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1 **A look into the toolbox of multi-talents: insect pathogenicity determinants of plant-beneficial**

2 **pseudomonads**

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5 Christoph Keel*

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7 Department of Fundamental Microbiology, University of Lausanne, Lausanne, Switzerland

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13 *For correspondence. Dr. Christoph Keel, Department of Fundamental Microbiology, Biophore

14 Building, Room 2310, University of Lausanne, CH-1015 Lausanne, Switzerland; E-mail

15 christoph.keel@unil.ch; Tel. +41 21 692 56 36; Fax +41 21 692 56 05.

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19 *Running Title:* Insect pathogenicity in plant-beneficial pseudomonads

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22 The *Pseudomonas fluorescens* group of bacteria is very diverse and comprises members that make part

23 of the beneficial rhizosphere microbiota that cooperates with the plant (Mendes *et al.*, 2013; Venturi

24 and Keel, 2016). Plant-beneficial activities of rhizosphere pseudomonads have been investigated since

25 several decades. They include stimulation of plant growth and defense, mobilization of soil nutrients,

26 and suppression of phytopathogenic fungi, protists and bacteria via antimicrobial compounds (Haas

27 and Défago, 2005). Recent research identified a phylogenetically distinct *P. fluorescens* subgroup,

28 specified by strains of *Pseudomonas protegens* and *Pseudomonas chlororaphis*, which exhibits potent

29 insecticidal activities as an extra (Péchy-Tarr *et al.*, 2008; Olcott *et al.*, 2010; Ruffner *et al.*, 2015;

30 Flury *et al.*, 2016). According to current knowledge, the insecticidal strains are capable of colonizing

31 and killing representatives of three major insect orders, i.e. Lepidoptera, Diptera and Hemiptera,

32 including several agricultural pests, following oral uptake (Olcott *et al.*, 2010; Kupferschmied *et al.*,

33 2013; Ruffner *et al.*, 2013; Flury *et al.*, 2016). In a typical course of infection, ingested

34 entomopathogenic pseudomonads colonize the gut, breach the intestinal epithelial barrier, invade the

35 hemocoel, proliferate, and eventually kill the insect (Kupferschmied *et al.*, 2013). While the molecular

36 mechanisms involved in plant-beneficial activities of these pseudomonads have been dissected in some

37 detail (Haas and Défago, 2005; Gross and Loper, 2009; Kupferschmied *et al.*, 2013), the virulence

38 factors and mechanisms contributing to their capacity to invade and kill insects currently are largely

39 unknown. A major insect virulence factor identified so far is the Fit toxin, which typically is produced

40 by strains of *P. protegens* and *P. chlororaphis* and is significant for their pathogenicity towards

41 Lepidopteran larvae (Péchy-Tarr *et al.*, 2008; Ruffner et al., 2013; Flury *et al.*, 2016). Production of

42 the insecticidal protein is specifically switched on in the insects, but not on plant roots (Péchy-Tarr *et*

43 *al.*, 2013; Kupferschmied *et al.*, 2014). Mutants lacking the Fit toxin retain substantial toxicity,

44 stressing that insect pathogenicity of these bacteria is multifactorial.

45   In this issue of Environmental Microbiology, Loper *et al.* (2016) report on the identification of

46 additional insect pathogenicity factors of a representative insecticidal pseudomonad, i.e. *P. protegens*

47 Pf-5, in an oral infection model. Their approach was inspired by the observation that *P. protegens*

48 mutants defective for the global regulator GacA are severely impaired in oral toxicity to Dipteran and

49 Lepidopteran insects (Olcott *et al.*, 2010; Ruffner *et al.*, 2013; Flury *et al.*, 2016). GacA is known to

50 positively control various traits contributing to beneficial and pathogenic activities of pseudomonads

51 (Haas and Défago, 2005). In their study, Loper *et al.* (2016) focused on GacA-controlled toxic and

52 lytic exoproducts of *P. protegens* and investigated their contribution to oral toxicity towards

53 *Drosophila melanogaster* using directed mutational analysis. Several of their findings significantly

54 expand our knowledge about the insect pathogenicity determinants contained in the rich toolbox of

55 these multi-talented bacteria (Figure 1). Loper *et al.* (2016) identified rhizoxin as a major factor in oral

56 toxicity of *P. protegens* Pf-5 towards *Drosophila*. The macrolide molecule interferes with mitosis in

57 eukaryotic cells, which enables broad antifungal, cytotoxic, and phytotoxic activities, and it has

58 demonstrated function in plant pathogen suppression (Loper *et al.*, 2008). Insect toxicity now emerges

59 as an additional feature of rhizoxin, illustrating that *P. protegens* can deploy certain toxic secondary

60 metabolites for plant-beneficial as well as for insect-pathogenic activities. Remarkably, the rhizoxin

61 gene cluster is only present in a subset of *P. protegens* strains and linked to the Fit toxin gene cluster,

62 and both clusters are part of a dynamic genomic region, which likely evolved via events involving

63 horizontal transfer (Ruffner *et al.*, 2015; Loper *et al.*, 2016). It remains to be determined whether

64 acquisition of the rhizoxin cluster promotes the insecticidal efficacy of these pseudomonads, e.g., by

65 extending their host range or by conferring increased toxicity.

66 Orfamide is another toxic metabolite that contributes to plant-beneficial (Ma *et al.*, 2016) as well

67 as insecticidal activities of *P. protegens* (Loper *et al.*, 2016), and there is evidence that this and other

68 lipopeptides have activity against representatives of different insect orders (Kupferschmied *et al.*,

69 2013; P. Flury *et al.*, unpublished). In the study of Loper *et al.* (2016), none of the known other *P.*

70 *protegens* metabolites having broad-spectrum toxicity (i.e. 2,4-diacetylphloroglucinol, pyoluteorin,

71 pyrrolnitrin, hydrogen cyanide, and toxoflavin) and a role in pathogen suppression (Gross and Loper,

72   2009) appeared to be involved in oral toxicity to *Drosophila*. More surprisingly, no role was observed

73   neither for the insecticidal Fit toxin, identified as an important virulence factor in Lepidoptera. Future

74   work should therefore investigate whether *P. protegens* activates specific subsets of its toxic

75   exoproducts during infection of different insect species and beneficial interaction with plants, and

76   regulates their biosynthesis accordingly. Varied expression levels in different insects and tight

77   regulation have recently been observed for the Fit toxin (Kupferschmied *et al.*, 2014). Different insect

78   species might also be differentially sensitive to the exoproducts of insecticidal pseudomonads.

79   Besides the toxic exoproducts, chitinase was required for full pathogenicity of *P. protegens*

80   towards *Drosophila* (Loper *et al.*, 2016). Chitinase, along with another exoenzyme (phospholipase),

81   was previously identified as an oral pathogenicity determinant towards lepidopteran insects, in a

82   comparative genomic analysis of *P. fluorescens* group bacteria, yielding a set of almost 200 genes that

83   are common and unique to insecticidal strains (Flury *et al.*, 2016). The lytic exoproduct is thought to

84   help the bacteria breach the peritrophic matrix, a chitinous layer that protects epithelial cells lining the

85   insect gut and restricts access of bacterial invaders to the hemocoel. Following ingestion, invading

86   pseudomonads have to cope not only with this physical barrier, but moreover with competition from

87   the resident gut microbiota and insect immune defences, including production of reactive oxygen

88   species, antimicrobial peptides, and phagocytic hemocytes (Buchon *et al.*, 2013; Engel and Moran,

89   2015). Recent research indicates that besides toxic and lytic exoproducts, certain cell surface

90   components, notably specific lipopolysaccharide O-antigen decorations, may help protect insecticidal

91   pseudomonads against the immune response of the insect and thus promote their virulence

92   (Kupferschmied *et al.*, submitted). Gut colonization in presence of the resident microbiota may require

93   type VI secretion-mediated competition (Cianfanelli *et al.*, 2016; Péchy-Tarr *et al.*, unpublished).

94   Loper *et al.* (2016) together with Flury *et al.* (2016) provide first detailed insights into the

95   molecular equipment that enables certain plant-beneficial pseudomonads to function also as insect

96   pathogens. There are still numerous questions for future work. How precisely do these bacteria use

97 and control their insecticidal equipment during the different stages of interaction with contrasting

98 insect hosts? How did they acquire this equipment? How widespread are these particular bacteria in

99 nature, which niches do they occupy and what is the ecological relevance of their insect-related

100 activities? Are they simply opportunists that due to their versatile equipment take advantage of any

101 nutrient source (plant, insect, etc.) offered, in a highly competitive manner, or do they follow specific

102 strategies, e.g., by exploiting insects as containers to persist, multiply, and travel (or feed on) as it has

103 been observed for various plant pathogens. Potent insecticidal activity occurs also in certain other

104 environmental pseudomonads, notably within the *Pseudomonas putida* (Opota *et al.*, 2011; Chen *et*

105 *al.*, 2014), *Pseudomonas syringae* (Nadarasah and Stavrinides, 2011) and *Pseudomonas aeruginosa*

106 (Kupferschmied *et al.*, 2013) groups, and it may be rewarding to compare strategies and toolboxes they

107 use. In a longer-term perspective, specific strains of these competitive plant colonizers displaying

108 activities both against plant pathogens and against herbivorous insects could be developed for

109 biotechnological applications such as control of agricultural pests.

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173   **Fig. 1. Insect pathogenicity determinants of plant-beneficial pseudomonads.** The insecticidal toxin

174   Fit, lipopeptides, certain secreted enzymes and the toxic metabolite rhizoxin contribute to the virulence

175   of entomopathogenic pseudomonads following oral infection of Lepidopteran or Dipteran insects. Cell

176   surface-exposed components such as lipopolysaccharide O-antigen and certain exopolysaccharides,

177   and the type VI secretion apparatus and effectors are suspected to play an important role during insect

178   infection as well. Virulence factors so far were mainly studied in model strains of *Pseudomonas*

179   *protegens* (CHA0 and Pf-5) and *Pseudomonas chlororaphis* (PCL1391).

Lipopeptides
(orfamide)

Exopolysaccharides

O antigen polysaccharides
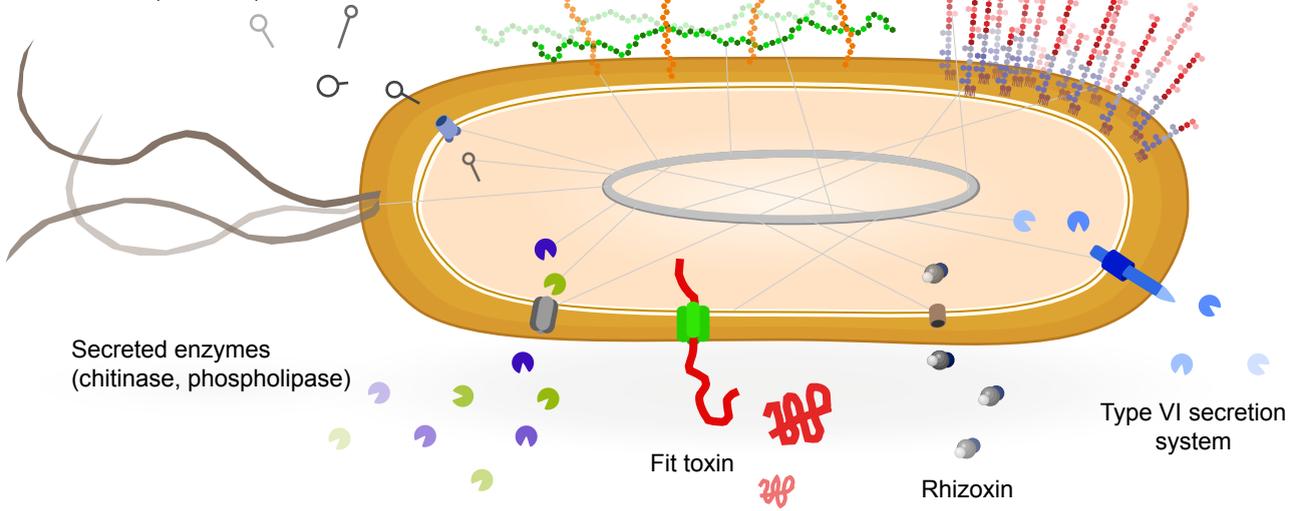
Secreted enzymes
(chitinase, phospholipase)

Fit toxin

Rhizoxin

Type VI secretion
system