretion: from structure to function. FEMS Microbiol Lett 255: 175-186.
53. Dalebroux ZD, Miller SI (2014) *Salmonellae* PhoPQ regulation of the outer membrane to resist innate immunity. Curr Opin Microbiol 17: 106-113.
54. Gooderham WJ, Gellatly SL, Sanschagrin F, McPhee JB, Bains M, et al. (2009) The sensor kinase PhoQ mediates virulence in *Pseudomonas aeruginosa*. Microbiology 155: 699-711.
55. Macfarlane EL, Kwasnicka A, Hancock RE (2000) Role of *Pseudomonas aeruginosa* PhoP-PhoQ in resistance to antimicrobial cationic peptides and aminoglycosides. Microbiology 146 (Pt 10): 2543-2554.
56. Lory S, Wolfgang M, Lee V, Smith R (2004) The multi-talented bacterial adenylate cyclases. Int J Med Microbiol 293: 479-482.
57. Cornelis P, Dingemans J (2013) *Pseudomonas aeruginosa* adapts its iron uptake strategies in function of the type of infections. Front Cell Infect Microbiol 3: 75.
58. Ghafoor A, Hay ID, Rehm BH (2011) Role of exopolysaccharides in *Pseudomonas aeruginosa* biofilm formation and architecture. Appl Environ Microbiol 77: 5238-5246.
59. Stutz E, Défago G, Kern H (1986) Naturally occurring fluorescent pseudomonads involved in suppression of black root rot of tobacco. Phytopathology 76: 181-185.
60. Chin-A-Woeng TFC, Bloemberg GV, van der Bij AJ, van der Drift KMGF, Schripsema J, et al. (1998) Biocontrol by phenazine-1-carboxamide-producing *Pseudomonas chlororaphis* PCL1391 of tomato

root rot caused by *Fusarium oxysporum* f. sp. *radicis-lycopersici*. Mol Plant Microbe Interact 11: 1069-1077.

61. Sambrook J, Russel DW (2001) Molecular Cloning: A Laboratory Manual. Cold Spring Harbor (New York): Cold Spring Harbor Laboratory Press.
62. Martínez-García E, de Lorenzo V (2011) Engineering multiple genomic deletions in Gram-negative bacteria: analysis of the multi-resistant antibiotic profile of *Pseudomonas putida* KT2440. Environ Microbiol 13: 2702-2716.
63. Kupferschmied P, Péchy-Tarr M, Imperiali N, Maurhofer M, Keel C (2014) Domain shuffling in a sensor protein contributed to the evolution of insect pathogenicity in plant-beneficial *Pseudomonas protegens*. PLoS Pathog 10: e1003964.
64. Bao Y, Lies DP, Fu H, Roberts GP (1991) An improved Tn*7*-based system for the single-copy insertion of cloned genes into chromosomes of Gram-negative bacteria. Gene 109: 167-168.

CHAPTER 5

Genetic basis, evolution and biological roles of O-polysaccharides in two insect-pathogenic pseudomonads

A trimmed version of this thesis chapter is currently in preparation for publication by Peter Kupferschmied, Tiancong Chai, Pascale Flury, Theo H.M. Smits, Jochen Blom, Alexander Goesmann, Monika Maurhofer, and Christoph Keel.

Tiancong Chai contributed to this chapter by performing experiments and analyzing data; Pascale Flury, Theo H.M. Smits, Jochen Blom, and Alexander Goesmann assembled the genomes, subjected them to automatic annotation and provided Peter Kupferschmied access to the genome data and analysis tools; Pascale Flury in addition performed the oral feeding assays.

Abstract

Some plant-beneficial *Pseudomonas* species display insect pathogenicity in addition to their ability of protecting roots from phytopathogenic fungi and oomycetes. Although the bacterial surface is the site where direct microbe-host interactions take place, its characteristics have barely been studied in these pseudomonads. Lipopolysaccharide (LPS) is the major constituent of the outer membrane of Gram-negative bacteria and generally consists of lipid A, core oligosaccharide and the distal and variable O-antigenic polysaccharide (O-PS, O-antigen). The O-PS moiety of LPS is an important virulence factor in many pathogenic bacteria, since it was suggested to play a role in host colonization and in evasion of or resistance to immune responses of the host organism. We explored the genetic basis of O-PS biosynthesis in recently sequenced, insect-pathogenic pseudomonads by a combined bioinformatic and genetic approach. Gene clusters that were identified as putative O-PS biosynthesis loci in these microorganisms were individually mutated in *Pseudomonas chlororaphis* PCL1391 and *Pseudomonas protegens* CHA0 and their involvement in LPS decoration was assessed by LPS extraction and detection. Insecticidal pseudomonads were found to produce at least two forms of O-antigen simultaneously. Reduction of existing O-PS gene clusters in some strains and their replacement with gene clusters acquired by horizontal gene transfer resulted in a great diversity of O-PS structures in these microbes, which might contribute to host adaptation. Mutant strains lacking O-antigens were significantly attenuated for virulence in *Plutella xylostella* feeding and *Galleria mellonella* injection assays, suggesting that these polysaccharides are vital for a successful oral and systemic infection of the insect host. Cationic antimicrobial peptides play a central role in the immune system of insects. We found that many insect-pathogenic *Pseudomonas* strains were highly resistant to a model antimicrobial peptide while non-insecticidal strains were susceptible. The potential of causing disease in insects might thus be correlated with the resistance to antimicrobial peptides in plant-beneficial pseudomonads. In the two model strains, high resistance to these antimicrobial compounds is dependent on the biosynthesis of the dominant form of O-PS in addition to modification of lipid A with aminoarabinose. This study provides the necessary basis for future research on the biological role, regulation and evolution of O-PS biosynthesis in plant-beneficial pseudomonads with insect-pathogenic properties and represents the first step in the investigation of how cell surface constituents modulate host interactions in these bacteria.

Introduction

Root-associated pseudomonads with plant-beneficial and insect-pathogenic properties are promising biocontrol agents that can be exploited as an alternative to chemical control of phytopathogenic fungi and pest insects in crops [1,2]. Their role in protection of plants against pathogenic fungi and the underlying mechanisms have been studied in detail over decades [2], yet we still have only limited knowledge about the interaction of these microorganisms with their insect hosts. Insect-pathogenic *Pseudomonas* strains are able to infect and cause disease in certain insect species upon oral or systemic infection [1,3,4]. The host-specific production of an insecticidal toxin termed Fit in the insect hemolymph contributes significantly to the pathogenicity in these bacteria [3-5]. However, we do not know the mechanisms that enable them to successfully colonize and invade insects and that make the immune system of the host organism fail to clear the infection.

The cell surface of Gram-negative bacteria consists largely of lipopolysaccharide (LPS), exopolysaccharides and some proteins and is the site where these microbes directly interact with their host organisms [6,7]. Despite their central role in host-microbe interactions, the genetic basis and composition of the cell surface constituents in plant-beneficial pseudomonads have rarely been investigated. LPS is the primary component of the outer membrane in Gram-negative bacteria [8-10]. It consists in general of three different regions: lipid A, core oligosaccharide and O-antigenic polysaccharide (O-PS, also termed O-antigen). LPS is anchored in the outer membrane by the lipid A moiety, a phosphoglycolipid which is essential for bacterial survival and growth and whose structure is highly conserved among species. The lipid A-core serves as an attachment site for the O-PS which consists of repeating units (O-units) of one to five sugar residues and is the exposed part of LPS. LPS is known as “smooth” when O-PS is attached to the lipid A-core and “rough” when uncapped. There is a large variety of monosaccharides that can be incorporated into O-units with different linkages, different stoichiometry and in different orders [11]. The sugar residues can further be modified, for example by methylation or acetylation, which gives rise to a tremendous diversity of O-PS structures in bacteria.

The lipid A moiety of LPS strongly stimulates the innate immune system of mammals via pattern recognition receptors (PRRs) such as the Toll-like receptor 4 (TLR4) complex [9,10,12,13]. Its immunostimulatory properties can result in a massive release of proinflammatory mediators upon recognition and as a consequence is highly toxic to mammalian species [13]. LPS is thus also called endotoxin. In contrast, LPS does not seem to be an immunostimulatory component in insects [14]. Their immune system recognizes bacteria mostly by their peptidoglycan structures [14].

Since O-PS often constitutes the outermost part of a bacterial cell and thus is at the interface between the bacterium and its surrounding environment, the LPS side chains play an important role in microbe-host interactions and are under high selective pressure [11,14,15]. O-PS contributes to pathogenicity and is

generally associated with the ability of a bacterium to colonize a respective host organism and to bypass or overcome host defense mechanisms [8,11,15,16]. The highly variable side chains of LPS have been shown to contribute to resistance to killing by host-derived antimicrobial compounds such as antimicrobial peptides (AMPs) [17-19], and by the complement system in mammalian hosts [17,20]. They were further suggested to protect microorganisms against phagocytosis [21] and to be important for the optimal function and correct localization of other virulence factors [20,22]. In certain bacteria, O-PS also plays a role in colonization of host tissues [8,11,15,16]. It does so by functioning as an adherence factor and by contributing to biofilm formation. While being a virulence factor in pathogenic bacteria, O-antigen can be important for commensal and symbiotic bacteria as well. There are for example indications that it is involved in the molecular communication between such bacteria and plants [11,23,24].

In mammalian pathogens, O-PS can further modulate the recognition of LPS by the innate immune system and thereby influence how these bacteria are perceived by their host organisms [11,16]. Some O-antigen structures have been found to be very similar to polysaccharides of host tissues and were thus suggested to play a role in molecular mimicry [11,25]. In contrast, the O-PS can also constitute an Achilles' heel for bacteria: they serve as receptors for bacteriophages [26] and bacteriocins [27].

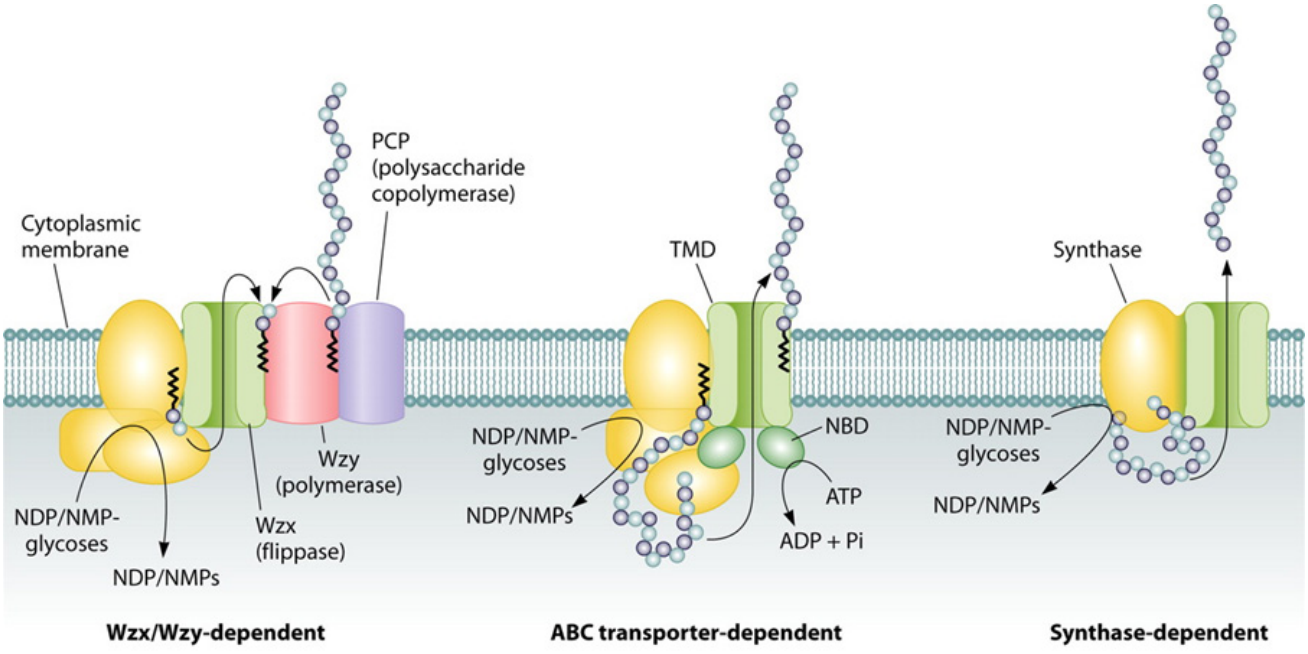

Figure 1: Models for the three pathways involved in the biosynthesis and translocation of O-polysaccharide (O-PS). Repeating units for the biosynthesis of O-PS can be assembled and translocated across the inner membrane by three different pathways. The Wzx/Wzy-dependent and the ABC transporter-dependent pathways are widespread in bacteria and have in common that the so-called O-units are synthesized in the cytoplasm by glycosyltransferases that use nucleotide sugars as donors and undecaprenyl diphosphate as a lipid carrier. In contrast to the ABC transporter-dependent pathway, the O-PS is assembled in the periplasm with the help of a polymerase in the Wzx/Wzy-dependent pathway. The rare synthase-dependent pathway has been reported only for a particular *Salmonella enterica* O-antigen. The figure was taken from [28].

The assembly and translocation of most O-PS occur via Wzx/Wzy-dependent and ATP-binding cassette (ABC) transporter-dependent pathways while a third pathway, which is dependent on a specific synthase, is only rarely found in bacteria (Figure 1) [9,29-32]. The two prominent pathways have in common that undecaprenyl diphosphate (und-PP) is used as a lipid anchor for the synthesis of individual O-units or the entire O-PS at the cytoplasmic face of the inner membrane. Repeating units are assembled by glycosyltransferases which use nucleotide-activated sugars, synthesized by specific enzymes, as precursors (Figure 1). Homopolymers (i.e., polysaccharides with a single monosaccharide component) are commonly synthesized via the ABC transporter-dependent pathway while heteropolymers are most often produced following the Wzx/Wzy-dependent pathway. In the latter pathway, individual und-PP-linked repeating units are transported across the inner membrane with the help of a flippase (Wzx) and are then assembled into O-PS by a polymerase (Wzy) [9,30]. The chain length of the O-PS can be controlled via one or several co-polymerases (Wzz). In the ABC transporter-pathway, the O-PS is completely synthesized in the cytoplasm and exported to the periplasm by an ABC transporter composed of the nucleotide-binding protein Wzt and the transmembrane protein Wzm [9,31]. Several mechanisms for chain length regulation are known for this pathway and seem to be species-dependent. The O-PS is then covalently linked to the lipid A-core in the periplasm by the O-antigen ligase WaaL, irrespective of the pathway it was assembled and translocated by. The LPS molecules are subsequently exported to the cell surface via a separate pathway.

Interestingly, the two prominent O-PS assembly pathways resemble those involved in the biosynthesis of capsular polysaccharide in species of the Enterobacteriaceae, pointing to common evolutionary origins [33]. However, capsular polysaccharides are usually not attached to the lipid A-core and are translocated across the periplasm and outer membrane by distinct pathways.

Genes for the biosynthesis of O-PS are generally organized as gene clusters and can typically be categorized into three groups: the nucleotide sugar pathway genes, sugar glycosyltransferase genes and O-PS processing genes [8,9]. The latter group comprises genes necessary for the transport, chain length determination and modification of O-PS. The presence of genes belonging to these different groups, a certain level of conservation of their nucleotide (and protein) sequences across species and their particular localization on the chromosome makes it possible to identify putative O-PS gene clusters in sequenced genomes.

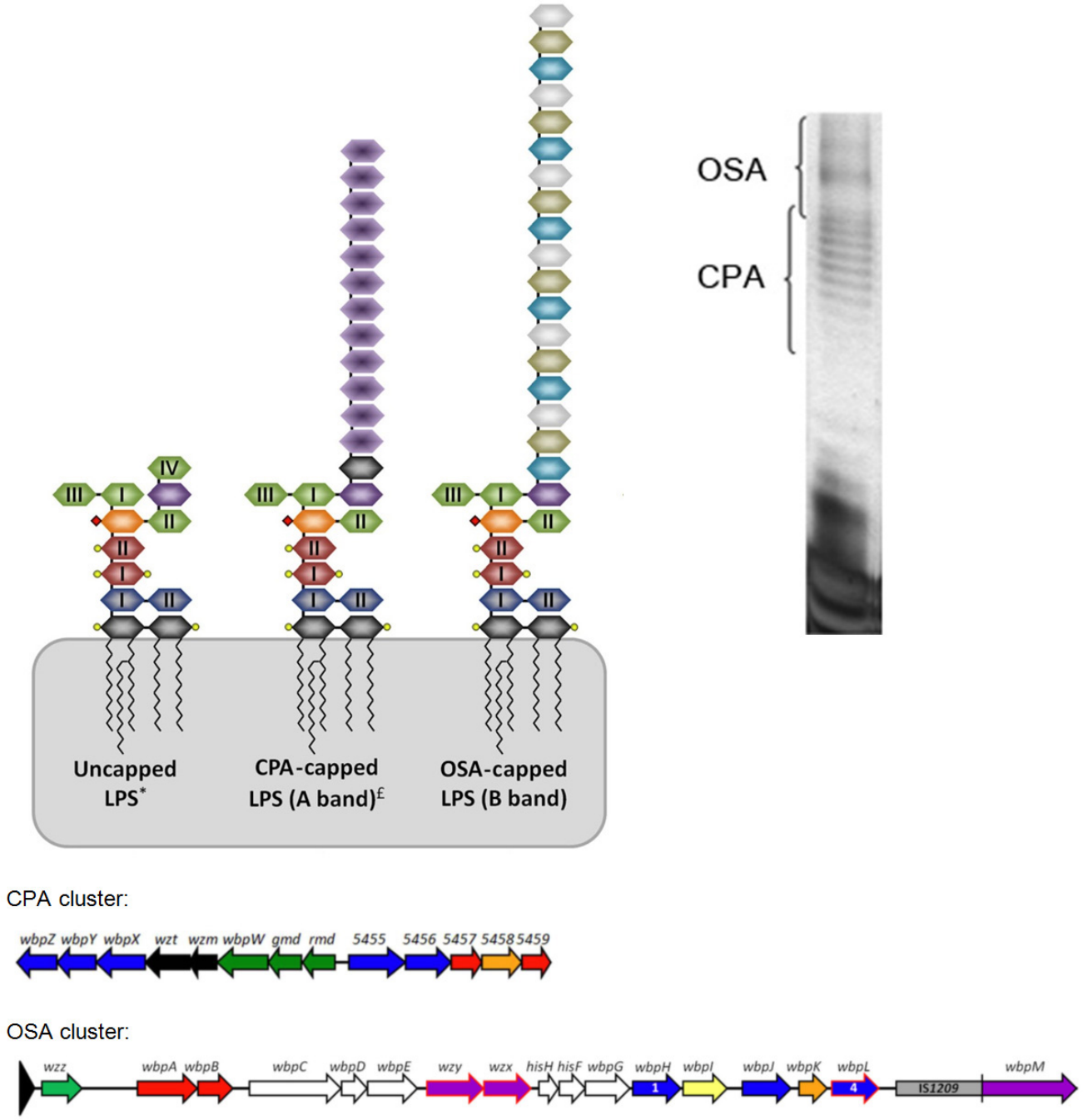

Figure 2: Biosynthesis of O-antigen in *Pseudomonas aeruginosa* PAO1. The opportunistic human pathogen is known to express two distinct forms of O-PS. While the common polysaccharide antigen (CPA; formerly A band) is synthesized by the ABC transporter-dependent pathway and consists of the monosaccharide D-rhamnose, the O-specific antigen (OSA; formerly B band) is produced via the Wzx/Wzy-dependent pathway and is a heteropolymer. Variations in chain lengths result in the typical LPS latter banding pattern observed on silver-stained polyacrylamide gels following SDS-PAGE. OSA and CPA are synthesized and exported by proteins encoded in two separate gene clusters in *P. aeruginosa*. The figure was adapted from [8].

The genetics of O-PS biosynthesis has been well studied in *Pseudomonas aeruginosa* (reviewed in [8]), which provides a prototype for the study of O-antigen biosynthesis in other pseudomonads (Figure 2). The opportunistic pathogen produces two distinct forms of O-antigen: a homopolymer composed of D-rhamnose

and termed common polysaccharide antigen (CPA; formerly A band) and a heteropolymer known as O-specific antigen (OSA; formerly B band) [8,34]. CPA is assembled and transported in *P. aeruginosa* strains via an ABC transporter-dependent pathway. OSA is synthesized via a Wzx/Wzy-dependent pathway and its chain length is controlled by two co-polymerases (Wzz1 and Wzz2). Most genes for the biosynthesis of these two O-PS types were found to be located in two distinct gene clusters (Figure 2). Most species of *Pseudomonas* seem to possess the OSA gene cluster while only *P. aeruginosa* and certain *P. fluorescens* strains were reported to have the gene cluster for the biosynthesis of CPA [35]. The OSA gene cluster might be conserved across species because it contains the gene for the initial glycosyltransferase WbpL, which was shown to be essential for the synthesis of both CPA and OSA [36]. Mutation of *wbpL* significantly reduced the virulence of *P. aeruginosa* in several model organisms [37,38], indicating that O-PS is an important virulence factor in this bacterium as well. *P. aeruginosa* isolates from patients with cystic fibrosis were repeatedly reported to partially lack or be completely devoid of O-antigen [8,39,40]. As a consequence, these O-PS-deficient strains can no longer be bound by O-antigen-specific antibodies, are more inflammatory (due to better recognition of the lipid A part by the innate immune system) but are also less virulent. Several studies suggested that O-PS are amongst others involved in immune evasion, resistance to complement-mediated killing, motility, and biofilm formation in this microorganism [8,41,42]. Resistance to AMPs has, however, not been directly linked to O-PS in pseudomonads to date, but mainly to modification of lipid A with aminoarabinose [43].

Protection of the cell envelope against attacks from AMPs might be critical for bacterial pathogenicity towards insects, as these animals massively produce such antimicrobial compounds for defense against invading microbes since they do not possess the complement system for clearing bacterial infections [14,44]. This prompted us to investigate whether AMP resistance is an intrinsic feature of insecticidal pseudomonads and whether it is associated with their ability to produce LPS with side chains. In the present study, we explored the genetic basis of O-PS biosynthesis in diverse *P. fluorescens* group strains. We discovered that many insect-pathogenic strains indeed are resistant to certain cationic AMPs and that this resistance is dependent on the presence of O-specific side chains on the surface of these bacteria. Loss of the ability to produce smooth LPS resulted in significantly attenuated virulence of a model strain in insect larvae, demonstrating the importance of O-PS biosynthesis for insect pathogenicity in these pseudomonads.

Results

Identification of putative O-polysaccharide biosynthesis genes

Genes encoding proteins that make part of the O-PS biosynthesis machinery (in particular glycosyltransferases, certain O-PS transporters and enzymes synthesizing sugar precursors) can often be detected in a given genome by BLAST searches and analysis of information from automatic and manual gene annotation. Because polysaccharide biosynthesis genes are mostly organized in gene clusters, in this study, the search for them in recently sequenced and publicly accessible genomes was focused on genomic regions where several of these genes were found in proximity. The search for putative O-PS genes was initially focused on the two model strains *Pseudomonas chlororaphis* PCL1391 (Flury *et al*., manuscript in preparation) and *Pseudomonas protegens* CHA0 [45]. A gene cluster flanked by the *ihfB* and *comEA* genes and similar to the OSA cluster of *P. aeruginosa* strains was identified in both PCL1391 (Figure 3) and CHA0 (Figure 4). While in PCL1391 this gene cluster is organized similarly to the one in *P. aeruginosa* PAO1 (including an additional putative chain-length regulator gene residing outside of the locus), it is remarkably reduced in CHA0 where only a few genes are present, a Wzy polymerase-encoding gene is absent and *wzz* is a pseudogene (Figure 4). In contrast to the *P. protegens* strain, PCL1391 furthermore possesses a gene cluster orthologous to the CPA cluster in PAO1 (Figure 3). The three genes *rmd*, *gmd* and *wbpW* which encode enzymes necessary for the biosynthesis of GDP-D-rhamnose in PAO1 [8] are however absent in PCL1391. For simplicity, the two newly identified gene clusters in PCL1391 and CHA0 are called OSA and CPA from here on, owing to their similarity with the *P. aeruginosa* O-antigen clusters.

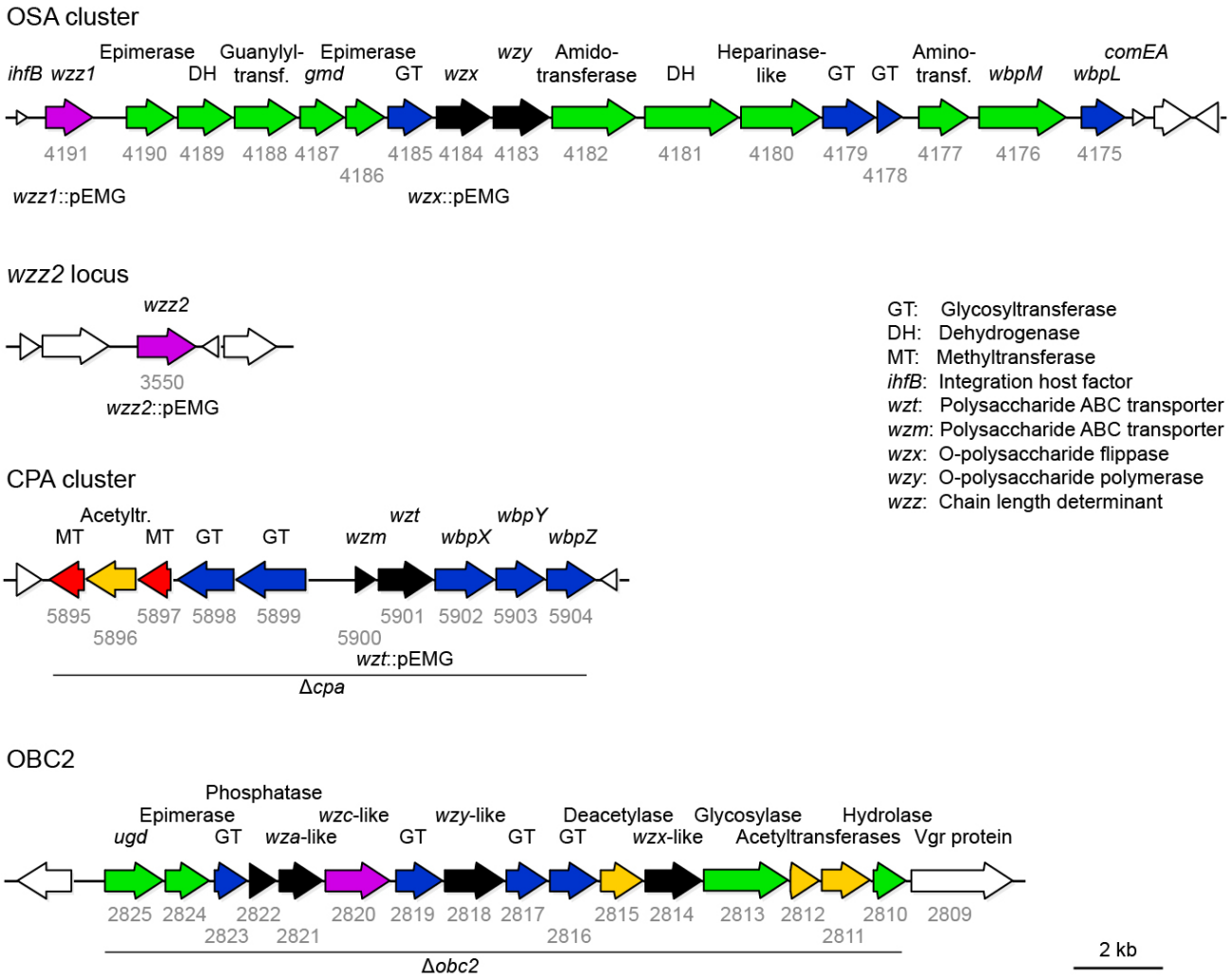

Figure 3: Gene clusters predicted to be involved in the biosynthesis of O-PS in *Pseudomonas chlororaphis* PCL1391. Depicted are genes and gene clusters that were predicted to contribute to O-PS biosynthesis in strain PCL1391. Putative functions of encoded proteins or gene names are indicated above the corresponding genes, information about the locus tags are given below. Genes are drawn to scale and are color-coded according to the function of the proteins they code for: black, transport and polymerization; blue, glycosyltransferases; green, nucleotide sugar biosynthesis and modification of sugars; red, methylation; yellow, (de)acetylation; violet, chain length determination; white, flanking genes. Mutations that were introduced in specific genes and gene clusters in the present study are depicted below each cluster.

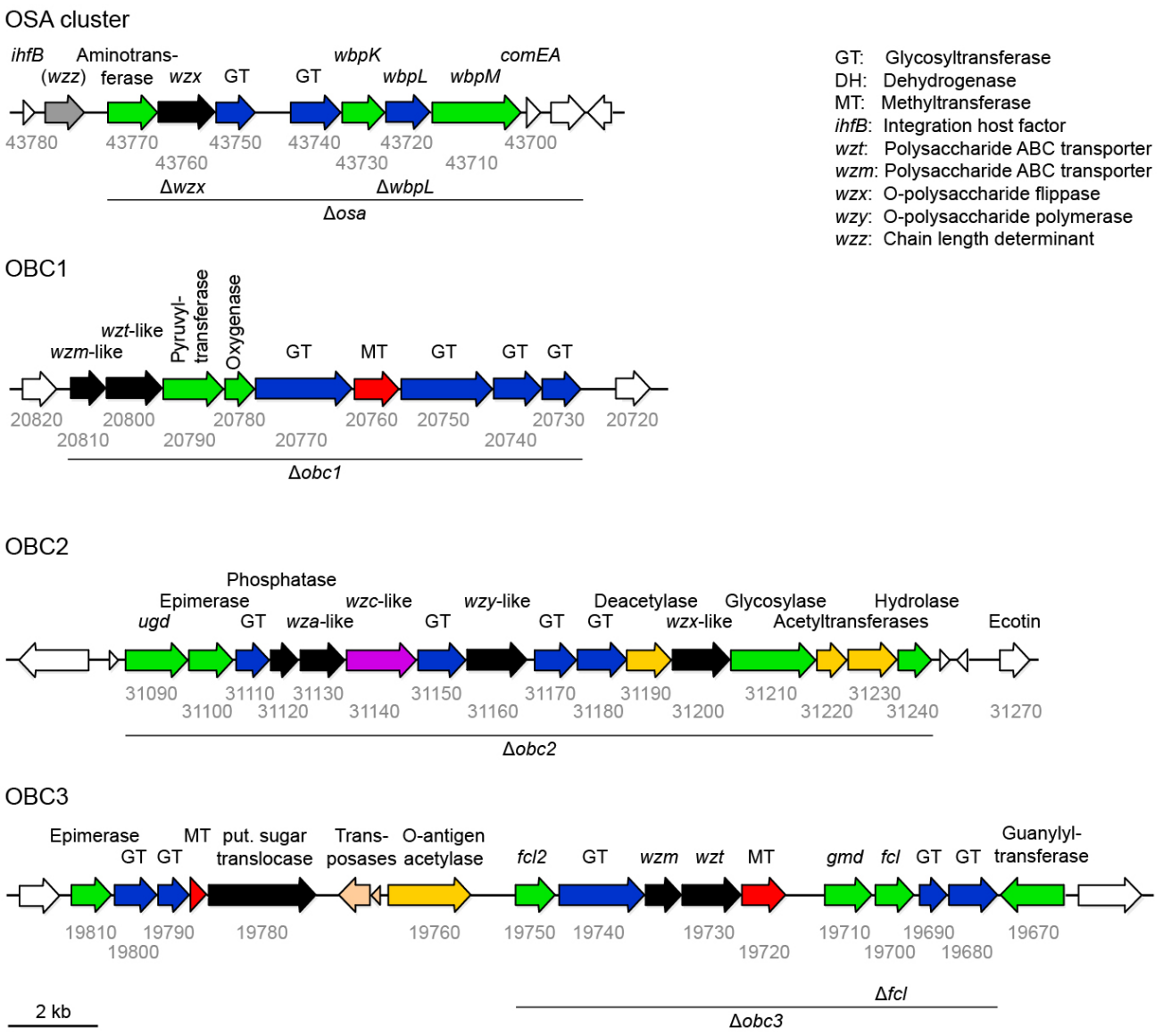

Figure 4: Gene clusters predicted to be involved in the biosynthesis of O-PS in *Pseudomonas protegens* CHA0. Depicted are genes and gene clusters that were predicted to contribute to O-PS biosynthesis in strain CHA0. Putative functions of encoded proteins or gene names are indicated above the corresponding genes, information about the locus tags are given below. Genes are drawn to scale and are color-coded according to the function of the proteins they code for: black, transport and polymerization; blue, glycosyltransferases; green, nucleotide sugar biosynthesis and modification of sugars; red, methylation; yellow, (de)acetylation; violet, chain length determination; gray, pseudogenes; light brown, transposition; white, flanking genes. Mutations that were introduced in specific genes and gene clusters in the present study are depicted below each cluster.

Three additional, so far uncharacterized gene clusters were found in CHA0 and termed O-PS biosynthesis cluster (OBC) 1, 2 and 3 (Figure 4). OBC1 and OBC3 contain genes that were predicted to code for ABC transporter proteins, suggesting an ABC-transporter-dependent biosynthesis of polysaccharides. In contrast, OBC2, which was also detected in the genome of PCL1391 (Figure 3), harbors genes that code for proteins with predicted functions similar to Wzx, Wzy and Wzz in PAO1, indicating the biosynthesis of polysaccharides via a Wzx/Wzy-dependent pathway (Figure 4). A closer analysis of the genetic organization and predictions of the function of the encoded proteins suggested that the OBC2 cluster allows for the biosynthesis of capsular polysaccharides. Capsules form protective structures on the surfaces of many bacteria [29,46], but have not been reported to be produced in pseudomonads before. Unlike O-PS they are not linked to the lipid A-core and are translocated to the cell surface by separate pathways.

The WaaL-encoding genes (*waaL*) were identified by protein BLAST and protein sequence analysis in the LPS core biosynthesis loci of PCL1391 (29% amino acid sequence identity) and CHA0 (42% amino acid sequence identity) (Figure S1). It should be pointed out that, in strain CHA0, *waaL* seems to have undergone a fusion with a gene that is coding for a putative phosphotransferase and is orthologous (49% amino acid sequence identity) to PA4998 in PAO1, and thus most likely encodes an O-PS ligase fused to a kinase domain at its C-terminus (Figure S1B). This gene fusion was observed for all *P. protegens* strains in this study and also in some additional *P. fluorescens* group lineages (data not shown).

Since the biosynthesis of different polysaccharides in bacteria involves similar proteins [33], the screening for O-PS genes revealed genes for the production of oligosaccharides and polysaccharides other than O-antigen as well. It thus brought up gene clusters predicted to be responsible for flagellar glycosylation, the biosynthesis of the LPS core oligosaccharide and the production of the exopolysaccharides Psl, Pel and alginate (Table S1). Remarkably, also an operon (*pgaABCD*) that most probably encodes proteins for the biosynthesis of the exopolysaccharide poly-*N*-acetylglucosamine (PNAG) was detected in many strains of the *P. fluorescens* group (Table S1). PNAG is produced for example by *Escherichia coli* and *Staphylococcus* species for which it is an important colonization and immune evasion factor [47-49], but has not been identified in *Pseudomonas* species so far.

Mutational analysis of O-PS biosynthesis

Putative O-PS biosynthesis genes and gene clusters identified in strains PCL1391 and CHA0 were mutated individually as depicted in Figures 3 and 4. The resulting LPS banding patterns were studied by extraction, separation and detection of LPS on polyacrylamide gels (Figure 5).

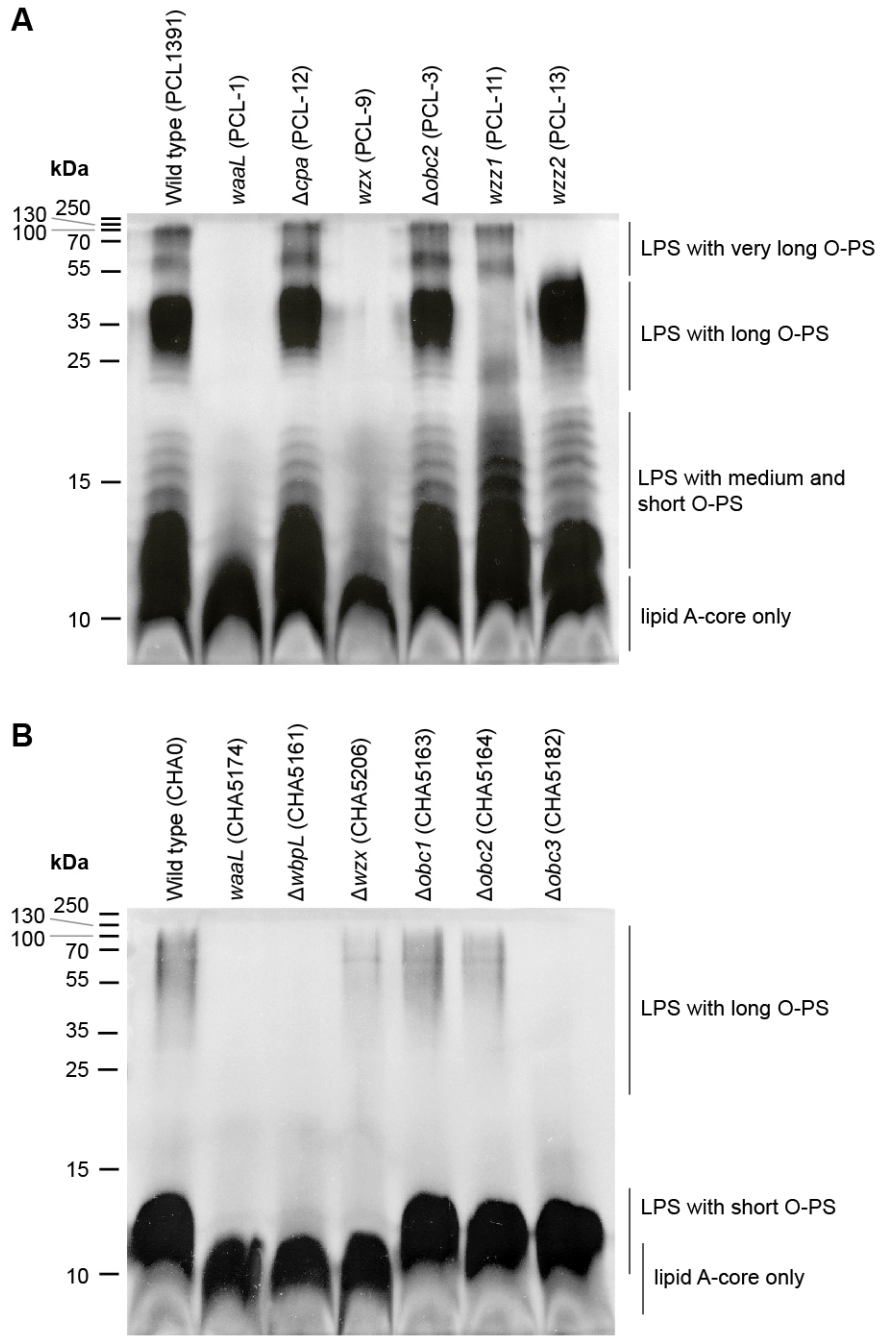

Figure 5: Mutation of O-PS biosynthesis genes in *Pseudomonas chlororaphis* PCL1391 (A) and *Pseudomonas protegens* CHA0 (B). SDS-PAGE of LPS extracted from PCL1391, CHA0 and their isogenic mutant strains grown at 25°C to late exponential growth phase in Lysogeny Broth. LPS was made visible by silver staining. Molecular weights in kDa are depicted on the left of each gel and predicted compositions of LPS molecules on the right.

P. chlororaphis PCL1391 displayed a complex LPS banding pattern with a modular length distribution (Figure 5A). Under the chosen growth conditions, the wild-type strain produced LPS with three distinct modules of bands with high to very high molecular weights (25 to over 100 kDa; indicated with “long” and “very long” in Figure 5A) and additionally several low-molecular-weight bands (10 to approx. 20 kDa). Mutation of *waaL* (PCL-1) resulted in the loss of most bands, except for the one with the lowest molecular weight which most

probably corresponds to the uncapped lipid A-core moiety of LPS. Since WaaL is essential in most Gram-negative bacteria to produce smooth LPS, this strongly indicated that bands visible above the lowest one on the gel corresponded to LPS molecules with covalently linked O-PS (or capsular polysaccharides). Re-introduction of *waaL* in strain PCL-1 rescued all bands visible in the wild-type strain, indicating that there was no polar effect caused by disruption of *waaL* in PCL1391 (Figure S2). Disruption of *wzx* encoding the putative flippase in the OSA cluster resulted in a similar LPS phenotype as in the *waaL* mutant, with only weak bands remaining above the lipid A-core band on the gel. The deletion of the CPA cluster did not result in a significant change in the LPS banding pattern, except for a decreased intensity of some bands just below 25 kDa. Extraction and visualization of LPS from the wild type and isogenic mutants grown at 30°C suggested that the CPA cluster significantly contributed to O-PS production at elevated temperatures by encoding proteins that are responsible for the biosynthesis of a *wzx*-independent form of O-PS in this strain (Figure S3). LPS banding patterns of the *obc2* deletion mutant resembled those of the wild type, indicating that OBC2 is not essential for O-PS biosynthesis in PCL1391. Mutagenesis of *waaL* and genes of the OSA cluster (except for *wzz1*) in PCL1391 resulted in mutant strains with obvious growth defects, while deletion or disruption of other genes did not seem to alter the growth rate of the bacterium considerably (Figure S4). Additionally, several attempts to delete genes in the OSA cluster failed. All these findings suggest that the OSA gene cluster is the dominant O-PS biosynthesis cluster in PCL1391 under the conditions tested.

Mutation of *wzz1* or *wzz2*, which code for putative O-PS chain length regulators, led to the disappearance of the dominant module with high-molecular-weight bands and the two modules with very-high-molecular-weight bands, respectively (Figure 5A). This result suggests that Wzz1 mediates the biosynthesis of long OSA-type O-PS chains, while Wzz2 is essential for the production of very long O-PS chains. The *wzz1* mutant of PCL1391 was further unable to swarm (Figure S4C), indicating that presence and correct modular size distribution of O-PS is essential for swarming motility.

P. protegens CHA0 displayed an LPS banding pattern distinct from PCL1391 (Figure 5B). Separation of the extracted LPS on polyacrylamide gels and subsequent silver staining revealed a dominant low-molecular-weight band between 10 and 15 kDa and a smear comprising molecules with high molecular weights of up to more than 100 kDa. The disruption of *waaL* and the deletion of *wbpL*, which encodes the putative initial glycosyltransferase essential for both types of O-PS in PAO1, both resulted in the loss of the high-molecular-weight bands and in a shift of the low-molecular-weight band of several kDa. Complementation of the *waaL* and *wbpL* mutants rescued the production of wild-type O-PS (Figure S2). This suggests that the smear with the high-molecular-weight bands and the dominant low-molecular-weight band consisted of smooth LPS molecules with long and short O-PS, respectively.

In-frame deletion of *wzx* encoding the putative flippase in CHA0 resulted in the shift of the low-molecular-weight band observed before in the *waaL* and Δ*wbpL* mutants, but not in the disappearance of the LPS

molecules with the long O-PS. In contrast, partial deletion of the OBC3 cluster (Figure 4) led to the loss of the LPS form with long O-PS without causing a shift in the low-molecular-weight band (Figure 5B). Deletion of either OBC1 or OBC2 did not alter the LPS banding pattern observed on the gels, nor did the double mutation in CHA5165 (data not shown). This indicates that, although they seemed to be expressed (Figure S5) and might somehow modify O-PS, the OBC1 and OBC2 gene clusters are not essential for O-PS biosynthesis in strain CHA0. These observations together suggest that genes in the OBC3 cluster are essential for the synthesis of smooth LPS with long O-PS in *P. protegens* CHA0, while the remaining genes in the OSA cluster are necessary for the production of LPS molecules capped with (very) short O-PS. WbpL, which is the putative initial glycosyltransferase encoded in the OSA cluster, seems to be essential for the biosynthesis of both forms of O-PS in strain CHA0. Since no gene coding for a Wzy polymerase was found in CHA0, it is likely that the short O-PS form consists of only one O-PS repeating unit as it was reported for a *wzy* mutant of PAO1 [8].

Although having different genetic bases for the biosynthesis of polysaccharides, both *P. chlororaphis* PCL1391 and *P. protegens* CHA0 are capable of synthesizing two distinct forms of O-PS simultaneously. The strains might achieve this by employing two different biosynthesis pathways, i.e. a flippase-dependent (in the case of OSA) and an ABC-transporter-dependent (in case of CPA and OBC3), in parallel. In both strains, the OSA-type capped form of LPS seemed to dominate under the chosen growth conditions.

Genetic diversity and evolution of O-PS biosynthesis

The available genome sequences (Flury *et al*., manuscript in preparation) and the knowledge gained about the genetic basis of O-PS biosynthesis in strains PCL1391 and CHA0 made it possible to analyze the diversity of genes and gene clusters predicted to be involved in the production of O-PS in biocontrol pseudomonads with insecticidal activity. Analysis of banding patterns of LPS extracted from selected *Pseudomonas* strains indicated that they all produce smooth forms of LPS (Figure 6A).

The presence and genetic composition of predicted O-PS biosynthesis gene clusters varied extensively among the insect-pathogenic *Pseudomonas* strains analyzed in this study (Figure 6B). Remarkably, only one strain (*P. chlororaphis* JF3835, isolated from fish [50]) in the focus of this study harbored the complete classical *P. aeruginosa*-type O-PS gene clusters. All other strains showed significant changes to the prototype of O-PS biosynthesis in pseudomonads.

All *P. protegens* strains in this study were found to possess an OSA gene cluster that is significantly reduced compared to the classical OSA cluster of *P. aeruginosa* and certain *P. chlororaphis* strains (Figure 6B, Table S1). This genetic reduction was even more extensive in the *P. protegens* strains K94.41, PF and Pf-5 where the OSA cluster consists of only *wbpL* and the two flanking genes. A similarly reduced OSA cluster could also

be detected in *P. chlororaphis* strains LMG1245, YL-1 and O6 as well as in *Pseudomonas* sp. CMR5c. The genome analysis also revealed that *P. protegens* strains not only have a reduced OSA cluster but also entirely lack the CPA cluster which was present in all other pseudomonads in the focus of this study (Figure 6B, Table S1).

Interestingly, all strains with a reduced OSA gene cluster were found to possess additional gene clusters (OBC3-7) harboring genes that are predicted to contribute to O-PS biosynthesis (Figures 6B and S6). Sequence comparisons using nucleotide BLAST suggested that these additional gene clusters (or at least parts of them) were acquired at one point by horizontal transfer across species and/or genus barriers, since orthologs of many genes of these clusters were absent in genomes of closely related *Pseudomonas* strains but detectable in polysaccharide biosynthesis clusters of various different bacterial species and genera (Figure S6). An example is the OBC3 gene cluster, which was found to be essential for the biosynthesis of long O-PS in CHA0 (Figure 5B). It was additionally identified in *P. protegens* PGNR1, BRIP, and Cab57 as well as in *Pseudomonas* sp. CMR5c (Figure 6B). The OBC3 cluster harbors several genes with relatively high sequence identities (67-75%) to genes of the O-PS biosynthesis locus of *Rhizobium etli* (Figures 7A and S6). Three of these genes encode enzymes (a GDP-mannose 4,6-dehydratase and a GDP-L-fucose synthase) and a glycosyltransferase (the fucosyltransferase WreE) that are sufficient for the synthesis of GDP-L-fucose from GDP-D-mannose and the covalent binding of the sugar to O-PS repeat units in *R. etli* CE3 ([51], Figure 7B). The homologous genes in CHA0 were at least 67% identical to the *Rhizobia* genes and seemed to be organized as an operon as well, indicating that CHA0 is capable of synthesizing GDP-L-fucose (Figure 7A). Deletion of *fcl*, which codes for a putative GDP-L-fucose synthase, in CHA0 (CHA5205) resulted in the loss of long O-PS (Figure 7C) as it was observed for the Δ*obc3* mutant before (Figure 5B). Re-introduction of *fcl* into the genome of CHA5205 rescued the biosynthesis of the long O-PS. Complementation of the same mutant strain with *fcl* from *R. etli* CE or *E. coli* K-12, which is able to synthesize GDP-L-fucose as well [52], similarly rescued the production of long O-PS, which, however, appeared to have lower molecular weights compared to the wild type. Nevertheless, it can be assumed that CHA0 *fcl* encodes a GDP-L-fucose synthase just as in *R. etli* and *E. coli*, since glycosyltransferases generally display strict substrate specificity [53]. It is therefore likely that an ancestor of *P. protegens* has acquired the ability to synthesize long O-PS containing fucose by horizontal transfer of genes from rhizobia.

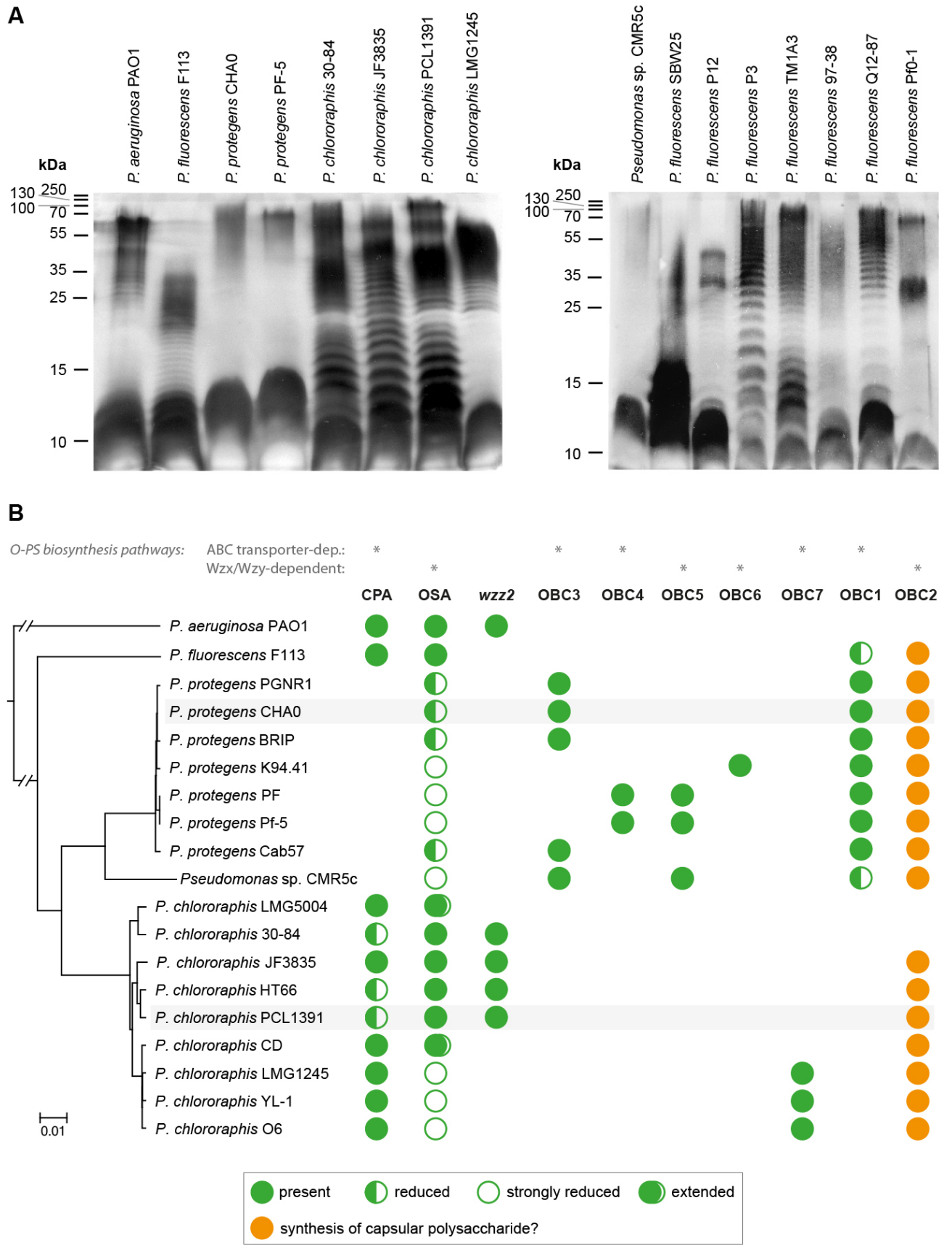

Figure 6: Genetic and phenotypical diversity of O-PS biosynthesis in selected pseudomonads. (A) SDS-PAGE of LPS extracted from diverse *Pseudomonas* strains grown until late exponential growth phase in Lysogeny Broth (LB) at 25°C. Silver staining was used to visualize LPS on the gels. Molecular weights in kDa are indicated on the left of each gel. **(B)** Phylogeny of selected pseudomonads,

based on the full core genome (Flury *et al*., manuscript in preparation), and the presence of identified gene clusters predicted to contribute to the biosynthesis of O-PS in these strains. Gene clusters lacking functionally important genes (CPA: *rmd*, *gmd* and/or *wbpW*; OSA: *wzz1*, *wzy*; OBC1: genes encoding proteins for nucleotide sugar biosynthesis at the 5' end) are depicted as "reduced" and the ones missing additional genes (OSA: *wzx*) are displayed as "strongly reduced"). Gene clusters that comprise genes coding for both ABC transporters and flippases are depicted as "extended". No role in O-antigen biosynthesis was found for OBC1. Locus tags for all genes and gene clusters are listed in Table S1.

Orthologs of genes of the OBC5 and OBC6 gene clusters were detected at the OSA locus (flanked by *ihfB* and *comEA*) of certain *Pseudomonas* strains (Figure S6 and data not shown). This suggests that the OBC5 and OBC6 gene clusters were originally residing within the OSA locus, encoding the proteins responsible for the biosynthesis of the major O-PS, in the donor bacteria before they were vertically acquired by ancestors of the respective *P. protegens* strains. Three genes of the OBC5 cluster (*rfbF*, *rfbG* and *rfbH* (Figure S6)) were similar (66% nucleotide sequence identity) to genes of the O-PS biosynthesis gene cluster *rfb* of *Salmonella enterica* subsp. *enterica*. They were predicted to code for enzymes that catalyze the production of CDP-D-abequose, a nucleotide sugar used for the synthesis of O-PS in certain *Salmonella* serovars [54,55]. This indicates that, at one point, these sugar biosynthesis genes have been acquired horizontally by pseudomonads from enterobacteria.

While one *P. chlororaphis* lineage appears to have lost functionally important genes of the OSA cluster, independent events in two other lineages of the same species seem to have led to the loss of genes in the CPA gene cluster necessary for the synthesis of GDP-rhamnose. Interestingly, strain 30-84 only lost the gene *rmd*, but still possesses the *gmd* and *wbpW* genes (data not shown), which pointed to a gradual loss of genes for GDP-rhamnose biosynthesis in *P. chlororaphis*. Since some indications were found in this study that PCL1391 is still able to synthesize O-PS with the remaining CPA cluster genes, it is likely that GDP-rhamnose was replaced by another nucleotide sugar for the synthesis of the CPA-type O-PS in *P. chlororaphis* strains with reduced CPA clusters.

The OBC2 cluster, for which no obvious role in O-PS biosynthesis was observed in PCL1391 and CHA0, was found to be generally well conserved in strains of the *P. fluorescens* group in terms of presence and composition (Figure 6). The observed conservation indicated that OBC2 has an important biological function in these bacteria, but, compared to the CPA and OSA gene clusters, does not seem to be under high selective pressure.

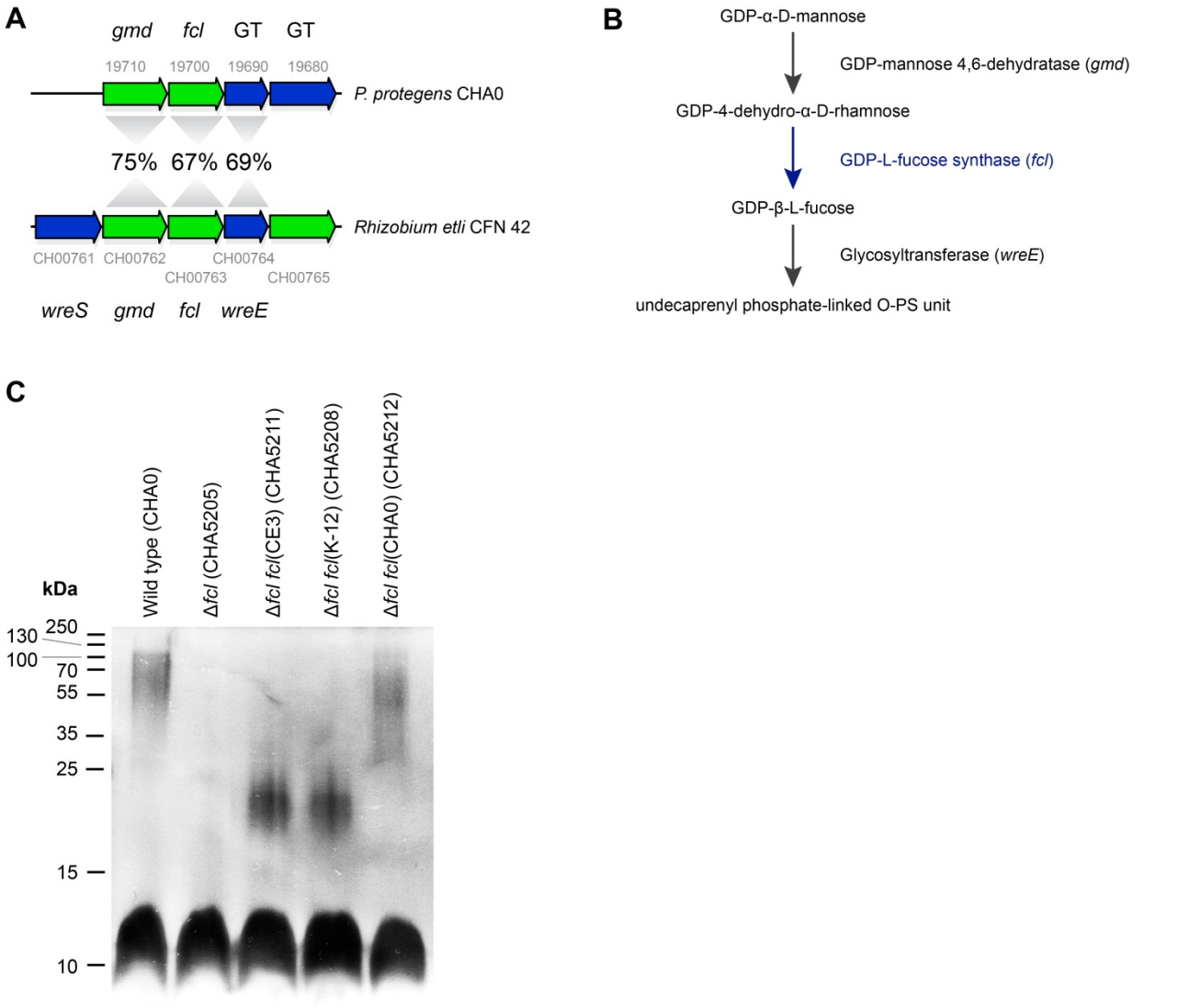

Figure 7: Genes for the biosynthesis of GDP-fucose possibly have been acquired by horizontal gene transfer in *Pseudomonas protegens*. (A) Certain genes of the OBC3 locus of *P. protegens* CHA0 are homologous to genes of the O-antigen biosynthesis locus of *Rhizobium etli* CE3. Three genes of the OBC3 gene cluster show sequence identities of at least 67% to genes in *R. etli* CE3 necessary for the biosynthesis of L-fucose-containing O-antigen [51]. **(B)** GDP-L-fucose is synthesized in various bacteria by conversion of GDP-D-mannose by a GDP-mannose dehydrogenase and a GDP-L-fucose synthase and can subsequently be used to assemble O-PS units [51,52]. **(C)** SDS-PAGE with LPS extracted from CHA0, its Δ*fcl* mutant and complemented strains. Molecular weights are indicated on the left of the gel in kDa. Long O-PS were lost in the Δ*fcl* mutant and (partly) rescued by complementation with *fcl* from CHA0, *E. coli* K-12 or *R. etli* CE3.

The OBC3-type O-polysaccharide acts as a bacteriophage receptor

A lytic bacteriophage isolated from a culture of *P. protegens* CHA0 and named GP100 was found to have a deleterious impact on the survival and biocontrol activity of the bacterial host [56]. Because O-PS frequently serve as receptors for bacteriophages of Gram-negative bacteria [26], the ability of the phage GP100 to infect constructed O-PS mutants of *P. protegens* CHA0 was investigated. Deletion of *fcl* but not *wzx* rendered strain CHA0 resistant to infection by GP100 (Figure 8), indicating that the long O-PS synthesized by proteins encoded in the OBC3 gene cluster is the receptor for the bacteriophage. Complementation *in cis* with *fcl* from CHA0 or *in trans* with *fcl* from *R. elti* CE3 or *E. coli* K-12 restored the susceptibility of CHA0 to GP100 (Figure 8). The observation that the bacteriophage was able to recognize and infect the Δ*fcl* mutant complemented *in trans* further supports our hypothesis that CHA0 is capable of synthesizing GDP-L-fucose and that it incorporates this particular sugar into its long O-PS chains.

It was reported that phage GP100 only infects a narrow range of *P. fluorescens* group strains [56], which can now be explained by the presence of the OBC3 cluster in only a limited number of *Pseudomonas* strains (Figure 6B). All strains possessing the OBC3 cluster available for testing in the laboratory were susceptible to infection by the bacteriophage, while *P. protegens* strains without it were resistant (Figure 8).

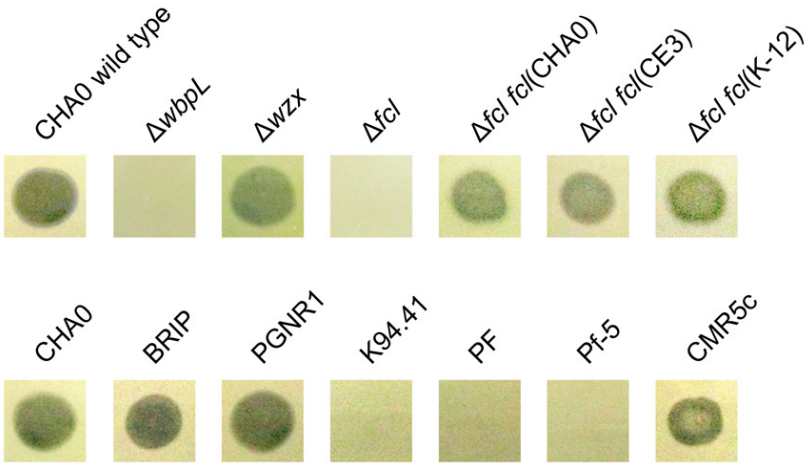

Figure 8: The OBC3-type O-polysaccharide serves as a receptor for bacteriophage GP100. The susceptibility of CHA0, its isogenic mutant strains and various *Pseudomonas* strains to infection by GP100 was assessed by inoculation of a soft agar layer containing the pseudomonads with some microliters of bacteriophage suspension. A clear zone indicated lysis of the bacteria due to infection by GP100, while phage-resistant bacteria were able to grow in the presence of the bacteriophage.

O-polysaccharides contribute to insect pathogenicity of *Pseudomonas protegens* CHA0

O-PS plays an important role as virulence factors in many pathogenic bacteria. Therefore, the contribution of these molecules to insect pathogenicity in *P. protegens* CHA0 and *P. chlororaphis* PCL1391 was investigated. To do so, mutant strains with no obvious growth defects (Figure S4) were selected for virulence and competition experiments. *P. protegens* CHA0 without O-PS (Δ*wbpL*, CHA5161) was strongly reduced in its ability to kill larvae of *Galleria mellonella* upon injection (Figure 9A). Complementation of the mutant strain (CHA5169) restored its insecticidal activity to wild-type level. Loss of the short and long O-PS (CHA5206 and CHA5205, respectively) both resulted in a significantly reduced virulence in strain CHA0, suggesting that both forms of O-PS contribute to the systemic virulence of the bacterium in *G. mellonella*. In contrast, mutation of *wzz1* and *wzz2* in *P. chlororaphis* PCL1391 did not cause a decrease in virulence in this infection model (Figure S7), indicating that regulation of O-PS chain lengths is not critical for virulence of PCL1391 during systemic infection of insects. The Δ*wzx* and the Δ*wbpL* mutants of *P. protegens* CHA0 were also significantly less virulent than the wild type when orally administered to *Plutella xylostella* larvae (Figure 9B), indicating that in this bacterium the short O-antigen also contributes to insect pathogenicity via the oral route of infection. In contrast to the assay with *G. mellonella*, the *wbpL* deletion mutant was more virulent than the Δ*wzx* mutant in this infection model. Moreover, the Δ*fcl* mutant was as lethal as the wild type in *P. xylostella*, suggesting that the long O-PS is not important for pathogenicity in this insect species upon ingestion.

For a competition assay, the mutant strains were tagged with mCherry and injected into *G. mellonella* as a 1:1 mixture with the respective GFP-tagged wild-type bacteria. Lack of the short, long or both types of O-PS significantly reduced the competitiveness of mutants of strain CHA0 in the insect larvae (Figure 9C). Production of O-PS thus might be crucial for efficient colonization of the insect host by *P. protegens* during systemic infection. Isogenic mutants of PCL1391 lacking functional Wzz1 or Wzz2 were in contrast as competitive as the wild type (Figure 9C). Therefore, specific regulation of O-PS length did not seem to be essential for colonization of *G. mellonella* by *P. chlororaphis* upon injection.

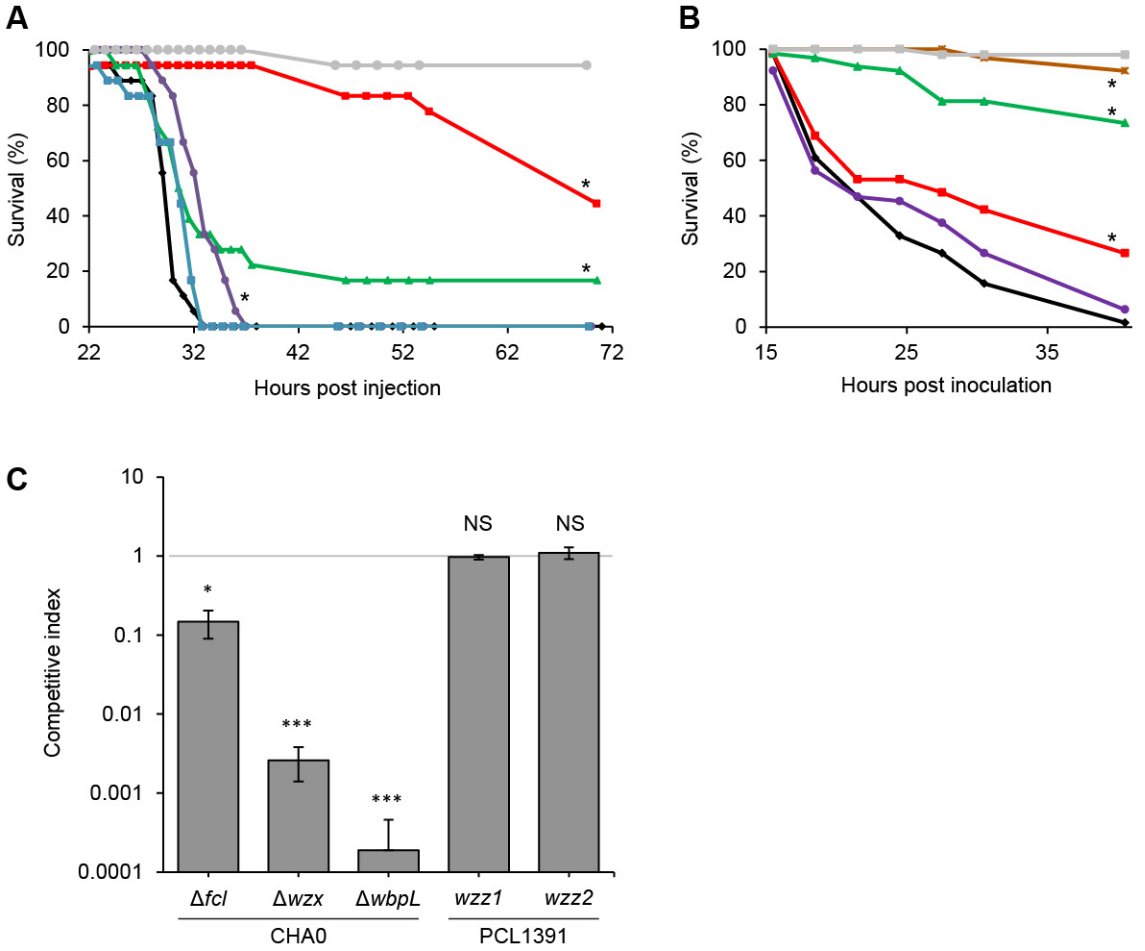

Figure 9: O-polysaccharides contribute to insect pathogenicity in *Pseudomonas protegens* CHA0. (A) Virulence of mutant strains of CHA0 was determined by injection of a low number of bacterial cells into larvae of *Galleria mellonella* and monitoring the survival of the insects over time. Deletion of *wbpL* (in red, CHA5161), *wzx* (in green, CHA5206) or *fcl* (in violet, CHA5205) significantly reduced the mortality of the larvae (*, p-value < 0.001; Log-rank test), while the complemented Δ*wbpL* mutant (in blue, CHA5169) was as virulent as the wild type (in black, CHA0). Saline solution served as a negative control (in gray). The experiment was repeated once with similar results. **(B)** The virulence for selected mutants (CHA5161, in red; CHA5205, in violet; CHA5206, in green) and the wild-type strain (in black) of *P. protegens* CHA0 upon oral infection was assessed using larvae of *Plutella xylostella*. The caterpillars were fed with bacteria-treated artificial diet and their survival was monitored over two days. Saline solution (in gray) and the *gacA* mutant of CHA0 (CHA89, in brown) served as a negative control. Treatments that were significantly different from the one with wild-type CHA0 are indicated with * (p-value < 0.005; Log-rank test). This experiment was repeated twice with similar results. **(C)** Competitiveness of mutant strains during insect infection was investigated by injecting equal numbers of fluorescently marked mutant and corresponding wild-type cells into larvae of *G. mellonella* and counting bacterial cells in hemolymph samples 20 to 24 hours after the injection by epifluorescence microscopy. The competitive index indicates the ratio between the cell numbers of the mutant and the wild type, normalized by the ratio in the inoculum. Significant differences between the competing strains are indicated with * (p-value < 0.05; one-sample *t* test) and *** (p-value < 0.005; one-sample *t* test). The experiment was repeated twice with similar results. NS, not significant.

The same reporter bacteria were also used for an analogous competition assay on roots of cucumber plants. All mutant strains colonized the root tips as competitively as their respective wild types (Figure 10), suggesting that O-PS are not critical for colonization of plant roots under axenic conditions. Furthermore, loss of O-PS did not seem to markedly affect the motility and biofilm formation in CHA0 (Figure S4), nor its hemolytic activity and production of antifungal metabolites (data not shown).

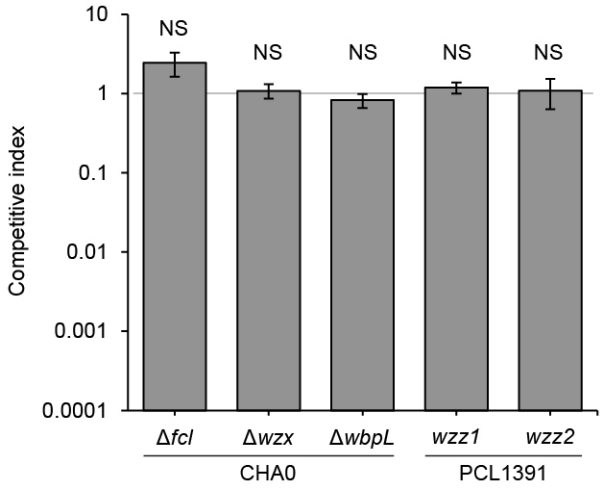

Figure 10: O-polysaccharide is not essential for colonization of plant roots by *Pseudomonas protegens* CHA0 under axenic conditions. Competitiveness of mutant strains during root colonization was studied by inoculation of cucumber plants with equal numbers of fluorescently marked mutant and corresponding wild-type cells and counting bacterial cells on root tips by epifluorescence microscopy 5 days after the inoculation. The competitive index indicates the ratio between the cell numbers of the mutant and the wild type, normalized by the ratio in the inoculum. No significant differences between the competing strains was observed (one-sample *t* test; $\alpha = 0.05$). The experiment was repeated once with similar results. NS, not significant.

The major O-polysaccharide contributes to antimicrobial peptide resistance

Because O-PS were suggested to be important for resistance to cationic AMPs [17,18], selected *Pseudomonas* strains were tested for their susceptibility to polymyxin B (PMB). PMB is a bacterial cationic AMP with a mode of action similar to that of AMPs of higher organisms and is therefore commonly used as an inexpensive model AMP [57-59].

Remarkably, strains of *P. protegens* and *Pseudomonas* sp. CMR5c were highly resistant to PMB, with visible growth observed at a concentration of 250 µg/ml (Figure 11A). Most *P. chlororaphis* strains also displayed resistance to this cationic AMP, while the other pseudomonads tested were susceptible to PMB. Isogenic mutants of CHA0 incapable of synthesizing LPS capped with short O-PS were significantly less resistant to PMB than the wild type (Figure 11B): no survival at 100 µg/ml PMB was observed when *wbpL*, *waaL*, or *wzx* was mutated whereas complementations restored wild-type resistance levels. The presence of long O-PS however did not seem to be important for AMP resistance, since the Δ*fcl* mutant was as resistant to PMB as the wild type. In contrast, mutation of *arnA*, required for the modification of lipid A with aminoarabinose,

resulted in a decreased level of polymyxin resistance resembling that of the Δ*wzx* mutant of CHA0. A *wbpL arnA* double mutant (CHA5214) was completely sensitive to PMB, suggesting that lipid A modification with aminoarabinose and capping LPS with short O-PS contributed to PMB resistance independently. Similar results were obtained with colistin (data not shown), another polymyxin AMP. Moreover, the presence of O-antigen seems to be essential for AMP resistance in *P. chlororaphis* PCL1391 as well (Figure S8). The length of the O side chains thereby seems to shape the degree of resistance to these antimicrobial compounds.

Since lipid A modification with aminoarabinose in *P. aeruginosa* is known to be dependent *in vitro* on limitation of magnesium (Mg^{2+}) in the growth medium [60], resistance of *P. protegens* CHA0 to PMB was also assessed in the presence of high concentrations (20 mM) of Mg^{2+}. Although the bacterium became less resistant to the action of PMB with increasing Mg^{2+} concentrations, the survival of the remaining cells was still dependent on the function of both *arnA* and *wbpL* (Figure S9). This further suggests that *P. protegens* is resistant to AMPs even in the presence of the high concentrations of Mg^{2+} occurring in the hemolymph of insect larvae [61-63] and that it needs both mechanisms to protect itself against AMPs during insect infection.

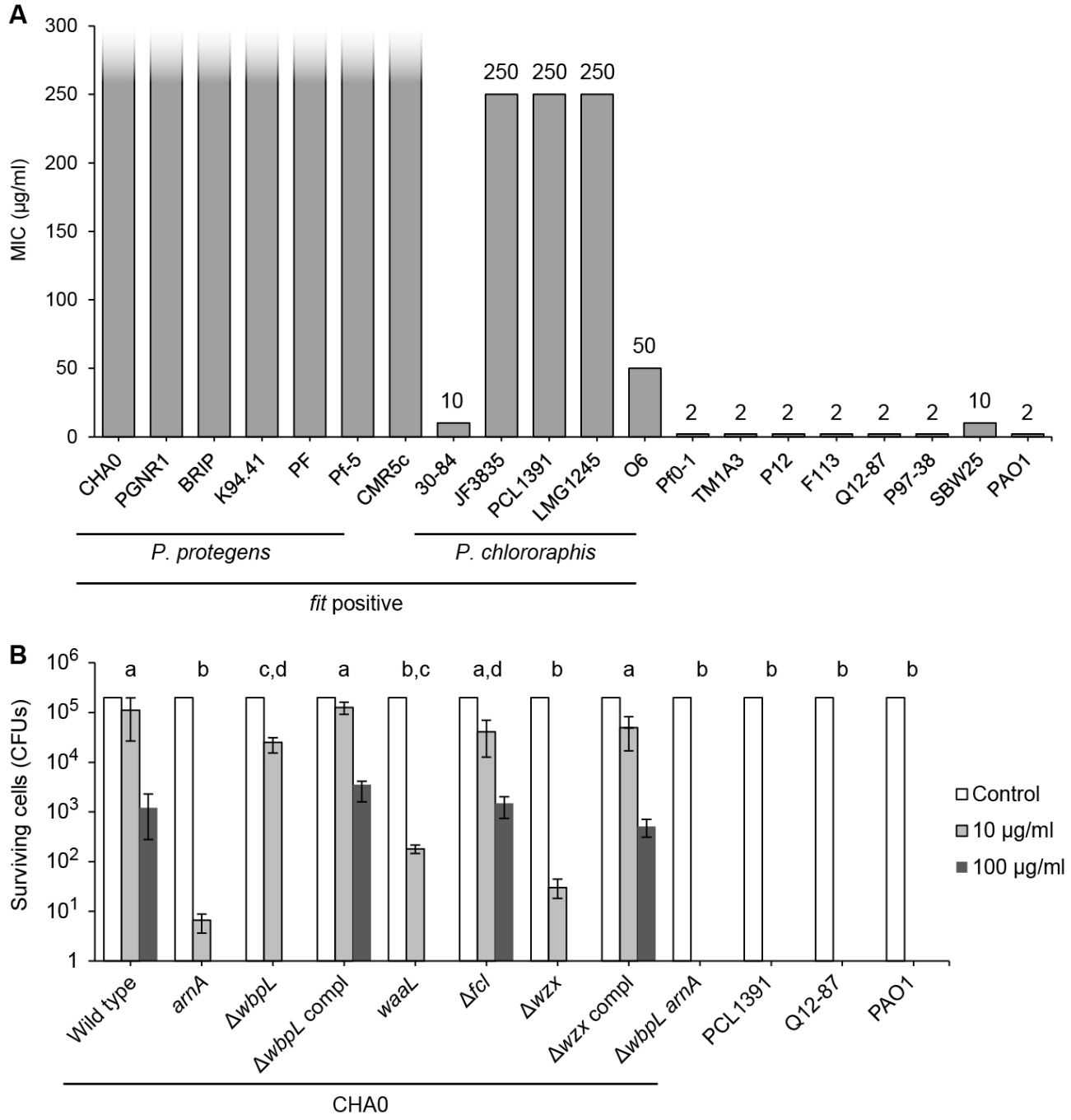

Figure 11: Occurrence of high antimicrobial peptide resistance in insect-pathogenic pseudomonads and underlying mechanisms in *Pseudomonas protegens* CHA0. (A) The minimum inhibitory concentration (MIC) of polymyxin B (PMB) was determined for selected *Pseudomonas* strains by growing them for 24 hours in Lysogeny Broth in presence of different concentrations of the antimicrobial peptide (2, 10, 50, 100, or 250 µg/ml PMB). The MIC was defined as the concentration at which no visible growth of the bacteria was observed. For *P. protegens* strains CHA0, PGNR1, BRIB, K94.41, PF, Pf-5, and *Pseudomons* sp. CMR5c growth was observed even at the highest concentration tested. Strains that possess the *fit* gene cluster are efficiently killing lepidopteran insect larvae. The experiment was repeated twice with similar results. **(B)** Cell suspensions of *P. protegens* CHA0 and derivatives were serially diluted and aliquots were spotted on nutrient agar plates containing either 10 or 100 µg/ml PMB. Colonies were counted after incubating the plates for 24 hours. Colony numbers were normalized by the number of colonies in the control plates without PMB, in order to compare them between strains. Shown are means and standard deviations from three independent assays with similar results. Strains marked with different characters were significantly different from each other in respect to PMB resistance (p-value < 0.05; two-way ANOVA with transformed data).

O-polysaccharides are adaptable cell surface structures

The genetics of O-PS biosynthesis in the two model strains CHA0 and PCL1391 was investigated under standard laboratory conditions. It has however been observed that bacteria change the composition and sizes of their O-PS according to the environment [11]. Therefore, this study also addressed the questions whether these pseudomonads are able to vary the sizes of their O-PS in response to different media and hosts.

When strain PCL1391 was grown in Grace's Insect Medium (GIM), which is a medium that mimics the physicochemical conditions found in lepidopteran insect larvae [5,64], its LPS banding pattern changed significantly compared to growth in Lysogeny Broth (LB) (Figure 12). The quantity of very long O-PS increased when PCL1391 was grown in GIM compared to LB. In CHA0, the production of the long and short O-PS observed in LB did not seem to change dramatically when the bacterium was grown in GIM, but some strong medium-size bands were visible on the gel (Figure 12A). Additional experiments are necessary to determine whether these bands consisted of further O-PS or of some other, LPS-unrelated polysaccharides.

Green fluorescence protein (GFP)-based reporter plasmids were constructed (Table S2) to assess whether the changes in O-PS length in PCL1391 could have arisen from differential expression of Wzz1 and Wzz2. The expression of *wzz2* and *wzz1* in PCL1391 changed in response to different environments (Figure 12B). Compared to LB, the chain-length regulator Wzz2 was more expressed in GIM, while the expression of Wzz1 slightly decreased. Based on the previous finding that in PCL1391 Wzz1 and Wzz2 are regulating the synthesis of long and very long O-PS, respectively (Figure 5A), it can be hypothesized that the observed increase of very long O-PS chains in GIM (Figure 12A) is due to an increased expression of *wzz2*. During an infection of *G. mellonella* larvae the expression level of *wzz2* in PCL1391 was higher than on cucumber roots, suggesting that the O-PS length distribution of PCL1391 during insect infection is different from the length distribution during root colonization. These results suggest that the bacterium is able to adjust the length of the LPS glycan chains in response to different environments by differential expression of *wzz1* and *wzz2*.

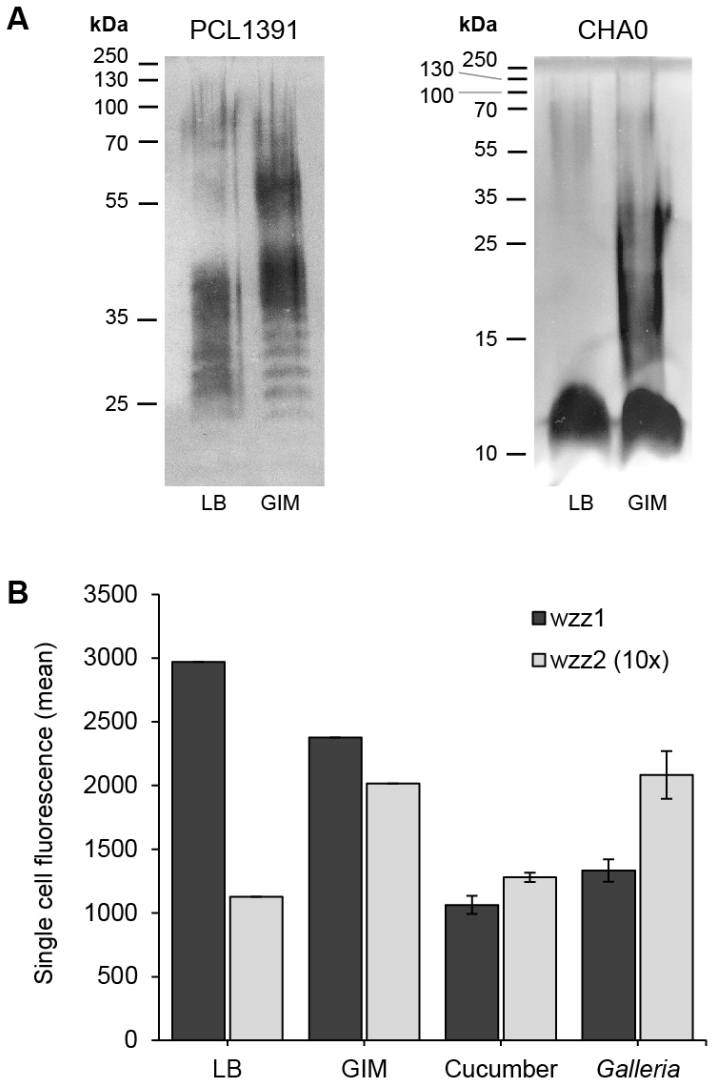

Figure 12: O-polysaccharides are adaptable cell surface structures. (A) SDS-PAGE with LPS extracted from *Pseudomonas chlororaphis* PCL1391 and *Pseudomonas protegens* CHA0 grown in Lysogeny Broth (LB) or Grace's Insect Medium (GIM). Polysaccharides were detected by SDS-PAGE and by silver staining. Bands on the CHA0 gel that are visible in GIM but not in LB are probably exopolysaccharides and not O-polysaccharides. Molecular weights are indicated on the left of the gel in kDa. The experiment was repeated once with similar results. **(B)** Single cell fluorescence intensities of *P. chlororaphis* PCL1391 carrying reporter plasmids (pME8396 for *wzz1* or pME11019 for *wzz2*) and grown in LB, GIM, insect larvae (*Galleria mellonella*) or on plant roots (cucumber) were determined by fluorescence microscopy. Shown are population means (LB and GIM) or means and standard deviations from three independent samples (cucumber and *Galleria*). Exposure times for bacteria carrying the *wzz2* reporter plasmid were 10-times longer than for *wzz1* to account for the difference in expression levels.

Discussion

High diversity of O-antigen gene clusters in insect-pathogenic pseudomonads

LPS O side chains are at the interface between the bacterial cell and the host organism and therefore play an important role in microbe-host interactions and are under high selective pressure [8-10,12,13,65]. Although the composition of these glycan chains was determined in some plant-beneficial pseudomonads [66-68], this study is the first to address the genetic basis and evolution of O-PS biosynthesis in these particular microorganisms.

Identification of genes and gene clusters putatively contributing to O-PS biosynthesis in the model strains *P. chlororaphis* PCL1391 and *P. protegens* CHA0 and their mutagenesis revealed both similarities and differences in the genetic basis of O-PS biosynthesis in these two bacteria (Figure 13). Both strains produce two distinct forms of O-PS, which seems to be a characteristic of pseudomonads [35]. Our data indicate that in PCL1391 O-specific side chains of LPS are synthesized in a similar way as previously reported for *P. aeruginosa* PAO1 [8] (Figure 13A). As in the prominent human pathogen, the major O-PS type of PCL1391 is most probably produced via the Wzx/Wzy-dependent pathway by proteins encoded in the OSA gene cluster and its length is regulated by two chain length determination proteins. Additionally, although slightly reduced, a functional CPA gene cluster was found to be responsible for the biosynthesis of a minor form of O-PS in this strain under certain conditions. While the regulation of OSA chain lengths by Wzz1 and Wzz2 was found to be important for the pathogenesis of *P. aeruginosa* [69], it did not seem to contribute to infection and killing of insect larvae by the *P. chlororaphis* strain in injection assays (Figures 9B and S7). However, a role of Wzz1 and Wzz2 in insect pathogenicity cannot be ruled out and needs to be addressed in future experiments, in particular by using an oral infection model. We for example observed that correct synthesis of OSA is essential for swarming motility in *P. chlororaphis* PCL1391. Because mutations in the OSA cluster of PCL1391 or the *waaL* gene negatively affected the growth rate of the bacterium, we did not investigate whether loss of O-antigen reduces the virulence of this particular pseudomonad.

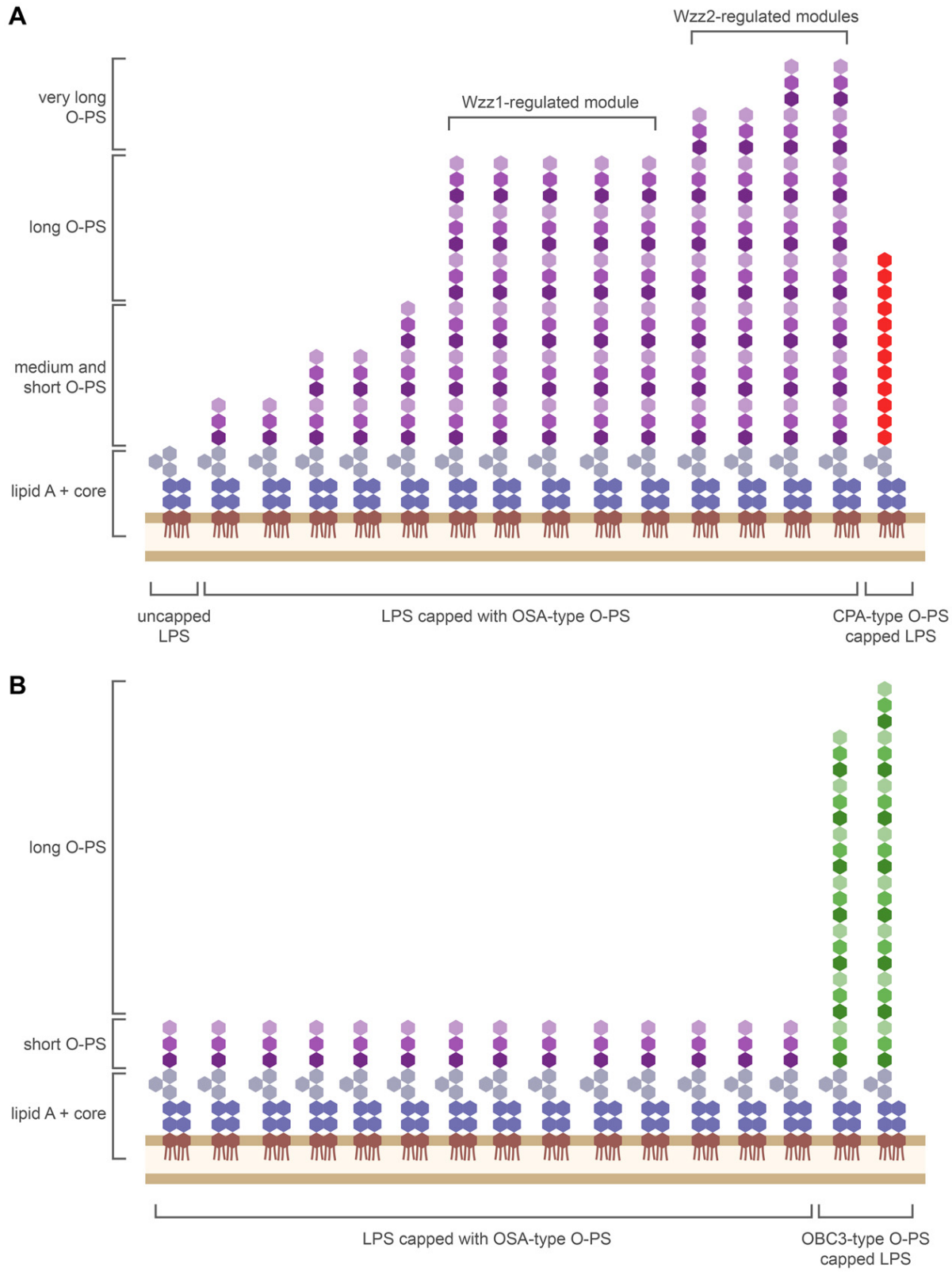

Figure 13: Models for the LPS structures in two plant-beneficial pseudomonads with insecticidal activity. *Pseudomonas chlororaphis* PCL1391 **(A)** and *Pseudomonas protegens* CHA0 **(B)** both produce LPS with two distinct forms of O-PS. The major type of O-PS is probably a heteropolymer (in violet) and synthesized by proteins encoded in the OSA gene cluster via the Wzx/Wzy-dependent pathway. In PCL1391, the chain lengths of this particular O-PS vary and are regulated by the chain length determination proteins Wzz1 and Wzz2. In CHA0, the major form of O-PS consists of a single repeating unit due to the lack of a polymerase. Both

strains produce a second, minor type of O-PS which is synthesized via the ATP-binding cassette (ABC) transporter-dependent pathway by proteins encoded in the CPA gene cluster in PCL1391 (in red) and the OBC3 gene cluster in CHA0 (in green). The CPA in PCL1391 is mainly produced at temperatures above 25°C and likely a homopolymer. In CHA0, most of the lipid A-core molecules are capped with O-PS while in PCL1391 also rough LPS (i.e. lipid A-core without O-PS) is produced and presented on the bacterial surface.

In contrast to PCL1391 and PAO1, the genetic basis of O-PS biosynthesis in *P. protegens* strains is characterized by reduction and replacement of gene clusters during the evolution of this species (Figures 4 and 6). The OSA gene cluster in *P. protegens* strains has been reduced to various degrees over time by loss of genes, including the *wzy* gene coding for the O-antigen polymerase. The lack of this polymerase most probably rendered *P. protegens* unable to synthesize OSA-type O-PS with more than one O-unit (Figure 13B). LPS capped with the resulting very short O-PS therefore structurally resembles the lipooligosaccharide of certain Gram-negative bacteria, such as *Haemophilus* and *Neisseria* species [70]. All *P. protegens* and *P. chlororaphis* strains that possess a reduced OSA gene cluster retain the same three genes: *wbpL* and its two flanking genes. These genes are most probably conserved because the bacteria absolutely need the initial glycosyltransferase WbpL to be able to synthesize O-PS. All *P. protegens* strains further lack the gene cluster for the biosynthesis of CPA, but possess additional gene clusters in return which enable them to synthesize O-PS with long sugar chains.

Changing the O-PS structure for better adaptation?

P. fluorescens group species other than *P. protegens* harbor OSA gene clusters with complex and diverse genetic compositions (data not shown). This particular locus therefore seems to be a hot spot for the acquisition of new genes in pseudomonads. High genetic diversity in the major O-PS gene cluster has been reported for other bacterial species such as *P. aeruginosa* and *Salmonella enterica* before [71-73]. Classically, this glycan diversity has been explained with the selective pressure exerted by host immunity, i.e. bacteria producing rare or novel types of O-antigen are more likely to avoid detection by the host immune system [74]. It is known that certain bacteria are mimicking surface structures of mammalian cells to better survive in the host by avoiding, reducing or manipulating host immunity [75]. Selection by the host immune system can however not explain why also non-pathogenic bacteria display high O-antigen diversity and it has become evident that also other factors contribute to the diversity of glycan structures in microorganisms. O-PS can, amongst others, be important for bacterial competition. McCaughey *et al.* reported that rhamnose-containing LPS molecules are the target of lectin-like bacteriocins from pseudomonads [27]. The reduction or loss of the CPA gene cluster thus could have rendered certain pseudomonads resistant to this kind of bacteriocins. It has further been suggested that infection by bacteriophages, which often use O-PS structures as receptors for cell entry, act as driving force [8,26]. Indeed, we discovered that the long O-PS synthesized by proteins encoded in the OBC3 gene cluster serve as a receptor for at least one type of phage (Figure 8).

Glycans can play an important role for long-term microbe-host interactions. Specific glycan structures might be important for shaping beneficial microbial communities on plant roots or in the intestines of animals [75]. Due to their short generation times and capability of exchanging genetic material across vast phylogenetic distances, microorganisms are able to quickly adapt to new environments and hosts. By lateral transfer of genes for the biosynthesis of O-PS bacteria can rapidly change the chemical composition of their surface decoration in response to a specific niche [75]. Bacteria that are able to change the chemical composition of their O-PS and as a consequence can better adapt to a particular environment thus might have a fitness advantage and are consequently selected for. The diversity of O-PS structures in biocontrol pseudomonads thus might reflect the adaptation to specific plant species or varieties and mediate strain-host specificity.

Emergence of a novel mechanism for exchanging surface glycans?

In the *P. fluorescens* group, reduction of the OSA gene cluster seems to have taken place uniquely in *P. protegens* and certain *P. chlororaphis* lineages (Figure 6; data not shown). The diversity of O-PS structures in these pseudomonads is no longer created by exchanging genes at the OSA locus, as it has been observed for other *Pseudomonas* bacteria [35,71], but by acquiring entire gene clusters via horizontal gene transfer at diverse chromosomal loci. Possession of additional O-PS gene clusters was not observed for *P. fluorescens* group species other than *P. protegens* and *P. chlororaphis* (data not shown; the OBC1 cluster was not found to contribute to the biosynthesis of O-PS). *P. protegens* strains are all very similar in terms of genome sequence (Flury *et al*., manuscript in preparation; [76]) and it is therefore striking that they display completely different glycan structures. Fast evolution of O-PS structures by acquisition of new O-PS genes outside of the OSA locus might represent a novel mechanism for rapidly changing the surface structure in pseudomonads.

It is interesting to note that all strains that harbor reduced OSA clusters possess new O-PS gene clusters and that those seem to replace the biosynthesis of long O-antigen sugar chains. This strongly indicates that reduction of the major O-antigen gene cluster in these bacteria is directly linked to the acquisition of new O-PS gene clusters. From our data, it is, however, impossible to deduce which of the two events happened first in these microbes. An advantage of reducing the OSA gene cluster instead of using it as a hot spot for lateral gene transfer might be that it restricts the dynamics at the OSA locus and that acquisition of genes at new loci can take place without changing the genetics of the major O-PS form. It thus enables *P. protegens* to change the long O-PS to adapt to different niches without affecting the biosynthesis of the OSA-type O-PS. This so far unappreciated mechanism might functionally disconnect the synthesis of the major form of O-PS, which has a kind of housekeeping function and is now buried instead of exposed, from the production of

minor types of O-PS with long sugar chains for host adaptation. It might accelerate the exchange of O-PS gene clusters and be the cause why we see so much diversity in phylogenetically closely related strains.
Diversification of glycan structures might have allowed *P. protegens* to successfully adapt to plant and insect hosts as one single bacterial species. The acquisition of genes that direct the biosynthesis of diverse nucleotide sugars by horizontal gene transfer might have enabled it to incorporate sugars such as fucose or abequose into O-PS, as reported for rhizobia and enterobacteria [51], instead of the for pseudomonads common rhamnose. Interestingly, fucose is an important constituent of polysaccharides in animal guts and on plant roots [77]. This particular sugar is important for the interaction between the gut microbiota and the host organism [78]. Many pathogenic bacteria produce polysaccharides containing fucose residues [78,79] and plant-beneficial pseudomonads were reported to produce fucose-containing polysaccharides before [68,80]. Certain rhizobia also produce O-antigens that contain L-fucose and influence the symbiosis with the plant [51]. It can therefore be speculated that fucose-containing O-PS somehow contributes to either recognition of *P. protegens* by the plant host or to evasion of recognition of the microorganism by the insect immune system. The chemical composition and structure of the particular O-PS needs however to be determined in the future to have more evidence for the presence of fucose and to investigate the roles of the chemical structure of these O-specific side chains in the interaction of the bacterium with plant and insect hosts.

It will be important to test the hypothesis of the proposed new mechanism for O-PS gene cluster exchange by performing experiments that allow following changes in O-PS gene clusters over long periods of time under different environmental conditions. In particular it would be interesting to inoculate different plant species in the field with these pseudomonads and analyze whether these bacteria effectively rapidly change their O-PS gene clusters in response to the plant host by re-isolation after several months or years. In case acquisition of new O-PS genes or gene clusters can be observed, whole genome sequencing would possibly give some valuable information about the underlying mechanisms of these changes.

OSA-capped LPS might protect pseudomonads against immune responses during insect infection

O-antigen has been reported in several bacteria to be important for virulence in insects [18,81-83]. We found that in particular the very short OSA-type O-PS contributes to the ability of *P. protegens* CHA0 to cause disease in insect larvae (Figure 9). Our results indicate that the bacterium is less virulent when it lacks the O-antigen possibly because it is less effective in bypassing or overcoming host defense mechanisms. The presence of O-PS on the cell surface likely renders the microbe more resistant to antimicrobial factors produced by the insects during systemic infection. There is little but increasing evidence that O-PS is

important in diverse bacteria for the resistance to AMPs [15,18,19] which play a central role in the immune system of insects [14,44]. We present evidence in this study that certain pseudomonads are naturally resistant against selected AMPs and that O-PS are crucial for resistance to attack by AMPs in both CHA0 and PCL1391 (Figures 11 and S8). In both strains we identified the OSA as the O-PS form responsible for the moderate to high resistance to PMB. It is possible that ligation of this kind of O-PS to the lipid A-core changes the overall charge of LPS molecules, which might confer resistance to AMP binding. Additionally, the frequency of O-PS-capped LPS seems to be very high in *P. protegens* strains and in *Pseudomonas* sp. CMR5c (Figure 5B and Figure 6), which might leave only very few LPS molecules uncapped on the cell surface and susceptible to binding by AMPs. In contrast, capping frequencies in *P. aeruginosa* were reported to be between 0.2 and 14% only [34]. Another explanation could be that OSA increases the resistance to AMPs indirectly, for example by acting as an anchor or stabilizer for proteases that neutralize AMPs such as AprA in *Pseudomonas entomophila* [84]. Although the exact mechanism remains unclear, the role of the short O-PS in hyper-resistance toward polymyxins suggests that the reduction of the OSA gene cluster somehow contributed to the development of high AMP resistance in *P. protegens*.

Strains of *P. protegens* and *P. chlororaphis* were shown to be able to colonize and kill certain insect species upon oral or systemic infection, while other strains of the *P. fluorescens* group (with the exception of SBW25-like strains) do not seem to be pathogenic to insects ([4] and Flury *et al*., manuscript in preparation). In general, resistance to PMB thus seems to correlate with the potential of bacteria of the *P. fluorescens* group to cause disease in lepidopteran insects (Figure 11A). Interestingly, resistance to AMPs was recently proposed to be a common feature of the human gut microbiota [59]. PMB resistance (i.e. visible growth at a concentration of 10 µg/ml or beyond) could therefore be used as a quantitative marker for oral insect pathogenicity in plant-beneficial pseudomonads, in addition to the presence of *fitD* on their chromosomes [85]. However, the genetic and molecular basis of the discovered PMB resistance in insecticidal pseudomonads remains vague. Modification of lipid A with aminoarabinose seems to be essential for the high resistance to PMB in CHA0 in addition to the presence of OSA (Figure 11B). Lipid A modification was further reported to play a central role in PMB resistance in *P. aeruginosa* [9,43]. It thus should be investigated whether strains resistant to PMB display higher levels of lipid A modification than pseudomonads susceptible to PMB.

This is the first report of hyper-resistance to polymyxins in pseudomonads. PMB and colistin are used in clinics to treat patients with *Pseudomonas* infections and considered as last-resort antibiotics [86,87]. Our results suggest that development of high resistance to these polymyxins could take place in *P. aeruginosa* as well, for example by modification of the OSA gene cluster, which likely would make such strains resistant to a broad range of AMPs. In the worst case, this event could also lead to cross-resistance to host AMPs [58].

It is possible that decreased AMP resistance is not the only reason why O-PS mutants of *P. protegens* CHA0 are significantly reduced in virulence in insects. O-PS might contribute to CHA0 having an extremely tight and stable outer membrane which would confer a general resistance to immune defenses beyond AMPs. It is tempting to speculate that the presence of smooth LPS protects insect-pathogenic pseudomonads against reactive oxygen species and lysozymes, which are produced by insects as antimicrobials in addition to AMPs [14], as well. This should be tested by exposing wild-type and O-antigen mutant strains of *P. protegens* CHA0 to compounds/proteins such as paraquat, hydrogen peroxide and chicken egg white lysozyme in assays similar to the ones described for AMPs.

Phagocytic hemocytes are contributing to the clearance of bacterial infections in the hemolymph as well [14]. That O-PS can protect against phagocytosis has been shown in other bacteria [21,88,89]. We observed that CHA0 is resistant to phagocytosis by hemocytes and it is thinkable that O-antigen contributes to this resistance in this microorganism as well. This could be assessed by conducting an *in vitro* assay using cell cultures of phagocytic hemocytes and O-PS mutant strains equipped with GFP cell tags for microscopic observations.

Possible directions for future research

We found bioinformatic evidence that genes in the well-conserved OBC2 cluster could allow the synthesis of capsular polysaccharide in pseudomonads, which to our best knowledge has not been reported before. Previous studies have indicated that capsular polysaccharides cannot be detected by silver staining [90]. Detection and characterization of these polysaccharides could be achieved by using an alternative dye such as Alcian blue [91]. Since capsules are important virulence factors for many pathogenic bacteria [46,92] we will investigate in a future study when they are produced and whether they contribute to insect pathogenicity in *P. protegens* and *P. chlororaphis*.

The observed changes in O-PS chain lengths (Figure 12) demonstrate that these polysaccharides are not rigid structures but are modified according to the surrounding conditions. Future research should address how LPS modifications are accomplished in the model strain PCL1391, i.e. which signals trigger these changes and what regulators control the modification of O-PS biosynthesis. It would further be important to study the composition and structure of the O-PS in different growth phases and under additional growth conditions (changes in pH, temperature, presence of AMPs, etc.). The GPF-based reporter strains created in this study could constitute valuable tools for such studies.

The present study has created the base for further studies of the role, evolution and regulation of O-PS in insecticidal pseudomonads. The notion that O-PS contributes to the protection of bacteria against AMPs is

currently still underappreciated [43,58,86]. Our study demonstrates for the first time the importance of O-PS in the resistance of *Pseudomonas* species to AMPs and supports similar findings in other bacteria from previous studies [17-19]. In the future, the resistance of selected strains to concrete AMPs from lepidopteran insect species should be tested to further demonstrate the relevance of AMP resistance for insect infection.

Our results presented in this study further point out that O-PS evolve rapidly in plant-beneficial pseudomonads and that this might be linked to host adaptation. This notion needs to be addressed in future studies as it possibly will have consequences for the successful application of these biocontrol bacteria for plant protection. In addition, we think that the high genetic diversity of polysaccharide biosynthesis in these pseudomonads and the many genome sequences that are available now make these bacteria an ideal model to study the evolution of genes implicated in the glycan decoration of bacterial surfaces.

Material and Methods

Bacterial strains, plasmids, media, and culture conditions

All strains and plasmids used in this study are listed in Table S2. Bacterial strains were routinely maintained on nutrient agar (NA) plates or cultured in LB (BD Difco) supplemented with appropriate antibiotics as needed. *Pseudomonas* strains were grown at 25°C while *E. coli* was cultured at 37°C. The following antibiotic concentrations were used: ampicillin, 100 µg/ml; chloramphenicol, 10 µg/ml; kanamycin, 25 µg/ml for *E. coli* and *P. protegens*, 50 µg/ml for *P. chlororaphis*; gentamicin, 10 µg/ml for *E. coli* and *P. protegens*, 20 µg/ml for *P. chlororaphis*; and tetracycline, 25 µg/ml or 125 µg/ml for *E. coli* and *Pseudomonas*, respectively. Isopropyl β-D-1-thiogalactopyranoside (IPTG) was added at a final concentration of 0.1 mM, if not otherwise stated.

Recombinant DNA techniques

DNA manipulations and PCRs were performed according to standard protocols [93]. Genomic DNA was extracted with the Promega Wizard Genomic DNA Purification Kit. Plasmid DNA preparations were performed using the QIAprep Spin Miniprep Kit (Qiagen) and the Genomed JETStar Plasmid Purification Midi Kit for small and large scale purifications, respectively. DNA gel extractions were conducted using the MinElute Gel Extraction Kit and the QIAquick Gel Extraction Kit (Qiagen). DNA restriction and modification enzymes were purchased from Promega and were used according to the manufacturer's recommendations. DNA enzyme reaction cleanups were conducted using the QIAquick PCR Purification Kit (Qiagen). PCR for molecular cloning was conducted according to the recommendations of the manufacturer with the

PrimeSTAR HS high-fidelity DNA polymerase kit (Takara Bio Inc.), while the DNA Polymerase kit (Promega) was used to perform PCRs for analytic purposes. The primers used in this study were synthesized by Microsynth AG (Balgach, Switzerland) and are listed in Table S3. DNA sequencing was conducted at GATC Biotech (Konstanz, Germany) and obtained sequences were analyzed using the DNASTAR Lasergene software suite.

Construction of in-frame deletion mutants

In-frame deletions of selected genes in *P. protegens* CHA0 and *P. chlororaphis* PCL1391 were performed based on homologous recombinations using the suicide vector pEMG and the I-SceI system [94]. For the construction of the suicide vectors (Table S2), upstream and downstream regions of 500–600 bp length flanking the region to be deleted were amplified by PCR using the primer pairs listed in Table S3 and chromosomal DNA from strains CHA0 or PCL1391 as DNA templates. Purified PCR products were digested using the corresponding restriction enzymes (indicated in Table S3) and subsequently cloned into the suicide vector pEMG via triple ligation. Correct insert sequences of the resulting plasmids were confirmed via DNA sequencing. The obtained suicide vectors then served to construct strains CHA5161, CHA5163, CHA5164, CHA5165, CHA5182, CHA5205, CHA5206, PCL-3, and PCL-12 (Table S2), respectively, using the I-SceI system as described previously [5].

Construction of gene disruption mutants

In cases where genes or gene clusters could not be deleted using the method described above, selected genes were mutated by sequence-specific insertion of pEMG-based suicide vectors via homologous recombination, which led to the disruption of the coding sequences (CDSs). Suicide vectors were constructed by PCR amplification of approximately 500-bp regions in the middle of the genes of interest, using the primer pairs listed in Table S3 and chromosomal DNA from the respective bacterial strain. The resulting fragments were digested with BamHI and EcoRI and cloned into pEMG opened with the same restriction enzymes. The insert sequences of the constructed vectors were verified by DNA sequencing and the plasmids were integrated into the CHA0 or PCL1391 chromosome by transformation of the bacteria via electroporation. Correct insertion of the suicide vectors in the chromosomes was confirmed by conducting specific PCR using the “check” primers specified in Table S3.

In order to introduce a frame shift in CHA0 *waaL*, two 350-bp fragments of the gene were amplified by PCR using primer pairs waaL-fs-1/waaL-fs-2 and waaL-fs-3/waaL-fs-4 and chromosomal DNA from CHA0. The obtained DNA fragments were digested with the corresponding restriction enzymes (Table S3) and cloned into pEMG opened with EcoRI and BamHI. The insert sequence of the resulting plasmid pME8383 was verified by DNA sequencing. The created vector was integrated into the chromosome of CHA0 by

electroporation of electrocompetent cells, resulting in strain CHA5174 (Table S2). Since several attempts to get frame shift mutants by selection for bacteria that underwent second homologous recombination failed, strain CHA5174 served as a gene disruption mutant for this study.

Complementation of mutant strains

For complementation of selected gene deletion and disruption mutants of CHA0 and PCL1391, the respective genes were cloned under the control of the $P_{tac/laclq}$ promoter and introduced into the unique chromosomal Tn*7* attachment site of corresponding mutant strains using the mini-Tn*7* delivery vector pME8300 [5] as follows. Primer pairs listed in Table S3 were used to amplify the CDS of selected genes of *P. protegens* CHA0, *P. chlororaphis* PCL1391, *E. coli* K-12, and *R. etli* CE3 by PCR using chromosomal DNA from the respective bacterial strains. Resulting PCR products were digested with the restriction enzymes indicated in Table S3 and consequently cloned individually into pME8300 opened with the same enzymes. The sequences of the inserts in the resulting plasmids were verified by DNA sequencing. The pME8300 derivatives and the Tn*7* transposition helper plasmid pUX-BF13 were co-electroporated into competent cells of the respective mutant strains to create strains CHA5169, CHA5207, CHA5208, CHA5211, CHA5212, PCL-8, and PCL-14 (Table S2).

Because the above described cloning strategy did not work for CHA0 *wzx*, CHA5206 was complemented as follows. A 3-kb region, comprising the CDS of *wzx*, the upstream-flanking gene and its native promoter, was amplified by PCR using the primer pair wzx-expr-F2/wzx-expr-R2 and chromosomal DNA from CHA0. The purified PCR product was digested with EcoRI and BamHI and subsequently ligated into pME4510 which was opened with the same restriction enzymes. The sequence of the insert of the resulting vector pME11024 (Table S2) was verified by DNA sequencing and the plasmid was used to transform CHA5206 by electroporation.

Bioinformatics

Genes and gene clusters putatively involved in the biosynthesis of O-PS in *P. protegens* strains CHA0, Pf-5 and Cab57 were identified by performing nucleotide (blastn) and protein BLAST on the NCBI website (http://blast.ncbi.nlm.nih.gov/) using sequences of genes and proteins (primarily glycosyltransferases, transporters, chain length regulators and the O-antigen ligase WaaL) reported to be important for the synthesis of O-PS in *P. aeruginosa* PAO1 [8]. Identification of such genes and gene clusters in a set of recently sequenced *Pseudomonas* strains (Flury *et al*., manuscript in preparation) and publicly available genome sequences was done similarly (with a minimum of 70% nucleotide sequence identity over 70% of the CDS) by using the information from gene annotation and pan-genome calculations in EDGAR [95], with standard settings as described in [96], and from their localization on the chromosomes in GenDB [97].

Detected genes and gene clusters were further analyzed by predicting the function of the encoded proteins using the NCBI Conserved Domain Database (CDD) Search [98] and InterPro [99] with default parameters and by finding homologous/orthologous genes in *P. aeruginosa* PAO1 by performing BLAST searches against this particular bacterial strain ([100]; http://pseudomonas.com/blast.jsp;). The presence of orthologs of newly identified *P. protegens* gene clusters (or individual genes thereof) in other bacteria was analyzed by conducting blastn searches on the NCBI website. The blastn hits were sorted by coverage (descending order) and the first three hits for each cluster were analyzed in more detail (sequences with at least 60% nucleotide identity and 300 nucleotides length were considered).

LPS extraction and visualization

Extraction of LPS was performed as previously described by Davis and Goldberg [101] with the following modifications. If not otherwise stated, bacteria were sampled for LPS extraction after growing them for 16 h (i.e. to late exponential growth phase) in 10 ml of LB at 25°C and 180 rpm as 1.5-ml suspensions with an optical density at 600 nm (OD_{600}) of 5. Because purification with phenol led to a loss of LPS with long O-PS in *P. protegens*, the samples were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) directly after the Proteinase K treatment. SDS-PAGE was performed with 15% acrylamide gels and the peqGOLD pre-stained Protein Marker V (PEQLAB Biotechnologie GmbH, Ehrlangen, Germany) was used as molecular mass standard. Silver staining of the gels was performed as described previously [102].

Growth curves, biofilm formation and motility

Growth defects in constructed mutant strains were assessed by performing growth curve assays. Therefore, five glass test tubes per strain containing 3 ml of LB each were inoculated with aliquots of 60 µl of a bacterial suspension at an OD_{600} of 1. The OD_{600} of each culture incubated at 25°C and 180 rpm was monitored over time by spectrometry.

For biofilm formation studies according to Coffey and Anderson [103], 200 µl of LB in a 96-well microplate (Greiner Bio-One, Kremsmünster, Austria) were inoculated 1:100 with bacterial suspension and incubated at 25°C for 24 h without agitation. The cell density at 600 nm was measured with a FLUOstar multidetection microplate reader (BMG Labtech GmbH, Offenburg, Germany). The supernatant was then removed and the plate was washed once with saline solution for 5 min at room temperature with agitation (500 rpm). The liquid was removed and 200 µl of a 0.1% crystal violet solution per well was added in order to stain the biofilm. The microplate was incubated for 15 min at room temperature without shaking and then washed three times with bi-distilled water. Two hundred microliters of ethanol were added into each well and the plate was incubated at room temperature for 15 min with shaking. The absorbance at 600 nm was determined using the FLUOstar multidetection microplate reader.

For studying the motility, aliquots of 5 µl of bacterial suspension at an OD_{600} of 1 was spotted in the middle of swimming and swarming agar plates as described previously [104]. The plates were incubated overnight at room temperature and the diameter of each bacterial colony was determined.

Hemolytic and antifungal activities

Changes in hemolytic activity upon mutation of selected O-PS biosynthesis genes was assessed by spotting 5 µl of bacterial suspension at an OD_{600} of 1 on sheep blood agar plates. Diameters of lysis zones were measured and compared between strains after incubating the plates for 2 days at 30°C. For analysis of fungal growth inhibition, 5 µl of bacterial suspension at an OD_{600} of 1 were spotted in a triangle form on a Malt Extract Agar (Oxoid) plate. With the aid of a cork borer, a piece of Malt Extract Agar containing *Pythium ultimum* was added to the middle of the plate, which was then incubated at 24°C for 24 h. Diameters of inhibitions zones were measured and compared between strains.

Bacteriophage infection

Bacteriophage GP100 was propagated in *P. protegens* CHA0 as described previously [56]. Infection of different *Pseudomonas* strains and isogenic mutants of CHA0 by this bacteriophage was assessed by a double layer assay. Briefly, 4 ml of liquid LB soft agar (5 g/L bacteriological agar (Oxoid)), containing IPTG if necessary, was mixed with 100 µl of bacterial suspension of a given strain that was grown overnight in 10 ml of LB at 25°C and 180 rpm, supplemented with antibiotics and IPTG if necessary, and poured onto a sterile NA plate. Seven microliters of a GP100 suspension (containing 3 x 10^7 plaque forming units/ml) was spotted on the solidified double layer. Plates were evaluated for lysis of bacteria after overnight incubation at room temperature.

Galleria mellonella virulence and competition assays

Injection assays for virulence determination using last-instar larvae of *G. mellonella* (Entomos AG, Grossdietwil, Switzerland) were performed as described before [105]. For the competition assay, O-PS mutant strains of CHA0 and PCL1391 were marked with a constitutively expressed mCherry tag using the Tn*7* delivery vector pME9407 as described previously [106] (Table S2). The PCL1391 wild-type strain was marked analogously with a constitutively expressed GFP tag using the plasmid pBK-miniTn7-*gfp1* (Table S2). The resulting strains and the GFP-tagged CHA0 wild-type strain [105] were grown overnight in 10 ml of LB at 25°C and 180 rpm. The cells were washed once in 0.9% NaCl solution and the OD_{600} of the bacterial suspension was adjusted to 1. Each mCherry-tagged mutant strain was mixed 1:1 with the corresponding GFP-marked wild-type strain and the resulting suspension was diluted 200-times in saline solution. Aliquots of 5 µl were injected into the last left pro-leg of *G. mellonella* larvae. After incubation at room temperature for

approximately 22 h, hemolymph of the larvae was collected and visualized by fluorescence microscopy as described before [105]. Mutant and wild-type cells were counted using the DsRed and GFP channels, respectively. The competitive index was calculated by first normalizing the counts for the wild type and the mutant with the ratio observed in the undiluted inoculum (to correct for variation within the inocula) and then dividing the number of mutant cells by the number of wild-type cells.

Plutella xylostella virulence assays

To test the ability of selected O-PS mutant strains to kill insects upon ingestion, feeding assays using larvae of the diamond back moth *P. xylostella* as an oral infection model were performed. Briefly, bacterial strains were grown overnight at 24°C and 180 rpm in LB. The cells were washed with 0.9% NaCl solution and the OD_{600} of the bacterial suspension was adjusted to 0.5. Pellets of artificial insect diet (25 g Adapta Bio Dinkel, 0.5 tab Santogen Gold-V.7, 7.75 g yeast extract, 3.75 g casamino acids, 0.125 g cholesterol, 0.25 mL maize germ oil, 250 mL dH_2O, 3.75 g agar) with a diameter of 3 mm were placed individually in wells of 128-cell Bio-Assay trays (Frontier Agricultural Sciences, Newark, USA) which were disinfected with 70% ethanol and equipped with filter papers beforehand. Ten microlitres of bacterial suspension were pipetted onto each food pellet. Sixty-four wells per treatment were prepared and saline solution was used as a negative control. Eggs of *P. xylostella* obtained from Syngenta Crop Protection (Stein, Switzerland) were hatched in a growth chamber set at 26°C with 60% relative humidity and 16 h of light (20 000 lux). The larvae were kept at 18°C in the dark for two days prior to experiment and then placed individually into the wells of the Bio-Assay trays containing the treated food pellets. The insects were incubated in the 26°C growth chamber and their survival was monitored for two days. Caterpillars that did not respond to repeated stimulus were scored as dead. To avoid drying-out of the food pellets, the filter papers were regularly wetted with 10 µl of sterile distilled H_2O.

Competitive root colonization

The assay to assess the competitiveness of mutant strains on cucumber roots was performed as described previously [5] with modifications. Briefly, three-day-old cucumber (*Cucumis sativus* cv. Chinese Snake) seedlings were grown axenically in 50-ml tubes (three plants per tube) containing 35-ml of 0.35% (w/v) water agar. Tubes with seedlings were incubated for three days in a growth chamber set to 80% relative humidity and 16 h with light (160 mE/m2/s) at 22°C, followed by an 8-h dark period at 18°C. Fluorescently marked wild-type and mutant strains constructed for the competition assay with *G. mellonella* were grown as mentioned above, washed in saline solution and their OD_{600} was adjusted to 1. Each mutant strain was mixed 1:1 with its corresponding wild-type strain and each plant was inoculated at the stem base with 30 µl of the bacterial mixture. After another 5 days of incubation in the growth chamber, bacteria were isolated

from root tips and counted by fluorescence microscopy as described before [5]. The competitive index was calculated as mentioned above.

Polymyxin resistance assays

Minimum inhibitory concentrations (MICs) for polymyxin B were determined for selected *Pseudomonas* strains by assessing their growth in LB in the presence of different concentrations of the antimicrobial peptide. Glass test tubes containing 3 ml of LB supplemented with 2, 10, 50, 100, or 250 µg/ml of polymyxin B (Sigma) were inoculated with 30 µl of bacterial suspension at an OD_{600} of 1 prepared from bacterial cultures grown overnight in 10 ml of LB at 25°C and 180 rpm. The MIC for a given strain was defined as the concentration at which no visible growth was observable after 24 h of incubation at 25°C and 180 rpm.

In order to investigate the percentage of cells in a population that survive and grow in the presence of a certain concentration of antimicrobial peptides, bacterial strains were grown on agar plates containing polymyxin B or colistin as an alternative polymyxin at two different concentrations. Therefore, bacterial suspension with an OD_{600} of 1 were prepared as described above and serially diluted to 10^{-4}. Ten microliters of each diluted bacterial suspension were spotted on NA plates supplemented with 10 or 100 µg/ml of polymyxin B or colistin sulfate (Sigma). The same dilutions were also spotted on NA plates without polymyxins as a negative control. The plates were incubated for 24 h at 30°C and colony forming units (CFU) per 10 µl were calculated for each strain. The CFU counts were normalized with the numbers obtained from the negative controls to account for variations between bacterial samples.

Expression studies

Fragments of approximately 700 bp containing the putative promoter regions located upstream of the respective gene or gene cluster were amplified from chromosomal DNA of CHA0 or PCL1391 by PCR using the primer pairs indicated in Table S3. The DNA fragments obtained were digested with BamHI and EcoRI and cloned into the GFP-based promoter probe vector pPROBE-TT [107] opened with the same restriction enzymes. Insert sequences of the resulting plasmids were verified by DNA sequencing. Competent cells of CHA0 or PCL1391 and their isogenic mutants were subsequently transformed with the constructed vectors by electroporation to create reporter strains for expression studies.

For expression assays *in vitro*, reporter bacteria were grown at 25°C and 180 rpm in 10 ml LB or Grace's Insect Medium (GIM, Sigma) [5] contained in 50-ml Erlenmeyer flasks. Samples were taken at different time points and single cell fluorescence intensities were measured by fluorescence microscopy as described previously for the Fit toxin [5], using the GFP channel.

For *in vivo* studies, the constructed reporter plasmids were used to transform CHA0 and PCL1391 strains which were previously marked with a constitutively expressed mCherry tag using the plasmid pME9407. To

study the expression of selected genes and gene clusters on roots, reporter strains were grown individually on cucumber roots analogous to the competitive root colonization assay described above. Bacteria were isolated and gene expression was determined by fluorescence microscopy as described before [5], using the DsRed channel for identification of the reporter bacteria and the GFP channel for quantification of fluorescence intensities. In order to investigate the expression of the O-PS biosynthesis genes and gene clusters during an infection of insects, reporter bacteria were grown and injected into larvae of *G. mellonella* as described previously for the virulence assay [105], but with higher cell densities (200-fold dilution of bacterial suspensions at an OD_{600} of 1). After an incubation of the larvae for 20 to 24 h at room temperature, hemolymph was obtained and analysed by fluorescence microscopy as described previously for the Fit toxin [105], using the DsRed channel for identification of the reporter bacteria and the GFP channel for quantification of fluorescence intensities.

Statistical analysis

Statistical analysis of experiments was performed in RStudio version 0.98.1091 (http://www.rstudio.com/). One-way or two-way analysis of variance (ANOVA) with Tukey's HSD test for post-hoc comparisons was performed when appropriate. The Log-Rank test of the *survival* package of R was used to calculate significant differences in insect toxicity between the wild type and isogenic mutant strains in the *Galleria* and *Plutella* virulence assay. One-sample *t* tests were performed with data from competition assays.

Acknowledgements

We would like to thank Dale Noel for providing us with *R. etli* strain CE3.

References

1. Kupferschmied P, Maurhofer M, Keel C (2013) Promise for plant pest control: root-associated pseudomonads with insecticidal activities. Front Plant Sci 4: 287.
2. Haas D, Défago G (2005) Biological control of soil-borne pathogens by fluorescent pseudomonads. Nat Rev Microbiol 3: 307-319.
3. Péchy-Tarr M, Bruck DJ, Maurhofer M, Fischer E, Keel C (2008) Molecular analysis of a novel gene cluster encoding an insect toxin in plant-associated strains of *Pseudomonas fluorescens*. Environ Microbiol 10: 2368-2386.
4. Ruffner B, Péchy-Tarr M, Ryffel F, Hoegger P, Obrist C, et al. (2013) Oral insecticidal activity of plant-associated pseudomonads. Environ Microbiol 15: 751-763.
5. Kupferschmied P, Péchy-Tarr M, Imperiali N, Maurhofer M, Keel C (2014) Domain shuffling in a sensor protein contributed to the evolution of insect pathogenicity in plant-beneficial *Pseudomonas protegens*. PLoS Pathog 10: e1003964.
6. Beveridge TJ (1999) Structures of gram-negative cell walls and their derived membrane vesicles. J Bacteriol 181: 4725-4733.
7. Lebeer S, Vanderleyden J, De Keersmaecker SC (2010) Host interactions of probiotic bacterial surface molecules: comparison with commensals and pathogens. Nat Rev Microbiol 8: 171-184.
8. Lam JS, Taylor VL, Islam ST, Hao Y, Kocincova D (2011) Genetic and functional diversity of *Pseudomonas aeruginosa* lipopolysaccharide. Front Microbiol 2: 118.
9. Raetz CR, Whitfield C (2002) Lipopolysaccharide endotoxins. Annu Rev Biochem 71: 635-700.
10. Trent MS, Stead CM, Tran AX, Hankins JV (2006) Diversity of endotoxin and its impact on pathogenesis. J Endotoxin Res 12: 205-223.
11. Lerouge I, Vanderleyden J (2002) O-antigen structural variation: mechanisms and possible roles in animal/plant-microbe interactions. FEMS Microbiol Rev 26: 17-47.
12. Erbs G, Newman MA (2012) The role of lipopolysaccharide and peptidoglycan, two glycosylated bacterial microbe-associated molecular patterns (MAMPs), in plant innate immunity. Mol Plant Pathol 13: 95-104.
13. Park BS, Lee JO (2013) Recognition of lipopolysaccharide pattern by TLR4 complexes. Exp Mol Med 45: e66.
14. Lemaitre B, Hoffmann J (2007) The host defense of *Drosophila melanogaster*. Annu Rev Immunol 25: 697-743.
15. Skurnik M, Bengoechea JA (2003) The biosynthesis and biological role of lipopolysaccharide O-antigens of pathogenic *Yersiniae*. Carbohydr Res 338: 2521-2529.
16. Moran AP (2001) Molecular structure, biosynthesis, and pathogenic roles of lipopolysaccharides. In: Mobley HLT, Mendz GL, Hazell SL, editors. *Helicobacter pylori*: Physiology and Genetics. Washington (DC).
17. Nesper J, Lauriano CM, Klose KE, Kapfhammer D, Kraiss A, et al. (2001) Characterization of *Vibrio cholerae* O1 El tor galU and galE mutants: influence on lipopolysaccharide structure, colonization, and biofilm formation. Infect Immun 69: 435-445.
18. Nehme NT, Liegeois S, Kele B, Giammarinaro P, Pradel E, et al. (2007) A model of bacterial intestinal infections in *Drosophila melanogaster*. PLoS Pathog 3: e173.
19. West NP, Sansonetti P, Mounier J, Exley RM, Parsot C, et al. (2005) Optimization of virulence functions through glucosylation of *Shigella* LPS. Science 307: 1313-1317.
20. Hölzer SU, Schlumberger MC, Jäckel D, Hensel M (2009) Effect of the O-antigen length of lipopolysaccharide on the functions of Type III secretion systems in *Salmonella enterica*. Infect Immun 77: 5458-5470.
21. Liang-Takasaki CJ, Makela PH, Leive L (1982) Phagocytosis of bacteria by macrophages: changing the carbohydrate of lipopolysaccharide alters interaction with complement and macrophages. J Immunol 128: 1229-1235.

22. Sandlin RC, Lampel KA, Keasler SP, Goldberg MB, Stolzer AL, et al. (1995) Avirulence of rough mutants of *Shigella flexneri*: requirement of O antigen for correct unipolar localization of IcsA in the bacterial outer membrane. Infect Immun 63: 229-237.
23. van Loon LC, Bakker PA, van der Heijdt WH, Wendehenne D, Pugin A (2008) Early responses of tobacco suspension cells to rhizobacterial elicitors of induced systemic resistance. Mol Plant Microbe Interact 21: 1609-1621.
24. Leeman M, Vanpelt JA, Denouden FM, Heinsbroek M, Bakker PAHM, et al. (1995) Induction of systemic resistance against fusarium-wilt of radish by lipopolysaccharides of *Pseudomonas fluorescens*. Phytopathology 85: 1021-1027.
25. Appelmelk BJ, Monteiro MA, Martin SL, Moran AP, Vandenbrouck-Grauls CMJE (2000) Why *Helicobacter pylori* has Lewis antigens. Trends Microbiol 8: 565-570.
26. Chaturongakul S, Ounjai P (2014) Phage-host interplay: examples from tailed phages and Gram-negative bacterial pathogens. Front Microbiol 5: 442.
27. McCaughey LC, Grinter R, Josts I, Roszak AW, Waloen KI, et al. (2014) Lectin-like bacteriocins from *Pseudomonas* spp. utilise D-rhamnose containing lipopolysaccharide as a cellular receptor. PLoS Pathog 10: e1003898.
28. Cuthbertson L, Kos V, Whitfield C (2010) ABC transporters involved in export of cell surface glycoconjugates. Microbiol Mol Biol Rev 74: 341-362.
29. Whitfield C, Trent MS (2014) Biosynthesis and export of bacterial lipopolysaccharides. Annu Rev Biochem 83: 99-128.
30. Kalynych S, Morona R, Cygler M (2014) Progress in understanding the assembly process of bacterial O-antigen. FEMS Microbiol Rev 38: 1048-1065.
31. Greenfield LK, Whitfield C (2012) Synthesis of lipopolysaccharide O-antigens by ABC transporter-dependent pathways. Carbohydr Res 356: 12-24.
32. Samuel G, Reeves P (2003) Biosynthesis of O-antigens: genes and pathways involved in nucleotide sugar precursor synthesis and O-antigen assembly. Carbohydr Res 338: 2503-2519.
33. Whitfield C (2006) Biosynthesis and assembly of capsular polysaccharides in *Escherichia coli*. Annu Rev Biochem 75: 39-68.
34. Rocchetta HL, Burrows LL, Lam JS (1999) Genetics of O-antigen biosynthesis in *Pseudomonas aeruginosa*. Microbiol Mol Biol Rev 63: 523-553.
35. Cunneen MM, Reeves PR (2011) Evolution of lipopolysaccharide biosynthesis genes. In: Knirel YA, Valvano MA, editors. Bacterial Lipopolysaccharides. Wien: Springer-Verlag. pp. 339-370.
36. Rocchetta HL, Burrows LL, Pacan JC, Lam JS (1998) Three rhamnosyltransferases responsible for assembly of the A-band D-rhamnan polysaccharide in *Pseudomonas aeruginosa*: a fourth transferase, WbpL, is required for the initiation of both A-band and B-band lipopolysaccharide synthesis. Mol Microbiol 28: 1103-1119.
37. Feinbaum RL, Urbach JM, Liberati NT, Djonovic S, Adonizio A, et al. (2012) Genome-wide identification of *Pseudomonas aeruginosa* virulence-related genes using a *Caenorhabditis elegans* infection model. PLoS Pathog 8: e1002813.
38. Lee DG, Urbach JM, Wu G, Liberati NT, Feinbaum RL, et al. (2006) Genomic analysis reveals that *Pseudomonas aeruginosa* virulence is combinatorial. Genome Biol 7: 10.1186/gb-2006-1187-1110-r1190.
39. Hancock RE, Mutharia LM, Chan L, Darveau RP, Speert DP, et al. (1983) *Pseudomonas aeruginosa* isolates from patients with cystic fibrosis: a class of serum-sensitive, nontypable strains deficient in lipopolysaccharide O side chains. Infect Immun 42: 170-177.
40. Lam MY, McGroarty EJ, Kropinski AM, MacDonald LA, Pedersen SS, et al. (1989) Occurrence of a common lipopolysaccharide antigen in standard and clinical strains of *Pseudomonas aeruginosa*. J Clin Microbiol 27: 962-967.
41. Pier GB (2007) *Pseudomonas aeruginosa* lipopolysaccharide: a major virulence factor, initiator of inflammation and target for effective immunity. Int J Med Microbiol 297: 277-295.
42. Lau PC, Dutcher JR, Beveridge TJ, Lam JS (2009) Absolute quantitation of bacterial biofilm adhesion and viscoelasticity by microbead force spectroscopy. Biophys J 96: 2935-2948.

43. Fernandez L, Alvarez-Ortega C, Wiegand I, Olivares J, Kocincova D, et al. (2013) Characterization of the polymyxin B resistome of *Pseudomonas aeruginosa*. Antimicrob Agents Chemother 57: 110-119.
44. Royet J, Dziarski R (2007) Peptidoglycan recognition proteins: pleiotropic sensors and effectors of antimicrobial defences. Nat Rev Microbiol 5: 264-277.
45. Jousset A, Schuldes J, Keel C, Maurhofer M, Daniel R, et al. (2014) Full-Genome Sequence of the Plant Growth-Promoting Bacterium *Pseudomonas protegens* CHA0. Genome Announc 2.
46. Willis LM, Whitfield C (2013) Structure, biosynthesis, and function of bacterial capsular polysaccharides synthesized by ABC transporter-dependent pathways. Carbohydr Res 378: 35-44.
47. Mack D, Fischer W, Krokotsch A, Leopold K, Hartmann R, et al. (1996) The intercellular adhesin involved in biofilm accumulation of *Staphylococcus epidermidis* is a linear beta-1,6-linked glucosaminoglycan: purification and structural analysis. J Bacteriol 178: 175-183.
48. Wang X, Preston JF, 3rd, Romeo T (2004) The *pgaABCD* locus of *Escherichia coli* promotes the synthesis of a polysaccharide adhesin required for biofilm formation. J Bacteriol 186: 2724-2734.
49. Kropec A, Maira-Litran T, Jefferson KK, Grout M, Cramton SE, et al. (2005) Poly-N-acetylglucosamine production in *Staphylococcus aureus* is essential for virulence in murine models of systemic infection. Infect Immun 73: 6868-6876.
50. Gobeli S, Goldschmidt-Clermont E, Frey J, Burr SE (2009) *Pseudomonas chlororaphis* strain JF3835 reduces mortality of juvenile perch, *Perca fluviatilis* L., caused by *Aeromonas sobria*. J Fish Dis 32: 597-602.
51. Ojeda KJ, Simonds L, Noel KD (2013) Roles of predicted glycosyltransferases in the biosynthesis of the *Rhizobium etli* CE3 O antigen. J Bacteriol 195: 1949-1958.
52. Andrianopoulos K, Wang L, Reeves PR (1998) Identification of the fucose synthetase gene in the colanic acid gene cluster of *Escherichia coli* K-12. J Bacteriol 180: 998-1001.
53. Rini J, Esko J, Varki A (2009) Glycosyltransferases and glycan-processing enzymes. In: Varki A, Cummings RD, Esko JD, Freeze HH, Stanley P et al., editors. Essentials of Glycobiology. 2nd ed. Cold Spring Harbor (NY).
54. Koropatkin NM, Cleland WW, Holden HM (2005) Kinetic and structural analysis of alpha-D-Glucose-1-phosphate cytidylyltransferase from *Salmonella typhi*. J Biol Chem 280: 10774-10780.
55. Micoli F, Ravenscroft N, Cescutti P, Stefanetti G, Londero S, et al. (2014) Structural analysis of O-polysaccharide chains extracted from different *Salmonella* Typhimurium strains. Carbohydr Res 385: 1-8.
56. Keel C, Ucurum Z, Michaux P, Adrian M, Haas D (2002) Deleterious impact of a virulent bacteriophage on survival and biocontrol activity of *Pseudomonas fluorescens* strain CHAO in natural soil. Mol Plant Microbe Interact 15: 567-576.
57. Vaara M (1992) Agents that increase the permeability of the outer membrane. Microbiol Rev 56: 395-411.
58. Anaya-López JL, López-Meza JE, Ochoa-Zarzosa A (2013) Bacterial resistance to cationic antimicrobial peptides. Crit Rev Microbiol 39: 180-195.
59. Cullen TW, Schofield WB, Barry NA, Putnam EE, Rundell EA, et al. (2015) Gut microbiota. Antimicrobial peptide resistance mediates resilience of prominent gut commensals during inflammation. Science 347: 170-175.
60. McPhee JB, Lewenza S, Hancock RE (2003) Cationic antimicrobial peptides activate a two-component regulatory system, PmrA-PmrB, that regulates resistance to polymyxin B and cationic antimicrobial peptides in *Pseudomonas aeruginosa*. Mol Microbiol 50: 205-217.
61. Chapman RF (1998) Circulatory systems, blood and immune systems. In: Chapman RF, editor. The Insects: Structure and Function. Cambridge: Cambridge University Press.
62. Pannabecker TL, Andrews F, Beyenbach KW (1992) A quantitative analysis of the osmolytes in the hemolymph of the larval gypsy moth, *Lymantria dispar*. J Insect Physiol 38: 823-830.
63. Wyatt GR (1961) Biochemistry of insect hemolymph. Annu Rev Entomol 6: 75-102.
64. Grace TD (1962) Establishment of four strains of cells from insect tissues grown in vitro. Nature 195: 788-789.

65. Wildschutte H, Wolfe DM, Tamewitz A, Lawrence JG (2004) Protozoan predation, diversifying selection, and the evolution of antigenic diversity in *Salmonella*. Proc Natl Acad Sci U S A 101: 10644-10649.
66. Pieretti G, Puopolo G, Carillo S, Zoina A, Lanzetta R, et al. (2011) Structural characterization of the O-chain polysaccharide from an environmentally beneficial bacterium *Pseudomonas chlororaphis* subsp. *aureofaciens* strain M71. Carbohydr Res 346: 2705-2709.
67. Veremeichenko SN, Zdorovenko GM (2004) [Structure and properties of the lipopolysaccharide of *Pseudomonas fluorescens* IMV 2366 (biovar III)]. Mikrobiologiia 73: 312-319.
68. de Weger LA, van Loosdrecht MCM, Klaassen HE, Lugtenberg B (1989) Mutational changes in physicochemical cell-surface properties of plant-growth-stimulating *Pseudomonas* spp do not influence the attachment properties of the cells. J Bacteriol 171: 2756-2761.
69. Kintz E, Scarff JM, DiGiandomenico A, Goldberg JB (2008) Lipopolysaccharide O-antigen chain length regulation in *Pseudomonas aeruginosa* serogroup O11 strain PA103. J Bacteriol 190: 2709-2716.
70. Preston A, Mandrell RE, Gibson BW, Apicella MA (1996) The lipooligosaccharides of pathogenic gram-negative bacteria. Crit Rev Microbiol 22: 139-180.
71. Raymond CK, Sims EH, Kas A, Spencer DH, Kutyavin TV, et al. (2002) Genetic variation at the O-antigen biosynthetic locus in *Pseudomonas aeruginosa*. J Bacteriol 184: 3614-3622.
72. Xiang SH, Haase AM, Reeves PR (1993) Variation of the *rfb* gene clusters in *Salmonella enterica*. J Bacteriol 175: 4877-4884.
73. Popoff MY (2001) Antigenic Formulas of the *Salmonella* Serovars. Paris: Institut Pasteur.
74. Reeves P (1995) Role of O-antigen variation in the immune response. Trends Microbiol 3: 381-386.
75. Varki A, Freeze HH, Gagneux P (2009) Evolution of glycan diversity. In: Varki A, Cummings RD, Esko JD, editors. Essentials of Glycobiology. Cold Spring Harbor (NY): Cold Spring Harbor Laboratory Press.
76. Takeuchi K, Noda N, Someya N (2014) Complete genome sequence of the biocontrol strain *Pseudomonas protegens* Cab57 discovered in Japan reveals strain-specific diversity of this species. PLoS ONE 9: e93683.
77. Ma B, Simala-Grant JL, Taylor DE (2006) Fucosylation in prokaryotes and eukaryotes. Glycobiology 16: 158R-184R.
78. Wang G, Ge Z, Rasko DA, Taylor DE (2000) Lewis antigens in *Helicobacter pylori*: biosynthesis and phase variation. Mol Microbiol 36: 1187-1196.
79. Rehm BH (2010) Bacterial polymers: biosynthesis, modifications and applications. Nat Rev Microbiol 8: 578-592.
80. Anderson AJ (1984) Differences between lipopolysaccharide compositions of plant pathogenic and saprophytic *Pseudomonas* species. Appl Environ Microbiol 48: 31-35.
81. Noonin C, Jiravanichpaisal P, Soderhall I, Merino S, Tomas JM, et al. (2010) Melanization and pathogenicity in the insect, *Tenebrio molitor*, and the crustacean, *Pacifastacus leniusculus*, by *Aeromonas hydrophila* AH-3. PLoS ONE 5: e15728.
82. Bender JK, Wille T, Blank K, Lange A, Gerlach RG (2013) LPS structure and PhoQ activity are important for *Salmonella* Typhimurium virulence in the *Galleria mellonella* infection model [corrected]. PLoS ONE 8: e73287.
83. Miyashita A, Iyoda S, Ishii K, Hamamoto H, Sekimizu K, et al. (2012) Lipopolysaccharide O-antigen of enterohemorrhagic *Escherichia coli* O157:H7 is required for killing both insects and mammals. FEMS Microbiol Lett 333: 59-68.
84. Liehl P, Blight M, Vodovar N, Boccard F, Lemaitre B (2006) Prevalence of local immune response against oral infection in a *Drosophila*/*Pseudomonas* infection model. PLoS Pathog 2: e56.
85. Ruffner B (2013) Insecticidal activity in plant-beneficial pseudomonads: Molecular basis and ecological relevance. PhD thesis; ETH Zurich, Zurich, Switzerland.
86. Olaitan AO, Morand S, Rolain JM (2014) Mechanisms of polymyxin resistance: acquired and intrinsic resistance in bacteria. Front Microbiol 5: 643.
87. Landman D, Georgescu C, Martin DA, Quale J (2008) Polymyxins revisited. Clin Microbiol Rev 21: 449-465.
88. March C, Cano V, Moranta D, Llobet E, Perez-Gutierrez C, et al. (2013) Role of bacterial surface structures on the interaction of *Klebsiella pneumoniae* with phagocytes. PLoS ONE 8: e56847.

89. Lindell K, Fahlgren A, Hjerde E, Willassen NP, Fallman M, et al. (2012) Lipopolysaccharide O-antigen prevents phagocytosis of *Vibrio anguillarum* by rainbow trout (*Oncorhynchus mykiss*) skin epithelial cells. PLoS ONE 7: e37678.
90. Preston MA, Penner JL (1987) Structural and antigenic properties of lipopolysaccharides from serotype reference strains of *Campylobacter jejuni*. Infect Immun 55: 1806-1812.
91. Karlyshev AV, Wren BW (2001) Detection and initial characterization of novel capsular polysaccharide among diverse *Campylobacter jejuni* strains using alcian blue dye. J Clin Microbiol 39: 279-284.
92. Cress BF, Englaender JA, He W, Kasper D, Linhardt RJ, et al. (2014) Masquerading microbial pathogens: capsular polysaccharides mimic host-tissue molecules. FEMS Microbiol Rev 38: 660-697.
93. Sambrook J, Russel DW (2001) Molecular Cloning: A Laboratory Manual. Cold Spring Harbor (New York): Cold Spring Harbor Laboratory Press.
94. Martínez-García E, de Lorenzo V (2011) Engineering multiple genomic deletions in Gram-negative bacteria: analysis of the multi-resistant antibiotic profile of *Pseudomonas putida* KT2440. Environ Microbiol 13: 2702-2716.
95. Blom J, Albaum SP, Doppmeier D, Puhler A, Vorholter FJ, et al. (2009) EDGAR: a software framework for the comparative analysis of prokaryotic genomes. BMC Bioinformatics 10: 154.
96. Smits TH, Rezzonico F, Kamber T, Blom J, Goesmann A, et al. (2010) Complete genome sequence of the fire blight pathogen *Erwinia amylovora* CFBP 1430 and comparison to other *Erwinia* spp. Mol Plant Microbe Interact 23: 384-393.
97. Meyer F, Goesmann A, McHardy AC, Bartels D, Bekel T, et al. (2003) GenDB--an open source genome annotation system for prokaryote genomes. Nucleic Acids Res 31: 2187-2195.
98. Marchler-Bauer A, Lu S, Anderson JB, Chitsaz F, Derbyshire MK, et al. (2011) CDD: a Conserved Domain Database for the functional annotation of proteins. Nucleic Acids Res 39: D225-229.
99. Mitchell A, Chang HY, Daugherty L, Fraser M, Hunter S, et al. (2014) The InterPro protein families database: the classification resource after 15 years. Nucleic Acids Res: 10.1093/nar/gku1243.
100. Winsor GL, Lam DK, Fleming L, Lo R, Whiteside MD, et al. (2011) Pseudomonas Genome Database: improved comparative analysis and population genomics capability for *Pseudomonas* genomes. Nucleic Acids Res 39: D596-600.
101. Davis MR, Jr., Goldberg JB (2012) Purification and visualization of lipopolysaccharide from Gram-negative bacteria by hot aqueous-phenol extraction. J Vis Exp 28: 10.3791/3916.
102. Zhu ZX, Cong WT, Ni MW, Wang X, Ma WD, et al. (2012) An improved silver stain for the visualization of lipopolysaccharides on polyacrylamide gels. Electrophoresis 33: 1220-1223.
103. Coffey BM, Anderson GG (2014) Biofilm formation in the 96-well microtiter plate. In: Filloux A, Ramos JL, editors. Pseudomonas Methods and Protocols. New York Heidelberg Dordrecht London: Springer. pp. 631-641.
104. Péchy-Tarr M, Bottiglieri M, Mathys S, Lejbølle KB, Schnider-Keel U, et al. (2005) RpoN (σ^{54}) controls production of antifungal compounds and biocontrol activity in *Pseudomonas fluorescens* CHA0. Mol Plant Microbe Interact 18: 260-272.
105. Péchy-Tarr M, Borel N, Kupferschmied P, Turner V, Binggeli O, et al. (2013) Control and host-dependent activation of insect toxin expression in a root-associated biocontrol pseudomonad. Environ Microbiol 15: 736-750.
106. Rochat L, Péchy-Tarr M, Baehler E, Maurhofer M, Keel C (2010) Combination of fluorescent reporters for simultaneous monitoring of root colonization and antifungal gene expression by a biocontrol pseudomonad on cereals with flow cytometry. Mol Plant Microbe Interact 23: 949-961.
107. Miller WG, Leveau JHJ, Lindow SE (2000) Improved *gfp* and *inaZ* broad-host-range promoter-probe vectors. Mol Plant Microbe Interact 13: 1243-1250.

Supporting Information

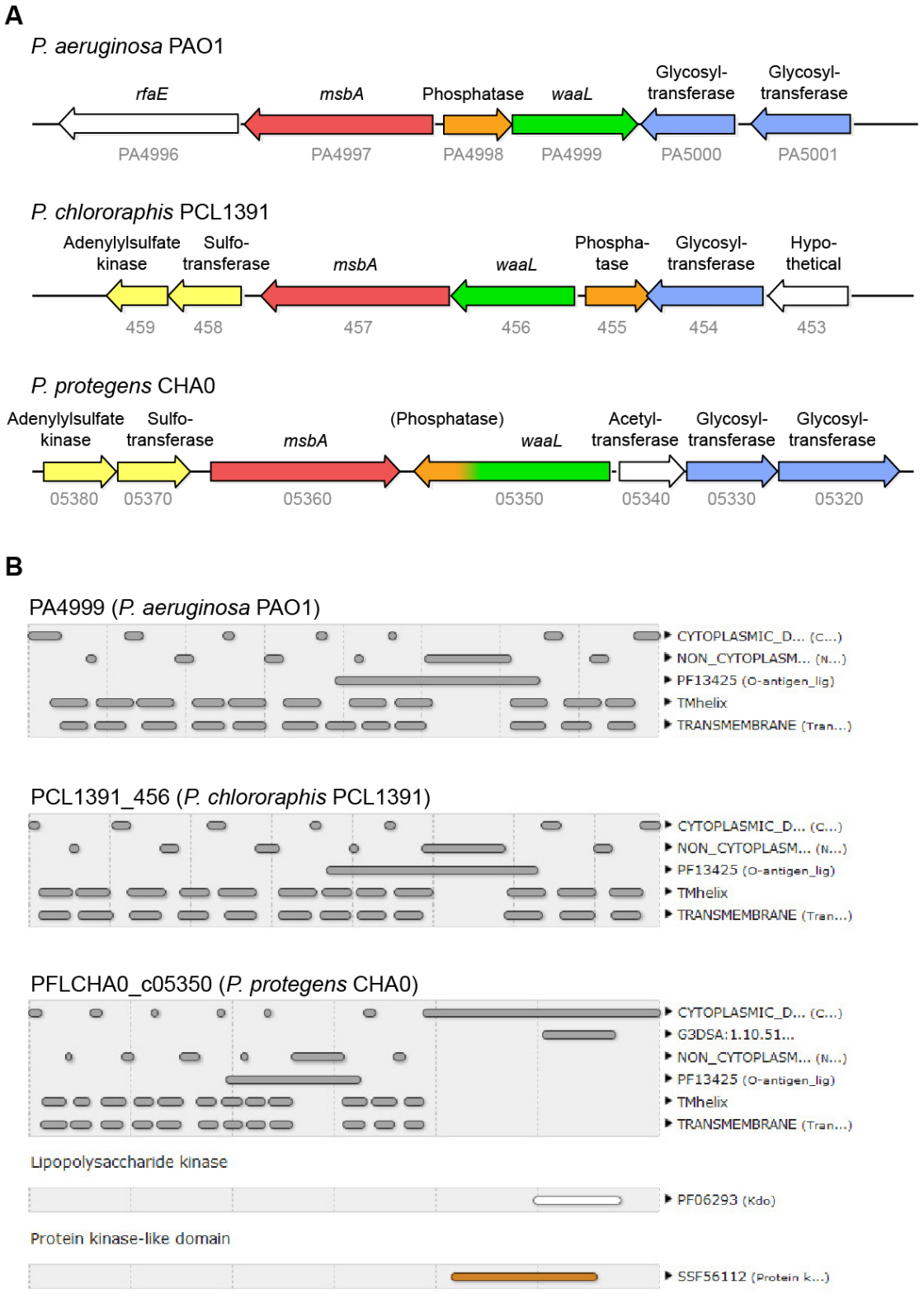

Figure S1: Identification of the *waaL* gene in *Pseudomonas chlororaphis* PCL1391 and *Pseudomonas protegens* CHA0. (A) Candidate genes for *waaL* were identified in strains PCL1391 and CHA0 by protein BLAST searches with the corresponding amino acid sequence from *Pseudomonas aeruginosa* PAO1. Shown are the genetic loci of the putative *waaL* genes. The candidate gene in strain CHA0

(PFLCHA0_c05350) seems to be the result of a fusion between the *waaL* gene and a gene coding for a phosphatase. **(B)** The proteins encoded by *waaL* in CHA0 and PCL1391 were predicted by InterPro protein sequence analysis to possess 12 transmembrane domains and a periplasmic loop like it has been shown for PAO1 WaaL by Islam *et al.* [1]. The WaaL protein in CHA0 is predicted to contain an additionally C-terminal kinase domain.

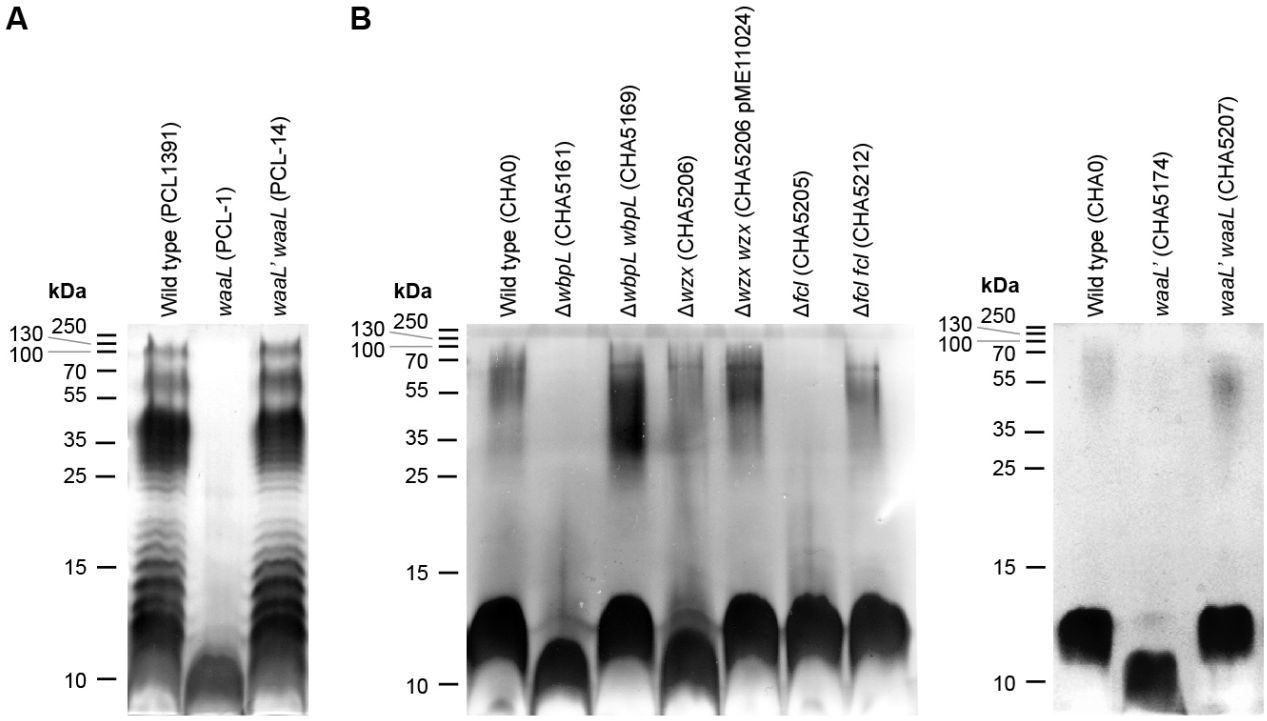

Figure S2: Complementation of selected mutants of *Pseudomonas chlororaphis* PCL1391 and *Pseudomonas protegens* CHA0 restored the expression of wild-type O-PS. SDS-PAGE of lipopolysaccharide (LPS) extracted from wild-type and selected mutant strains of *P. chlororaphis* PCL1391 and *P. protegens* CHA0 grown at 25°C to late exponential growth phase in Lysogeny Broth. LPS was made visible by silver staining. Molecular weights in kDa are depicted on the left of the gel. The experiment was repeated once with similar results.

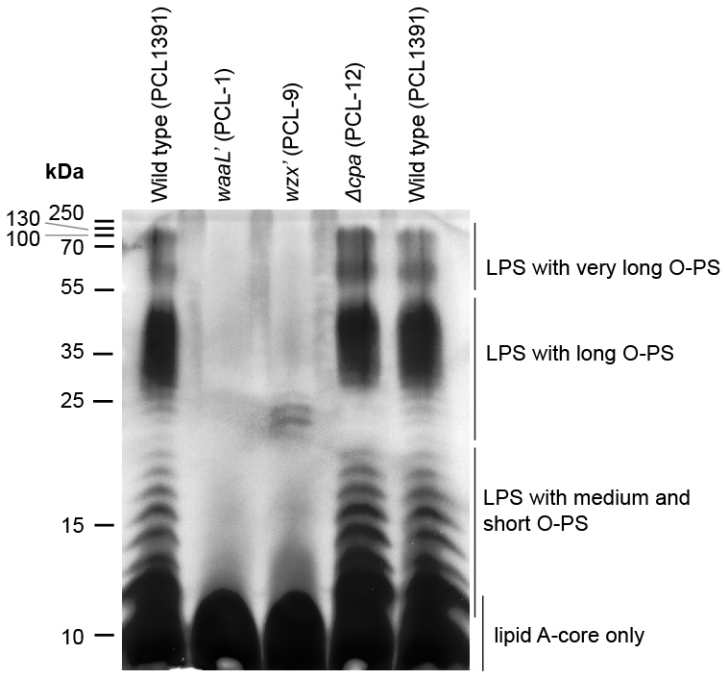

Figure S3: Mutation of O-PS biosynthesis genes in *Pseudomonas chlororaphis* PCL1391. SDS-PAGE of lipopolysaccharide (LPS) extracted from PCL1391 and isogenic mutant strains grown at 30°C to late exponential growth phase in Lysogeny Broth. LPS was made visible by silver staining. Molecular weights in kDa are depicted on the left of the gel. The experiment was repeated once with similar results.

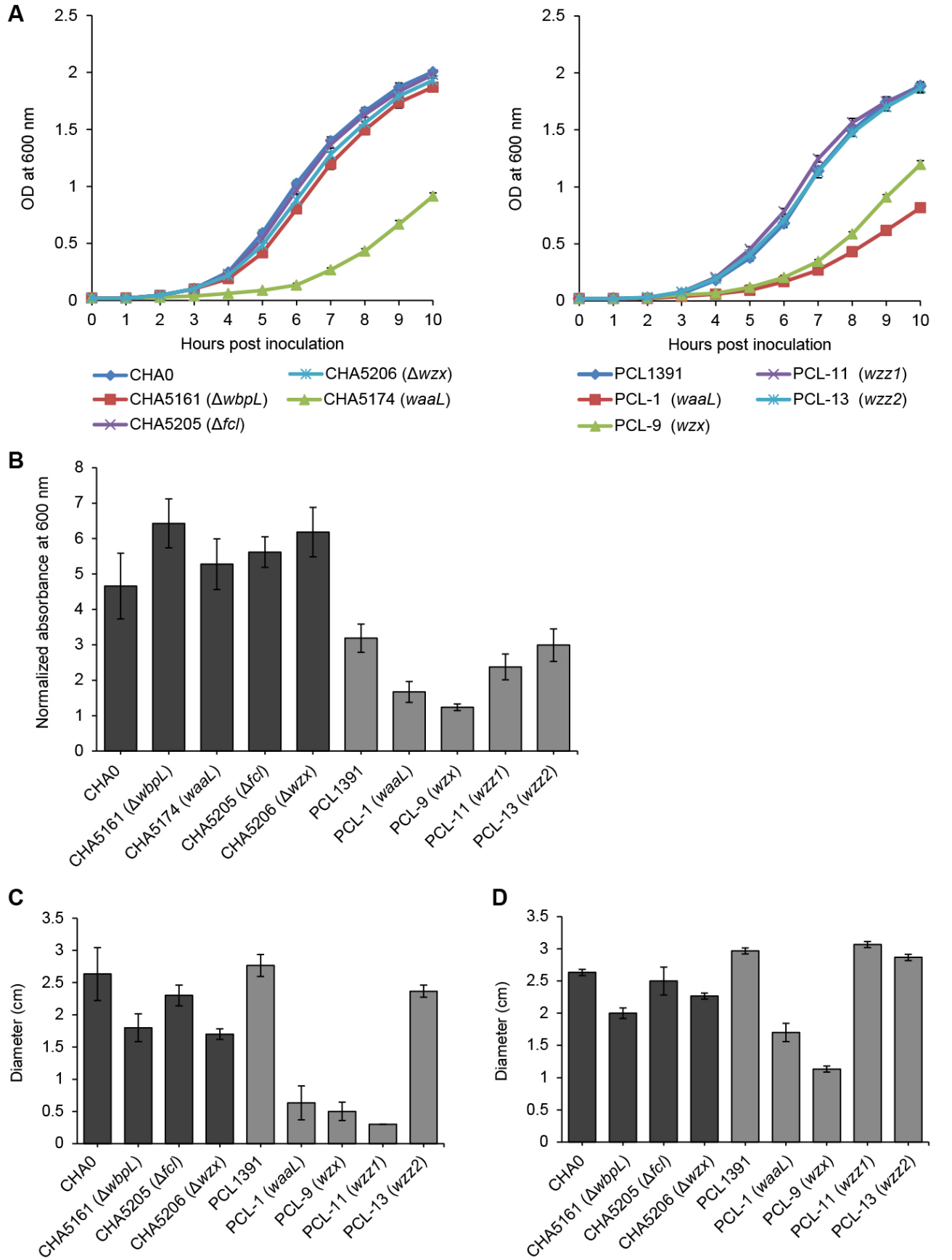

Figure S4: Characterization of constructed O-PS mutants. (A) Growth curves of *Pseudomonas protegens* CHA0, *Pseudomonas chlororaphis* PCL1391, and their isogenic O-polysaccharide (O-PS) mutants in Lysogeny Broth at 25°C. Shown are means and standard deviations of five independent cultures per bacterial strain. **(B)** Quantification of biofilm formation of the same strains in Grace's

Insect Medium contained in 96-well microplates by performing crystal violet staining. Shown are mean values and standard deviations of the absorbance at 600 nm of eight wells per strain, corrected for differences in growth rate. The absorbance correlates with the amount of biofilm formed on the walls of the wells. **(C)** Swarming motility. Shown are means and standard deviations of the diameter of bacterial displacement of three replicates per strain. *P. chlororaphis* PCL-11 was observed to be immobile on this particular semi-solid medium. **(D)** Swimming motility. Shown are means and standard deviations of diameters of three replicates per strain. The swimming diameters for strains PCL-1 and PCL-9 might be smaller due to the lower growth rate of these strains. All experiments were repeated at least once with similar results.

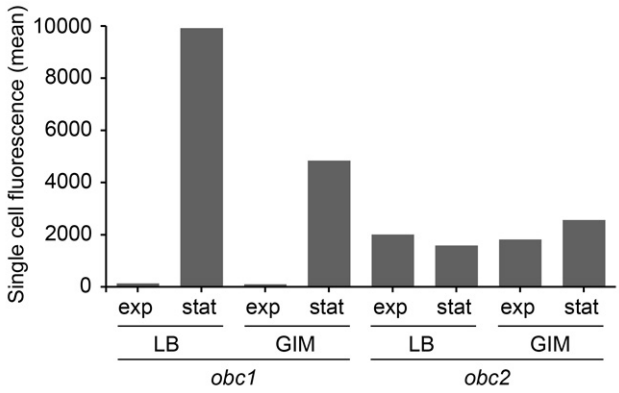

Figure S5: Genes in the OBC1 and OBC2 gene clusters of *Pseudomonas protegens* CHA0 seem to be expressed. *P. protegens* CHA0 was equipped with a GFP-based reporter plasmid (pME8369 for *obc1* or pME8370 for *obc2*) and grown in Lysogeny Broth (LB) or Grace's Insect Medium (GIM) at 25°C. Samples were taken 8 hours (exponential phase) and 24 hours (stationary phase) after inoculation and fluorescence intensities of individual cells were quantified by fluorescence microscopy. Shown are population averages of single cell fluorescence intensities, corrected for background and autofluorescence of the bacteria.

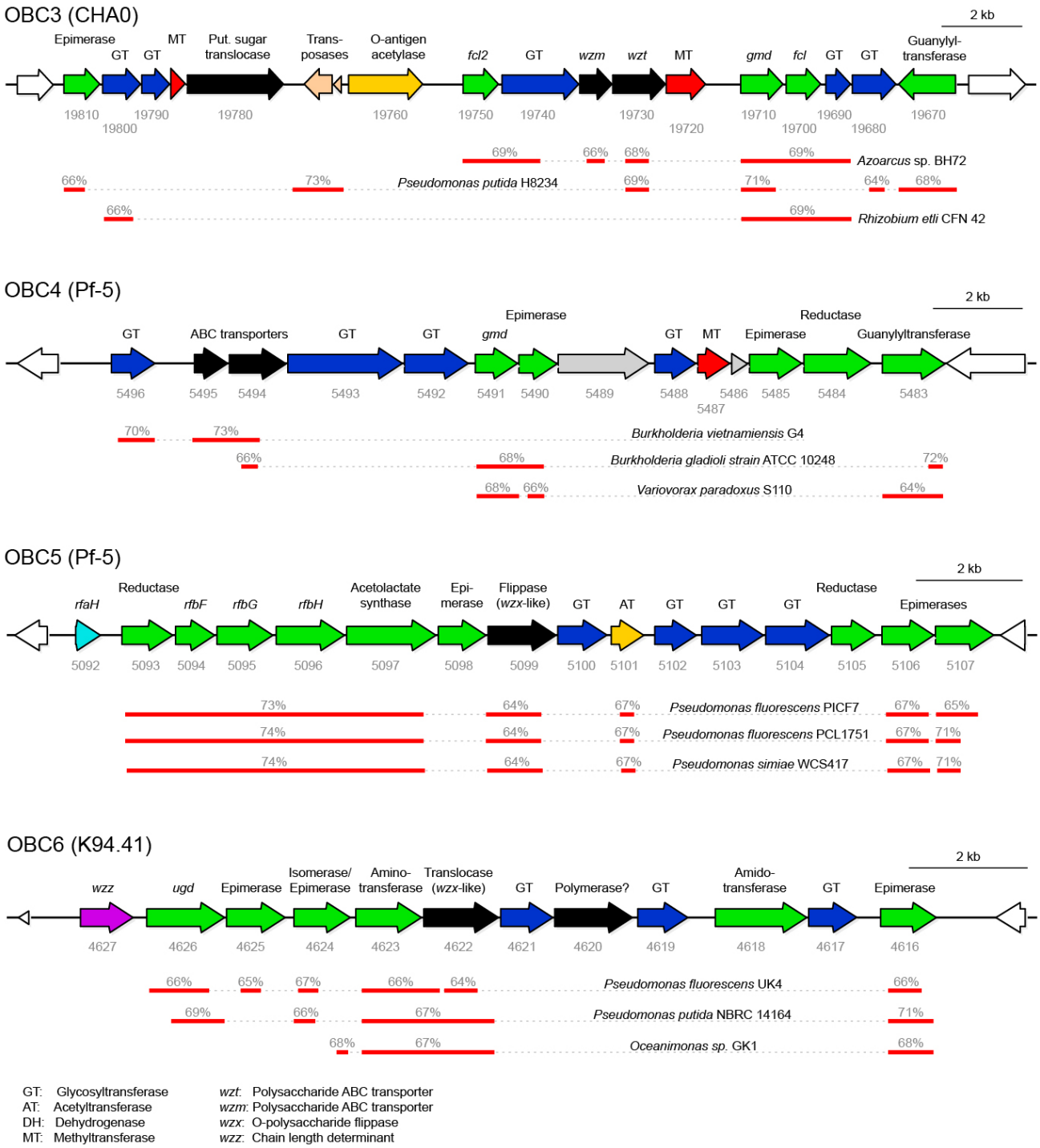

Figure S6: Gene clusters predicted to be involved in the biosynthesis of O-PS in different *Pseudomonas protegens* strains and search for homology in other species. Depicted are newly identified genes and gene clusters that were predicted to contribute to O-PS biosynthesis in different strains of *Pseudomonas protegens*. Putative functions of encoded proteins or gene names are indicated above the corresponding genes, information about the locus tags are given below. Genes are drawn to scale and are color-coded according to the function of the proteins they code for: black, transport and polymerization; blue, glycosyltransferases; green, nucleotide sugar biosynthesis and modification of sugars; red, methylation; yellow, (de)acetylation; violet, chain length determination; light blue, transcriptional regulation; gray, unknown function; light brown, transposition; white, flanking genes. The nucleotide sequences of the strains that are indicated in brackets were used to conduct blastn searches. The top three hits (bacterial strains, sorted by coverage in descending order) are shown below each cluster. Regions with at least 60% nucleotide identity and 300 nucleotides length are indicated with red bars. The numbers above the red bars correspond to nucleotide sequence identities.

Genome accession numbers: *Azoarcus* sp, AM406670.1; *Burkholderia gladioli*, CP009323.1; *Burkholderia vietnamiensis*, CP000614.1; *Oceanimonas* sp., CP003171.1; *Pseudomonas fluorescens* PCL1751, CP010896.1; *P. fluorescens* PICF7, CP005975.1; *P. fluorescens* UK4, CP008896.1; *Pseudomonas putida* H8234, CP005976.1; *P. putida* NBRC 14164, AP013070.1; *Pseudomonas simiae*, CP007637.1; *Rhizobium etli*, CP000133.1; *Variovorax paradoxus*, CP001635.1.

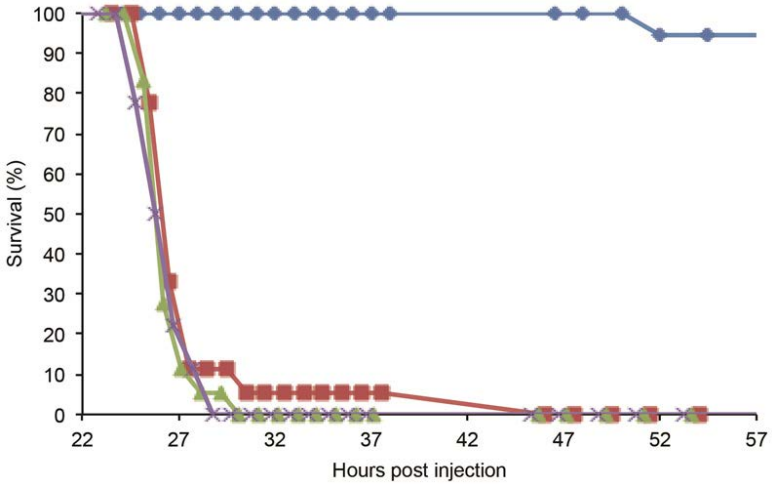

Figure S7: Regulation of O-PS chain length is not essential for virulence of *Pseudomonas chlororaphis* PCL13191 upon injection. Virulence of PCL1391 wild-type and mutant strains was determined by injection of low cell numbers into larvae of *Galleria mellonella* and monitoring the survival of the insects over time. Mutation of *wzz1* (in green, PCL-11) and *wzz2* (in red, PCL-13) did not significantly reduce the mortality of the larvae compared to the wild type (in violet) (p-value > 0.05; Log-rank test). Saline solution served as a negative control (in blue).

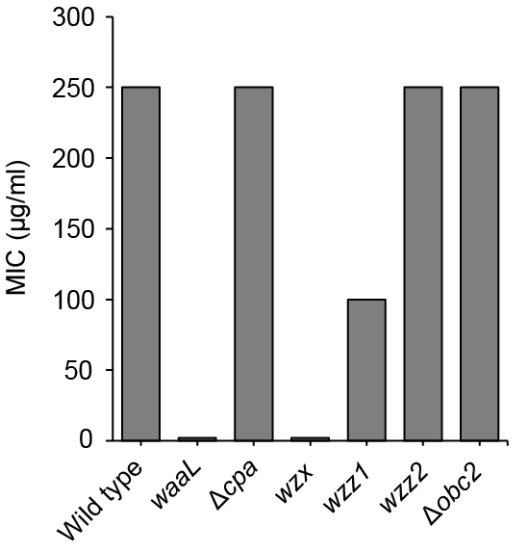

Figure S8: Resistance of *Pseudomonas chlororaphis* PCL1391 to Polymyxin B depends on the presence of the OSA-type O-antigen. The minimum inhibitory concentration (MIC) of polymyxin B (PMB) was determined for *P. chlororaphis* PCL1391 and isogenic mutant strains (PCL-1, *waaL*; PCL-3, Δ*obc2*; PCL-9, *wzx*; PCL-11, *wzz1*; PCL-12, Δ*cpa*; and PCL-13, *wzz2*) by growing them in Lysogeny Broth in presence of different concentrations of the antimicrobial peptide (2, 10, 50, 100, or 250 µg/ml PMB). The MIC was defined as the concentration at which no visible growth of the bacteria was observed 24 hours after inoculation. The experiment was repeated once with similar results.

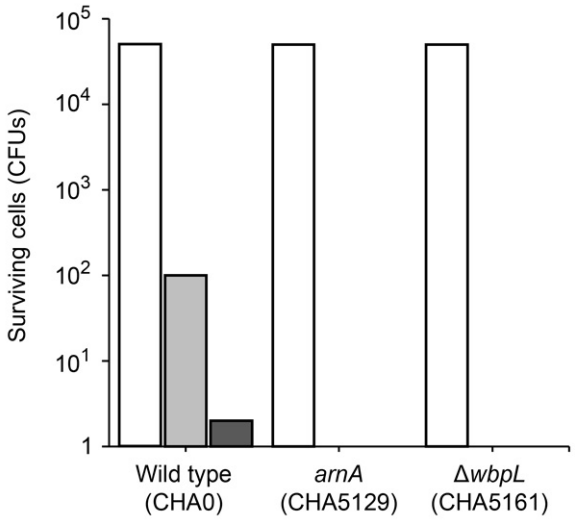

Figure S9: Resistance of *Pseudomonas protegens* CHA0 to polymyxin B depends on lipid A modification and presence of the short O-PS under high magnesium concentrations. *P. protegens* strains CHA0, CHA5129, and CHA5161 were grown overnight in Lysogeny Broth supplemented with 20 mM $MgCl_2$. The cells were washed and aliquots of the suspensions were exposed to polymyxin B at a concentration of 10 µg/ml (in gray) or 100 µg/ml (in black) for three hours in saline solution. The bacterial suspensions were subsequently serially diluted and plated on Nutrient Agar plates to assess the number of cells that survived the treatment with the antimicrobial peptide. Shown are the numbers of colony forming units (CFUs) per 10 µl for each treatment, normalized by the value for the negative control (no polymyxin B, in white).

Table S1: Polysaccharide biosynthesis gene clusters identified in selected *Pseudomonas* strains

Strain	Gene/gene cluster	Locus tag(s)	Remarks / description
CHA0	OSA	43710-43770	Synthesis of very short O-antigen
	OBC1	20730-20810	Unknown function
	OBC2	31090-31240	Putative capsular polysaccharide (CPS) gene cluster
	OBC3	19670-19810	Synthesis of long O-antigen
	psl	42710-42820	Psl biosynthesis
	pel	30140-30210	Pel biosynthesis
	alg	10330-10440	Alginate biosynthesis
	pga	01630-01660	PNAG biosynthesis
Cab57	OSA	4407-4413	O-antigen synthesis (prediction)
	OBC1	2048-2056	Unknown function
	OBC2	3106-3121	Putative CPS gene cluster
	OBC3	1981-1998	O-antigen synthesis (prediction)
	psl	4306-4317	Psl biosynthesis
	pel	3008-3015	Pel biosynthesis
	alg	1051-1062	Alginate biosynthesis
	pga	0165-0168	PNAG biosynthesis
PGNR1	OSA	4406-4409	O-antigen synthesis (prediction)
	OBC1	2119-2127	Unknown function
	OBC2	3145-3160	Putative CPS gene cluster
	OBC3	2007-2023	O-antigen synthesis (prediction)

	psl	4305-4316	Psl biosynthesis
	pel	3052-3059	Pel biosynthesis
	alg	1079-1090	Alginate biosynthesis
	pga	222-225	PNAG biosynthesis
BRIP	OSA	4339-4342	O-antigen synthesis (prediction)
	OBC1	2039-2047	Unknown function
	OBC2	3076-3091	Putative CPS gene cluster
	OBC3	1926-1942	O-antigen synthesis (prediction)
	psl	4238-4249	Psl biosynthesis
	pel	2984-2991	Pel biosynthesis
	alg	970-981	Alginate biosynthesis
	pga	113-116	PNAG biosynthesis
Pf-5	OSA	4305-4307	O-antigen synthesis (prediction)
	OBC1	2024-2032	Unknown function
	OBC2	3078-3093	Putative CPS gene cluster
	OBC4	5480-5496	O-antigen synthesis (prediction)
	OBC5	5092-5107	O-antigen synthesis (prediction)
	psl	4208-4219	Psl biosynthesis
	pel	2971-2978	Pel biosynthesis
	alg	1013-1024	Alginate biosynthesis
	pga	0161-0164	PNAG biosynthesis
PF	OSA	4481-4483	O-antigen synthesis (prediction)
	OBC1	2168-2176	Unknown function
	OBC2	3230-3245	Putative CPS gene cluster
	OBC4	5663-5680	O-antigen synthesis (prediction)
	OBC5	5290-5303	O-antigen synthesis (prediction)
	psl	4381-4392	Psl biosynthesis
	pel	3124-3131	Pel biosynthesis
	alg	1161-1172	Alginate biosynthesis
	pga	312-315	PNAG biosynthesis
K94.41	OSA	4400-4402	O-antigen synthesis (prediction)
	OBC1	2013-2021	Unknown function
	OBC2	3089-3104	Putative CPS gene cluster
	OBC6	4616-4627	O-antigen synthesis (prediction)
	psl	4299-4310	Psl biosynthesis
	pel	3002-3009	Pel biosynthesis
	alg	973-984	Alginate biosynthesis
	pga	113-116	PNAG biosynthesis
CMR5c	OSA	2610-2612	O-antigen synthesis (prediction)
	OBC1	6059-6065	Unknown function
	OBC2	0652-0667	Putative CPS gene cluster

	OBC3-A	3747-3756	O-antigen synthesis (prediction)
	OBC3-B	5999-6004	O-antigen synthesis (prediction)
	OBC5	1744-1759	O-antigen synthesis (prediction)
	psl	2510-2521	Psl biosynthesis
	alg	3365-3376	Alginate biosynthesis
	pga	2427-2430	PNAG biosynthesis
PCL1391	CPA	5895-5904	Synthesis of O-antigen of medium length
	OSA	4175-4191	Synthesis of O-antigen of various lengths
	wzz2	3550	Chain length determination
	OBC2	2810-2825	Putative CPS gene cluster
	psl	5052-5063	Psl biosynthesis
	alg	927-938	Alginate biosynthesis
	pga	126-129	PNAG biosynthesis
LMG1245	CPA	0135-0147	O-antigen synthesis (prediction)
	OSA	4629-4632	O-antigen synthesis (prediction)
	OBC2	3112-3127	Putative CPS gene cluster
	OBC7	0185-0190	O-antigen synthesis (prediction)
	psl	5501-5512	Psl biosynthesis
	alg	1184-1195	Alginate biosynthesis
	pga	0359-0362	PNAG biosynthesis
30-84	CPA	5929-5941	O-antigen synthesis (prediction)
	OSA	4169-4186	O-antigen synthesis (prediction)
	wzz2	3541	Chain length regulation
	psl	5061-5072	Psl biosynthesis
	alg	1006-1017	Alginate biosynthesis
	pga	0182-0185	PNAG biosynthesis
LMG5004	CPA	5952-5964	O-antigen synthesis (prediction)
	OSA	4268-4276	O-antigen synthesis (prediction)
	psl	5111-5122	Psl biosynthesis
	pel	3097-3104	Pel biosynthesis
	alg	922-933	Alginate biosynthesis
	pga	125-128	PNAG biosynthesis
JF3835	CPA	180-192	O-antigen synthesis (prediction)
	OSA	4713-4733	O-antigen synthesis (prediction)
	wzz2	4044	Chain length regulation
	OBC2	3184-3199	Putative CPS gene cluster
	psl	5584-5595	Psl biosynthesis
	alg	1229-1240	Alginate biosynthesis
	pga	407-410	PNAG biosynthesis
HT66	CPA	3323-3332	O-antigen synthesis (prediction)
	OSA	1779-1795	O-antigen synthesis (prediction)
	wzz2	696	Chain length regulation
	OBC2	5341-5356	Putative CPS gene cluster
	psl	3965-3976	Psl biosynthesis
	alg	2808-2819	Alginate biosynthesis
	pga	4843-4846	PNAG biosynthesis
CD	CPA	139-151	O-antigen synthesis (prediction)
	OSA	4388-4402	O-antigen synthesis (prediction)
	OBC2	3059-3074	Putative CPS gene cluster
	psl	5266-5277	Psl biosynthesis
	alg	1162-1173	Alginate biosynthesis

	pga	358-361	PNAG biosynthesis
YL-1	CPA	6922-6934	O-antigen synthesis (prediction)
	OSA	5232-5235	O-antigen synthesis (prediction)
	OBC2	3883-3898	Putative CPS gene cluster
	OBC7	6880-6884	O-antigen synthesis (prediction)
	psl	6197-6186	Psl biosynthesis
	alg	1987-1998	Alginate biosynthesis
	pga	1149-1152	PNAG biosynthesis
O6	CPA	6277-6289	O-antigen synthesis (prediction)
	OSA	4422- 4425	O-antigen synthesis (prediction)
	OBC2	3083-3098	Putative CPS gene cluster
	OBC7	0005-0009	O-antigen synthesis (prediction)
	psl	5328-5339	Psl biosynthesis
	alg	1018-1029	Alginate biosynthesis
	pga	0181-0184	PNAG biosynthesis

Table S2: Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype, phenotype or relevant characteristics	Reference or source
Pseudomonas protegens		
BRIP	Wild type	[2]
CHA0	Wild type	[3]
CHA0-*gfp2*	CHA0::attTn*7*-*gfp2*; Gm^r	[4]
CHA89	*gacA*::ΩKm^r	[5]
CHA5129	*arnA*::pEMG (PFLCHA0_c30730); Km^r	This study
CHA5161	Δ*wbpL* (PFLCHA0_c43720)	This study
CHA5161-*mChe*	CHA5161::attTn*7*-*mcherry*; Gm^r	This study
CHA5163	Δ*obc1*	This study
CHA5164	Δ*obc2*	This study
CHA5165	Δ*obc1* Δ*obc2*	This study
CHA5169	CHA5161::attTn*7*-$P_{tac/laclq}$-*wbpL*(CHA0); Gm^r	This study
CHA5174	*waaL*::pEMG (PFLCHA0_c05350); Km^r	This study
CHA5182	Δ*obc3*	This study
CHA5205	Δ*fcl* (PFLCHA0_19700)	This study
CHA5205-*mChe*	CHA5205::attTn*7*-*mcherry*; Gm^r	This study
CHA5206	Δ*wzx* (PFLCHA0_43760)	This study
CHA5206-*mChe*	CHA5206::attTn*7*-*mcherry*; Gm^r	This study
CHA5207	CHA5174::attTn*7*-$P_{tac/laclq}$-*waaL*(CHA0); Km^r, Gm^r	This study
CHA5208	CHA5205::attTn*7*-$P_{tac/laclq}$-*fcl*(K-12); Gm^r	This study
CHA5211	CHA5205::attTn*7*-$P_{tac/laclq}$-*fcl*(CE3); Gm^r	This study
CHA5212	CHA5205::attTn*7*-$P_{tac/laclq}$-*fcl*(CHA0); Gm^r	This study
CHA5214	CHA5161 *arnA*::pEMG (PFLCHA0_c30730); Km^r	This study
K94.41	Wild type	[6]
PF	Wild type	[7]
Pf-5	Wild type	[8]
PGNR1	Wild type	[9]
Pseudomonas chlororaphis		
30-84	Wild type	[10]
CD	Wild type	[2]

HT66	Wild type	[11]
JF3835	Wild type	[12]
LMG1245	Wild type	BCCM
LMG5004	Wild type	BCCM
O6	Wild type	[13]
PCL1391	Wild type	[14]
PCL1391-*gfp1*	PCL1391::attTn*7*-*gfp1*; Km^r	This study
PCL-1	*waaL*::pEMG (PCL1391_456); Km^r	This study
PCL-3	Δ*obc2*	This study
PCL-8	PCL-1:: attTn*7*-$P_{tac/laclq}$-*waaL*(CHA0); Km^r Gm^r	This study
PCL-9	*wzx*::pEMG (PCL1391_4184); Km^r	This study
PCL-11	*wzz1*::pEMG (PCL1391_4191); Km^r	This study
PCL-11-*mChe*	PCL-11::attTn*7*-*mcherry*; Gm^r	This study
PCL-12	Δ*cpa*	This study
PCL-13	*wzz2*::pEMG (PCL1391_3550); Km^r	This study
PCL-13-*mChe*	PCL-13::attTn*7*-*mcherry*; Gm^r	This study
PCL-14	PCL-1::attTn*7*-$P_{tac/laclq}$-*waaL*(PCL1391); Km^r Gm^r	This study
YL-1	Wild type	[15]
Pseudomonas spp.		
CMR5c	Wild type	[16]
P. aeruginosa PAO1	Wild type	[17]
P. brassicacearum TM1A3	Wild type	[18]
P. fluorescens 97-38	Wild type	[18]
P. fluorescens F113	Wild type	[19]
P. fluorescens Pf0-1	Wild type	[20]
P. fluorescens Q12-87	Wild type	[21]
P. fluorescens SBW25	Wild type	[22]
P. fluorescens P3	Wild type	[23]
P. kilonensis P12	Wild type	[9]
Escherichia coli		
DH5α, DH5α λpir, HB101, K-12	Laboratory strains	[24]
Rhizobium etli		
CE3	Wild type; Sm^r	[25]

Plasmids		
pBK-miniTn7-*gfp1*	pUC19-based delivery plasmid for miniTn7-*gfp1*; *mob*$^+$; Km^r, Cm^r, Ap^r	[26]
pEMG	pSEVA212S; *ori*R6K, *lacZα* MCS flanked by two I-SceI sites; Km^r, Ap^r	[27]
pME497	Mobilizing plasmid; Ap^r	[28]
pME8300	Carrier plasmid for Tn*7* for $P_{tac/laclq}$ controlled target gene expression; Gm^r, Ap^r	[29]
pME4510	Broad host range promoter-probe plasmid vector for Gram-negative bacteria; Gm^r	[30]
pME8362	pEMG-Δ*wbpL*(CHA0); suicide plasmid for the in-frame deletion of PFLCHA0_c43720 (*wbpL*) in CHA0; Km^r	This study
pME8366	pEMG-Δ*obc1*(CHA0); suicide plasmid for the deletion of the OBC1 cluster in CHA0; Km^r	This study
pME8367	pEMG-Δ*obc2*(CHA0); suicide plasmid for the deletion of the OBC2 cluster in CHA0; Km^r	This study

pME8368	pME8300-$P_{tac/lacIq}$-*waaL*(CHA0); IPTG-inducible expression of CHA0 *waaL* (PFLCHA0_c05350); Gm^r, Ap^r	This study
pME8369	pPROBE-TT-P_{obc1}(CHA0); Tc^r	This study
pME8370	pPROBE-TT-P_{obc2}(CHA0); Tc^r	This study
pME8378	pME8300-$P_{tac/lacIq}$-*wbpL*(CHA0); IPTG-inducible expression of CHA0 *wbpL* (PFLCHA0_ c43720); Gm^r, Ap^r	This study
pME8379	pEMG-*waaL*(PCL1391); suicide plasmid for the disruption of PCL1391 *waaL* (PCL1391_456); Km^r	This study
pME8382	pME8300-$P_{tac/lacIq}$-*waaL*(PCL1391); IPTG-inducible expression of PCL1391 *waaL* (PCL1391_456); Gm^r, Ap^r	This study
pME8383	pEMG-*waaL*(CHA0); suicide plasmid for the disruption of CHA0 *waaL* (PFLCHA0_c05350); Km^r	This study
pME8388	pEMG-Δ*obc2*(PCL1391); suicide plasmid for the deletion of the OBC2 cluster in PCL1391; Km^r	This study
pME8393	pEMG-Δ*cpa*(PCL1391); suicide plasmid for the deletion of the CPA cluster in PCL1391; Km^r	This study
pME8394	pEMG-Δ*obc3*(CHA0); suicide plasmid for the deletion of the OBC3 cluster in CHA0; Km^r	This study
pME8396	pPROBE-TT-P_{wzz1}(PCL1391); Tc^r	This study
pME8399	pEMG-*wzx*(PCL1391); suicide plasmid for the disruption of PCL1391 *wzx* (PCL1391_4184); Km^r	This study
pME11001	pEMG-*wzz1*(PCL1391); suicide plasmid for the disruption of PCL1391 *wzz1* (PCL1391_4191); Km^r	This study
pME11007	pEMG-Δ*fcl*(CHA0); suicide plasmid for the in-frame deletion of PFLCHA0_c19700 (*fcl*) in CHA0; Km^r	This study
pME11009	pEMG-Δ*wzx*(CHA0); suicide plasmid for the in-frame deletion of PFLCHA0_c43760 (*wzx*) in CHA0; Km^r	This study
pME11012	pEMG-*wzz2*(PCL1391); suicide plasmid for the disruption of PCL1391 *wzz2* (PCL1391_3550); Km^r	This study
pME11014	pME8300-$P_{tac/lacIq}$-*fcl*(K-12); IPTG-inducible expression of *E. coli* K-12 *fcl* (EO53_07645); Gm^r, Ap^r	This study
pME11019	pPROBE-TT-P_{wzz2}(PCL1391); Tc^r	This study
pME11021	pME8300-$P_{tac/lacIq}$-*fcl*(CE3); IPTG-inducible expression of *R. etli* CE3 *fcl* (RHE_CH00763); Gm^r, Ap^r	This study
pME11022	pME8300-$P_{tac/lacIq}$-*fcl*(CHA0); IPTG-inducible expression of CHA0 *fcl* (PFLCHA0_19700); Gm^r, Ap^r	This study
pME11024	pME4510-*wzx*-locus; expression of PFLCHA0_c43760 (*wzx*) under the control of its native promoter; Gm^r	This study
pME9407	pUC19-based delivery plasmid for miniTn7-*mcherry*; mob^+; Gm^r, Cm^r, Ap^r	[31]
pPROBE-TT	Promoter-probe vector based on eGFP; Tc^r	[32]
pSW-2	*ori*RK2, *xylS*, P_m::*I-sce*I; Gm^r	[27]
pUX-BF13	Helper plasmid encoding Tn*7* transposition functions; R6K-replicon; Ap^r	[33]

Abbreviations: Ap^r, ampicillin resistance; BCCM, Belgian Coordinated Collections of Microorganisms; Cm^r, chloramphenicol resistance; Gm^r, gentamicin resistance; Km^r, kanamycin resistance; Sm^r, streptomycin resistance; Tc^r, tetracycline resistance.

Table S3: Primers used in this study

Name	Sequence 5' → 3', restriction enzyme(s)[1]	Purpose
arnA-im-1	CGGGATCCAACTGGGTGCTGGTCAAG, BamHI	Disruption of CHA0 *arnA*
arnA-im-2	GGAATTCGTCCACCAGGCCCAGTTC, EcoRI	Disruption of CHA0 *arnA*
CE3.fcl-expr-F	GACTAGTATGGACAAGACATCGAAGATTTATG, SpeI	Expression of CE3 *fcl*
CE3.fcl-expr-R	GACTAGTAAGCGGTTCGATCATGTTTCAG, SpeI	Expression of CE3 *fcl*
fcl-del-1	GGAATTCCCTTCGTCACTCGCAAGATTAC, EcoRI	Deletion of CHA0 *fcl*

fcl-del-2	CCCAAGCTTGATCGCAGAGCCAGCCATAC, HindIII	Deletion of CHA0 *fcl*
fcl-del-3	CCCAAGCTTACGCAACTGCGTGAAGGTATAG, HindIII	Deletion of CHA0 *fcl*
fcl-del-4	CGGGATCCAGTCGCCACCAAAGATCGTAG, BamHI	Deletion of CHA0 *fcl*
fcl-expr-F	GACTAGTGTGGATAAACGCGCCAAAATATAC, SpeI	Expression of CHA0 *fcl*
fcl-expr-R	CCCAAGCTTCATTCAGTGGCGATCCAGAC, HindIII	Expression of CHA0 *fcl*
K12.fcl-expr-F	GACTAGTGATCGCGCTGGAGTCATAAGC, SpeI	Expression of K-12 *fcl*
K12.fcl-expr-R	CCCAAGCTTCATTACCCCCGAAAGCGGTC, HindIII	Expression of K-12 *fcl*
obc1-del-1	CGGGATCCGCATCCTCTTCAGCTTCGAC, BamHI	Deletion of CHA0 *obc1*
obc1-del-2	CCCAAGCTTGGTACTTCCGGTCAACTCC, HindIII	Deletion of CHA0 *obc1*
obc1-del-3	CCCAAGCTTGTCCTCTCCTGGAGCATCT, HindIII	Deletion of CHA0 *obc1*
obc1-del-4	GGAATTCCCCAATCATAGCCACTCAC, EcoRI	Deletion of CHA0 *obc1*
obc2-del-1	CGGAATTCATGGGCACGGATTGAGTAAGG, EcoRI	Deletion of CHA0 *obc2*
obc2-del-2	CCCAAGCTTGAGGGTGGTTCTCACCTTCT, HindIII	Deletion of CHA0 *obc2*
obc2-del-3	CCCAAGCTTATTGCCTACAGCGAGCTGTAA, HindIII	Deletion of CHA0 *obc2*
obc2-del-4	GGGATCCAACGAGCTGATTGCCGAATTG, BamHI	Deletion of CHA0 *obc2*
obc3-del-1	GGAATTCTGTTACCCTTCCCCCATACAG, EcoRI	Deletion of CHA0 *obc3*
obc3-del-2	GACTAGTACAGCAGCCTGGTTGGTAAG, SpeI	Deletion of CHA0 *obc3*
obc3-del-3	GACTAGTCCGCAAGAAGCATTGGTAATGG, SpeI	Deletion of CHA0 *obc3*
obc3-del-4	CGGGATCCGACCATGCCCAAGACATCAAG, BamHI	Deletion of CHA0 *obc3*
PCL.cpa-del-1	CGGAATTCGGTGATCAGGGTTGCTGAAG, EcoRI	Deletion of PCL1391 *cpa*
PCL.cpa-del-2	CCCAAGCTTCGAGTTGTTCACCGGTTTTCC, HindIII	Deletion of PCL1391 *cpa*
PCL.cpa-del-5	CCCAAGCTTCCACCGGTTTCTCCTACAG, HindIII	Deletion of PCL1391 *cpa*
PCL.cpa-del-6	GGGATCCAGACGATGGCGTAGCTCAGT, BamHI	Deletion of PCL1391 *cpa*
PCL.obc2-del-1	CGGAATTCCTTGGATGTCAGCGTTTTTGG, EcoRI	Deletion of PCL1391 *obc2*
PCL.obc2-del-2	CCCAAGCTTACAGCTCGCTCAACTGCTG, HindIII	Deletion of PCL1391 *obc2*
PCL.obc2-del-3	CCCAAGCTTCCATATCGACTCCCATCAGC, HindIII	Deletion of PCL1391 *obc2*
PCL.obc2-del-4	GGGATCCGATCGTCAGGGAAATATGGG, BamHI	Deletion of PCL1391 *obc2*
PCL.osa-P1-F	CGGGATCCGCTTGGGCTGTTCAAAATCTCC, BamHI	Reporter for PCL1391 *wzz1*
PCL.osa-P1-R	GGAATTCTCCTGCAGATCCACTTCCTC, EcoRI	Reporter for PCL1391 *wzz1*
PCL.Pwzz2-F	CGGGATCCACTCCGCGAAAGGACTGGTC, BamHI	Reporter for PCL1391 *wzz2*
PCL.Pwzz2-R	GGAATTCCAGAGTACAGCCAATTACCAGC, EcoRI	Reporter for PCL1391 *wzz2*
PCL.waaL-check-F	GGGCCTGATCAGTCTGTTGT	Disruption of PCL1391 *waaL*
PCL.waaL-check-R	TGATCGAACGAAAACCCTTCAG	Disruption of PCL1391 *waaL*
PCL.waaL-expr-F	GACTAGTATGCAGGCCACACGTTGGG, SpeI	Expression of PCL1391 *waaL*
PCL.waaL-expr-R	CCCAAGCTTGTCGAGCTCACTGTTTAGGAG, HindIII	Expression of PCL1391 *waaL*
PCL.waaL-im-1	GGAATTCCTGTTGTTTTTCCCGGTTTTCG, EcoRI	Disruption of PCL1391 *waaL*
PCL.waaL-im-2	CGGGATCCAACATCGCCAGTAGCAATG, BamHI	Disruption of PCL1391 *waaL*
PCL.wzx-check-1	CGGGTAGTCTAGGGATGCAA	Disruption of PCL1391 *wzx*
PCL.wzx-check-2	CAACATTGTTCCGCAAGAGAGC	Disruption of PCL1391 *wzx*
PCL.wzx-im-1	CGGGATCCTGGTCGTGCTCACCAACTTAC, BamHI	Disruption of PCL1391 *wzx*
PCL.wzx-im-2	CGGGATCCCAAGAACGAGGCTCAATATG, BamHI	Disruption of PCL1391 *wzx*
PCL.wzz2-check-1	GGTACGGCATGACAGGGTTA	Disruption of PCL1391 *wzz2*
PCL.wzz2-check-2	CTGCAGCTCCTTACCGATTTC	Disruption of PCL1391 *wzz2*
PCL.wzz2-im-1	GGAATTCGCTCGGAAGTCTACAAACTG, EcoRI	Disruption of PCL1391 *wzz2*
PCL.wzz2-im-2	CGGGATCCTTGCGCTGTTCTTTCAACTG, BamHI	Disruption of PCL1391 *wzz2*
PCL.wzz-check-1	AGTGGATCTGCAGGAGCTTG	Disruption of PCL1391 *wzz1*
PCL.wzz-check-2	GTAGCTCACGCAAACGATCAG	Disruption of PCL1391 *wzz1*
PCL.wzz-im-1	GGAATTCGTAGTGCCTCCGACACAAAAAG, EcoRI	Disruption of PCL1391 *wzz1*
PCL.wzz-im-2	CGGGATCCAATTTGCTGCTCGACGTTTCG, BamHI	Disruption of PCL1391 *wzz1*
Pobc1-F	CGGGATCCGACCTGCTGGTGCTTTCCAT, BamHI	Reporter for CHA0 *obc1*
Pobc1-R	GGAATTCGCCTATCACTTCGCGTTCGATC, EcoRI	Reporter for CHA0 *obc1*

Pobc2-F	CGGGATCCGAAAGTCCCGGTTGAAGTAG, BamHI	Reporter for CHA0 *obc2*
Pobc2-R	GGAATTCGACTTCCAAGGGAACGACCT, EcoRI	Reporter for CHA0 *obc2*
waaL-expr-F	GACTAGTATGCAACCCAATGCCCTTCAC, SpeI	Expression of CHA0 *waaL*
waaL-expr-R	CCCAAGCTTCCCTGTGTAGCTGTCATGGA, HindIII	Expression of CHA0 *waaL*
waaL-fs-1	GGAATTCTGCCGATCGGTTATCTACTG, EcoRI	Disruption of CHA0 *waaL*
waaL-fs-2	CCCAAGCTTGAGAGCAACGATCGCAGCAC, HindIII	Disruption of CHA0 *waaL*
waaL-fs-3	CCCAAGCTTTCGCCTCGATCTTCAACCTG, HindIII	Disruption of CHA0 *waaL*
waaL-fs-4	CGGGATCCAGAAGCATCTGCGAGAAC, BamHI	Disruption of CHA0 *waaL*
wbpL-del-1	CGGGATCCGCCTTGCACGACCTTATTGTG, BamHI	Deletion of CHA0 *wbpL*
wbpL-del-2	GACTAGTCCAGTACACATGGCTCATACG, SpeI	Deletion of CHA0 *wbpL*
wbpL-del-3	GACTAGTGCAAGAGAGAACTGAGATGCG, SpeI	Deletion of CHA0 *wbpL*
wbpL-del-4	GGAATTCAGCGCCTGCTCCATAAATAG, EcoRI	Deletion of CHA0 *wbpL*
wbpL-expr-F	GACTAGTATGAGCCATGTGTACTGGG, SpeI	Expression of CHA0 *wbpL*
wbpL-expr-R	CCCAAGCTTCGCATCTCAGTTCTCTCTTGC, HindIII	Expression of CHA0 *wbpL*
wzx-del-1	GGAATTCGTCATTCGAGAGAAAGGGACGA, EcoRI	Deletion of CHA0 *wzx*
wzx-del-2	GACTAGTAGAGGCGACAGTTAAAGCAGC, SpeI	Deletion of CHA0 *wzx*
wzx-del-3	GACTAGTGTGGTTGCCGTATCAGGTTTT, SpeI	Deletion of CHA0 *wzx*
wzx-del-4	CGGGATCCACAGCCTTTCCTTCGGGAAT, BamHI	Deletion of CHA0 *wzx*
wzx-expr-F2	GGAATTCATCCTGATTGAGGTTCTCGTAG, EcoRI	Expression of CHA0 *wzx*
wzx-expr-R2	CGGGATCCCTCATGCAAACGAGAGTAC, BamHI	Expression of CHA0 *wzx*

[1] Restriction sites are underlined

Protocols

Whole cell LPS extraction

Modified after Davis and Goldberg (2012) JOVE

1. Overnight cultures of the bacterial strains (10 ml LB, 25°C and 180 rpm).
2. Inoculation of 10 ml LB in a 50-ml Erlenmeyer flask with 100 µl bacterial culture and incubation for 16 hours (overnight) at 25°C and 180 rpm.
3. OD_{600} reading in a spectrophotometer (optionally wash the cells once with NaCl solution).
4. Centrifuge 1.5 ml of bacterial suspension with an OD_{600} = 5 and remove the supernatant (pellet can be stored at -20°C at this point).
5. Resuspend the pelleted bacteria in 100 µl of bi-distilled water (do not vortex!) and add 100 µl of 2x SDS-buffer. (Ensure that the pellet is completely resuspended by pipetting the suspension up and down slowly. Do not vortex!)
6. Boil the suspended bacteria in a water bath for 10 minutes. Allow the solution to cool at room temperature for some minutes.
7. Add 5 µl of both DNase I and RNase solutions (10 mg/ml stocks). Incubate the samples at 37°C for 30 minutes.
8. Add 10 µl of Proteinase K solution (10 mg/ml stock). Incubate the samples at 56°C for at least 3 hours. (This step can be performed overnight, if there are time constraints.)

Solutions and chemicals:

- 2x SDS buffer: Make a 50 mL solution of 4% β-mercaptoethanol (BME), 4% SDS and 20% glycerol in 0.1 M Tris-HCl, pH 6.8 (put the bottle on a shaker to dissolve the chemicals). Add a pinch of bromophenol blue to dye the solution. This can be stored at room temperature.

- DNAse (10 mg/ml), RNAse (10 mg/ml), and Proteinase K (10 mg/ml)

Silver Staining of LPS Gels

According to Zhu *et al.* 2012

1. Perform SDS PAGE (let the samples migrate for about 2 hours at 150 V on a 15% minigel)
2. Fixation: 10 min in 50 ml of fixation solution
3. Washing: 2x 5 min in 50 ml deionized water
4. Impregnation: 5 min in 50 ml of impregnation solution
5. Washing: 2x 20 sec in deionized water
6. Development: 5-8 min in 50-100 ml of freshly prepared (!!) development solution
7. Stopping: 1 min in stopping solution and wash twice with distilled water

Solutions (for two minigels):

Fixation solution (100 ml):	0.7 g periodic acid (= iodic(VII) acid) 30 ml ethanol 10 ml acetic acid 60 ml dH_2O
Impregnation solution (100 ml):	0.2 g silver nitrate 100 ml dH_2O
Development solution (200 ml): *(add the water just after starting the staining!)*	6 g sodium carbonate 0.04 g ascorbic acid 0.08 g sodium thiosulfate 0.1 g sodium hydroxide (= 1 pellet) 200 ml dH_2O
Stopping solution (100 ml):	10 ml acetic acid 90 ml dH_2O

References

1. Islam ST, Taylor VL, Qi M, Lam JS (2010) Membrane topology mapping of the O-antigen flippase (Wzx), polymerase (Wzy), and ligase (WaaL) from *Pseudomonas aeruginosa* PAO1 reveals novel domain architectures. MBio 1: e00189-00110.
2. Ruffner B (2013) Insecticidal activity in plant-beneficial pseudomonads: Molecular basis and ecological relevance. PhD thesis; ETH Zurich, Zurich, Switzerland.
3. Stutz E, Défago G, Kern H (1986) Naturally occurring fluorescent pseudomonads involved in suppression of black root rot of tobacco. Phytopathology 76: 181-185.
4. Péchy-Tarr M, Borel N, Kupferschmied P, Turner V, Binggeli O, et al. (2013) Control and host-dependent activation of insect toxin expression in a root-associated biocontrol pseudomonad. Environ Microbiol 15: 736-750.
5. Laville J, Voisard C, Keel C, Maurhofer M, Défago G, et al. (1992) Global control in *Pseudomonas fluorescens* mediating antibiotic synthesis and suppression of black root rot of tobacco. Proc Natl Acad Sci U S A 89: 1562-1566.
6. Wang CX, Ramette A, Punjasamarnwong P, Zala M, Natsch A, et al. (2001) Cosmopolitan distribution of *phlD*-containing dicotyledonous crop-associated biocontrol pseudomonads of worldwide origin. FEMS Microbiol Ecol 37: 105-116.
7. Levy E, Gough FJ, Berlin KD, Guiana PW, Smith JT (1992) Inhibition of *Septoria tritici* and other phytopathogenic fungi and bacteria by *Pseudomonas fluorescens* and its antibiotics. Plant Pathol 44: 335-341.
8. Howell CR, Stipanovic RD (1979) Control of *Rhizoctonia solani* on cotton seedlings with *Pseudomonas fluorescens* and with an antibiotic produced by the bacterium. Phytopathology 69: 480-482.
9. Keel C, Weller DM, Natsch A, Défago G, Cook RJ, et al. (1996) Conservation of the 2,4-diacetylphloroglucinol biosynthesis locus among fluorescent *Pseudomonas* strains from diverse geographic locations. Appl Environ Microbiol 62: 552-563.
10. Pierson LS, 3rd, Thomashow LS (1992) Cloning and heterologous expression of the phenazine biosynthetic locus from *Pseudomonas aureofaciens* 30-84. Mol Plant Microbe Interact 5: 330-339.
11. Chen Y, Shen X, Peng H, Hu H, Zhang X (2015) Comparative genomic analysis and phenazine production of *Pseudomonas chlororaphis*, a plant growth-promoting rhizobacterium. Genomics Data 4: 33-42.
12. Gobeli S, Goldschmidt-Clermont E, Frey J, Burr SE (2009) *Pseudomonas chlororaphis* strain JF3835 reduces mortality of juvenile perch, *Perca fluviatilis* L., caused by *Aeromonas sobria*. J Fish Dis 32: 597-602.
13. Tucker B, Radtke C, Kwon SI, Anderson AJ (1995) Suppression of bioremediation by *Phanerochaete chrysosporium* by soil factors. J Hazard Mater 41: 251-265.
14. Chin-A-Woeng TFC, Bloemberg GV, van der Bij AJ, van der Drift KMGF, Schripsema J, et al. (1998) Biocontrol by phenazine-1-carboxamide-producing *Pseudomonas chlororaphis* PCL1391 of tomato root rot caused by *Fusarium oxysporum* f. sp. *radicis-lycopersici*. Mol Plant Microbe Interact 11: 1069-1077.
15. Liu Y, Lu SE, Baird SM, Qiao J, Du Y (2014) Draft genome sequence of *Pseudomonas chlororaphis* YL-1, a biocontrol strain suppressing plant microbial pathogens. Genome Announc 2.
16. Perneel M, Heyrman J, Adiobo A, De Maeyer K, Raaijmakers JM, et al. (2007) Characterization of CMR5c and CMR12a, novel fluorescent *Pseudomonas* strains from the cocoyam rhizosphere with biocontrol activity. J Appl Microbiol 103: 1007-1020.
17. Holloway BW, Krishnapillai V, Morgan AF (1979) Chromosomal genetics of *Pseudomonas*. Microbiol Rev 43: 73-102.
18. Frapolli M, Défago G, Moënne-Loccoz Y (2007) Multilocus sequence analysis of biocontrol fluorescent *Pseudomonas* spp. producing the antifungal compound 2,4-diacetylphloroglucinol. Environ Microbiol 9: 1939-1955.
19. Fenton AM, Stephens PM, Crowley J, O'Callaghan M, O'Gara F (1992) Exploitation of gene(s) involved in 2,4-diacetylphloroglucinol biosynthesis to confer a new biocontrol capability to a *Pseudomonas* strain. Appl Environ Microbiol 58: 3873-3878.

20. Compeau G, Al-Achi BJ, Platsouka E, Levy SB (1988) Survival of rifampin-resistant mutants of *Pseudomonas fluorescens* and *Pseudomonas putida* in soil systems. Appl Environ Microbiol 54: 2432-2438.
21. Vincent MN, Harrison LA, Brackin JM, Kovacevich PA, Mukerji P, et al. (1991) Genetic analysis of the antifungal activity of a soilborne *Pseudomonas aureofaciens* strain. Appl Environ Microbiol 57: 2928-2934.
22. Silby MW, Cerdeno-Tarraga AM, Vernikos GS, Giddens SR, Jackson RW, et al. (2009) Genomic and genetic analyses of diversity and plant interactions of *Pseudomonas fluorescens*. Genome Biol 10: R51.
23. Meyer JB, Frapolli M, Keel C, Maurhofer M (2011) Pyrroloquinoline quinone biosynthesis gene pqqC, a novel molecular marker for studying the phylogeny and diversity of phosphate-solubilizing pseudomonads. Appl Environ Microbiol 77: 7345-7354.
24. Sambrook J, Russel DW (2001) Molecular Cloning: A Laboratory Manual. Cold Spring Harbor (New York): Cold Spring Harbor Laboratory Press.
25. Noel KD, Sanchez A, Fernandez L, Leemans J, Cevallos MA (1984) *Rhizobium phaseoli* symbiotic mutants with transposon Tn*5* insertions. J Bacteriol 158: 148-155.
26. Koch B, Jensen LE, Nybroe O (2001) A panel of Tn*7*-based vectors for insertion of the *gfp* marker gene or for delivery of cloned DNA into Gram-negative bacteria at a neutral chromosomal site. J Microbiol Methods 45: 187-195.
27. Martínez-García E, de Lorenzo V (2011) Engineering multiple genomic deletions in Gram-negative bacteria: analysis of the multi-resistant antibiotic profile of *Pseudomonas putida* KT2440. Environ Microbiol 13: 2702-2716.
28. Voisard C, Bull C, Keel C, Laville J, Maurhofer M, et al. (1994) Biocontrol of root diseases by *Pseudomonas fluorescens* CHA0: current concepts and experimental approaches. In: O'Gara F, Dowling D, Boesten B, editors. Molecular ecology of rhizosphere microorganisms: biotechnology and the release of GMOs. Weinheim: VCH Publishers. pp. 67-89.
29. Kupferschmied P, Péchy-Tarr M, Imperiali N, Maurhofer M, Keel C (2014) Domain shuffling in a sensor protein contributed to the evolution of insect pathogenicity in plant-beneficial *Pseudomonas protegens*. PLoS Pathog 10: e1003964.
30. Rist M, Kertesz MA (1998) Construction of improved plasmid vectors for promoter characterization in *Pseudomonas aeruginosa* and other Gram-negative bacteria. FEMS Microbiol Lett 169: 179-183.
31. Rochat L, Péchy-Tarr M, Baehler E, Maurhofer M, Keel C (2010) Combination of fluorescent reporters for simultaneous monitoring of root colonization and antifungal gene expression by a biocontrol pseudomonad on cereals with flow cytometry. Mol Plant Microbe Interact 23: 949-961.
32. Miller WG, Leveau JHJ, Lindow SE (2000) Improved *gfp* and *inaZ* broad-host-range promoter-probe vectors. Mol Plant Microbe Interact 13: 1243-1250.
33. Bao Y, Lies DP, Fu H, Roberts GP (1991) An improved Tn*7*-based system for the single-copy insertion of cloned genes into chromosomes of Gram-negative bacteria. Gene 109: 167-168.

CHAPTER 6

Identification and characterization of the virulence regulatory system PhoP-PhoQ in insect-pathogenic pseudomonads

Abstract

Pseudomonads are fascinating bacteria with usually multiple lifestyles due to their ability to adapt to several different environments. *Pseudomonas protegens* and *Pseudomonas chlororaphis* are plant-protecting rhizobacteria which promote plant growth, compete with and inhibit phytopathogenic fungi by producing a cocktail of antifungal compounds, induce systemic resistance in their host plants, and display insect pathogenicity. To infect and kill insect larvae, these bacteria need to deal with the host's immune system by evading detection, suppressing its defense reactions or being resistant to its antimicrobial responses. The two-component regulatory system PhoP-PhoQ is well-known to regulate resistance to antimicrobial peptides (AMPs), which kill invading microorganisms and are central to the insect innate immunity, and to contribute to virulence in pathogenic bacteria. In this study, we identified the PhoP-PhoQ system in *P. protegens* CHA0 and *P. chlororaphis* PCL1391 and characterized its role in regulating AMP resistance genes and virulence. The sensor protein PhoQ was required for full virulence of *P. protegens* CHA0 in a systemic infection model and influenced the expression of an insecticidal toxin. Its cognate response regulator PhoP did not seem to be required for virulence but was essential for the strain's intrinsic resistance toward the model AMP polymyxin B. The PhoP-PhoQ system controls the expression of the *arn* gene cluster, which is responsible for the addition of 4-aminoarabinose to lipid A and contributes significantly to AMP resistance in strain CHA0, in a magnesium-dependent manner. We additionally found first indications that the presence of AMPs can induce the expression of resistance genes in this bacterium. This study revealed that more knowledge about the interaction of entomopathogenic pseudomonads with the insect innate immunity is necessary in order to understand which strategies these bacteria evolved to evade or survive the host's immune defenses and whether AMP resistance is required for their virulence.

Introduction

Certain *Pseudomonas* species are of special interest for agriculture because they are able to promote plant growth and protect crop plants against diverse pests and diseases [1,2]. Strains of *Pseudomonas protegens* and *Pseudomonas chlororaphis* are insect pathogens in addition to being capable of protecting plants against phytopathogenic fungi and promoting plant growth [1-6]. Research on their insecticidal activities may lead to the development of novel biological insecticides based on selected *Pseudomonas* strains which could help to reduce the amount of harmful chemical pesticides used for crop protection [2]. The model bacteria *P. protegens* CHA0 and *P. chlororaphis* PCL1391 were shown to efficiently infect and kill larvae of several agriculturally important lepidopteran pest insects via the oral infection route [2,5]. Apart from an insecticidal toxin termed Fit [4,5] and the biosynthesis of lipopolysaccharide (LPS) O-antigen (Chapter 5), it is still unclear which additional features contribute to the insect pathogenicity in these rhizobacteria. Our research aims at a better understanding of how these pseudomonads are able to cause disease in insects.

Bacteria that colonize and infect insects have to interact with the host's immune defenses. To persist and establish residency within the host, they have to repress the immune responses, evade detection by the immune system and/or mediate resistance factors. The epithelial tissues constitute the first line of defense, termed epithelial immunity, and prevent microorganisms from invading the hemocoel by forming a physical barrier [7] (Figure 1). Gut epithelial cells synthesize certain antimicrobial peptides (AMPs), lytic proteins and reactive oxygen species (ROS) constitutively without any immune challenge [7,8]. The second line of defense is comprised of responses of the systemic immunity which can be divided into cellular and humoral reactions and are induced upon detection of invading microbes [7,9]. The cellular immunity is mediated mainly by hemocytes (blood cells), whose numbers increase during an infection, and consists of phagocytosis, nodulation and encapsulation. The hallmark of the humoral responses is the production and secretion of AMPs by the fat body (the equivalent of the vertebrate liver) and to a lower extent by hemocytes and epithelial cells. Additional reactions, such as melanization, contribute to responses of the innate immune system. Invading pseudomonads thus need to be resistant to phagocytosis and the action of defense molecules or to prevent the activation of these immune responses in order to survive within the hemolymph, once they crossed the epithelial barrier.

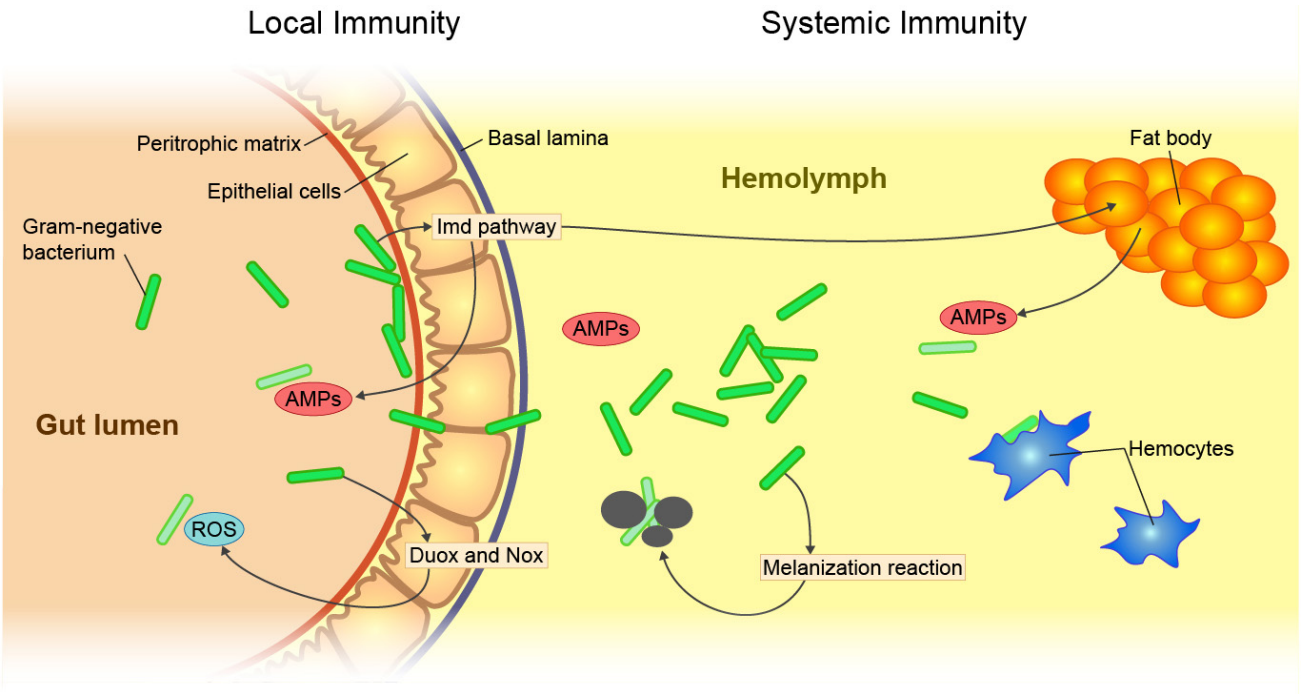

Figure 1: Insect immune defenses in response to bacterial infection. The local immunity constitutes the first line of defense against invading microbes, such as Gram-negative bacteria (in green). The peritrophic matrix and epithelial cells build a physical barrier against invading bacteria. Antimicrobial peptides (AMPs) and reactive oxygen species (ROS) are produced constitutively or upon detection of pathogenic bacteria and are secreted into the gut lumen. ROS are produced in insects mainly by the Nox and Duox enzymes. Recognition of invading bacteria via the Imd or other pathways results in a massive production of AMPs, which play a central role in systemic immunity, in the fat body. Bacterial infection can also be cleared by hemocytes, which amongst others phagocytize the pathogens, or via melanization catalyzed by phenoloxidases in the hemolymph.

Insects synthesize a powerful array of AMPs which play a key role in insect immunity [7,10,11]. AMPs are typically small cationic peptides that kill and clear pathogens by inserting into and disrupting microbial membranes. Synthesis of AMPs is not restricted to insects; also plants, microorganisms and other animals including mammals are able to produce them. These antimicrobial compounds can display activity against bacteria, fungi, parasites and even enveloped viruses [12,13]. In insects, certain AMPs are produced constitutively whereas others are synthesized only upon detection of invading pathogens via Toll, Immune-deficiency (Imd) and other signaling pathways [7]. While the Toll pathway is activated in response to infection with fungi or Gram-positive bacteria, the Imd pathway is mainly involved in the detection and clearing of Gram-negative bacteria [7].

Some entomopathogenic bacteria were found to interfere with the activation of these signaling pathways and thus repress the production of host AMPs. The nematode symbiont *Xenorhabdus nematophila*, for example, was reported to suppress the production of a particular AMP, cecropin, during systemic infection of the beet armyworm, *Spodoptera exigua*, via unknown mechanisms [14]. The same bacterial species was previously suggested to suppress the insect immune responses also directly by proteolytic inactivation of AMPs [15]. The insect-pathogenic *Pseudomonas entomophila* was suggested to similarly degrade AMPs in

the gut of *Drosophila* by expressing and secreting the protease AprA [16]. Because negatively charged surface phospholipids such as LPS in Gram-negative bacteria bind most AMPs, some species gain resistance by modifying the structure of the lipid A moiety in order to reduce the overall net negative charge of LPS [13,17]. This can be achieved by covalent addition of, for example, phosphoethanolamine or aminoarabinose (4-amino-4-deoxy-L-arabinose) to lipid A.

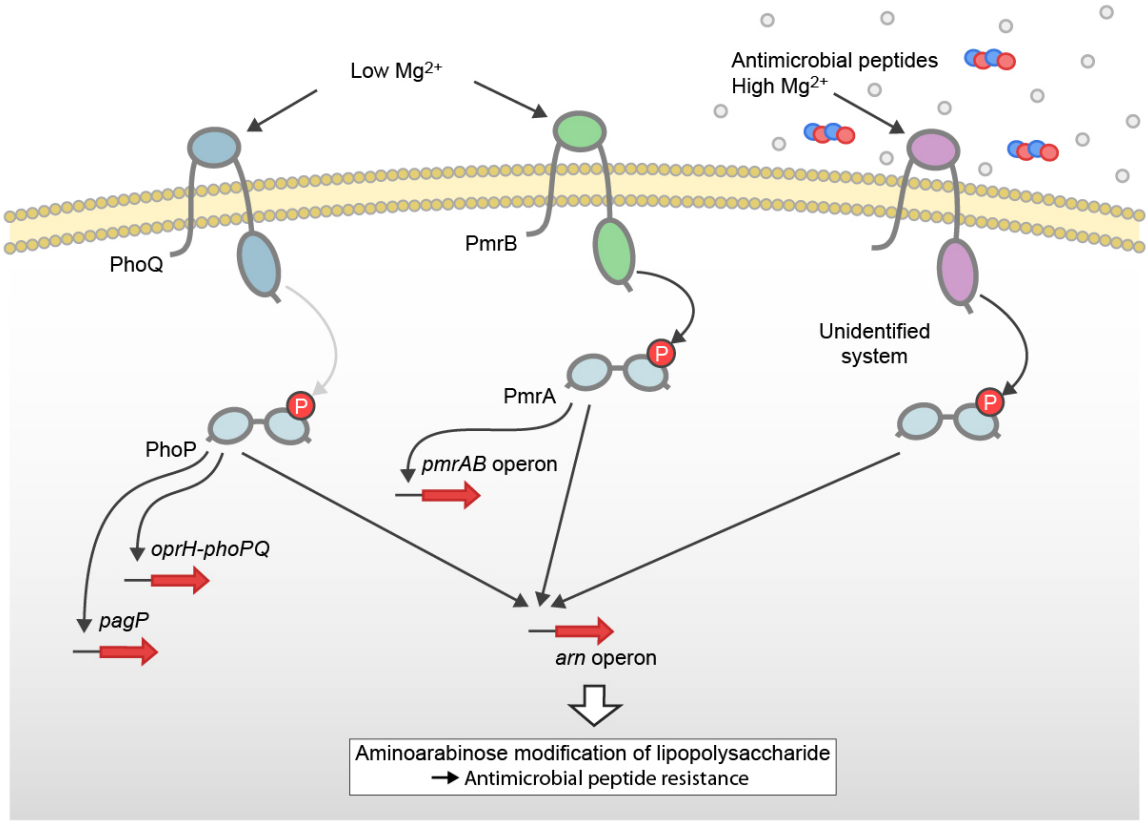

Figure 2: Regulation of antimicrobial peptide (AMP) resistance by the PhoP-PhoQ and PmrA-PmrB two-component regulatory system in *Pseudomonas aeruginosa*. In response to magnesium limitation, the PhoP-PhoQ and PmrA-PmrB systems activate the expression of the *arn* operon in *P. aeruginosa*. The Arn proteins covalently add 4-aminoarabinose to the lipid A part of lipopolysaccharide. This reduces the overall negative charge of the outer membrane and thereby reduces the attraction of cationic AMPs, thus increases the resistance of *P. aeruginosa* to AMPs. The regulatory systems further autoregulate their expression and PhoP in addition activates the expression of the palmitoyltransferase PagP which also contributes to AMP resistance in this bacterium. It has been proposed that one or several so far unidentified regulatory systems induce the expression of the *arn* gene cluster in the host when AMPs are present. This figure was adapted from W.J. Gooderham and R.E.W. Hancock (2009) [18].

The PhoP-PhoQ two-component regulatory system was identified in many proteobacteria and generally regulates properties of the outer membrane barrier that increase bacterial resistance to AMPs and other responses of the innate immunity (Figure 2) [17,19-21]. In several pathogenic bacteria, including *Salmonella enterica*, *Pseudomonas aeruginosa* and *Photorhabdus luminescens*, it was additionally shown to be a major virulence regulator [19,22-24]. It consists of the inner membrane sensor kinase PhoQ and its cognate

response regulator PhoP [25,26], and responds to low concentrations of divalent cations, such as magnesium (Mg^{2+}) (Figure 2). In the intracellular pathogen *S. enterica*, where the regulatory system was first described, deletion of either *phoP* or *phoQ* leads to reduced AMP resistance and to attenuation in virulence [19,21]. The PhoP-PhoQ system was demonstrated to be required for invasion of macrophages and intracellular survival in this bacterium.

In *P. aeruginosa*, the two-component system PhoP-PhoQ seems to work in a slightly different way than canonical two-component regulatory systems. PhoQ was suggested to predominantly act as a phosphatase of PhoP in this opportunistic pathogen and therefore to mainly inactivate the response regulator [18,20]. The resistance to AMPs in this bacterium is mostly mediated through the addition of 4-aminoarabinose to lipid A via enzymes encoded in the *arn* operon (also referred to as *pmr* operon in certain species) [17,27,28]. When extracellular divalent cations are limited, PhoP is in its phosphorylated state (due to inactivity of PhoQ) and activates the transcription of the *arn* operon (Figure 2) [18]. Under Mg^{2+}-replete conditions, PhoQ dephosphorylates its cognate response regulator and therefore represses the expression of the Arn proteins. As a consequence, deletion of *phoQ* highly increases the bacterium's resistance to AMPs even when divalent cations are not limited, but at the same time reduces its virulence [20,24]. *P. aeruginosa* isolates from patients with cystic fibrosis were often found to show dysregulation of the PhoP-PhoQ system (often due to loss-of-function mutations in *phoQ*) and thus displayed high resistance to AMPs such as polymyxin B (PMB) due to constitutive modification of lipid A with 4-aminoarabinose [28,29]. The importance of PhoQ for the virulence of *P. aeruginosa* has been explained with the observation that the sensor protein regulates the expression of many genes outside the PhoP regulon [24]. Under Mg^{2+}-replete conditions, PhoQ amongst others controls the expression of type II secretion genes, iron-scavenging-related genes, energy-metabolism-related genes, and other global regulators, such as AlgR, independent of PhoP. The effect of PhoQ on virulence thus seems to be multifactorial. Mutation of *phoQ* in *P. aeruginosa* resulted in highly attenuated virulence in a rat model of chronic lung infection, reduced cytotoxicity toward human bronchial epithelial cells, reduced biofilm formation, and impairment in twitching motility [24].

In contrast to PhoQ, PhoP regulates the expression of only a very small set of genes in *P. aeruginosa* [24]. In addition to the *arn* operon, the transcriptional regulator directly controls the expression of the *oprH-phoP-phoQ* operon and thereby autoregulates its expression (Figure 2) [30]. The outer membrane protein OprH is likely to interact directly with LPS and thereby contributes to AMP resistance in this pathogenic bacterium [31]. Additionally, PhoP also regulates the expression of PagP, which is responsible for the transfer of palmitate to lipid A and also contributes to resistance to AMPs, in a Mg^{2+}-dependent manner [30,32]. However, PhoP-dependent lipid A modification does not seem to be required for full virulence in *P. aeruginosa* because the *phoP* mutant was as virulent and competitive as the wild type [24].

In addition to the PhoP-PhoQ system, protection through 4-aminoarabinose addition to lipid A is also controlled by the PmrA-PmrB two-component system in *P. aeruginosa* (Figure 2) [27,33]. It has been

suggested that additional regulatory systems induce the expression of the *arn* operon in the host in response to attacks by AMPs under Mg^{2+}-replete conditions [18].

P. protegens and most *P. chlororaphis* strains seem to be intrinsically resistant to at least certain AMPs (Chapter 5). AMP resistance in these bacteria seems to depend on the presence of LPS O-antigen and modification of lipid A with 4-aminoarabinose, and is likely to contribute to their insect pathogenicity. The goals of this study were to identify the PhoP-PhoQ regulatory system in *P. protegens* CHA0 and *P. chlororaphis* PCL1391 and to investigate whether it regulates AMP resistance and virulence in these bacteria.

Results

Identification of the PhoP-PhoQ system and its role in antimicrobial peptide resistance

The sequences of PhoP (PA1179) and PhoQ (PA1180) of *P. aeruginosa* PAO1 were used to identify candidates for functional orthologs in *P. protegens* CHA0 via BLAST searches. The putative *phoQ* gene (PFLCHA0_c45400; 75% nucleotide sequence identity to PAO1 *phoQ*) was predicted to code for a membrane-bound histidine kinase with a periplasmic sensor domain and a HAMP linker domain (Figure 3A). The predicted *phoP* gene (PFLCHA0_c45410) most likely encodes a DNA-binding response regulator. The gene directly upstream showed sequence similarity to PAO1 *oprH*.

Deletion of PFLCHA0_c45410 in CHA0 (CHA5134) resulted in the loss of the strain's intrinsic resistance to PMB, similar to the *arnA* mutant strain CHA5129, irrespective of the Mg^{2+} availability (Figure 3B and C). Complementation of the mutation by re-introducing the wild-type *phoP* gene (CHA5153) into the chromosome restored the resistance to this AMP (Figure 3B), indicating that no polar effects were caused by the deletion of the gene. Under Mg^{2+}-limited conditions, in-frame deletion of PFLCHA0_c45400 (CHA5133) led to a slight decrease in resistance to this AMP when at a low concentration (Figure 3B). In contrast, the mutation in this gene increased the strain's resistance under high Mg^{2+} concentrations (Figure 3C). The sequence similarities to PAO1 PhoP and PhoQ and their role in PMB resistance strongly indicate that PFLCHA0_c45400 and PFLCHA0_c45410 code for PhoQ and PhoP in *P. protegens* CHA0, respectively. Since these genes are organized similarly to their orthologs in *P. aeruginosa* PAO1, it is likely that *phoP* and *phoQ* are co-transcribed in CHA0 with *oprH* and form a three-gene operon, too (Figure 3A).

The deletion of *phoP* and *phoQ* did not seem to affect the growth rate of CHA0 in the absence of AMPs (Figure 3B and C). However, the induced expression of these genes at the Tn*7* attachment locus, which might have resulted in an overexpression of *phoP* and *phoQ*, influenced the growth of this bacterium, indicating that high expression of genes under the control of this regulatory system might be costly to the microbe.

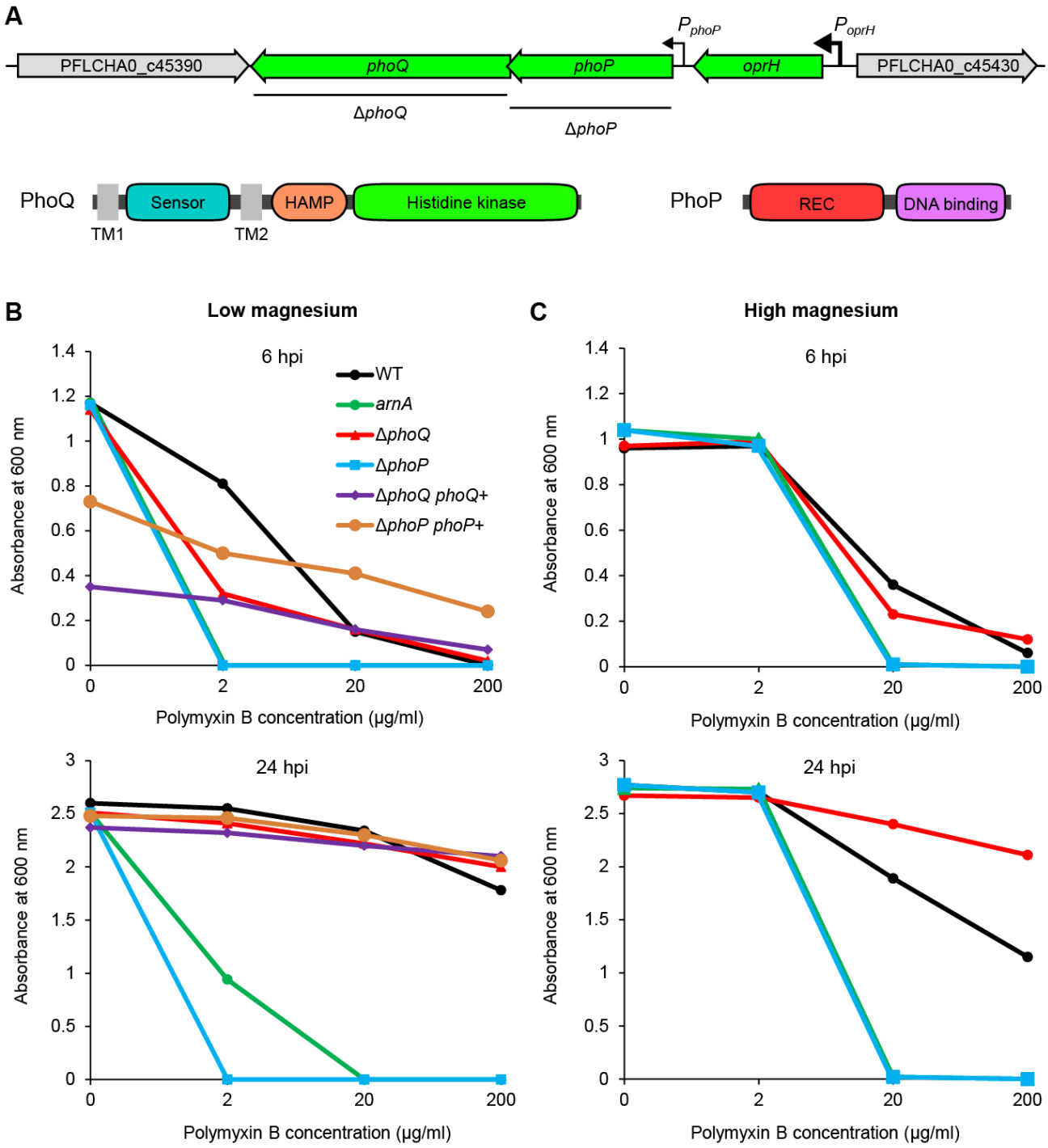

Figure 3: Identification of the PhoP-PhoQ regulatory system in *Pseudomonas protegens* CHA0. (A) Organization of the locus predicted to code for the PhoP-PhoQ two-component system in *P. protegens* CHA0. Based on findings from *P. aeruginosa*, the *phoP-phoQ* genes are probably co-transcribed with *oprH*, which encodes an outer membrane protein, under the control of the P_{oprH} promoter. An additional weak promoter (P_{phoP}) directly upstream of *phoP* might ensure a basal expression level of the PhoP-PhoQ system. The predicted functional domains of the *phoPQ*-encoded proteins are depicted below. HAMP, alpha-helical linker domain; REC, receiver domain; TM, transmembrane domain. **(B)** CHA0 and its isogenic mutant strains CHA5129 (*arnA*), CHA5133 (Δ*phoQ*), CHA5134 (Δ*phoP*), CHA5138 (Δ*phoQ phoQ+*), and CHA5153 (Δ*phoP phoP+*) were grown at 25°C in Lysogeny Broth (LB) at different concentrations of polymyxin B. The optical density of the cultures at 600 nm was determined in the exponential (6 hours post inoculation (hpi)) and stationary growth phase (24 hpi). The curves at 6 hpi of CHA5129 (*arnA*) and CHA5134 (Δ*phoP*) are overlapping. **(C)** The same assay as described in (B), but without the complementation strains CHA5138 and CHA5153, was

performed with LB supplemented with 20 mM $MgCl_2$. The curves of CHA5129 (*arnA*) and CHA5134 (Δ*phoP*) are overlapping. Both experiments were repeated once with similar results.

A locus orthologous to CHA0 *oprH-phoPQ* was also found in the genome of *P. chlororaphis* PCL1391 (Figure 4A). As already previously observed (Chapter 5), PCL1391 was less resistant to PMB than *P. protegens* CHA0 (Figure 4B and C). Increasing the concentration of Mg^{2+} in the growth medium resulted in a higher resistance of strain PCL1391 to low levels of PMB. However, at higher doses of PMB, PCL1391 became more sensitive to this antimicrobial compound when the Mg^{2+} concentration was high. Deletion of PCL1391_4349, the *phoQ* ortholog in *P. chlororaphis* PCL1391, increased the strain's resistance to PMB at high Mg^{2+} concentrations but decreased it when Mg^{2+} was limited. This indicates that PCL1391_4349 is coding for PhoQ in PCL1391 and that the sensor kinase is able to increase and decrease the bacterium's resistance to AMPs, depending on the availability of divalent cations in its surrounding.

Taken together, these data suggest that both *P. protegens* CHA0 and *P. chlororaphis* PCL1391 possess the two-component regulatory system PhoP-PhoQ and that it is involved in the regulation of AMP resistance in these microbes.

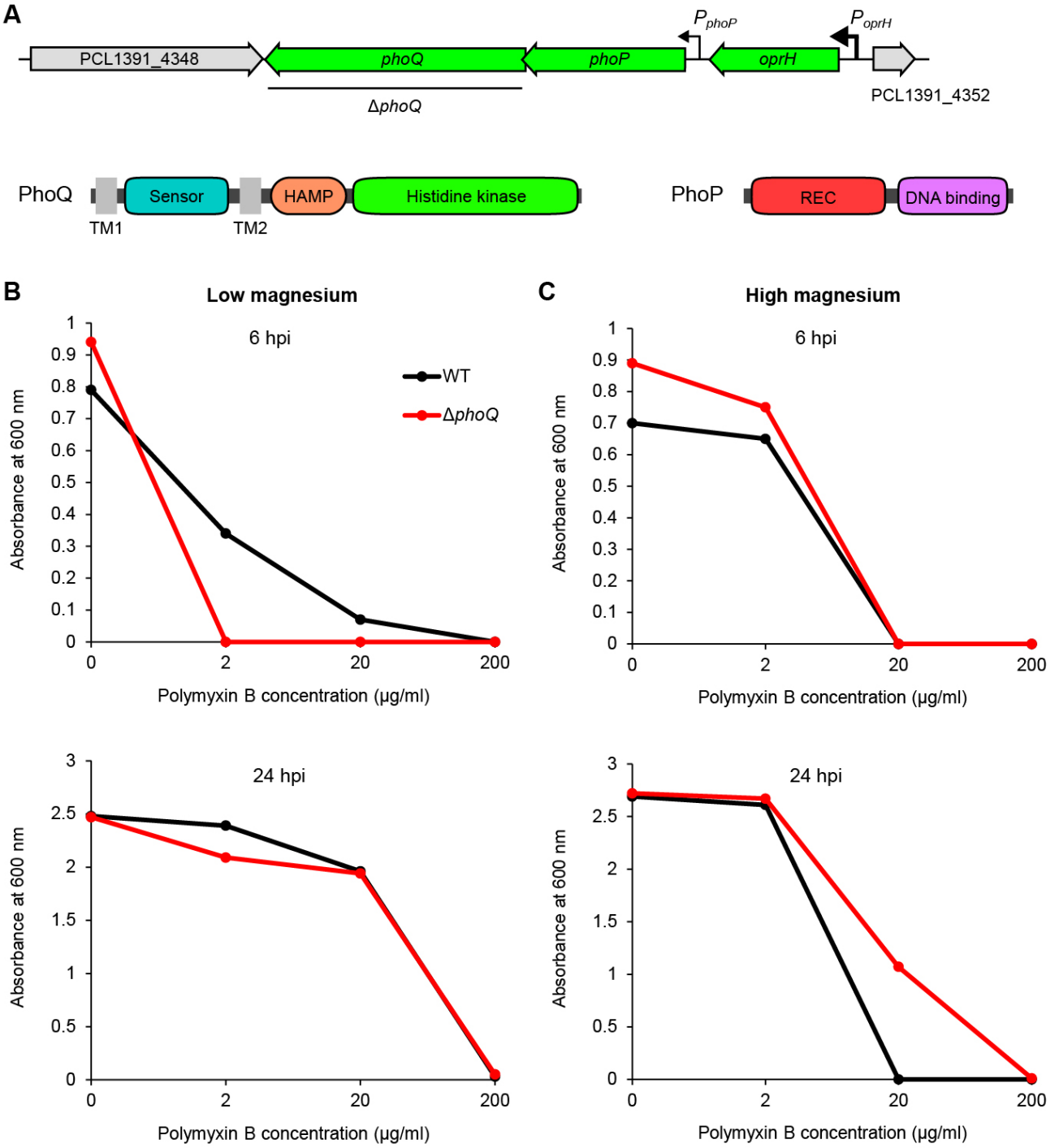

Figure 4: Identification of the PhoP-PhoQ regulatory system in *Pseudomonas chlororaphis* PCL1391. (A) Organization of the locus predicted to code for the PhoP-PhoQ two-component system in *P. chlororaphis* PCL1391. Based on findings from *P. aeruginosa*, the *phoP-phoQ* genes are probably co-transcribed with *oprH*, which encodes an outer membrane protein, under the control of the P_{oprH} promoter. An additional weak promoter (P_{phoP}) directly upstream of *phoP* might ensure a basal expression level of the PhoP-PhoQ system. The predicted functional domains of the *phoPQ*-encoded proteins are depicted below. HAMP, alpha-helical linker domain; REC, receiver domain; TM, transmembrane domain. **(B and C)** *P. chlororaphis* PCL1391 and its isogenic *phoQ* deletion mutant (PCL-10) were grown at 25°C in Lysogeny Broth (LB), with (B) or without 20 mM $MgCl_2$ (C), at different concentrations of polymyxin B. The optical density of the cultures at 600 nm was determined in the exponential (6 hours post inoculation (hpi)) and stationary growth phase (24 hpi). The experiment was repeated once with similar results.

Aminoarabinose modification of lipid A is dependent on magnesium availability and PhoP-PhoQ

In Gram-negative bacteria, resistance to AMPs is amongst others mediated through covalent addition of 4-aminoarabinose to lipid A via enzymes encoded in the *arn* gene cluster [17]. This modification neutralizes the negative charge of the lipid A 4'-phosphate group and thereby reduces the binding of AMPs to the bacterial outer membrane. In this study, a gene cluster orthologous to the *arn* gene cluster of *P. aeruginosa* PAO1 was identified in *P. protegens* CHA0 (with an overall nucleotide sequence identity of 78.5%) and *P. chlororaphis* PCL1391 (78.5% overall nucleotide sequence identity) (Figure 5). The *arn* genes in PCL1391 and CHA0 seem to be organized as an operon and are thus most probably co-transcribed. In order to study the role of PhoP-PhoQ in the regulation of the expression of the *arn* gene cluster in *P. protegens* CHA0, a plasmid-based transcriptional *arnB-gfp* reporter fusion (pME8348) was constructed. The *arnB* gene was highly expressed in wild-type CHA0 when the bacteria were grown under low Mg^{2+} conditions in Lysogeny Broth (LB) (Figure 6). In contrast, the expression of *arnB* in the wild type was comparatively low in LB when supplemented with 22 mM $MgCl_2$ and in Grace's Insect Medium (GIM), which has an equal concentration of Mg^{2+} and mimics the insect hemolymph. This indicates that Mg^{2+} availability plays a role in the expression of the *arn* gene cluster.

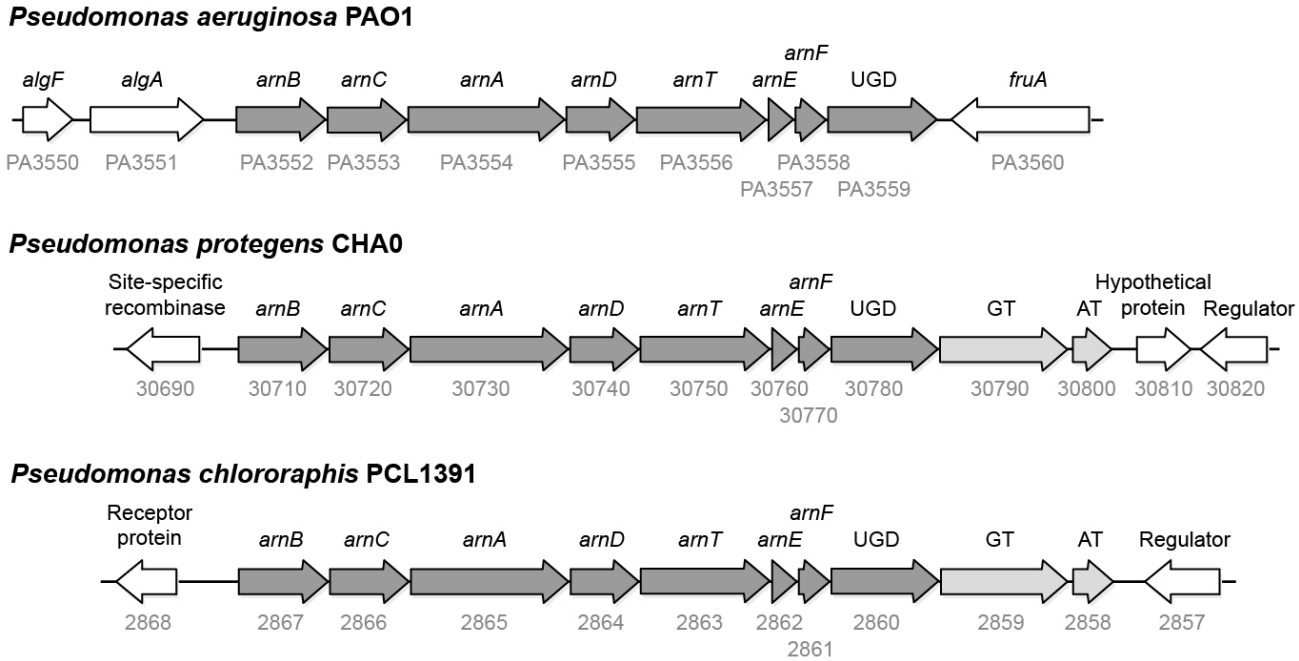

Figure 5: The *arn* gene cluster in *Pseudomonas aeruginosa* PAO1, *Pseudomonas protegens* CHA0 and *Pseudomonas chlororaphis* PCL1391. Genes displayed in dark gray are encoding enzymes necessary for the modification of lipid A with 4-aminoarabinose in *P. aeruginosa* PA01 or are orthologs identified in *P. protegens* CHA0 and *P. chlororaphis* PCL1391. Gene names or putative functions of encoded proteins are indicated above the corresponding genes, information about the locus tags is given below (locus tags for PCL1391 are based on the draft genome and temporal). Genes in gray are most probably co-transcribed. Flanking genes are displayed in white. Genes are drawn to scale. AT, acetyltransferase; GT, glycosyltransferase; UGD, putative UDP-glucose 6-dehydrogenase.

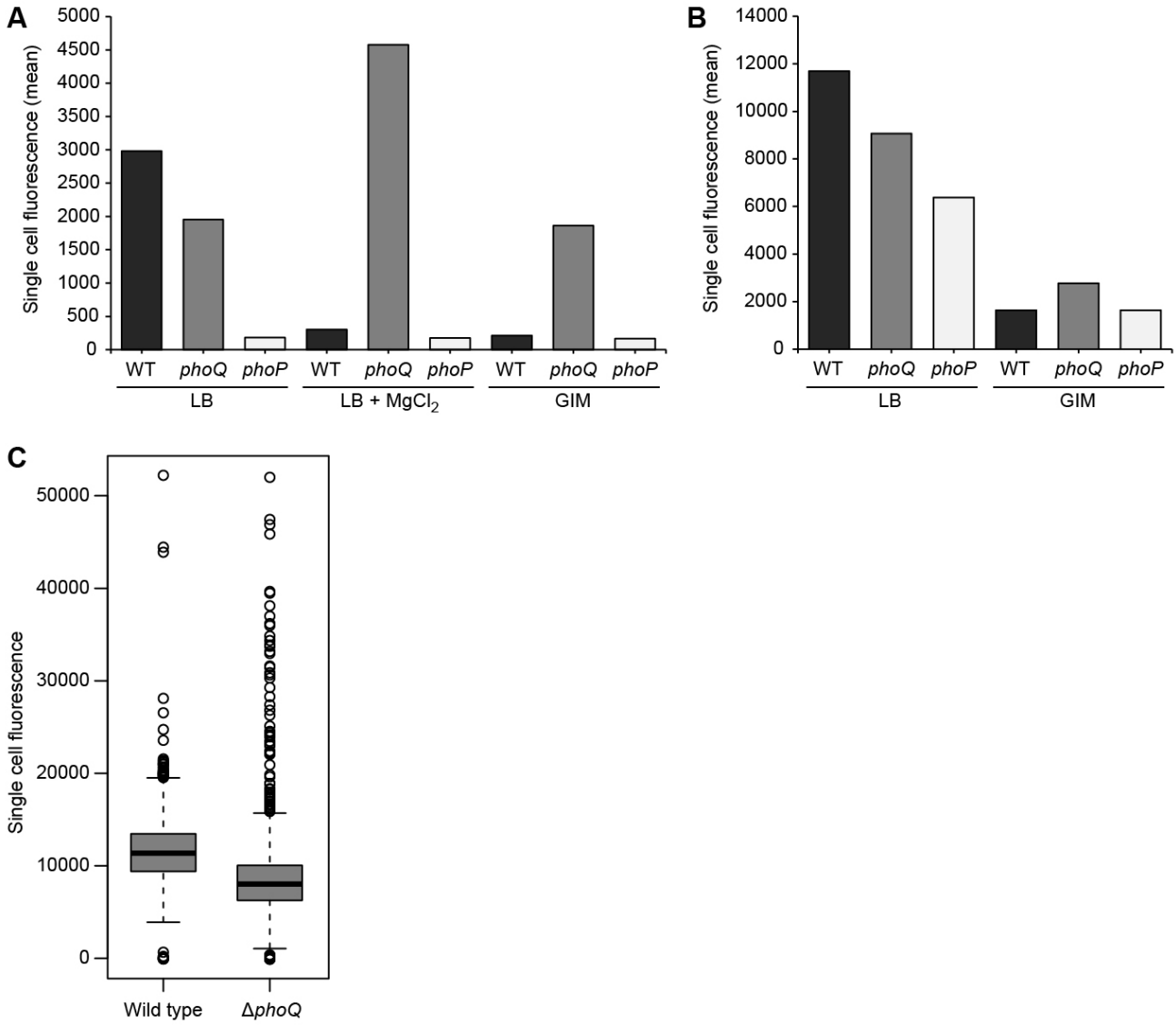

Figure 6: The expression of the *arn* gene cluster is induced under magnesium-limited conditions and controlled by the PhoP-PhoQ regulatory system in *Pseudomonas protegens*. CHA0, CHA5133 (Δ*phoQ*) and CHA5134 (Δ*phoP*) harboring the reporter plasmid pME8348 (P_{arnB}-*gfp*) were grown at 25°C in Lysogeny Broth (LB), LB supplemented with 22 mM $MgCl_2$ or Grace's Insect Medium (GIM). Green fluorescence intensities of single cells were quantified **(A)** in the late exponential growth phase (6 hours post inoculation) and **(B)** in early stationary phase (24 hours post inoculation) by fluorescence microscopy (n=300-1300 cells analyzed per strain and condition). Shown are averages single cell fluorescence intensities per culture, corrected for background and autofluorescence using strain CHA0 carrying the empty pPROBE-TT vector as the control. **(C)** Single cell intensities of CHA0 and CHA5133 in LB shown in (A) are displayed as boxplots. The number of cells analyzed per strains was similar (n=1244 for CHA0, n=1175 for CHA5133). The experiment was repeated once with similar results.

P. protegens CHA0 missing a functional *phoP* gene (CHA5134) displayed a strongly decreased expression of *arnB* (Figure 6A and B). During the exponential growth phase, the expression level in this mutant was similar to the expression level of the wild-type strain under Mg^{2+}-replete conditions (Figure 6A), which suggests that PhoP is required for the activation of *arn* gene expression when Mg^{2+} is limited. In-frame deletion of *phoQ* (CHA5133) resulted in an increased activity of the P_{arnB} promoter at high Mg^{2+} concentrations. In contrast,

the expression of *arnB* was lower in the Δ*phoQ* mutant than in the wild type when Mg^{2+} was limited in the medium. These observations suggest that PhoQ is repressing the expression of the *arn* gene cluster when Mg^{2+} concentrations are high but contributes to induction of their expression under Mg^{2+}-limited conditions. The mutation of *phoQ*, however, caused that in LB a higher proportion of the bacterial population expressed *arnB* at very high levels in comparison to wild type CHA0 (Figure 6C). In summary, these findings indicate that modification of lipid A via Arn proteins takes place mainly at low Mg^{2+} concentrations and is controlled by the PhoP-PhoQ regulatory system in *P. protegens* CHA0, which seems to respond to divalent cations in this bacterium as in *P. aeruginosa* [18,20,34].

Since the expression of AMP resistance genes was reported to be induced in some bacteria in the presence of AMPs, the effect of PMB on the expression of *arnB* was investigated. When this AMP was present at sublethal concentrations, the expression of the *arn* gene cluster seemed to be induced in a small subpopulation of *P. protegens* CHA0 under Mg^{2+}-replete conditions (Figure 7). This indicates that in *P. protegens* the presence of AMPs induces the expression of at least this resistance mechanism.

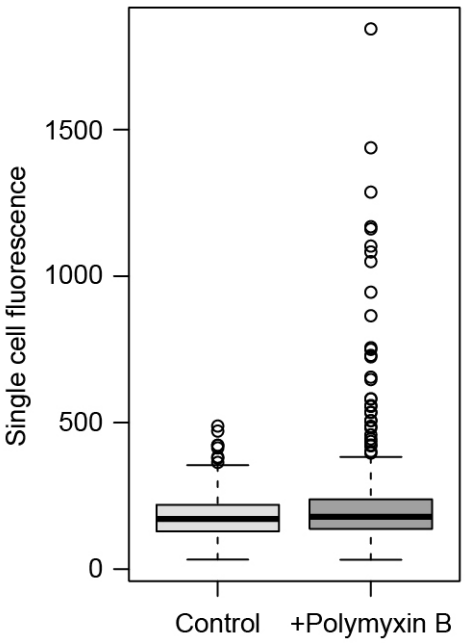

Figure 7: Polymyxin B induces the expression of the *arn* gene cluster in a subpopulation of *Pseudomonas protegens* CHA0 at sublethal concentrations. The *P. protegens* strain CHA0 equipped with the GFP-based reporter plasmid pME8348 was grown at 25°C in Lysogeny Broth supplemented with 20 mM $MgCl_2$ in the presence or absence of polymyxin B at a sublethal concentration (2 µg/ml). The green fluorescence intensities of single cells were quantified by epifluorescence microscopy in the exponential phase (6 hours post inoculation) and the values are displayed as boxplots. Equal numbers of cells were analyzed for each treatment (n= approx. 400 cells per treatment).

The PhoP-PhoQ regulatory system controls the expression of *oprH*

To investigate whether PhoP-PhoQ also regulates the expression of *oprH*, a P_{oprH}-*gfp* reporter plasmid (pME11004) was constructed. As for the *arn* cluster, the expression of *oprH* was high in LB and repressed under high Mg^{2+} conditions and under the control of the PhoP-PhoQ two-component regulatory system (Figure 8). In contrast to the *arn* gene cluster, the differences in the expression level of *oprH* between the wild type and the isogenic PhoP and PhoQ defective mutants were larger in the stationary phase than in the exponential growth phase. If *phoP* and *phoQ* are co-transcribed with *oprH* in CHA0 as reported for *P. aeruginosa* PAO1 [20], the two-component regulatory system most likely regulates its own expression via PhoP. High expression of the *arn* operon in the Δ*phoQ* mutant under high Mg^{2+} conditions might thus be a consequence of the overexpression of *phoP* in this mutant strain.

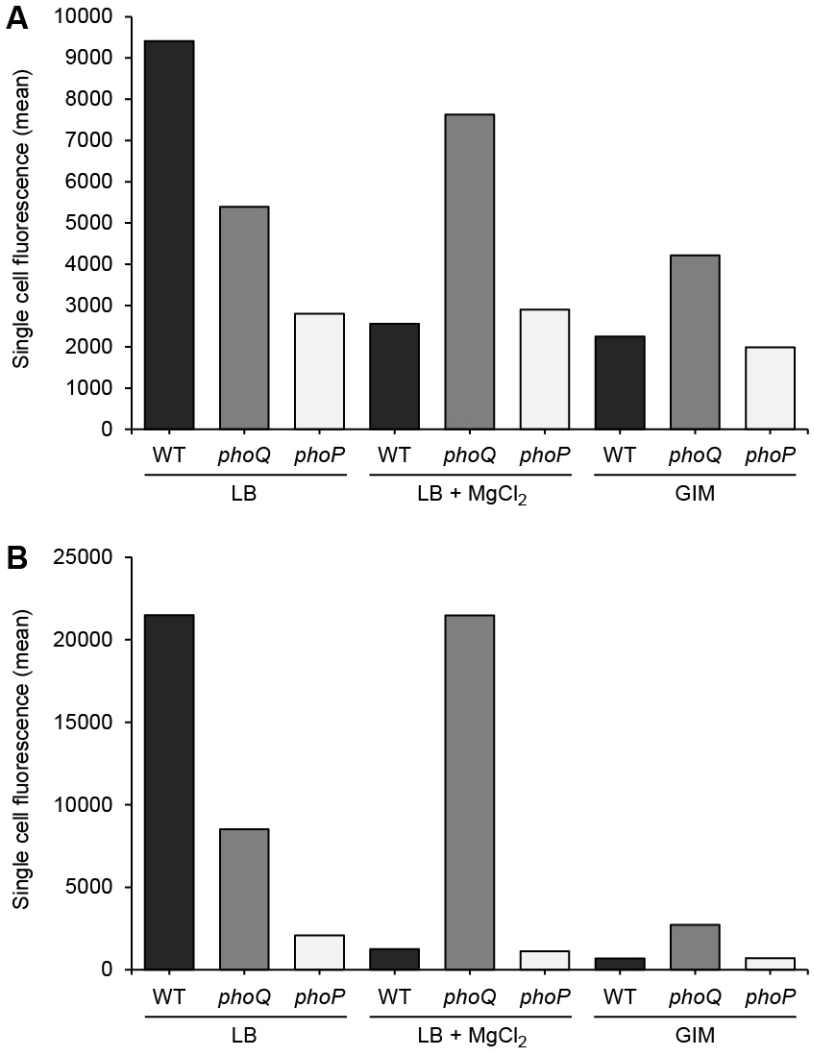

Figure 8: The expression of *oprH* is induced under magnesium-limited conditions and controlled by the PhoP-PhoQ regulatory system in *Pseudomonas protegens*. CHA0, CHA5133 (Δ*phoQ*) and CHA5134 (Δ*phoP*) harboring the reporter plasmid pME11004 (P_{oprH}-*gfp*) were grown at 25°C in Lysogeny Broth (LB), LB supplemented with 22 mM $MgCl_2$ or Grace's Insect Medium (GIM). Green fluorescence intensities of single cells were quantified **(A)** in the exponential growth phase (6 hours post inoculation) and **(B)** in early

stationary phase (24 hours post inoculation) by fluorescence microscopy (n=250-1300 cells analyzed per strain and condition). Shown are average single cell fluorescence intensities per culture, corrected for background and autofluorescence using strain CHA0 carrying the empty pPROBE-TT vector as control.

PhoQ is important for virulence of *Pseudomonas protegens* in *Galleria mellonella*

Virulence and competition assays with larvae of *G. mellonella* were carried out to determine whether PhoP-PhoQ is important for insect pathogenicity in *P. protegens* CHA0. The Δ*phoQ* mutant of CHA0 was significantly reduced in virulence in *Galleria* larvae upon injection (Figure 9A). In contrast, the Δ*phoP* mutant was as virulent as the wild type in this systemic infection model. A Δ*phoPQ* double mutant displayed a reduced virulence as well, however, not to the extent of the Δ*phoQ* mutant. Whereas deletion of *phoQ* resulted in significantly reduced competitiveness during infection of *G. mellonella*, mutation of *phoP* rendered CHA0 slightly more competitive compared to wild-type bacteria (Figure 9B).

These observations indicate that, in contrast to PhoP, the sensor kinase PhoQ is essential for full virulence of *P. protegens* CHA0 upon systemic infection. Factors controlled by PhoQ outside of the PhoP regulon thus might contribute to insect pathogenicity of CHA0. However, it cannot be excluded that both components of this regulatory system modulate the virulence of CHA0 since deletion of either gene led to an altered competitiveness in *Galleria* larvae.

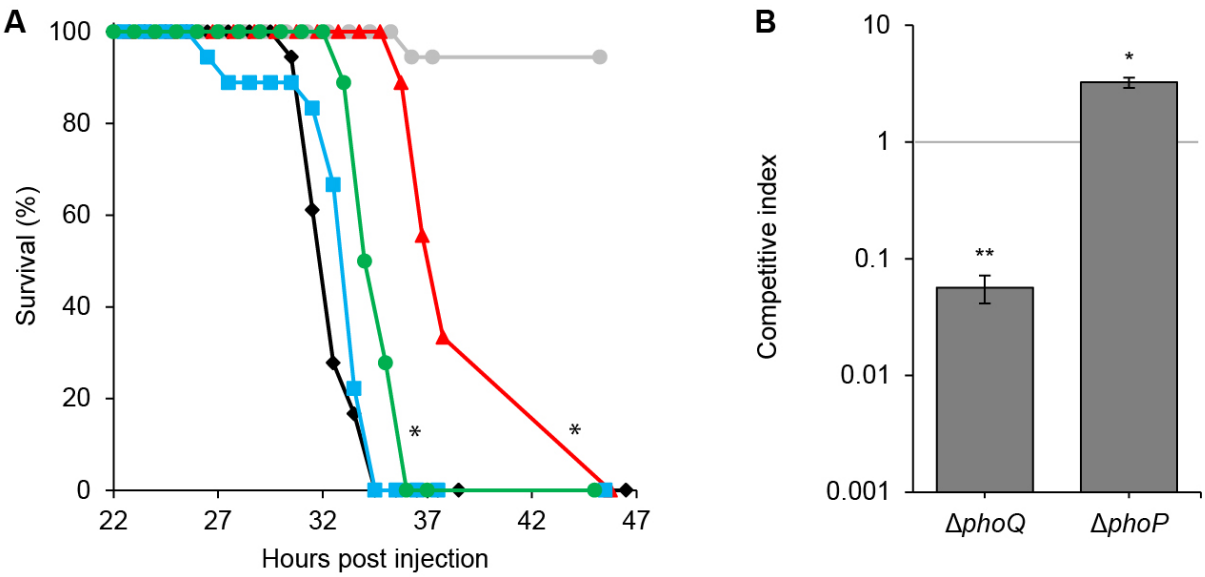

Figure 9: PhoQ contributes to insect pathogenicity in *Pseudomonas protegens* CHA0. (A) Virulence of the PhoP and PhoQ defective mutant strains of CHA0 was determined by injection of low cell numbers into larvae of *Galleria mellonella* and monitoring the survival of the insects over time. Deletion of *phoQ* (in red, CHA5133) and *phoPQ* (in green, CHA5204) significantly reduced the virulence of the bacterium (p-value < 0.0001; Log-Rank test with Kaplan Meier estimations), while the Δ*phoP* mutant (in blue, CHA5134) was as virulent as the wild type (in black, CHA0). Sterile saline solution served as a negative control (in gray). The experiment was repeated once with similar results. **(B)** Competitiveness of the Δ*phoQ* and Δ*phoP* mutant strains during systemic insect infection was investigated by injecting equal numbers of fluorescently tagged mutant (CHA5133-*mChe* and CHA5134-*mChe*,

respectively) and corresponding wild-type cells (CHA0-*gfp2*) into larvae of *G. mellonella* and counting bacterial cells in hemolymph samples 20 to 24 hours after the injection by epifluorescence microscopy. The competitive index indicates the ratio between the cell numbers of the mutant and the wild type, normalized by the ratio in the inoculum. Asterisks indicate significant differences between the mutants and the wild type (*, p-value < 0.05; **, p-value < 0.001; one-sample *t* test).

PhoQ represses Fit toxin production

The *phoQ* and *phoP* genes were deleted individually in the FitD-mCherry reporter strain CHA1163 [3] to investigate whether the two-component regulatory system has any influence on the production of the Fit toxin in *P. protegens* CHA0. Deletion of *phoQ*, but not *phoP*, increased the expression of FitD-mCherry when the reporter bacteria were grown in GIM (Figure 10A). This suggests that PhoQ represses the production of Fit toxin in CHA0 in insect hemolymph, probably through a PhoP-independent pathway. Because the two-component system was activated by Mg^{2+} limitation in CHA0 (Figure 8), the expression of FitD was further studied in minimal medium with low or high concentration of Mg^{2+}. Minimal medium was chosen because, in contrast to LB, Fit toxin expression is induced in *P. protegens* CHA0 when grown in this medium (Chapter 3) and, in contrast to GIM, it is possible to change the concentration of Mg^{2+}. Expression of the insecticidal toxin increased approximately 3-fold in the Δ*phoQ* mutant compared to the wild type regardless of the amount of Mg^{2+} in the growth medium, indicating that repression of Fit toxin production by PhoQ is independent of the Mg^{2+} availability (Figure 10).

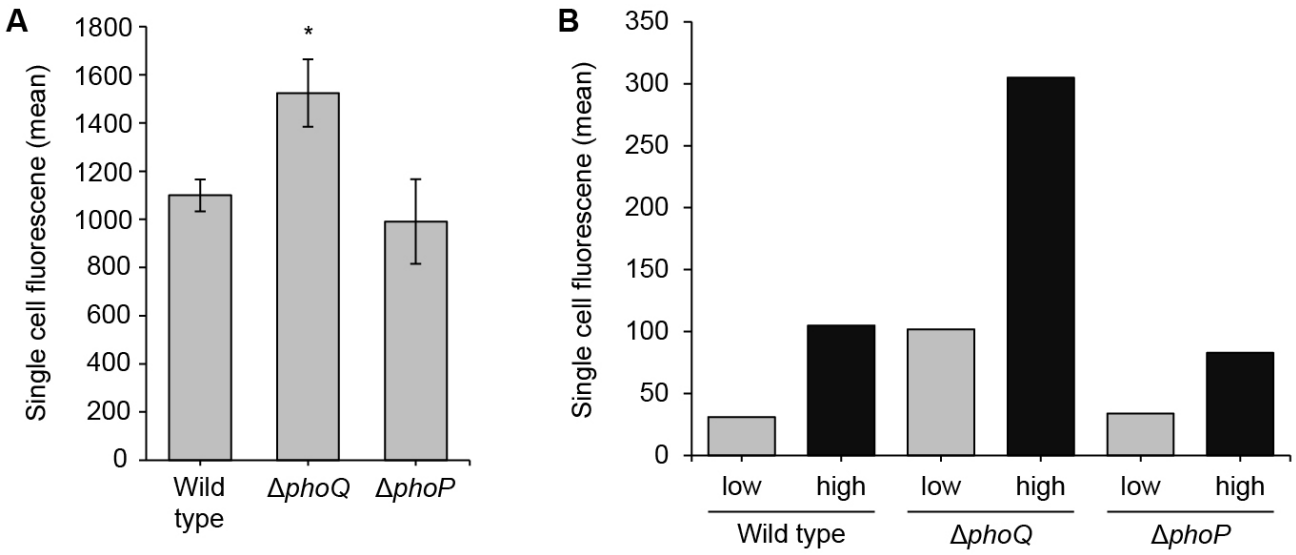

Figure 10: PhoQ represses Fit toxin expression in *Pseudomonas protegens* independent of the magnesium availability. (A) The *fitD-mCherry* fusion reporter strain of CHA0 (CHA1163) and isogenic mutant strains with a deletion in *phoQ* (CHA5135) or *phoP* (CHA5136) were grown for 24 hours in Grace's Insect Medium as three independent cultures. Red fluorescence intensity of single cells was quantified by epifluorescence microscopy. Shown are the means and standard deviations per treatment. Asterisks indicate strains which were significantly different from the wild type in (A) (p-value < 0.02; one-way ANOVA with Tukey HSD post hoc test). **(B)** The same assay was also performed in low phosphate minimal medium with 2 mM (low) or 40 mM (high) Mg^{2+}, in order to study

the effect of magnesium on the expression of the Fit toxin, and with only one culture per strain and condition. The experiment was repeated once with similar results.

The aminoarabinose modification genes are conserved in insect-pathogenic pseudomonads

Genome comparisons revealed that not all *P. fluorescens* group strains possess the *arn* gene cluster (Figure 11). A more detailed study of the different genomes showed that all *P. protegens* and *P. chlororaphis* strains have a fully conserved gene cluster, while most strains without insecticidal activities seemed to have reduced or completely lost it over time (Figures 5 and 11). The lineages with a reduced *arn* operon lack the *arnB* gene which is generally highly conserved across species and essential for 4-aminoarabinose modification of lipid A [35,36]. Interestingly, whereas the gene sequences are highly conserved, the promoter sequence of the *arn* gene cluster differs significantly between *P. protegens* and *P. chlororaphis* (data not shown), which might lead to a higher (or constitutive) expression of these genes in the former bacterial species. Certain strains seem to possess an ortholog of *P. aeruginosa* PAO1 *pagP* (Figure 11) which is a lipid A acylation protein and might contribute to PMB resistance in addition to lipid A modification with 4-aminoarabinose [30,32].

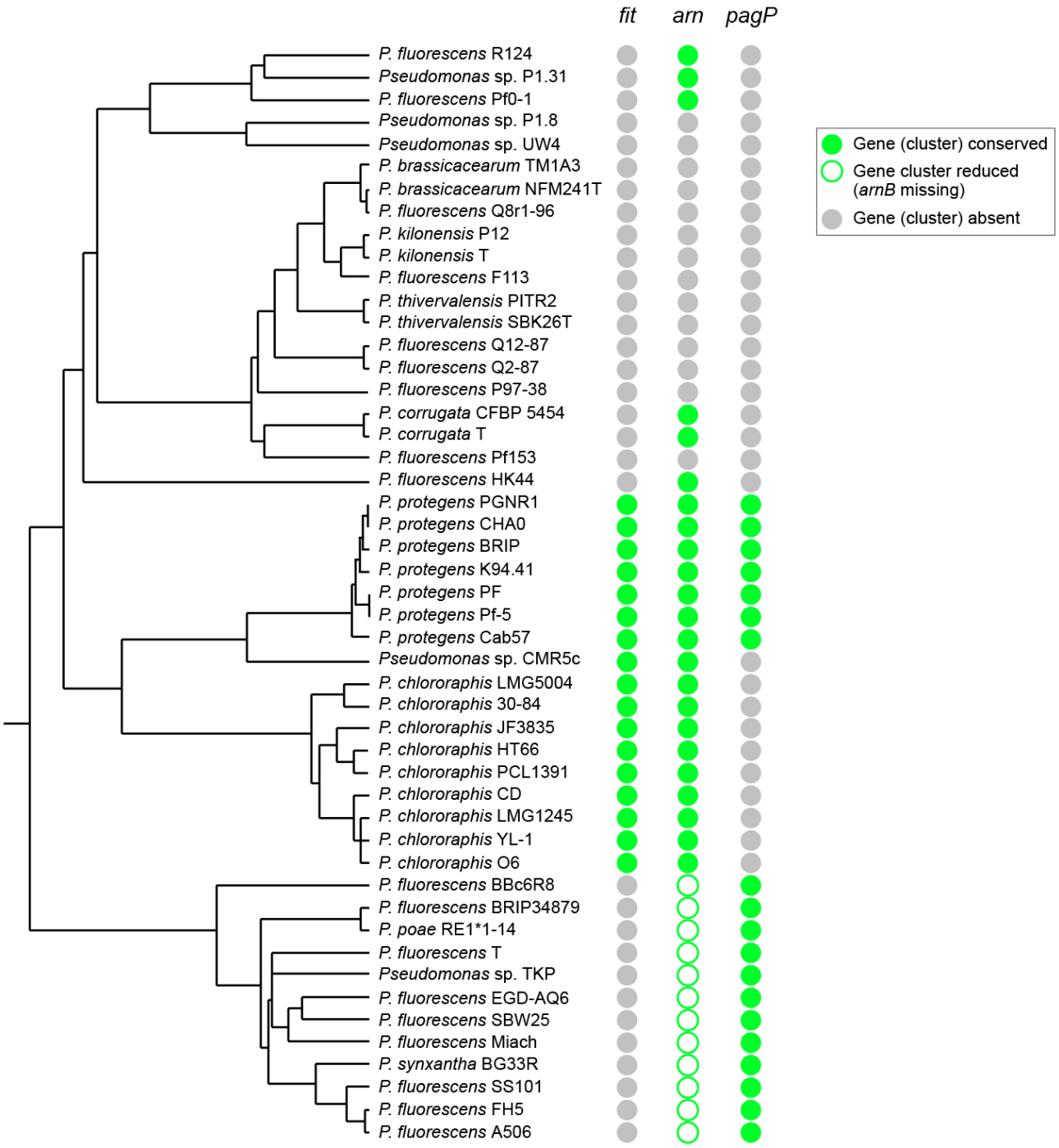

Figure 11: Conservation of the *arn* gene cluster in insecticidal *Pseudomonas protegens* and *Pseudomonas chlororaphis*. The presence or absence of the *fit* gene cluster (encoding the Fit toxin as well as transport and regulatory proteins), the *arn* gene cluster (coding for enzymes for the covalent modification of lipid A with 4-aminoarabinose and for resistance to cationic antimicrobial peptides (AMPs)) and the putative *pagP* gene (encoding a lipid A acylation protein putatively contributing to AMP resistance) is indicated for selected strains of the *P. fluorescens* group. Strains that possess the *fit* gene cluster are efficiently killing lepidopteran insect larvae. The phylogenic tree is based on sequences of the core genome (Flury *et al.*, manuscript in preparation). Incomplete *arn* clusters (i.e. the absence of the *arnB* gene) are indicated as open circles in the figure.

Discussion

PhoP-PhoQ regulates AMP resistance in entomopathogenic pseudomonads

This study identified the PhoP-PhoQ regulatory system in two insecticidal pseudomonads with biocontrol properties and provides evidence that it plays an important role in AMP resistance and virulence in these bacteria. Although some of the results presented here are preliminary and several experiments need to be repeated in triplicates, the present study gives a first insight into the possible roles of this complex regulatory system in two related bacterial species.

The response regulator PhoP activates the expression of *oprH* and *arn* genes which likely both contribute to PMB resistance in pseudomonads (Figure 12). Unlike in *P. aeruginosa*, where AMP resistance is only increased in *phoQ* mutants [18,34], these genes seem to be highly transcribed under Mg^{2+}-limited conditions in *P. protegens* wild type CHA0. In contrast, the expression of these PhoP-regulated genes is repressed in this bacterium when the availability of the divalent cation is high. The sensor kinase PhoQ seems to be the regulator that controls the Mg^{2+}-dependent switch in *oprH* and *arn* expression. Results in this study suggest that, in *P. protegens* CHA0 and *P. chlororaphis* PCL1391, PhoQ contributes to the activation of the expression of these genes under low Mg^{2+} concentrations, but represses their transcription under Mg^{2+}-replete conditions. The sensor kinase has been suggested to act predominantly as a phosphatase in *P. aeruginosa* [18,20] whereas in *Salmonella* it activates PhoP by phosphorylation [21,25]. The PhoP-PhoQ system in the pseudomonads tested in the present study is thus a good example for the general assumption that PhoQ can be both a phosphatase and a phosphorylase of its cognate response regulator in the same bacterium.

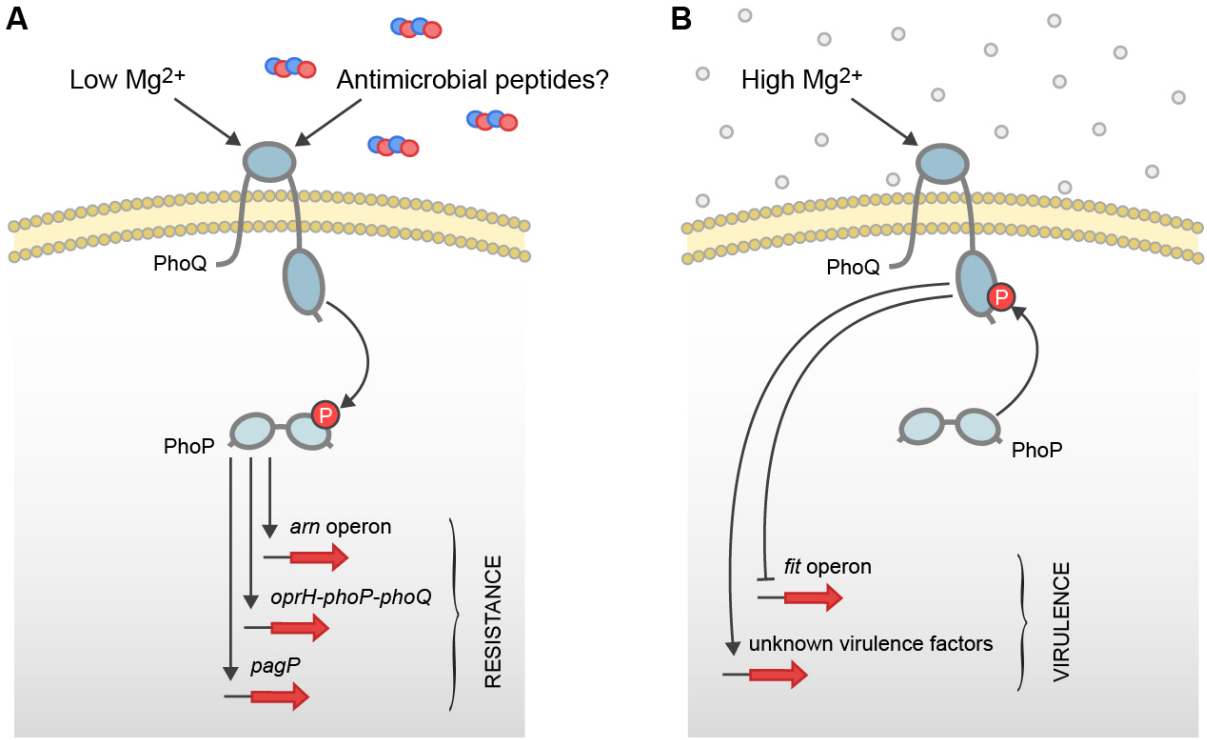

Figure 12: Model for the PhoP-PhoQ two-component regulatory system in *Pseudomonas protegens* CHA0. (A) Under magnesium-limited conditions or when antimicrobial peptides (AMPs) are present in the environment, the sensor kinase PhoQ activates its cognate response regulator PhoP by phosphorylation in *P. protegens* CHA0. The transcriptional regulator subsequently activates the expression of proteins that contribute to AMP resistance. These include the Arn proteins which are responsible for the addition of 4-aminoarabinose to lipid A, the outer membrane protein OprH, and the palmitoyltransferase PagP. It is also possible that the expression of these proteins is induced by AMPs indirectly via or independent of PhoP-PhoQ. **(B)** Under magnesium-replete conditions, PhoQ dephosphorylates PhoP and thereby inactivates the response regulator and the expression of AMP resistance genes. In addition, the sensor kinase regulates the expression of genes that are contributing to virulence of *P. protegens* CHA0, such as the Fit insect toxin, independent of PhoP.

The role of the PhoP-PhoQ system in regulating the expression of the Arn proteins directly translated into the bacterial resistance to PMB. Both PhoP and the Arn proteins were essential for high resistance to this particular AMP under all conditions tested. Based on the observed lower *arn* expression at high Mg^{2+} concentrations in strain CHA0 and the literature about *P. aeruginosa* PhoP-PhoQ, one would expect that 4-aminoarabinose modification of lipid A does not play a significant role in AMP resistance under high Mg^{2+} conditions as occurring for instance in insect hemolymph [37-39]. Our results, however, strongly indicate that PhoP-dependent 4-aminoarabinose modification of lipid A is also taking place in presence of AMPs, at least in a subpopulation, and contributing to AMP resistance when Mg^{2+} is not limited. It is possible that the presence of AMPs (for example in the insect gut and hemolymph) induces the expression of the *arn* operon and other AMP resistance genes (at least in a subpopulation), which has been suggested to happen in many other bacteria (Figure 2) [40-42]. Moreover, in *P. aeruginosa* it was previously found that binding to epithelial cells highly induces the PhoP-PhoQ regulatory system under Mg^{2+}-replete conditions [43]. It is

further important to note that the expression of genes necessary for AMP resistance via lipid A modification are generally not solely controlled by the PhoP-PhoQ system. In *Salmonella* and *P. aeruginosa*, the PmrA-PmrB two-component system also contributes, amongst other regulators, to *arn* gene regulation (Figure 2) [27,33]. The induction of the *arn* genes could thus theoretically also take place in a Δ*phoP* mutant. However, since the PmrA-PmrB regulatory is not conserved in *P. protegens* and *P. chlororaphis* (data not shown), this may involve further, as yet to be identified regulatory systems.

Resistance to AMPs most probably is mediated by several factors. We have previously shown that resistance to PMB is also dependent on O-antigen (Chapter 5) whose synthesis might not be controlled by PhoP-PhoQ. We noticed that *P. protegens* strains further possess a gene coding for the putative palmitoyltransferase PagP whose expression might also contribute to AMP resistance [30,32]. Under high Mg^{2+} conditions, *P. protegens* CHA0 was resistant to low doses of polymyxin independent of PhoP and Arn proteins. This could be attributed to the presence of such other resistance factors. Another explanation could be that the negatively charged lipid A moieties were masked by Mg^{2+} under these conditions, which would have protected these pseudomonads against attacks by PMB.

PhoQ is important for insect pathogenicity

By using *G. mellonella* as a systemic infection model we found evidence that a functional PhoQ is essential for full virulence and competition of *P. protegens* CHA0 in insects. The studies on the regulation of *oprH* and the *arn* operon by PhoP-PhoQ indicate that deletion of *phoQ* caused constitutive expression of the regulatory system and PhoP-regulated genes. As it has been suggested by others before [18,20,34], a Δ*phoQ* mutant could thus display the phenotypes of a *phoP* overexpressing mutant, which could lead to false conclusions on the role of the sensor kinase. We therefore created a *phoP-phoQ* double mutant, which was sensitive to polymyxin in the same way as the Δ*phoP* mutant strain (data not shown), and assessed the resulting virulence in the insect model. Because the deletion of both *phoP* and *phoQ* significantly reduced the virulence of the bacterium as well, we conclude that PhoQ is important for the insect pathogenicity of CHA0.

In contrast, deletion of *phoP* did not affect the virulence of CHA0 upon injection into the hemocoel of *G. mellonella*. Mutation of *phoP* even seemed to increase competitiveness of CHA0 in this insect model. It is therefore possible that PhoP-controlled modifications of lipid A are not important for *P. protegens* to infect and kill the insect host upon injection or that regulators other than PhoP are important for the expression of the *arn* genes during insect infection. It is further possible that *P. protegens* interferes with the production of AMPs in *G. mellonella*, as it has been observed in *Drosophila* for *P. aeruginosa* [44], or evades detection by the host's immune system. If this was true, high resistance to AMPs might be unnecessary for persistence of CHA0 in the hemolymph of the insect host when injected, and even cause a decrease in fitness. Since

lepidopteran insects constitutively synthesize AMPs in their guts [7], resistance to these antimicrobials might alternatively be vital for a successful infection of the host via the oral route. It is also plausible that during an infection by ingestion the host organism has more time and ways to detect the bacterium and mount a protective systemic immune response. Mutation of the *arn* gene cluster in *Salmonella*, for example, reduced its virulence only when mice were infected orally but not when the bacteria were administered intraperitoneally [36].

The finding that PhoP is not required for systemic virulence does, however, not exclude that CHA0 is resistant to AMPs during insect interaction by other mechanisms, such as protection by O-antigen. Genome comparisons indicated that certain *P. fluorescens* group lineages possess proteases similar to *P. entomophila* AprA that could degrade AMPs when secreted into the environment (Flury *et al.*, manuscript in preparation). As discussed above, the expression of lipid A modification genes could also be induced in insect hemolymph independent of PhoP. Costechareyre *et al.* reported that the *arn* operon is important for virulence of the plant pathogenic bacterium *Dickeya dadantii* in the pea aphid, *Acyrthosiphon pisum* [45]. They observed that AMP resistance genes, including the *arn* gene cluster, were upregulated during *D. dadantii* infection. Preliminary results of an experiment with the CHA0 *arnB-gfp* reporter strains in *G. mellonella*, however, suggest that this is not the case in *P. protegens* (data not shown). In addition, expression of *arnB* was observed to be relatively low in the insect medium compared to LB. Further experiments are necessary to clarify whether and how well the *arn* operon is expressed by CHA0 during insect infection and if expression of the Arn proteins contributes to the virulence in this bacterial strain, in particular during infection via the oral route. Interestingly, the *arn* gene cluster is conserved in all *P. protegens* and *P. chlororaphis* strains while it has been reduced or lost in most other *P. fluorescens* group strains inspected. This could help to explain why only insect-pathogenic pseudomonads were found to be naturally resistant to PMB. It can be speculated that AMP resistance might not have been conserved in non-insecticidal strains because of the lack of interaction with insects.

It can be argued that results obtained with the model AMP PMB cannot be generalized for insect derived AMPs. Modification of lipid A might thus not confer resistance to all AMPs synthesized by insects. However, in previous studies a correlation between resistance to polymyxins and resistance to host AMPs has been observed [13,46], suggesting that PMB is a good model AMP. Studies using insect-derived AMPs would be expensive since the costs for the purification or synthesis of these antimicrobial compounds are very high. Alternatively, it could be interesting to investigate whether certain lepidopteran AMPs can be heterologously expressed in *Escherichia coli*, yeast cells or an insect cell line and be used for inhibition tests on agar plates. Amino acid sequences of several lepidopteran AMPs are available [47] and similar experiments with plant AMPs have been published previously [48].

Similar to *P. protegens* CHA0, it has been reported that only PhoQ is required for virulence of *P. aeruginosa*, but not PhoP [24]. This discrepancy has been explained with the finding that PhoQ controls the expression of

numerous genes outside the PhoP regulon [24]. PhoQ controls, amongst others, the expression of the type II secretion genes, iron-scavenging-related genes, energy-metabolism-related genes, and other global regulators independent of PhoP [24]. The reduced virulence of the CHA0 Δ*phoQ* mutant thus could be attributed to dysregulation of genes outside the PhoP regulon as well. We found evidence that PhoQ influences the expression of the Fit toxin, an important virulence factor of *P. protegens* CHA0 [3,5]. Because the sensor protein seemed to repress the expression of this insecticidal toxin, it does, however, not explain the reduced virulence of the CHA0 Δ*phoQ* mutant in *Galleria*. The virulence of the PCL1391 Δ*phoQ* mutant remains to be tested to assess whether the sensor kinase plays a similar role in virulence in *P. chlororaphis* as in *P. protegens*. PhoQ could influence virulence in PLC1391 via affecting the quorum sensing systems. The PhoP-PhoQ system was previously suggested to influence quorum sensing in *P. fluorescens* 2P24 [49]. For future studies it could therefore be interesting to identify additional genes controlled by PhoP-PhoQ in these bacteria, for example by an RNA sequencing approach.

Conclusions and future directions

The present study identified PhoP-PhoQ as a further two-component global regulatory system, in addition to GacS-GacA [5], that regulates virulence in *P. protegens* CHA0. In contrast to canonical two-component systems its components seem to regulate two different sets of genes. With a few exceptions, the PhoP-PhoQ system of *P. protegens* and *P. chlororaphis* is similar to its equivalent in *P. aeruginosa*. So far, only the sensor kinase PhoQ was found to be required for the virulence of strain CHA0. The response regulator PhoP in contrast seems to be necessary for the bacterium's inherent resistance to AMPs under laboratory conditions. PhoP-dependent modification of lipid A does not seem to be essential for persistence in the hemocoel of *G. mellonella* upon injection. This challenges the hypothesis previously formulated in Chapter 5 that AMP resistance is important for CHA0 to systemically infect insect larvae. Resistance to AMPs might be more important for surviving antimicrobial responses of epithelial immunity during colonization of the insect gut and conferred by multiple factors, some of which are expressed independent of the PhoP-PhoQ system. Additional resistance mechanisms, such as the expression of proteases and efflux pumps and the synthesis of exopolysaccharides, could be identified in strain CHA0 with a transposon sequencing approach by growing a mutant library in a specific medium supplemented with PMB or another model AMP. Deletion of *oprH* and *pagP* will further reveal whether their encoded proteins contribute to AMP resistance in CHA0. Insect feeding assays with several AMP-sensitive strains should be performed in order to test the hypothesis that resistance to AMPs contributes to oral insecticidal activity in pseudomonads. Future studies should in addition aim at the identification of PhoQ-regulated genes that contribute to virulence of CHA0.

The results of this study point out that we need to learn more about the interaction of entomopathogenic pseudomonads with the insect's immune system. It is still completely unknown which strategy these bacteria

evolved to counteract the immune defenses of the insect host, which makes it very difficult to tell if their natural resistance to AMPs is required in order to successfully infect and kill insect larvae. Thus, the next big challenge will be to answer the question whether these bacteria are able to suppress, evade or resist the host's production of these key players of innate immunity.

Material and Methods

Bacterial strains, plasmids, media, and culture conditions

All strains and plasmids used in this study are listed in Table 1. Bacterial strains were routinely maintained on nutrient agar (NA) plates or cultured in LB (LB Broth Miller, BD Difco) supplemented with appropriate antibiotics as needed. *Pseudomonas* strains were grown at 25°C while *E. coli* was cultured at 37°C. The following antibiotic concentrations were used: ampicillin, 100 µg/ml; chloramphenicol, 10 µg/ml; kanamycin, 25 µg/ml for *E. coli* and *P. protegens*, 50 µg/ml for *P. chlororaphis*; gentamicin, 10 µg/ml for *E. coli* and *P. protegens*, 20 µg/ml for *P. chlororaphis*; and tetracycline, 25 µg/ml or 125 µg/ml for *E. coli* and *Pseudomonas*, respectively. Isopropyl β-D-1-thiogalactopyranoside (IPTG) was added at a final concentration of 0.1 mM, if not otherwise stated.

Table 1: Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype, phenotype or relevant characteristics	Reference or source
Pseudomonas protegens		
CHA0	Wild type	[50]
CHA0-*gfp2*	CHA0::attTn*7*-*gfp2*; Gm^r	[3]
CHA1163	CHA0::*fitD-mcherry*	[3]
CHA5129	CHA0 *arnA*::pEMG (PFLCHA0_c30730); Km^r	This study
CHA5133	CHA0 Δ*phoQ* (PFLCHA0_c45400)	This study
CHA5133-*mChe*	CHA5133::attTn*7*-*mcherry*; Gm^r	This study
CHA5134	CHA0 Δ*phoP* (PFLCHA0_c45410)	This study
CHA5134-*mChe*	CHA5134::attTn*7*-*mcherry*; Gm^r	This study
CHA5135	CHA5133::*fitD-mcherry*	This study
CHA5136	CHA5134::*fitD-mcherry*	This study
CHA5138	CHA5133::attTn*7*-$P_{tac/laclq}$-*phoQ*; Gm^r	This study
CHA5153	CHA5134::attTn*7*-$P_{tac/laclq}$-*phoP*; Gm^r	This study
CHA5204	CHA0 Δ*phoPQ*	This study
Pseudomonas chlororaphis		
PCL1391	Wild type	[51]
PCL-10	PCL1391 Δ*phoQ* (PCL1391_4349)	This study
Escherichia coli		
DH5α, DH5α λpir	Laboratory strains	[52]

Plasmids		
pEMG	pSEVA212S; *ori*R6K, *lacZα* MCS flanked by two I-SceI sites; Km^r, Ap^r	[53]
pME8300	Carrier plasmid for Tn*7* for $P_{tac/laclq}$ controlled target gene expression; Gm^r, Ap^r	[54]
pME8340	pEMG-*arnA'*; suicide plasmid for the disruption of *arnA* (PFLCHA0_c30730) in CHA0; Km^r	This study
pME8345	pEMG-Δ*phoQ*; suicide plasmid for the in-frame deletion of *phoQ* (PFLCHA0_c45400) in CHA0; Km^r	This study
pME8346	pEMG-Δ*phoP*; suicide plasmid for the in-frame deletion of *phoP* (PFLCHA0_c45410) in CHA0; Km^r	This study
pME8348	pPROBE-TT-P_{arnB}-*gfp*; Tc^r	This study
pME8349	pME8300-$P_{tac/laclq}$-*phoQ*; IPTG-inducible expression of CHA0 *phoQ* (PFLCHA0_c45400); Gm^r, Ap^r	This study
pME8353	pME8300-$P_{tac/laclq}$-*phoP*; IPTG-inducible expression of CHA0 *phoP* (PFLCHA0_c45410); Gm^r, Ap^r	This study
pME11003	pEMG-Δ*phoPQ*; suicide plasmid for the deletion of *phoP* (PFLCHA0_c45410) and *phoQ* (PFLCHA0_c45400) in CHA0; Km^r	This study
pME11004	pPROBE-TT-P_{oprH}-*gfp*; Tc^r	This study
pME11005	pEMG-Δ*phoQ*(PCL1391); suicide plasmid for the in-frame deletion of *phoQ* (PCL1391_4349) in PCL1391; Km^r	This study
pME9407	pUC19-based delivery plasmid for miniTn*7*-*mcherry*; *mob*$^+$; Gm^r, Cm^r, Ap^r	[55]
pPROBE-TT	Promoter-probe vector based on eGFP; Tc^r	[56]
pSW-2	*ori*RK2, *xylS*, P_m::*I-sce*I; Gm^r	[53]
pUX-BF13	Helper plasmid encoding Tn*7* transposition functions; R6K-replicon; Ap^r	[57]

Abbreviations: Ap^r, ampicillin resistance; Gm^r, gentamicin resistance; Km^r, kanamycin resistance; Tc^r, tetracycline resistance. Locus tags of newly identified genes are indicated in brackets.

Bioinformatics

The genes putatively coding for PhoP, PhoQ and the Arn proteins in *P. protegens* CHA0 were identified via protein BLAST searches performed on the Pseudomonas Genome Database website (http://pseudomonas.com/blast.jsp) using protein sequences of characterized genes (i.e., PA1179, PA1180 and PA3552-PA3558) from *P. aeruginosa* PAO1. Detected genes and gene clusters were further analyzed by predicting the function of the encoded proteins using the NCBI Conserved Domain Database [58] and InterPro [59] with default parameters. The ortholog of *phoQ* in *P. chlororaphis* PCL1391 was identified by local BLASTn search using the nucleotide sequence of CHA0 *phoQ* [60]. Orthologs of genes of the *arn* cluster were identified in *P. fluorescens* group strains by pan-genome calculations in EDGAR [61] with standard settings as described in [62].

Recombinant DNA techniques

DNA manipulations and PCRs were performed according to standard protocols [52]. Genomic DNA was extracted with the Promega Wizard Genomic DNA Purification Kit. Plasmid DNA preparations were performed using the QIAprep Spin Miniprep Kit (Qiagen) and the Genomed JETStar Plasmid Purification Midi Kit for small and large scale purifications, respectively. DNA gel extractions were conducted using the

MinElute Gel Extraction Kit and the QIAquick Gel Extraction Kit (Qiagen). DNA restriction and modification enzymes were purchased from Promega and were used according to the manufacturer's recommendations. DNA enzyme reaction cleanups were conducted using the QIAquick PCR Purification Kit (Qiagen). PCR for molecular cloning was conducted according to the recommendations of the manufacturer with the PrimeSTAR HS high-fidelity DNA polymerase kit (Takara Bio Inc.), while the DNA Polymerase kit (Promega) was used to perform PCRs for analytic purposes. The primers used in this study were synthesized by Microsynth AG (Balgach, Switzerland) and are listed in Table 2. DNA sequencing was conducted at GATC Biotech (Konstanz, Germany) and obtained sequences were analyzed using the DNASTAR Lasergene software suite.

Table 2: Primers used in this study

Name	Sequence 5' → 3', restriction enzyme(s)[1]	Purpose
arnA-im-1	CGGGATCCAACTGGGTGCTGGTCAAG, BamHI	Disruption of CHA0 *arnA*
arnA-im-2	GGAATTCGTCCACCAGGCCCAGTTC, EcoRI	Disruption of CHA0 *arnA*
ParnB-F	CGGGATCCGTGACATGCTCGGGTTG, BamHI	Reporter for CHA0 *arnB*
ParnB-R	GGAATTCGATCCAGCCGGAGCGCAG, EcoRI	Reporter for CHA0 *arnB*
PCL.phoQ-del-1	CGGGATCCTCAGACCGAGCAGTTCAACC, BamHI	Deletion of PCL1391 *phoQ*
PCL.phoQ-del-2	CCCAAGCTTGCGTAACGATCGGATCATCGG, HindIII	Deletion of PCL1391 *phoQ*
PCL.phoQ-del-3	CCCAAGCTTCTCTTCCCGACGATTTGACCG, HindIII	Deletion of PCL1391 *phoQ*
PCL.phoQ-del-4	GGAATTCGAGAGCCTGCTGGTGATCTG, EcoRI	Deletion of PCL1391 *phoQ*
phoP-del-1	GCGGATCCTGGCGAACTCAT, BamHI	Deletion of CHA0 *phoP*
phoP-del-2	GACTAGTAACGAGCGTTGCCGATGATTC, SpeI	Deletion of CHA0 *phoP*
phoP-del-3	GACTAGTCCAGGCAAAATTCGACATCTCC, SpeI	Deletion of CHA0 *phoP* and *phoPQ*
phoP-del-4	GGAATTCGATATGTTCCTGCCGGTGAC, EcoRI	Deletion of CHA0 *phoP* and *phoPQ*
phoP-expr-F	GACTAGTATGTCGAATTTTGCCTGGGAGAG; SpeI	Expression of CHA0 *phoP*
phoP-expr-R	CGGGATCCGAATCATCGGCAACGCTCG, BamHI	Expression of CHA0 *phoP*
phoQ-del-1	CGGGATCCTACGACCTGCTTTTCCCTTGC, BamHI	Deletion of CHA0 *phoQ* and *phoPQ*
phoQ-del-2	GACTAGTCACTTTCCGGCCCTGTAGG, SpeI	Deletion of CHA0 *phoQ* and *phoPQ*
phoQ-del-3	GACTAGTGCGCAGCGATCGAATCATCG, SpeI	Deletion of CHA0 *phoQ*
phoQ-del-4	GGAATTCAGACGTTCCCGATCCTCATC, EcoRI	Deletion of CHA0 *phoQ*
phoQ-expr-F	GACTAGTATGATTCGATCGCTGCGCCTG, SpeI	Expression of CHA0 *phoQ*
phoQ-expr-R	CCCAAGCTTCCTACAGGGCCGGAAAGTG, HindIII	Expression of CHA0 *phoQ*
PoprH-F	CGGGATCCGCACCGTGGAAACTCACCAC, BamHI	Reporter for CHA0 *oprH*
PoprH-R	GGAATTCGACAACCCAACGAAGTTGCTG, EcoRI	Reporter for CHA0 *oprH*

[1] Restriction sites are underlined

Construction of in-frame deletion and gene disruption mutants

In-frame deletions of selected genes in *P. protegens* CHA0 and *P. chlororaphis* PCL1391 were performed based on homologous recombinations using the suicide vector pEMG and the I-SceI system. For the construction of the suicide vectors (Table 1), upstream and downstream regions of 500–600 bp length flanking the region to be deleted were amplified by PCR using the primer pairs listed in Table 2 and

chromosomal DNA from strains CHA0 or PCL1391 as DNA templates. Purified PCR products were digested using the corresponding restriction enzymes (indicated in Table 2) and subsequently cloned into the suicide vector pEMG via triple ligation. Correct insert sequences of the resulting plasmids were confirmed via DNA sequencing. The obtained suicide vectors then served to construct strains CHA5133, CHA5134, CHA5204, and PCL-10 (Table 1), respectively, using the I-SceI system as described previously [54].

For the disruption of the *arnA* gene in *P. protegens* CHA0, a pEMG-based suicide vector was constructed and inserted sequence-specifically via homologous recombination. To do so, a 500-bp region in the middle of the *arnA* was amplified by PCR, using the primers arnA-im-1 and arnA-im-2 (Table 2) and chromosomal DNA from strain CHA0. The resulting BamHI-EcoRI fragment was digested with BamHI and EcoRI and cloned by ligation into pEMG opened with the same restriction enzymes. The insert sequence of the resulting vector pME8340 was verified by DNA sequencing and, for constructing strain CHA5129, the plasmid was integrated into the CHA0 chromosome by transformation of competent bacteria via electroporation.

Complementation of mutant strains

In order to complement the Δ*phoQ* mutation in strain CHA5133, the 1348 nt coding sequence (CDS) of *phoQ* was amplified by PCR using primers phoQ-expr-F and phoQ-expr-R (Table 2) and chromosomal DNA from *P. protegens* CHA0. After digestion with SpeI and HindIII, the fragment was ligated into plasmid pME8300 (Table 1) opened with the same restriction enzymes. Correct insert sequence of the resulting plasmid pME8349 (Table 1) was verified via DNA sequencing. The obtained vector was subsequently used for constructing strain CHA5138 by electroporation of competent CHA5133 cells with pME8349 and pUX-BF13 (Table 1).

A similar approach was chosen to complement the Δ*phoP* mutation in strain CHA5134. Using chromosomal DNA from CHA0 as a template, the 708 nt long fragment CDS of *phoP* was amplified by PCR using the primers phoP-expr-F and phoP-expr-R. The purified PCR product was digested with SpeI. The vector pME8300 was opened with HindIII, blunt-ended with T4 DNA polymerase and digested with SpeI after a purification step. The digested *phoP* PCR product was cloned into the opened plasmid via ligation. Correct insert sequence of the resulting vector pME8353 (Table 1) was verified via DNA sequencing and the plasmid was subsequently used for constructing strain CHA5153 by electroporation of competent CHA5134 cells with pME8353 and pUX-BF13 (Table 1).

Polymyxin resistance assays

To test the resistance of different *P. protegens* and *P. chlororaphis* strains toward cationic AMPs, the bacterial strains were exposed to increasing concentrations of polymyxin B (Sigma) (ranging from 2 µg/ml to 200 µg/ml). The bacteria were grown overnight at 25°C and 180 rpm in 10 ml LB supplemented with

kanamycin and IPTG if necessary. The cells were washed once with 0.9% NaCl solution and the optical density at 600 nm (OD_{600}) of the bacterial suspension was adjusted to 1. Three milliliter of LB (supplemented with 20 mM $MgCl_2$, if indicated) in glass test tubes were supplemented with 0, 2, 20, or 200 µg/ml of polymyxin B, and inoculated with 60 µl of bacterial suspension. The cultures were incubated at 25°C with a constant agitation of 180 rpm and the OD_{600} was measured 6 h and 24 h after inoculation.

Gene expression studies

For studying the expression of the *oprH-phoPQ* and *arn* operons, fragments of approximately 740 bp containing the putative promoter regions located upstream of the *arnB* and *oprH* gene were amplified from chromosomal DNA of *P. protegens* CHA0 by PCR using the primer pairs indicated in Table 2. The DNA fragments obtained were digested with BamHI and EcoRI and cloned into the GFP-based promoter probe vector pPROBE-TT [56] opened with the same restriction enzymes. Insert sequences of the resulting plasmids were verified by DNA sequencing. Competent cells of CHA0 and their isogenic mutants were subsequently transformed with the constructed vectors by electroporation to create reporter strains for expression studies. Reporter strains were grown at 25°C and 180 rpm in 10 ml LB, LB supplemented with 22 mM $MgCl_2$ or Grace's Insect Medium (GIM, Sigma) [54] contained in 50-ml Erlenmeyer flasks. Samples were taken at different time points and single cell fluorescence intensities were measured by fluorescence microscopy as described previously for the Fit toxin [54], using the GFP channel.

Assays for measuring the expression of the Fit toxin by fluorescence microscopy in strains CHA1163, CHA5135 and CHA5136 were performed in GIM as described previously [54]. For studying the effect of Mg^{2+}, the reporter strains were grown in M9 minimal medium (as described in [54]) with a low phosphate content (7.4 mM K_2HPO_4; low phosphate concentration to lower pH in order to avoid precipitation of salts) and with or without 40 mM $MgCl_2$.

Galleria mellonella virulence and competition assays

Injection assays for virulence determination using last-instar larvae of *G. mellonella* (Entomos AG, Grossdietwil, Switzerland) were performed as described before [3]. For the competition assay, strains CHA5133 and CHA5134 were marked with a constitutively expressed mCherry tag using the Tn*7* delivery vector pME9407 and the helper plasmid pUX-BF13 as described previously [55]. The resulting strains CHA5133-mChe and CHA5134-mChe (Table 1) and the GFP-tagged CHA0 wild-type strain (CHA0-*gfp2*) were grown overnight in 10 ml of LB at 25°C and 180 rpm. The cells were washed once in 0.9% NaCl solution and the OD_{600} of the bacterial suspension was adjusted to 1. Each mCherry-tagged mutant strain was mixed 1:1 with CHA0-*gfp2* and the resulting suspension was diluted 200-times in saline solution. Aliquots of 5 µl were injected into the last left pro-leg of *G. mellonella* larvae which were then incubated at room temperature for

approximately 22 h. Hemolymph was collected and visualized by fluorescence microscopy as described before [3]. Mutant and wild-type cells were counted using the DsRed and GFP channel, respectively. The competitive index was calculated by first normalizing the counts for wild type and mutant with the ratio observed in the undiluted inoculum (to correct for variation within the inocula) and then dividing the number of mutant cells by the number of wild-type cells.

Statistical analysis

Statistical analysis of experiments was performed in RStudio version 0.98.1091 (http://www.rstudio.com/). One-way or two-way analysis of variance (ANOVA) with Tukey's HSD test for post-hoc comparisons was performed when appropriate. The Log-Rank test of the *survival* package of R was used to calculate significant differences in insect toxicity between the wild type and isogenic mutant strains in the *Galleria* virulence assay. One-sample *t* tests were performed with data from competition assays.

References

1. Haas D, Défago G (2005) Biological control of soil-borne pathogens by fluorescent pseudomonads. Nat Rev Microbiol 3: 307-319.
2. Kupferschmied P, Maurhofer M, Keel C (2013) Promise for plant pest control: root-associated pseudomonads with insecticidal activities. Front Plant Sci 4: 287.
3. Péchy-Tarr M, Borel N, Kupferschmied P, Turner V, Binggeli O, et al. (2013) Control and host-dependent activation of insect toxin expression in a root-associated biocontrol pseudomonad. Environ Microbiol 15: 736-750.
4. Péchy-Tarr M, Bruck DJ, Maurhofer M, Fischer E, Keel C (2008) Molecular analysis of a novel gene cluster encoding an insect toxin in plant-associated strains of *Pseudomonas fluorescens*. Environ Microbiol 10: 2368-2386.
5. Ruffner B, Péchy-Tarr M, Ryffel F, Hoegger P, Obrist C, et al. (2013) Oral insecticidal activity of plant-associated pseudomonads. Environ Microbiol 15: 751-763.
6. Haas D, Keel C (2003) Regulation of antibiotic production in root-colonizing *Peudomonas* spp. and relevance for biological control of plant disease. Annu Rev Phytopathol 41: 117-153.
7. Lemaitre B, Hoffmann J (2007) The host defense of *Drosophila melanogaster*. Annu Rev Immunol 25: 697-743.
8. He Y, Cao X, Li K, Hu Y, Chen YR, et al. (2015) A genome-wide analysis of antimicrobial effector genes and their transcription patterns in *Manduca sexta*. Insect Biochem Mol Biol: 10.1016/j.ibmb.2015.1001.1015.
9. Kounatidis I, Ligoxygakis P (2012) *Drosophila* as a model system to unravel the layers of innate immunity to infection. Open Biol 2: 120075.
10. Kanost MR, Jiang H, Yu XQ (2004) Innate immune responses of a lepidopteran insect, *Manduca sexta*. Immunol Rev 198: 97-105.
11. Ganz T (2003) The role of antimicrobial peptides in innate immunity. Integr Comp Biol 43: 300-304.
12. Klotman ME, Chang TL (2006) Defensins in innate antiviral immunity. Nat Rev Immunol 6: 447-456.
13. Anaya-López JL, López-Meza JE, Ochoa-Zarzosa A (2013) Bacterial resistance to cationic antimicrobial peptides. Crit Rev Microbiol 39: 180-195.

14. Ji D, Kim Y (2004) An entomopathogenic bacterium, *Xenorhabdus nematophila*, inhibits the expression of an antibacterial peptide, cecropin, of the beet armyworm, *Spodoptera exigua*. J Insect Physiol 50: 489-496.
15. Caldas C, Cherqui A, Pereira A, Simoes N (2002) Purification and characterization of an extracellular protease from *Xenorhabdus nematophila* involved in insect immunosuppression. Appl Environ Microbiol 68: 1297-1304.
16. Liehl P, Blight M, Vodovar N, Boccard F, Lemaitre B (2006) Prevalence of local immune response against oral infection in a *Drosophila*/*Pseudomonas* infection model. PLoS Pathog 2: e56.
17. Raetz CR, Reynolds CM, Trent MS, Bishop RE (2007) Lipid A modification systems in gram-negative bacteria. Annu Rev Biochem 76: 295-329.
18. Gooderham WJ, Hancock RE (2009) Regulation of virulence and antibiotic resistance by two-component regulatory systems in *Pseudomonas aeruginosa*. FEMS Microbiol Rev 33: 279-294.
19. Fields PI, Groisman EA, Heffron F (1989) A *Salmonella* locus that controls resistance to microbicidal proteins from phagocytic cells. Science 243: 1059-1062.
20. Macfarlane EL, Kwasnicka A, Ochs MM, Hancock RE (1999) PhoP-PhoQ homologues in *Pseudomonas aeruginosa* regulate expression of the outer-membrane protein OprH and polymyxin B resistance. Mol Microbiol 34: 305-316.
21. Dalebroux ZD, Miller SI (2014) *Salmonellae* PhoPQ regulation of the outer membrane to resist innate immunity. Curr Opin Microbiol 17: 106-113.
22. Miller SI, Kukral AM, Mekalanos JJ (1989) A two-component regulatory system (*phoP phoQ*) controls *Salmonella typhimurium* virulence. Proc Natl Acad Sci U S A 86: 5054-5058.
23. Derzelle S, Turlin E, Duchaud E, Pages S, Kunst F, et al. (2004) The PhoP-PhoQ two-component regulatory system of *Photorhabdus luminescens* is essential for virulence in insects. J Bacteriol 186: 1270-1279.
24. Gooderham WJ, Gellatly SL, Sanschagrin F, McPhee JB, Bains M, et al. (2009) The sensor kinase PhoQ mediates virulence in *Pseudomonas aeruginosa*. Microbiology 155: 699-711.
25. Castelli ME, Garcia Vescovi E, Soncini FC (2000) The phosphatase activity is the target for Mg^{2+} regulation of the sensor protein PhoQ in *Salmonella*. J Biol Chem 275: 22948-22954.
26. Groisman EA (2001) The pleiotropic two-component regulatory system PhoP-PhoQ. J Bacteriol 183: 1835-1842.
27. Moskowitz SM, Ernst RK, Miller SI (2004) PmrAB, a two-component regulatory system of *Pseudomonas aeruginosa* that modulates resistance to cationic antimicrobial peptides and addition of aminoarabinose to lipid A. J Bacteriol 186: 575-579.
28. Barrow K, Kwon DH (2009) Alterations in two-component regulatory systems of *phoPQ* and *pmrAB* are associated with polymyxin B resistance in clinical isolates of *Pseudomonas aeruginosa*. Antimicrob Agents Chemother 53: 5150-5154.
29. Miller AK, Brannon MK, Stevens L, Johansen HK, Selgrade SE, et al. (2011) PhoQ mutations promote lipid A modification and polymyxin resistance of *Pseudomonas aeruginosa* found in colistin-treated cystic fibrosis patients. Antimicrob Agents Chemother 55: 5761-5769.
30. McPhee JB, Bains M, Winsor G, Lewenza S, Kwasnicka A, et al. (2006) Contribution of the PhoP-PhoQ and PmrA-PmrB two-component regulatory systems to Mg^{2+}-induced gene regulation in *Pseudomonas aeruginosa*. J Bacteriol 188: 3995-4006.
31. Edrington TC, Kintz E, Goldberg JB, Tamm LK (2011) Structural basis for the interaction of lipopolysaccharide with outer membrane protein H (OprH) from *Pseudomonas aeruginosa*. J Biol Chem 286: 39211-39223.
32. Thaipisuttikul I, Hittle LE, Chandra R, Zangari D, Dixon CL, et al. (2014) A divergent *Pseudomonas aeruginosa* palmitoyltransferase essential for cystic fibrosis-specific lipid A. Mol Microbiol 91: 158-174.
33. Gunn JS, Lim KB, Krueger J, Kim K, Guo L, et al. (1998) PmrA-PmrB-regulated genes necessary for 4-aminoarabinose lipid A modification and polymyxin resistance. Mol Microbiol 27: 1171-1182.
34. Macfarlane EL, Kwasnicka A, Hancock RE (2000) Role of *Pseudomonas aeruginosa* PhoP-PhoQ in resistance to antimicrobial cationic peptides and aminoglycosides. Microbiology 146 (Pt 10): 2543-2554.

35. Noland BW, Newman JM, Hendle J, Badger J, Christopher JA, et al. (2002) Structural studies of *Salmonella typhimurium* ArnB (PmrH) aminotransferase: a 4-amino-4-deoxy-L-arabinose lipopolysaccharide-modifying enzyme. Structure 10: 1569-1580.
36. Gunn JS, Ryan SS, Van Velkinburgh JC, Ernst RK, Miller SI (2000) Genetic and functional analysis of a PmrA-PmrB-regulated locus necessary for lipopolysaccharide modification, antimicrobial peptide resistance, and oral virulence of *Salmonella enterica* serovar *typhimurium*. Infect Immun 68: 6139-6146.
37. Chapman RF (1998) Circulatory systems, blood and immune systems. In: Chapman RF, editor. The Insects: Structure and Function. Cambridge: Cambridge University Press.
38. Pannabecker TL, Andrews F, Beyenbach KW (1992) A quantitative analysis of the osmolytes in the hemolymph of the larval gypsy moth, *Lymantria dispar*. J Insect Physiol 38: 823-830.
39. Wyatt GR (1961) Biochemistry of insect hemolymph. Annu Rev Entomol 6: 75-102.
40. Fernandez L, Gooderham WJ, Bains M, McPhee JB, Wiegand I, et al. (2010) Adaptive resistance to the "last hope" antibiotics polymyxin B and colistin in *Pseudomonas aeruginosa* is mediated by the novel two-component regulatory system ParR-ParS. Antimicrob Agents Chemother 54: 3372-3382.
41. Groisman EA, Mouslim C (2006) Sensing by bacterial regulatory systems in host and non-host environments. Nat Rev Microbiol 4: 705-709.
42. Bader MW, Sanowar S, Daley ME, Schneider AR, Cho U, et al. (2005) Recognition of antimicrobial peptides by a bacterial sensor kinase. Cell 122: 461-472.
43. Gellatly SL, Needham B, Madera L, Trent MS, Hancock RE (2012) The *Pseudomonas aeruginosa* PhoP-PhoQ two-component regulatory system is induced upon interaction with epithelial cells and controls cytotoxicity and inflammation. Infect Immun 80: 3122-3131.
44. Apidianakis Y, Mindrinos MN, Xiao W, Lau GW, Baldini RL, et al. (2005) Profiling early infection responses: *Pseudomonas aeruginosa* eludes host defenses by suppressing antimicrobial peptide gene expression. Proc Natl Acad Sci U S A 102: 2573-2578.
45. Costechareyre D, Chich JF, Strub JM, Rahbe Y, Condemine G (2013) Transcriptome of *Dickeya dadantii* infecting *Acyrthosiphon pisum* reveals a strong defense against antimicrobial peptides. PLoS ONE 8: e54118.
46. Cullen TW, Schofield WB, Barry NA, Putnam EE, Rundell EA, et al. (2015) Gut microbiota. Antimicrobial peptide resistance mediates resilience of prominent gut commensals during inflammation. Science 347: 170-175.
47. Bulet P, Stöcklin R (2005) Insect antimicrobial peptides: structures, properties and gene regulation. Protein Pept Lett 12: 3-11.
48. Garcia AN, Ayub ND, Fox AR, Gomez MC, Dieguez MJ, et al. (2014) Alfalfa snakin-1 prevents fungal colonization and probably coevolved with rhizobia. BMC Plant Biol 14: 248.
49. Yan Q, Gao W, Wu XG, Zhang LQ (2009) Regulation of the PcoI/PcoR quorum-sensing system in *Pseudomonas fluorescens* 2P24 by the PhoP/PhoQ two-component system. Microbiology 155: 124-133.
50. Stutz E, Défago G, Kern H (1986) Naturally occurring fluorescent pseudomonads involved in suppression of black root rot of tobacco. Phytopathology 76: 181-185.
51. Chin-A-Woeng TFC, Bloemberg GV, van der Bij AJ, van der Drift KMGF, Schripsema J, et al. (1998) Biocontrol by phenazine-1-carboxamide-producing *Pseudomonas chlororaphis* PCL1391 of tomato root rot caused by *Fusarium oxysporum* f. sp. *radicis-lycopersici*. Mol Plant Microbe Interact 11: 1069-1077.
52. Sambrook J, Russel DW (2001) Molecular Cloning: A Laboratory Manual. Cold Spring Harbor (New York): Cold Spring Harbor Laboratory Press.
53. Martínez-García E, de Lorenzo V (2011) Engineering multiple genomic deletions in Gram-negative bacteria: analysis of the multi-resistant antibiotic profile of *Pseudomonas putida* KT2440. Environ Microbiol 13: 2702-2716.
54. Kupferschmied P, Péchy-Tarr M, Imperiali N, Maurhofer M, Keel C (2014) Domain shuffling in a sensor protein contributed to the evolution of insect pathogenicity in plant-beneficial *Pseudomonas protegens*. PLoS Pathog 10: e1003964.

55. Rochat L, Péchy-Tarr M, Baehler E, Maurhofer M, Keel C (2010) Combination of fluorescent reporters for simultaneous monitoring of root colonization and antifungal gene expression by a biocontrol pseudomonad on cereals with flow cytometry. Mol Plant Microbe Interact 23: 949-961.
56. Miller WG, Leveau JHJ, Lindow SE (2000) Improved *gfp* and *inaZ* broad-host-range promoter-probe vectors. Mol Plant Microbe Interact 13: 1243-1250.
57. Bao Y, Lies DP, Fu H, Roberts GP (1991) An improved Tn*7*-based system for the single-copy insertion of cloned genes into chromosomes of Gram-negative bacteria. Gene 109: 167-168.
58. Marchler-Bauer A, Lu S, Anderson JB, Chitsaz F, Derbyshire MK, et al. (2011) CDD: a Conserved Domain Database for the functional annotation of proteins. Nucleic Acids Res 39: D225-229.
59. Mitchell A, Chang HY, Daugherty L, Fraser M, Hunter S, et al. (2014) The InterPro protein families database: the classification resource after 15 years. Nucleic Acids Res: 10.1093/nar/gku1243.
60. Jousset A, Schuldes J, Keel C, Maurhofer M, Daniel R, et al. (2014) Full-Genome Sequence of the Plant Growth-Promoting Bacterium *Pseudomonas protegens* CHA0. Genome Announc 2.
61. Blom J, Albaum SP, Doppmeier D, Puhler A, Vorholter FJ, et al. (2009) EDGAR: a software framework for the comparative analysis of prokaryotic genomes. BMC Bioinformatics 10: 154.
62. Smits TH, Rezzonico F, Kamber T, Blom J, Goesmann A, et al. (2010) Complete genome sequence of the fire blight pathogen *Erwinia amylovora* CFBP 1430 and comparison to other *Erwinia* spp. Mol Plant Microbe Interact 23: 384-393.

CHAPTER 7

General Discussion

Advancing the understanding of insect pathogenicity in plant-beneficial pseudomonads

Plant-beneficial pseudomonads have been studied for decades primarily because of their abilities to protect crop plants from phytopathogens and promote plant growth [1,2]. Eventually this led to the development and commercialization of several biofungicides [1,3] that are based on formulations of these rhizobacteria and help to reduce the amount of harmful chemical fungicides used for plant protection worldwide. Since *Pseudomonas* bacteria are well-known to thrive on various substrates and under many different conditions, it is not surprising that these microorganisms have often been detected in association with insects as well [1]. Moreover, we could show that strains of defined lineages of root-associated pseudomonads are in fact true insect pathogens as they are able to persist within the gut of certain insect species and subsequently kill them by invading their hemocoel and causing septicemia. While the molecular basis and ecology of their antifungal activities have been dissected in some detail [1], we have only just started to unravel the underlying mechanisms, regulation, ecological roles, and evolution of their insect pathogenicity. This thesis provided new insights into the regulation of an insecticidal toxin and suggests that a defined surface structure is critical for these bacteria to successfully infect the insect host. More precisely, we could demonstrate that *Pseudomonas protegens* produces its Fit toxin, which contributes to virulence of this entomopathogen [4,5], only during interaction with insects (Chapter 2). A follow-up study on the membrane-bound sensor protein FitF, which tightly controls the expression of this insecticidal toxin, brought up a possible explanation for how detection of the insect host works and evolved in this bacterium (Chapter 3). We further found evidence that the outermost and most variable part of lipopolysaccharide, the O-antigen, is vital for virulence of *P. protegens* and contributes amongst others to the protection against attacks by host antimicrobial peptides (AMPs) (Chapter 5). We discovered that many insecticidal strains of plant-beneficial *Pseudomonas* species are resistant to certain AMPs. We provided evidence that the resistance toward these insect-derived antimicrobials is multifactorial and could be critical for a successful infection of insects, as AMPs constitute an important immune response to clear bacterial infections (Chapter 6). These achievements were amongst others made possible by developing new methods and tools for the quantification of single cell fluorescence by microscopy and for faster and more efficient mutation of genes and gene clusters in our model bacteria.

This research project did therefore answer some of our initial questions regarding the molecular basis and regulation of insect pathogenicity in these microbes. It however also made us realize that in a way we were just scratching the surface and need to pursue with more in-depth studies in order to adequately answer our central research question: How do entomopathogenic pseudomonads with plant-beneficial properties survive within, invade and eventually kill the insect host? Some observations, hypotheses and possible approaches to further answer this question will be discussed in the following.

Interaction with the host's innate immunity

P. protegens strain CHA0 is able to invade and kill larvae of the large white, *Pieris brassicae*, upon ingestion within a few days while larvae of the greater wax moth, *Galleria mellonella*, seem to be resistant to oral infection (Chapter 2). One could conclude that *P. protegens* accidently infects susceptible insect species. However, our findings on the presence and host-specific expression of an insecticidal toxin and the resistance to AMPs suggest otherwise. This plant-beneficial bacterium and related strains seem to have adapted to the life within insects and to specifically produce virulence factors upon infection. The more we known about the underlying mechanisms and adaptations, the less it looks like accidental infections and more like these bacteria have evolved into insect pathogens over time. But why are these bacteria able to infect and kill certain insect species while others seem to be resistant?

The evolutionary success of insects has amongst others been attributed to their highly-developed innate immune systems [6]. To survive its defense reactions, pathogens need to evolve counterstrategies. These include resistance to antimicrobial compounds and phagocytosis, camouflage to avoid detection by the innate immune system, suppression of immune reactions, or combinations thereof [6,7]. In addition, they have to find a way to get across the gut epithelial barrier if they need to invade the insect in order to kill it and use it as a nutrient source. The strategy that entomopathogenic pseudomonads chose to interact with the insect immunity might limit the range of insect species they can successfully infect and kill. Knowing the infection tactic of these bacteria would certainly help us to answer our central question stated above.

We observed that strain CHA0 is able to invade the hemocoel of *P. brassicae* within one day after oral infection and to kill the insect within a few days, even when low doses of the bacterium were ingested [1,4]. It is thus likely that *P. protegens* follows a hit-and-run strategy which probably leaves the insects with not enough time to mount a protective immune response. To test if fast infection, which is a prerequisite for a hit-and-run strategy, is indeed essential for *P. protegens* to kill the insect host is challenging. One could engineer a bacterial strain whose growth is artificially slowed down, without affecting its ability to express virulence factors, and test if it can still cause mortality via the oral route of infection (with an expected delay). Even if the larvae failed to induce the necessary systemic immune reactions, invading bacteria would still have to deal with AMPs and reactive oxygen species (ROS) that are produced constitutively by the epithelial immunity and secreted into the gut (Chapter 6: Figure 1). Although we have no direct experimental evidence, O-antigen and lipid A modifications could amongst others provide the necessary protection against AMPs during these first steps of infection. AMP resistance was demonstrated to be an important factor for pathogenicity of other bacteria, such as *Staphylococcus epidermidis* [8]. The types of AMPs produced by insects vary considerably between insect species and orders [9-12]. It is thus possible that resistance to a

certain set of AMPs but susceptibility to others shapes the host range of *P. protegens*. The expression of catalase and superoxide dismutases might additionally confer resistance to host-derived ROS [13].

In contrast to *Pseudomonas entomophila*, which primarily colonizes the gut and can kill insects without necessarily invading the hemocoel [7,14], some of our observations suggest that *P. protegens* is not an efficient gut colonizer and probably needs to get across the gut epithelial barrier into the hemocoel in order to survive within the insect host. Hence, in addition to protection against ROS and AMPs, entomopathogenic pseudomonads also need a strategy to translocate to the hemocoel across the physical barrier composed of epithelial tissues and the peritrophic matrix (a chitin and glycoprotein layer [15]) [16]. To date, we can only speculate about how this is achieved by these microbes. It is imaginable that they produce and secrete certain enzymes, such as proteases and chitinases, or harmful secondary metabolites, such as hydrogen cyanide, that cause enough damage to the epithelial tissue to disrupt the integrity of the epithelial barrier which allows the translocation to the hemocoel (Figure 1). It needs to be determined whether the Fit toxin contributes to hemocoel invasion in *P. protegens*. A protein toxin of *Pseudomonas taiwanensis* was for example reported to cause damage to intestinal cells when ingested by larvae of the lepidopteran *Plutella xylostella* [17]. A similar mechanism was proposed for an insecticidal toxin (Monalysin) of *P. entomophila* [18]. However, our data predict that *P. protegens* expresses the Fit toxin only in hemolymph (Chapter 2 and 3). Pseudomonads could alternatively also disrupt the integrity of the gut epithelial barrier by promoting an extensive inflammation in the gut, which, however, might take several days to occur. Intracellular pathogens, such as *Salmonella* and *Yersinia*, exploit M cells for transepithelial transport [19]. Since pseudomonads are generally extracellular pathogens and the bacteria in the focus of our research lack the type III secretion system, which seems to be required for such a strategy [20], crossing the physical barrier passively with the help of host cells is rather unlikely for these pseudomonads.

Interestingly, most pathogenic bacteria that infect insects orally are not known to translocate across the epithelial tissue to kill insects via septicemia [11]. The tactic that *P. chlororaphis* and *P. protegens* use to invade the insect host thus might present a novel mechanism of tissue translocation.

According to the current knowledge about the innate immunity of insects, bacteria are recognized by the immune system mostly via detection of their peptidoglycan [7,21,22]. This macromolecule is essential for bacteria to grow and is released into their surroundings during cell division [22]. It might therefore be possible that limited proliferation in the gut is part of the proposed hit-and-run strategy of *P. protegens* to avoid detection and minimize the induction of the systemic immune reactions during the first steps of infection. Cell division could be suppressed or reduced until the bacteria are in the hemocoel where they grow faster than the immune system is able to clear the systemic infection. Alternatively it could be speculated that these bacteria found a way to modify their peptidoglycan structures as a kind of camouflage to reduce its detection by host receptors which has been observed in other bacteria [22-24]. Changes in the

composition of the peptidoglycan backbone could be determined by purification and analysis of the macromolecule via spectrometric methods.

To date, we do not know whether susceptible insect larvae are able to initiate the production of AMPs in the fat body upon infection with *P. protegens*. By conducting an RNA sequencing, real-time quantitative PCR or microarray experiment on the host side, or choosing a proteomics approach, one would get insights into which immune genes are up or downregulated during infection [25] and thus find out if and how the animal host reacts to invading pseudomonads. One might have to include a well-selected microbe or immunostimulant that triggers the induction of the insect immune system as a control in this experiment to see whether *P. protegens* is able to avoid detection or suppress defense reactions. *P. entomophila* was for example reported to cause a translational blockage in *Drosophila*, which inhibits immune responses that would otherwise clear the infection [25]. In case AMPs are synthesized and secreted into the hemolymph, O-antigen and lipid A modifications could play a vital role in the protection of *P. protegens* against these antimicrobial compounds during systemic infection as well.
The biosynthesis of O-antigens and exopolysaccharides, such as alginate, Psl and poly-*N*-acetylglucosamine (PNAG), as well as the Fit toxin could further protect the bacterium against phagocytosis via hemocytes. This could be studied *in vitro* using cell cultures of phagocytic hemocytes and the corresponding mutant strains, optionally equipped with GFP cell tags for observations by fluorescence microscopy.

Identification of additional factors contributing to virulence in these entomopathogens might also give valuable information about how these bacteria interact with the insect immune system and breach the gut epithelial barrier. Possible approaches to do so will be discussed in the following part.

It's on the outside that matters: looking for additional virulence-associated factors on the cell surface

Whole-genome screenings for putative virulence factors often result in a list of genes that are related to the metabolism or cell cycle of the pathogen [26]. It is however at the cell surface where direct microbe-host interactions take place and surface structures are thus particularly interesting to characterize with respect to contributions to virulence. If we better understood how the surface of our bacteria of interest looks like and what role it plays during the infection, we might also better understand how these plant-associated microorganisms evolved into insect pathogens. The fact that gene clusters for the biosynthesis of two surface molecules that have not been associated with *Pseudomonas* before (i.e. PNAG and the putative capsular polysaccharide (Chapter 5)) were detected in this research project without specifically searching for them, tells us that we hardly know what the surface is composed of in these rhizobacteria. If we keep

studying it, we might learn things even beyond the interactions with insects, since surface decoration most probably is also critical for symbiotic interactions with plants.

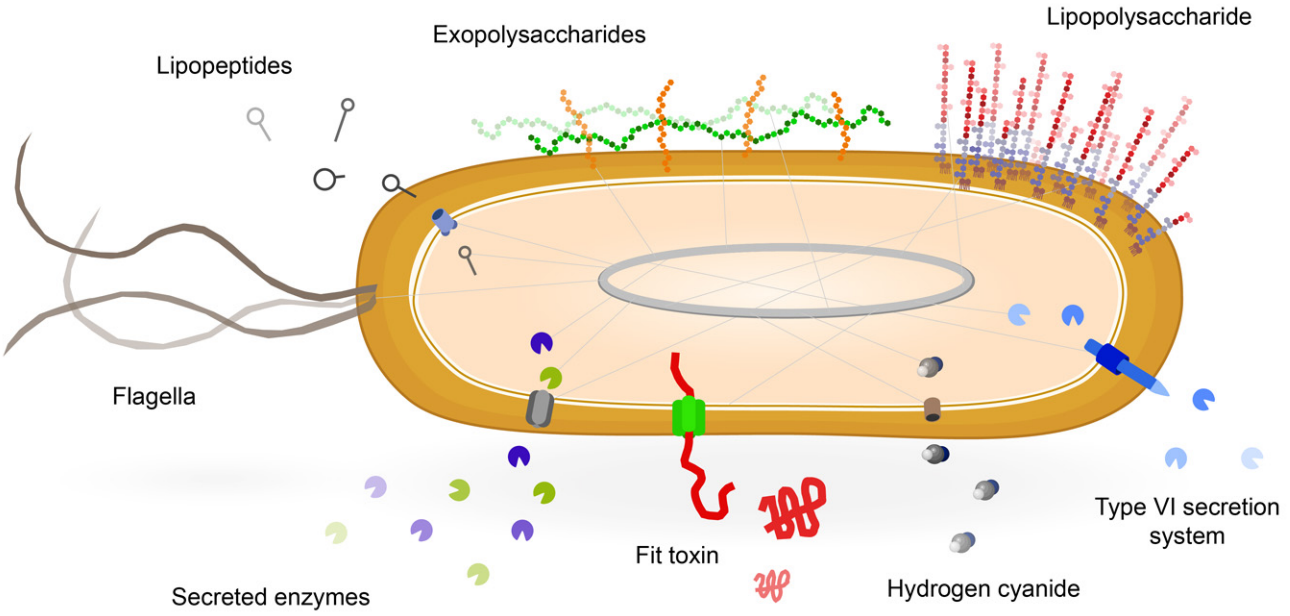

Figure 1: Virulence factors of plant-beneficial pseudomonads displaying insect pathogenicity. The insecticidal toxin Fit, lipopolysaccharide O-antigen, the type VI secretion system[1], hydrogen cyanide[2], lipopeptides[2], and certain secreted enzymes[2] contribute to the virulence of *P. protegens* CHA0 ([1] M. Péchy-Tarr, personal communication; [2] P. Flury *et al.*, in preparation). Flagella and certain exopolysaccharides, such as Pel[1], possibly play an important role during insect infection as well.

The targeted screening for new virulence factors in *P. protegens* and *P. chlororaphis* (Chapter 4) and the follow-up study on O-antigen (Chapter 5) clearly showed that at least one surface component is important for successful infections of insects in these bacteria. There are however many more surface-related candidate virulence factors that remain to be investigated in the future. It would for example be interesting to test whether motility and chemotaxis are necessary for invasion of insects (Figure 1). At this point, it cannot be excluded that also non-motile pseudomonads are capable of causing a systemic infection. It is also imaginable that these bacteria need to actively move to a particular location within the gut (which might require chemotaxis or a kind of tissue tropism) where they are able to breach the epithelial barrier. The genetic background of flagellar biogenesis and chemotaxis in bacteria is well known and this information could be used to generate specific mutants.

We found genes for the biosynthesis of several exopolysaccharides (alginate, Psl, Pel, PNAG, putative capsular polysaccharide, etc.) in the genomes of *P. protegens* and *P. chlororaphis* strains, but lack knowledge about their expression and contribution to virulence. They might play a role during the infection as colonization factor, protection against antimicrobial compounds and phagocytosis, as camouflage for evasion of immune detection, or for competition with the resident microbiota [8,27,28].

It is of course also possible to identify novel virulence factors by conducting a whole-genome random mutagenesis screening and performing RNA sequencing experiments. Such studies allow the identification of factors that contribute to pathogenicity without having necessarily been described as such in other bacteria before. Besides cell surface-related genes it most probably will also detect genes encoding for secreted proteins, which can be as important for virulence as the right surface decoration (Figure 1).

Perception of and adaptation to the insect host

We demonstrated that a local regulatory system, composed of FitF, FitH and FitG, controls Fit toxin expression in a host-specific manner in *P. protegens* CHA0, ensuring that the insecticidal toxin is only produced when needed [29,30]. We can ask ourselves whether *P. protegens* and *P. chlororaphis* also evolved similar regulatory systems to adaptively induce the expression of resistance genes and protect themselves against the attack by AMPs. Such systems have been reported for several pathogenic bacteria. *S. epidermidis* possesses the so-called Aps system to sense AMPs and regulate AMP resistance mechanisms [8,31]. The PhoP-PhoQ system in *Salmonella* was suggested to have a similar function [32]. In *Pseudomonas aeruginosa*, the two-component regulatory system ParR-ParS is attributed to activate the expression of AMP resistance genes while the PhoP-PhoQ system does not appear to play a major role in peptide-mediated adaptive resistance [33,34]. We have some indications from expression studies with sublethal doses of Polymyxin B that *P. protegens* CHA0 induces the expression of lipid A modification genes when exposed to AMPs. It would consequently make sense to search for such regulatory systems in our model bacteria. A possible experiment in this direction would be to conduct a screening with a transposon sequencing approach. Bacteria of a mutant library created by high density random transposon insertion mutagenesis could be exposed to synthetically produced AMPs of a lepidopteran insect species in Grace's Insect Medium. Mutants that display reduced survival could subsequently be identified by transposon sequencing. The advantage of this approach is that also novel resistance determinants can be identified and that it provides quantitative data about their individual contributions to AMP resistance. The challenge will be to produce the AMP for this experiment because this kind of peptides often requires post-translational modifications, such as glycosylation [12,35].

Significance of this research topic

Knowledge about the molecular basis and regulation of insecticidal activities and the host range of these interesting pseudomonads offers valuable information for the development and successful registration of novel bioinsecticides for crop protection. If we can confirm that these bacteria are mostly active against pest insects feeding on above-ground plant parts, development of products based on these entomopathogens should consequently be targeted at these insect species and not root feeders. Our research further could

help to isolate and select optimal strains for achieving a high efficiency in the field, which is a major challenge for the successful registration and commercialization of a new biocontrol product. For example, this might implicate that selected isolates need to survive well in the phyllosphere. Information about how these bacteria persist in the gut and manage to invade the hemocoel might additionally be critical for optimal formulation of these microorganisms. Moreover, this knowledge is of high importance for assessing the biosafety of these bacteria. It is not only important to know about the presence of virulence-associated genes but also when they are expressed in these microbes.

By doing basic research on the insect-associated lifestyles of pseudomonads we can also learn more about insects, a highly successful class of animals. Their enormous diversity has kept us in the dark about their many ways of dealing with pathogens. The insect immune system has almost exclusively been studied in a single species, *Drosophila melanogaster*. Owing to the now available genetic and molecular tools, we are just about to realize the differences in caterpillars and aphids [10,36-38], which are far more relevant to agriculture than the fly model. Knowing about how pest insects protect themselves against pathogens and how these mechanisms evolve will hopefully help us to use entomopathogenic microorganisms more efficiently for biological control of these pests.

Functions of virulence factors (and their regulators) are often conserved across species and even genera. What we discover about virulence determinants in our model bacteria thus could also make significant contributions to medical research. Studying the underlying mechanisms of intrinsic AMP resistance in *P. protegens* could for example be important for the treatment of patients with chronic *Pseudomonas aeruginosa* infections. Polymyxins are widely used as last-hope antibiotics against the human pathogen [39,40]. Development of resistance can, however, also occur against this novel class of antibiotics and a few resistance determinants have already been characterized in *P. aeruginosa* [33]. Our research illustrates that additional factors could be involved, how resistance to these AMPs can evolve in pseudomonads and that one has to be careful with applying polymyxin AMPs for the treatment of bacterial infections because of possible cross-resistance to host AMPs. Basic research on the molecular basis and regulation of AMP resistance and any other factor that contributes to virulence in *P. protegens* and *P. chlororaphis* could thus have impacts beyond a better understanding of the entomopathogenicity of these fascinating bacteria.

References

1. Kupferschmied P, Maurhofer M, Keel C (2013) Promise for plant pest control: root-associated pseudomonads with insecticidal activities. Front Plant Sci 4: 287.
2. Haas D, Défago G (2005) Biological control of soil-borne pathogens by fluorescent pseudomonads. Nat Rev Microbiol 3: 307-319.
3. Berg G (2009) Plant-microbe interactions promoting plant growth and health: perspectives for controlled use of microorganisms in agriculture. Appl Microbiol Biotechnol 84: 11-18.
4. Ruffner B, Péchy-Tarr M, Ryffel F, Hoegger P, Obrist C, et al. (2013) Oral insecticidal activity of plant-associated pseudomonads. Environ Microbiol 15: 751-763.
5. Péchy-Tarr M, Bruck DJ, Maurhofer M, Fischer E, Keel C (2008) Molecular analysis of a novel gene cluster encoding an insect toxin in plant-associated strains of *Pseudomonas fluorescens*. Environ Microbiol 10: 2368-2386.
6. Rolff J, Reynolds SE (2009) Insect Infection and Immunity: Evolution, Ecology, and Mechanisms; Rolff J, Reynolds S, editors. Oxford: Oxford University Press.
7. Lemaitre B, Hoffmann J (2007) The host defense of *Drosophila melanogaster*. Annu Rev Immunol 25: 697-743.
8. Otto M (2009) *Staphylococcus epidermidis* - the 'accidental' pathogen. Nat Rev Microbiol 7: 555-567.
9. Zhang S, Gunaratna RT, Zhang X, Najar F, Wang Y, et al. (2011) Pyrosequencing-based expression profiling and identification of differentially regulated genes from *Manduca sexta*, a lepidopteran model insect. Insect Biochem Mol Biol 41: 733-746.
10. Wu S, Zhang X, He Y, Shuai J, Chen X, et al. (2010) Expression of antimicrobial peptide genes in *Bombyx mori* gut modulated by oral bacterial infection and development. Dev Comp Immunol 34: 1191-1198.
11. Vallet-Gely I, Lemaitre B, Boccard F (2008) Bacterial strategies to overcome insect defences. Nat Rev Microbiol 6: 302-313.
12. Bulet P, Stöcklin R (2005) Insect antimicrobial peptides: structures, properties and gene regulation. Protein Pept Lett 12: 3-11.
13. Farr SB, Kogoma T (1991) Oxidative stress responses in *Escherichia coli* and *Salmonella typhimurium*. Microbiol Rev 55: 561-585.
14. Dieppois G, Opota O, Lalucat J, Lemaitre B (2015) *Pseudomonas entomophila*: a versatile bacterium with entomopathogenic properties. In: Ramos JL, Goldberg JB, Filloux A, editors. *Pseudomonas*: New Aspects of *Pseudomonas* Biology. Heidelberg: Springer.
15. Hegedus D, Erlandson M, Gillott C, Toprak U (2009) New insights into peritrophic matrix synthesis, architecture, and function. Annu Rev Entomol 54: 285-302.
16. Buchon N, Broderick NA, Lemaitre B (2013) Gut homeostasis in a microbial world: insights from *Drosophila melanogaster*. Nat Rev Microbiol 11: 615-626.
17. Chen WJ, Hsieh FC, Hsu FC, Tasy YF, Liu JR, et al. (2014) Characterization of an insecticidal toxin and pathogenicity of *Pseudomonas taiwanensis* against insects. PLoS Pathog 10: e1004288.
18. Opota O, Vallet-Gely I, Vincentelli R, Kellenberger C, Iacovache I, et al. (2011) Monalysin, a novel beta-pore-forming toxin from the *Drosophila* pathogen *Pseudomonas entomophila*, contributes to host intestinal damage and lethality. PLoS Pathog 7: e1002259.
19. Neutra MR, Frey A, Kraehenbuhl JP (1996) Epithelial M cells: gateways for mucosal infection and immunization. Cell 86: 345-348.
20. Ham H, Sreelatha A, Orth K (2011) Manipulation of host membranes by bacterial effectors. Nat Rev Microbiol 9: 635-646.
21. Leulier F, Parquet C, Pili-Floury S, Ryu JH, Caroff M, et al. (2003) The *Drosophila* immune system detects bacteria through specific peptidoglycan recognition. Nat Immunol 4: 478-484.
22. Mengin-Lecreulx D, Lemaitre B (2005) Structure and metabolism of peptidoglycan and molecular requirements allowing its detection by the *Drosophila* innate immune system. J Endotoxin Res 11: 105-111.
23. Davis KM, Weiser JN (2011) Modifications to the peptidoglycan backbone help bacteria to establish infection. Infect Immun 79: 562-570.

24. Boneca IG, Dussurget O, Cabanes D, Nahori MA, Sousa S, et al. (2007) A critical role for peptidoglycan N-deacetylation in *Listeria* evasion from the host innate immune system. Proc Natl Acad Sci U S A 104: 997-1002.
25. Chakrabarti S, Liehl P, Buchon N, Lemaitre B (2012) Infection-induced host translational blockage inhibits immune responses and epithelial renewal in the *Drosophila* gut. Cell Host Microbe 12: 60-70.
26. Turner KH, Everett J, Trivedi U, Rumbaugh KP, Whiteley M (2014) Requirements for *Pseudomonas aeruginosa* acute burn and chronic surgical wound infection. PLoS Genet 10: e1004518.
27. Franklin MJ, Nivens DE, Weadge JT, Howell PL (2011) Biosynthesis of the *Pseudomonas aeruginosa* extracellular polysaccharides, alginate, Pel, and Psl. Front Microbiol 2: 167.
28. Ghafoor A, Hay ID, Rehm BH (2011) Role of exopolysaccharides in *Pseudomonas aeruginosa* biofilm formation and architecture. Appl Environ Microbiol 77: 5238-5246.
29. Kupferschmied P, Péchy-Tarr M, Imperiali N, Maurhofer M, Keel C (2014) Domain shuffling in a sensor protein contributed to the evolution of insect pathogenicity in plant-beneficial *Pseudomonas protegens*. PLoS Pathog 10: e1003964.
30. Péchy-Tarr M, Borel N, Kupferschmied P, Turner V, Binggeli O, et al. (2013) Control and host-dependent activation of insect toxin expression in a root-associated biocontrol pseudomonad. Environ Microbiol 15: 736-750.
31. Li M, Lai Y, Villaruz AE, Cha DJ, Sturdevant DE, et al. (2007) Gram-positive three-component antimicrobial peptide-sensing system. Proc Natl Acad Sci U S A 104: 9469-9474.
32. Bader MW, Sanowar S, Daley ME, Schneider AR, Cho U, et al. (2005) Recognition of antimicrobial peptides by a bacterial sensor kinase. Cell 122: 461-472.
33. Fernandez L, Gooderham WJ, Bains M, McPhee JB, Wiegand I, et al. (2010) Adaptive resistance to the "last hope" antibiotics polymyxin B and colistin in *Pseudomonas aeruginosa* is mediated by the novel two-component regulatory system ParR-ParS. Antimicrob Agents Chemother 54: 3372-3382.
34. McPhee JB, Lewenza S, Hancock RE (2003) Cationic antimicrobial peptides activate a two-component regulatory system, PmrA-PmrB, that regulates resistance to polymyxin B and cationic antimicrobial peptides in *Pseudomonas aeruginosa*. Mol Microbiol 50: 205-217.
35. Anaya-López JL, López-Meza JE, Ochoa-Zarzosa A (2013) Bacterial resistance to cationic antimicrobial peptides. Crit Rev Microbiol 39: 180-195.
36. International Aphid Genomics Consortium (2010) Genome sequence of the pea aphid *Acyrthosiphon pisum*. PLoS Biol 8: e1000313.
37. He Y, Cao X, Li K, Hu Y, Chen YR, et al. (2015) A genome-wide analysis of antimicrobial effector genes and their transcription patterns in *Manduca sexta*. Insect Biochem Mol Biol: 10.1016/j.ibmb.2015.1001.1015.
38. Johnston PR, Makarova O, Rolff J (2014) Inducible defenses stay up late: temporal patterns of immune gene expression in *Tenebrio molitor*. G3 (Bethesda) 4: 947-955.
39. Olaitan AO, Morand S, Rolain JM (2014) Mechanisms of polymyxin resistance: acquired and intrinsic resistance in bacteria. Front Microbiol 5: 643.
40. Landman D, Georgescu C, Martin DA, Quale J (2008) Polymyxins revisited. Clin Microbiol Rev 21: 449-465.

Curriculum vitae

Peter Kupferschmied

Born 1st May 1986, Citizen of Eggiwil (BE), Switzerland

Education

PhD in Life Sciences, University of Lausanne	2011 to 2015
Master of Science in Biology (*summa cum laude*), ETH Zurich	2009 to 2011
Bachelor of Science in Biology, ETH Zurich	2006 to 2009
Grammar school, KZU Bülach	1999 to 2005
Primary school, Bülach	1993 to 1999

Professional experience

Research assistant, University of Lausanne	2011 to present
Technical assistant, FMS Force Measuring Systems AG	2006 to 2011
Teaching assistant, ETH Zurich	2008 to 2010

Publications

Kupferschmied P, Péchy-Tarr M, Imperiali N, Maurhofer M, Keel C (2014) Domain shuffling in a sensor protein contributed to the evolution of insect pathogenicity in plant-beneficial P*seudomonas protegens*. PLoS Pathogens. 10: e1003964.

Kupferschmied P, Keel C (2014) Insecticidal activity of plant root-associated bacteria. pp. 52-58 in Bertrand C (ed), Natural Products and Biocontrol. Presses Universitaires de Perpignan. ISBN 978-2-35412-234-8.

Kupferschmied P, Maurhofer M, Keel C (2013) Promise for plant pest control: root-associated pseudomonads with insecticidal activities. Frontiers in Plant Science. 4: 287.

Péchy-Tarr M, Borel N, **Kupferschmied P**, Turner V, Binggeli O, Radovanovic D, Maurhofer M, Keel C (2013) Control and host-dependent activation of insect toxin expression in a root-associated biocontrol pseudomonad. Environmental Microbiology. 15: 736-750.

Dengler V, Meier PS, Heusser R, **Kupferschmied P**, Fazekas J, Friebe S, Staufer SB, Majcherczyk PA, Moreillon P, Berger-Bächi B, McCallum N (2012) Deletion of hypothetical wall teichoic acid ligases in *Staphylococcus aureus* activates the cell wall stress response. FEMS Microbiology Letters. 333:109-20.

Over B, Heusser R, McCallum N, Schulthess B, **Kupferschmied P**, Gaiani JM, Sifri CD, Berger-Bächi B, Stutzmann Meier P (2011) LytR-CpsA-Psr proteins in *Staphylococcus aureus* display partial functional redundancy and the deletion of all three severely impairs septum placement and cell separation. FEMS Microbiology Letters. 320:142-51.

Conferences and Presentations

2011 (4)

D.Day 2011 (28.04.2011, UNIL, Lausanne, Switzerland)

Poster: "Molecular basis and regulation of insecticidal activity in plant root-associated pseudomonads"; receiving the Best Poster Award by the *Société vaudoise des sciences naturelles* (SVSN)

FEMS Congress 2011 (26.-30.06.2011, Geneva, Switzerland)

Participation without contribution

CUSO Annual Retreat 2011 (8.-9.09.2011, Villars-sur-Ollon, Switzerland)

Poster & oral presentation: "When *Pseudomonas fluorescens* CHA0 launches its insect toxin production"

DMF Impromptu (04.11.2011, UNIL, Lausanne, Switzerland)

Participation without contribution

2012 (10)

DMF Friday Seminar (10.02.2012, UNIL, Lausanne, Switzerland)

Oral presentation: "When *Pseudomonas fluorescens* CHA0 launches its insect toxin production"

Spring Meeting of the Swiss Society of Phytiatry (08.03.2012; Basel, Switzerland)

Oral presentation: "Geschichten aus dem Untergrund: Wie nützliche Bakterien mit Pflanzen, Pathogenen und Schädlingen interagieren" (presentation for a broad audience)

D.Day 2012 (19.04.2012, UNIL, Lausanne, Switzerland)

Poster: "When *Pseudomonas fluorescens* CHA0 launches its insect toxin production"

Swiss Society of Microbiology Meeting 2012 (21.-22.06.2012, St. Gallen, Switzerland)

Oral presentation & poster: "When *Pseudomonas fluorescens* CHA0 launches its insect toxin production"

International congress on invertebrate pathology and microbial control 2012 (05.-09.08.2012, Buenos Aires, Argentina)

Oral presentation: "Insecticidal Activity of Plant Root-Associated Pseudomonads: Host-Specific Expression of the Fit Insect Toxin"

CUSO Annual Retreat 2012 (05.-06.09.2012, Villars-sur-Ollon, Switzerland)

Short oral presentation & poster: "Fancy Microscopy for Fancy Bacteria: Visualization and Study of a Toxin at the Protein and Single Cell Level"

SWIMM Symposium 2012 (06.-07.09.2012, Villars-sur-Ollon, Switzerland)

Oral presentation & poster: "Fancy Microscopy for Fancy Bacteria: Visualization and Study of a Toxin at the Protein and Single Cell Level"

Natural Products and Biocontrol 2012 (19.-21.09.2012, Perpignan, France)

Oral presentation: “Insecticidal Activity of Plant Root-Associated Bacteria: Host-Specific Expression of the Fit Insect Toxin” (presentation for a broad audience)

IMUL-DMF Joint Seminar (05.10.2012, CHUV, Lausanne, Switzerland)

Oral presentation: “Why start from scratch? The role and evolution of an insect toxin-regulating sensor protein”

DMF Impromptu (15.11.2012, UNIL, Lausanne, Switzerland)

Participation without contribution

2013 (4)

DMF Friday Seminar (12.04.2013, UNIL, Lausanne, Switzerland)

Oral presentation: “Evolution is cleverer than you are - The story of the sensor protein FitF”

14th International Conference on Pseudomonas (7.-11.09.2013, Lausanne, Switzerland)

Poster: “Evolution of a sensor protein: domain shuffling for tight control of toxin expression in *Pseudomonas protegens*”

Autumn Meeting of the Swiss Society of Phytiatry (26.09.2013, Changins, Switzerland)

Poster: “Wurzelassoziierte Pseudomonaden mit toxischer Aktivität gegenüber Insekten”; awarded with a poster prize from the society

DMF Impromptu (29.11.2013, UNIL, Lausanne, Switzerland)

Participation without contribution

2014 (2)

International congress on invertebrate pathology and microbial control 2014 (03.-08.08.2014, Mainz, Germany)

Oral presentation: “Evolution of a Sensor Protein Controlling Production of an Insecticidal Toxin in Plant-Beneficial *Pseudomonas protegens*”; awarded with the SIP Student Travel Award

DMF Impromptu (07.11.2014, UNIL, Lausanne, Switzerland)

Participation without contribution

2015 (5)

Spring Meeting of the Swiss Society of Phytiatry (12.02.2015, Zurich, Switzerland)

Participation without contribution

DMF Friday Seminar (17.04.2015, UNIL, Lausanne, Switzerland)

Oral presentation: “Live and Let Die: How Do Plant-Beneficial Pseudomonads Infect and Kill Insects?”

Swiss Society of Microbiology Meeting 2015 (28.-29.05.2015, Lugano, Switzerland)

Oral presentation / poster: “The sweetest trick: O-polysaccharides protect plant-beneficial pseudomonads against antimicrobial peptides during insect infection”

PGPR Workshop 2015 (16.-19.06.2015, Liège, Belgium)

Oral presentation: “The sweetest trick: O-polysaccharides protect plant-beneficial pseudomonads against antimicrobial peptides during insect infection”

DMF-IMUL'sion (23.06.2015, Renens, Switzerland)

Workshop participation