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FEMS Microbiology Ecology 45 (2003) 71–81

FEMS MICROBIOLOGY Ecology

www.fems-microbiology.org

Degradation of pathogen quorum-sensing molecules by soil bacteria: a preventive and curative biological control mechanism

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Received 19 December 2002; received in revised form 6 April 2003; accepted 14 April 2003

First published online 17 May 2003

Abstract

The plasmid pME6863, carrying the *aiiA* gene from the soil bacterium *Bacillus* sp. A24 that encodes a lactonase enzyme able to degrade *N*-acyl-homoserine lactones (AHLs), was introduced into the rhizosphere isolate *Pseudomonas fluorescens* P3. This strain is not an effective biological control agent against plant pathogens. The transformant *P. fluorescens* P3/pME6863 acquired the ability to degrade AHLs. In planta, *P. fluorescens* P3/pME6863 significantly reduced potato soft rot caused by *Erwinia carotovora* and crown gall of tomato caused by *Agrobacterium tumefaciens* to a similar level as *Bacillus* sp. A24. Little or no disease reduction was observed for the wild-type strain P3 carrying the vector plasmid without *aiiA*. Suppression of potato soft rot was observed even when the AHL-degrading *P. fluorescens* P3/pME6863 was applied to tubers 2 days after the pathogen, indicating that biocontrol was not only preventive but also curative. When antagonists were applied individually with the bacterial plant pathogens, biocontrol activity of the AHL degraders was greater than that observed with several *Pseudomonas* 2,4-diacetylphloroglucinol-producing strains and with *Pseudomonas chlororaphis* PCL1391, which relies on production of phenazine antibiotic for disease suppression. Phenazine production by this well characterized biological control strain *P. chlororaphis* PCL1391 is regulated by AHL-mediated quorum sensing. When *P. chlororaphis* PCL1391 was co-inoculated with *P. fluorescens* P3/pME6863 in a strain mixture, the AHL degrader interfered with the normally excellent ability of the antibiotic producer to suppress tomato vascular wilt caused by *Fusarium oxysporum* f. sp. *lycopersici*. Our results demonstrate AHL degradation as a novel biocontrol mechanism, but also demonstrate the potential for non-target interactions that can interfere with the biocontrol efficacy of other strains.

Keywords: Autoinducer; Biocontrol; Microbial signaling; Quorum sensing; *Chromobacterium violaceum*; *Erwinia carotovora*

1. Introduction

Many Gram-negative bacteria utilize autoinducers such as *N*-acyl homoserine lactones (AHLs) to coordinate gene expression in a population density-dependent manner, a process referred to as quorum sensing. At low population densities, cells produce a basal level of AHL via the activity of AHL synthase. As cell density increases, AHLs accumulate in the growth environment. When a critical threshold concentration is reached, the AHL molecule diffuses into the cell and binds to its cognate receptor, which in turn activates or represses the coordinated expression of particular sets of genes that enhance the ecological competence of the bacterium [1]. In animal and plant pathogenic bacteria, AHLs regulate genes critical for dissemination and virulence. Important examples of factors that are regulated include bioluminescence in *Vibrio* species [2,3], conjugal transfer of the Ti plasmid in *Agrobacterium tumefaciens* [4–6], swarming motility in *Serratia liquefaciens* [7], diverse enzymes and toxins in *Burkholderia cepacia* [8], *Erwinia carotovora* [9,10], *E. chrysanthemi* [11], *Pantoea stewartii* [12], *Pseudomonas aeruginosa* [13], and *Xenorhab-*

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0168-6496/03/$22.00
doi:10.1016/S0168-6496(03)00125-9

dus nematophilus [14]. AHLs also play a role in the formation by *Aeromonas hydrophila* [15], *B. cepacia* [16,17] and *P. aeruginosa* [18] of biofilms that complicate disinfestation efforts.

Given the pivotal role quorum sensing plays in pathogenic interactions [19,20], inactivation or degradation of AHLs presents an attractive target for designing innovative disease control strategies. Several plant and microbial compounds have been reported to have such activity against AHLs. Halogenated furanones from the marine red alga, *Delisea pulchra*, inhibit luminescence and virulence in *Vibrio harveyi* by binding competitively to the AHL receptor protein, the LuxR transcription factor [21]. Antimicrobial triclosans suppress AHL biosynthesis by inhibiting the reaction catalyzed by the enoyl-acyl carrier protein reductase [22]. Pea and crown vetch seedlings have been reported to exude as yet uncharacterized substances that mimic bacterial AHLs and interfere with AHL-induced synthesis of violacein in the reporter bacterium, *Chromobacterium violaceum* [23]. Recently, two bacterial proteins that degrade AHLs have been described. In soil isolates of *Bacillus* species, AHL-lactonase encoded by the *aiiA* gene has been identified [24–27]. Another soil bacterium, *Variovorax paradoxus*, has been identified which neutralizes AHLs by cleaving the fatty acid tail and mineralizing the homoserine lactone ring, presumably via production of an aminoacylase [28].

The first application of disrupting quorum sensing for the purpose of disease control was the introduction of the *aiiA* gene cloned from *Bacillus* sp. into transgenic tobacco and potato plants [24]. Expression of this gene and production of AHL-lactonase by the genetically modified plants paralyzed quorum-sensing systems of the phytopathogenic bacterium, *E. carotovora*, resulting in increased plant disease resistance. Another approach has been to construct transgenic tobacco lines that express the *E. carotovora* AHL gene, *expI* [29]. Ectopic production of bacterial AHL by the transgenic plants tricks the pathogen into prematurely secreting virulence factors, such as pectinolytic enzymes, when cell populations are insufficient for infection. This is thought to trigger host plant defenses resulting in the observed disease resistance. Although apparently effective, there is little hope for commercial deployment of such transgenic plants in the near future because of prevailing public opposition in Europe and elsewhere to genetically engineered crops. A more acceptable strategy in the near term would be the application of microorganisms with natural ability to degrade AHLs.

The main objective of our work was to evaluate the efficacy of microbial-based AHL degradation for biological control of *E. carotovora* and other plant pathogens. We developed a transgenic microbial model to demonstrate the specific role for AHL degradation in disease suppression. We then demonstrated that a wild-type isolate of *Bacillus* sp. with AHL degrading ability offers significant disease protection to potato and tomato.

AHLs are also critical regulatory elements in the biosynthesis of antimicrobial compounds (e.g. antibiotics) by plant-beneficial non-pathogenic *Pseudomonas chlororaphis* (syn. *P. aureofaciens*) applied in agriculture for suppression of plant diseases [30]. Blocking the AHL signal abolishes much of the biocontrol activity of these strains [31]. A further objective of our work was to investigate the potential non-target impact of AHL degradation by our strains on another biocontrol agent applied in a strain mixture.

2. Materials and methods

2.1. Microorganisms and culture conditions

The bacterial and fungal strains used in this work are described in Table 1. *A. tumefaciens*, *Bacillus* sp. A24, *C. violaceum* CV026 [32] and *E. carotovora* were routinely grown on Luria–Bertani (LB) agar [33] or Difco nutrient agar (Becton Dickinson, Sparks, MD, USA). *Pseudomonas* strains were routinely grown on LB agar or King's medium B (KB) agar [34]. *F. oxysporum* f. sp. *lycopersici* was grown in 2% malt extract broth (w/v) (Oxoid, Basingstoke, Hampshire, UK).

2.2. In vitro assay for degradation of synthetic homoserine lactone

Degradation by cell lysates was determined using overnight cultures of *P. fluorescens* strains P3/pME6000 and P3/pME6863 grown at 30°C in 200 ml of nutrient-broth yeast-extract media [35]. Cultures were centrifuged and washed once at 4°C and 10 000 rpm for 10 min. Cell pellets were resuspended in 2 ml of CL buffer [potassium phosphate buffer (pH 7), 10 mM $MgCl_2$, 1 mM dithiothreitol and 10% glycerol (v/v)]. Cells were lysed with six cycles of sonication for 20 s each. After centrifugation, the supernatant containing cell lysates was collected and 100 μl was added to Eppendorf tubes containing 890 μl CL buffer and 10 μl of 20 mM *N*-butyryl-L-homoserine lactone (BHL). CL buffer with 10 μM BHL without added cell lysates served as a negative control. Tubes were incubated for 2 h at 30°C. Suspensions were adjusted to pH 5 and BHL was extracted with dichloromethane. The quantity of BHL remaining in suspensions was evaluated using thin-layer chromatography on a Merck TLC silica gel RP-C18 plate eluted with methanol:water (60:40, v/v). The presence of BHL was determined by overlaying plates with the AHL biosensor *C. violaceum* CV026, as previously described [32].

2.3. Bioassay for interference of AHL-dependent gene regulation in C. violaceum CV026

The AHL biosensor *C. violaceum* CV026 was streaked

in a line on plates of LB agar. The AHL donor *E. carotovora* was applied in spots 16–17 mm from the *C. violaceum* CV026 line. Test bacteria were spotted in between the biosensor and the AHL donor at a distance 6–7 mm from the *C. violaceum* CV026 line. Plates were incubated at 28°C for 2 days. Migration of AHL from the donor *E. carotovora* was confirmed by production of the purple-pigmented antibiotic violacein in the biosensor.

2.4. *In vitro assay to demonstrate that AHL produced by* E. carotovora *traverses the membrane of* P. fluorescens

Overnight cultures of *E. carotovora* grown at 27°C in 200 ml of LB medium were centrifuged at 4°C and 10 000 rpm for 10 min. The supernatant fraction was filtered to remove cells and AHLs were extracted as described previously [32]. The total extracted *E. carotovora* AHLs were added to 200 ml LB broth inoculated with *P. fluorescens* P3/pME6000, *P. fluorescens* P3/pME6863 or *Bacillus* A24. Controls consisted of AHLs added to non-inoculated LB. Cultures were incubated overnight, centrifuged, and AHLs extracted from the supernatant as described above. In addition, AHL that had entered cells was determined by washing cell pellets three times in 20 ml of potassium phosphate buffer (25 mM, pH 7), lysing cells with six cycles of sonication lasting 20 s each, centrifuging and then extracting AHLs from the supernatant containing cell lysates [32].

2.5. *Glucose 6-phosphate dehydrogenase assay*

The cytoplasmic marker enzyme glucose 6-phosphate dehydrogenase (EC 1.1.1.43) assay [36] is based on the reduction of NADP (nicotinamide adenine dinucleotide phosphate) to NADPH. The reaction mixture contained 10 mM $MgCl_2$, 1 mM glucose 6-phosphate, 0.4 mM NADP, 33.3 mM Tris–HCl, pH 7.5 and an appropriate volume of culture supernatant or cell extract, equivalent to 0.05–5 ml of culture. The reaction was carried out at room temperature and initiated by the addition of substrate. NADPH formation was monitored at OD_{340} for 5–60 min. One unit of activity was measured as an increase of 0.001 per min. Background dehydrogenase activity measured in the absence of added substrate was subtracted in each case.

2.6. *Bioassay for interference of AHL-dependent gene regulation in* E. carotovora

Overnight LB cultures with or without the addition of different concentrations of Fe_3Cl (10 or 20 μM) of *E. carotovora* grown alone or in combination with test strain *P. fluorescens* P3/pME6000 or *P. fluorescens* P3/pME6863 were spotted onto plates of skim milk agar [37] supplemented or not with Fe_3Cl (20 μM). After 16 h incubation at 30°C, protease production by *E. carotovora* was evaluated by the presence of a clear zone in the agar. Cellular growth of *E. carotovora* and of the *Pseudomonas* strains was evaluated by comparing dilution plate counts of total bacteria on KB medium with counts of *Pseudomonas* on KB amended with 100 μg ml^{-1} tetracycline.

2.7. *Biocontrol assay for potato tuber rot caused by* E. carotovora

Biocontrol of *E. carotovora* was evaluated in a potato (*Solanum tuberosum* L.) cv. Nicola tuber assay following

Table 1
Bacterial, fungal strains and plasmids used in this study

Strain	Relevant characteristic	Reference
Bacteria		
Agrobacterium tumefaciens A334	Crown gall pathogen, AHL producer	This study
Bacillus sp. A24	Soil bacterium, AHL degrader	[27]
Chromobacterium violaceum CV026	Double mini-Tn*5* mutant derived from ATCC31532, Km^R, AHL biosensor, produces violacein pigment only in the presence of exogenous AHLs	[32]
Erwinia carotovora 852	Potato soft rot pathogen, AHL producer	This study
Pseudomonas chlororaphis PCL1391	Biocontrol agent, AHL producer	[47]
P. fluorescens P3	Soil bacterium	[45]
P. fluorescens P3/pME6000	P3 transformant with pME6000 plasmid	This study
P. fluorescens P3/pME6863	P3 transformant with pME6863, AHL degrader	This study
Pseudomonas sp. PITR2	DAPG producer, HCN producer	[55]
Pseudomonas sp. Q2-87	DAPG producer, HCN producer	[56]
Fungi		
Fusarium oxysporum f. sp. *lycopersici* Fol 8	Tomato vascular wilt pathogen	[40]
Plasmids		
pME6000	Broad-host-range cloning vector, Tc^R	[45]
pME6863	pME6000 carrying the *aiiA* gene from *Bacillus* sp. A24 under the constitutive P_{lac} promoter	[27]

Km^R and Tc^R indicate resistance to kanamycin and tetracycline, respectively. AHL = *N*-acyl-homoserine-lactone; DAPG = 2,4-diacetylphloroglucinol; HCN = hydrogen cyanide.

the protocol described by Wang et al. [38]. Two wells (5×5 mm) were cut in these tubers. One well was inoculated with 10 μl *E. carotovora* alone. The other well was inoculated with equal parts of the pathogen plus a test strain (i.e. *Bacillus* sp. A24, *P. fluorescens* P3/pME6000, *P. fluorescens* P3/pME6863, *Pseudomonas* sp. PITR2, *Pseudomonas* sp. Q2-87 or *P. chlororaphis* PCL1391) applied together. Also, in another set of experiments, some of these test strains (*P. fluorescens* P3/pME6000, *P. fluorescens* P3/pME6863 or *P. chlororaphis* PCL1391) were added either 2 days before or 2 days after the pathogen. Controls consisted of the pathogen added alone to both wells. Petri dishes were hermetically sealed in individual 400 cm^2 plastic bags, and incubated at 15°C during 4 days from the inoculation of the pathogen. Treatments consisted of eight replicate potato slices in separate glass jars, and the experiment was repeated three times. Treatment means were compared with linked *t*-tests.

2.8. Biocontrol assay for crown gall of tomato caused by A. tumefaciens

Seeds of tomato (*Lycopersicum esculentum* L.) cv. Ailsa Craig (provided by D. Fray, Nottingham University, UK) were surface-sterilized by submersion in a 1% solution of sodium hypochlorite for 30 min and pre-germinated for 3 days on 0.8% agar plates at 24°C in darkness. Pre-germinated seeds were grown in sterile quartz sand for 2 weeks. Before transplanting seedlings to rock-wool blocks (Grodania, Hedehusene, Denmark), 1/3 of the roots were pruned. Biocontrol bacteria were applied by dipping roots in a saline suspension with 10^6 CFU ml^{-1} of *Bacillus* sp. A24, *P. fluorescens* P3/pME6000 or *P. fluorescens* P3/pME6863 for 30 min, and then blot-drying the roots with sterile paper towels. Plants were inoculated with *A. tumefaciens* by dipping the roots for 30 min in a bacterial suspension of 10^6 CFU ml^{-1} and then blow-drying the roots. After inoculation, plants were transferred to fresh rock-wool cubes placed in plastic trays and kept moist with Knopp nutrient solution [39]. Plants were grown for 3 weeks in a growth chamber with a light period of 16 h at 26°C and a dark period of 8 h at 22°C. Biocontrol was evaluated as a reduction in the total weight of crown gall tumors on roots. Treatments consisted of eight plants per container, with three replicate containers per experiment. The experiment was repeated three times.

2.9. Interaction between AHL-degrading bacteria and P. chlororaphis in biocontrol of tomato vascular wilt

Tomato cv. Ailsa Craig seedlings were grown in quartz sand for 2 weeks, and then without cutting roots plants were inoculated with the vascular wilt fungus, *Fusarium oxysporum* f. sp. *lycopersici* Fol 8, by dipping in a suspension of 10^6 microconidia ml^{-1} as previously described [40]. Bacterial treatments were administered together with the pathogen, by adding 10^6 CFU ml^{-1} bacteria to the dipping suspension. Bacterial treatments were the biocontrol strain *P. chlororaphis* PCL1391 alone or in combination with AHL-degrading *Bacillus* sp. A24, AHL-degrading *P. fluorescens* P3/pME6863, or the non-AHL-degrading *P. fluorescens* P3/pME6000. After treatment with the pathogen and bacteria, seedlings were transplanted into rock-wool cubes and grown for 25 days as described above. Biocontrol activity was evaluated as a reduction in the percentage of plants per container exhibiting clear disease symptoms. Each treatment consisted of eight plants per container with three replicates. The experiment was repeated three times.

3. Results

3.1. Bacterial degradation of pathogen AHL in vitro

The plasmid pME6863 [27], carrying the *aiiA* gene under the control of the constitutive promoter P_{lac}, was introduced into P3 to give P3/pME6863. P3 carrying the vector without *aiiA*, P3/pME6000, was constructed for use as a non-AHL-degrading control.

Degradation of synthetic BHL autoinducer was evaluated using *C. violaceum* strain CV026 as a biosensor. Lysed cell extracts from overnight cultures of the model strain P3/pME6863 completely degraded the autoinducer after 2 h incubation, indicated by the absence of violacein induction in the biosensor (Fig. 1A). In contrast, no degradation was observed with lysates from the control strain P3/pME6000 carrying the vector plasmid but not the lactonase gene *aiiA*, as indicated by the equally strong induction of violacein biosynthesis in the biosensor by lysates of this strain and the BHL control.

Degradation of *E. carotovora*-excreted AHL autoinducer was evaluated on LB agar, again using CV026 as a biosensor. No induction of violacein by the biosensor strain was observed when P3/pME6863 was grown in between the donor *E. carotovora* and CV026; donor AHL was degraded (Fig. 1B). Degradation of AHL was also observed, but to a lesser extent as indicated by slighter purple color formation in the biosensor, when the wild-type *Bacillus* sp. A24 was grown in between the biosensor and the donor. In contrast, when the strain without the *aiiA* gene, P3/pME6000, was grown in between the biosensor and the donor, no AHL degradation was observed, and the biosensor became bright purple.

A third test was conducted to demonstrate the degradation of AHL by strains carrying the *aiiA* gene. When *E. carotovora* was grown on skim milk agar, excretion of exoproteases that are regulated by an AHL-dependent pathway was evident as the development of a clear halo around the colony (Fig. 2A, inset). Co-inoculation of the pathogen with *P. fluorescens* P3/pME6863 nearly abolished the clearing in the agar, indicating interference

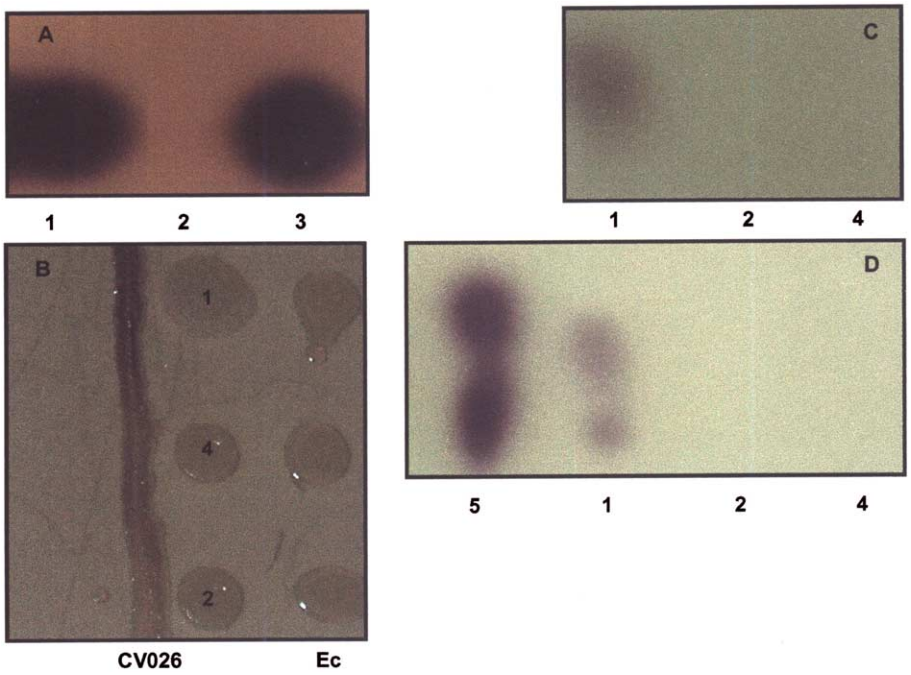

Fig. 1. Autoinducer degradation by bacteria carrying the *aiiA* lactonase encoding gene. Degradation of synthetic autoinducer BHL by cell lysates of *P. fluorescens* strains P3/pME6000 (1) and P3/pME6863 (2), without and with the *aiiA* gene inserted on a plasmid, respectively (A). Lysates were analyzed with TLC. Plates were overlayed with the biosensor *C. violaceum* CV026 (CV026) and BHL was detected based on autoinducer-dependent production of violacein. BHL incubated without bacterial lysates served as a negative control for degradation (3). Degradation of AHL autoinducer produced by the donor bacterium *E. carotovora* (Ec) by *Bacillus* sp. A24 carrying naturally the *aiiA* gene (4), P3/pME6000 or P3/pME6863 was evaluated by growing bacteria together on LB agar for 2 days (B). Degradation of AHL diffusing from the donor was determined by reduction or inhibition of violacein induction in the biosensor CV026. The persistence or degradation of the *E. carotovora* autoinducer added to growing cultures of P3/pME6000, P3/pME6863, A24 or non-inoculated LB medium (5) was detected by TLC analysis in cytoplasmic (C) and supernatant (D) fraction extractions using the biosensor CV026.

with *E. carotovora* protease activity. Measurements of the clear zone diameters indicated a significant reduction with P3/pME6863 compared to the pathogen inoculated alone (Fig. 2A). In contrast, the observed protease activity when the pathogen was co-inoculated with P3/pME6000 was smaller but not significantly different from when the pathogen was inoculated alone. This slight reduction of protease production was due to growth inhibition of the pathogen by *P. fluorescens*. Plate counts of the strain mixtures indicated that co-inoculation with either of the P3 derivatives reduced *E. carotovora* growth compared to the pathogen inoculated alone (Fig. 2B). *P. fluorescens* P3 produces siderophores that could sequester the available iron in the media. Addition of iron to the inoculum or the skim milk agar media did not affect the protease activity or the population size of *E. carotovora* when co-inoculated with the *P. fluorescens* P3 derivatives. The fact that the level of pathogen inhibition was the same for both P3/pME6863 and P3/pME6000 indicates that the major factor responsible for loss of protease activity in *E. carotovora* when co-inoculated with P3/pME6863 was expression of the AHL-degrading gene.

AiiA appears to be a cytoplasmic enzyme. No hydrophobic signal peptide has been found at its N-terminus and AHL autoinducer-inactivation activity was shown to be absent from supernatants of *Bacillus* sp. 240B1 and A24 [27,41]. How can the AiiA protein produced in the cytoplasm of the transgenic *P. fluorescens* P3/pME6863 degrade AHL autoinducer molecules which are excreted by neighboring bacteria like *E. carotovora*? AHL molecules can move in and out of cell membranes through diffusion or active transportation [42,43] and it is conceivable that AHLs that are produced by neighboring bacteria pass through P3/pME6863 where they are degraded in the cytoplasm. This ultimately leads to the elimination of AHL from the surroundings of P3/pME6863 without the need for AiiA to be secreted. This assumption was confirmed as follows. When AHL-degrading strains P3/pME6863 and A24 were grown in media supplemented with a physiological concentration of *E. carotovora* autoinducer, there was a decrease in residual AHL remaining in the culture supernatant (Fig. 1C) and in the cellular content (Fig. 1D). Violacein production by the biosensor strain was not induced, demonstrating degradation of the signal molecule produced by *E. carotovora*. The intensity of the purple spots produced by the biosensor with AHL supernatant extracts obtained from cultures of the non-AHL-degrading P3/pME6000 was markedly less than with the non-inoculated control (Fig. 1C), and residual AHLs were detected in the cellular cytoplasm (Fig. 1D).

We also verified that autoinducer inactivation observed with whole cells of P3/pME6863 was not simply due to cell

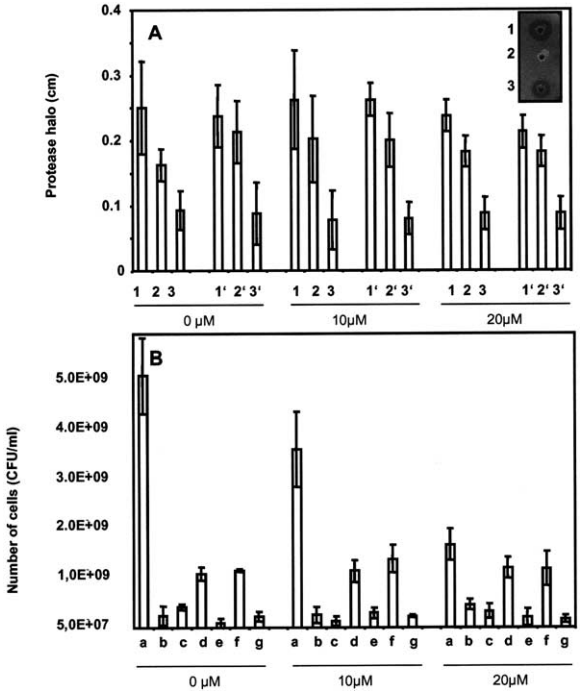

Fig. 2. A: Inhibition of AHL regulated extra-cellular protease activity of *E. carotovora*. The potato soft rot pathogen *E. carotovora* was cultivated alone (1), or co-inoculated with the non-AHL-degrading *P. fluorescens* P3/pME6000 (2) or P3/pME6863 carrying the *aiiA* gene (3) in the presence or absence of different concentrations of $FeCl_3$ (10 or 20 μM). These cultures were spot-inoculated onto skim milk agar supplemented (primed numbers) or not with $FeCl_3$ 20 μM. Protease activity was indicated by manifestation of clear zones around colonies (inset). The radius of the clear zones was measured after 16 h incubation. B: Cellular numbers per spot inoculated onto the skim agar medium were determined in the case of the pathogen inoculated alone (a), co-inoculated with P3/pME6000 (d), or co-inoculated with P3/pME6863 (f). Also, the numbers of P3/pME6000 inoculated alone (b) or in the presence of *E. carotovora* (e) and P3/pME6863 inoculated alone (c) or in the presence of the pathogen (g) were determined. Values represent the mean of three trials with three treatment replications per trial. Bars indicate standard deviation of the mean.

lysis which would liberate the cytoplasmic AiiA protein. This was done as follows. *P. fluorescens* P3/pME6000 and P3/pME6863 were cultivated in the presence of 10 μM BHL for 48 h. Aliquots of culture supernatants were extracted and analyzed for their BHL content. As expected, BHL was quantitatively degraded by P3/pME6863, but not by the control strain P3 carrying the empty vector pME6000 (data not shown). To evaluate whether cell lysis had occurred during growth, we measured the activity of the cytoplasmic marker enzyme glucose 6-phosphate dehydrogenase, in cell extracts and in cell-free culture supernatants. Whereas activities of 10.8 U and 10.4 U per mg of total protein (which is equivalent to 400 U and 480 U per ml of culture) were measured in cell extracts prepared from P3/pME6000 and P3/pME6863, no activity (less than 20 U per ml) was detectable in the corresponding culture supernatants, indicating that no substantial cell lysis (less than 5%) had occurred during growth of these cultures.

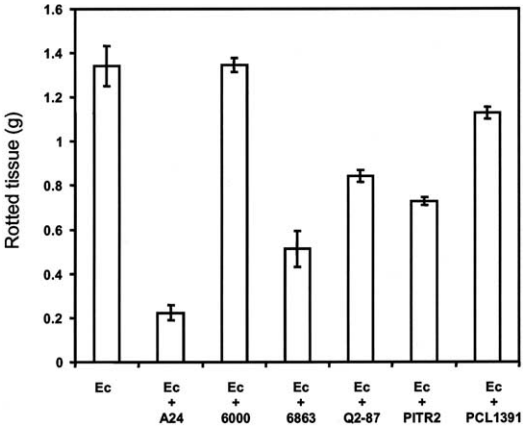

Fig. 3. Biocontrol of potato tuber soft rot caused by *E. carotovora*. Wells were cut into opposite sides of potato slices and inoculated with either the pathogen alone (Ec) or at the same time as a mixture with one putative biocontrol strain, *Bacillus* sp. A24 (Ec+A24), *P. fluorescens* P3/pME6863 (Ec+P3/pME6863), *P. fluorescens* P3/pME6000 (Ec+P3/pME6000), *Pseudomonas* sp. Q2-87 (Ec+Q2-87), *Pseudomonas* sp. PITR2 (Ec+PITR2), or *P. chlororaphis* PCL1391 (Ec+PCL1391). Potato slices were incubated in individual covered Petri dishes for 4 days. The extent of rot was quantified by comparing the weight difference of potato slices before and after washing away rotted tissue. Values represent the mean of three trials with eight treatment replications per trial. Bars indicate standard deviation of the mean.

3.2. Biocontrol of potato tuber soft rot using AHL-degrading bacteria

Inoculation of potato tubers with *E. carotovora* resulted in extensive tissue rot. Co-inoculation with the wild-type

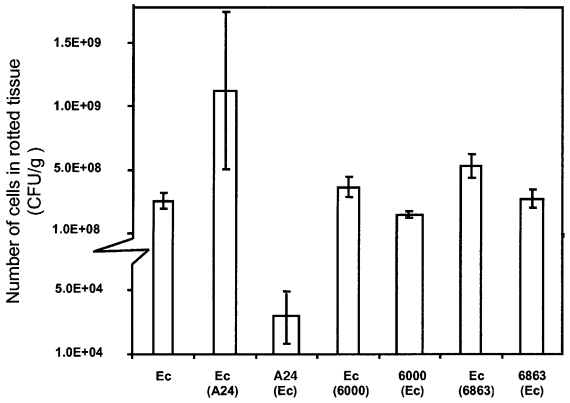

Fig. 4. Effect of AHL-degrading strains on the cell number of *E. carotovora* in potato tubers. In an experiment similar to that mentioned in Fig. 3, potato tubers were inoculated with *E. carotovora* alone or co-inoculated with this strain and *Bacillus* A24, *Pseudomonas fluorescens* P3/pME6863, or *P. fluorescens* P3/pME6000. Cell numbers of the pathogen inoculated alone (Ec), co-inoculated with *Bacillus* A24 [Ec (A24)], with *P. fluorescens* P3/pME6000 [Ec (6000)], or *Pseudomonas fluorescens* P3/pME6863 [Ec (6863)] were determined. Also, the number of the putative biocontrol *Bacillus* A24 [A24 (Ec)], *P. fluorescens* P3/pME6863 [6863 (Ec)], or the control *P. fluorescens* P3/pME6000 [6000 (Ec)] was determined in the presence of the pathogen. Values represent the mean of three trials with three treatment replications per trial. Bars indicate standard deviation of the mean.

AHL-degrading strain *Bacillus* sp. A24 or with the genetically engineered AHL-degrading strain P3/pME6863 provided a substantial reduction in tissue rot compared to the pathogen alone (Fig. 3). The biocontrol activity of the transgenic AHL-degrading strain was somewhat less effective than the protective activity of the wild-type *Bacillus* A24 (Fig. 3). In this experiment we included antibiotic-producing *Pseudomonas* strains, PCL1391, PITR2 and Q2-87, and were thus able to compare the relative efficacy of AHL degradation vs. antibiotic production as biocontrol mechanisms. The co-inoculation of these *Pseudomonas* strains with *E. carotovora* produced a significant reduction of tuber rot compared to the tuber rot area when potatoes were inoculated with the pathogen alone (Fig. 3). However, the level of protection offered by these antibiotic-producing strains was significantly less than that offered by the AHL-degrading strains. In contrast, the non-AHL-degrading control strain P3/pME6000 had no beneficial effect on reducing tissue rot. Pathogen growth inhibition by *Bacillus* A24 or P3/pME6863 was not observed in the potato tubers (Fig. 4). This indicates that in this disease system, disease protection was due to expression of the *aiiA* gene in A24 and P3/pME6863.

3.3. *Preventive and curative biocontrol activity of AHL-degrading bacteria against potato soft rot caused by* E. carotovora

In order to evaluate the curative potential for AHL degradation, tuber inoculation with the pathogen and the biocontrol strains was staggered. When the AHL degrader P3/pME6863 was applied 2 days before the pathogen, rot was almost completely prevented (Fig. 5A). When the AHL degrader was applied 2 days after the pathogen, there was more rot evident because of the head start given to the pathogen. However, the rotting was stopped when P3/pME6863 was applied, resulting in substantially less disease than in treatments with P3/pME6000 or the pathogen alone (Fig. 5B). This demonstrates curative as well as preventive biocontrol activity.

In this experiment we included the antibiotic producer PCL1391 and were thus able to compare the relative efficacy of AHL degradation vs. antibiotic production as biocontrol mechanisms. Whether PCL1391 was inoculated before or after the pathogen, tuber rot was significantly reduced compared to the tuber area inoculated with the pathogen alone (Fig. 5). However, the level of protection offered by PCL1391 was significantly less than that offered by the AHL-degrading P3/pME6863. The non-AHL-degrading control P3/pME6000 offered a slight degree of protection, as indicated by a reduction in rot when applied either before or after the pathogen, but this was not to the level of either the antibiotic producer or the AHL degrader (Fig. 5). In this case, competition or growth inhibition apparently made a minor contribution to overall disease protection offered by all biocontrol treatments.

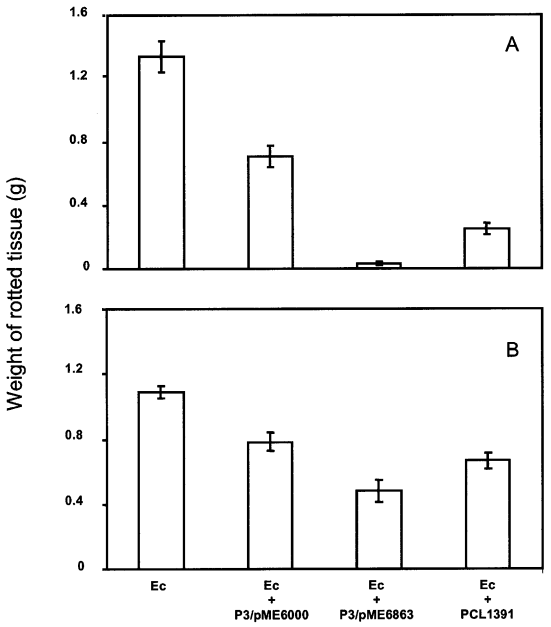

Fig. 5. Demonstration of preventive and curative biocontrol activity of AHL-degrading bacteria against potato tuber soft rot caused by *E. carotovora* (Ec). Using the potato slice assay described in Fig. 3, putative biocontrol strains were inoculated into wells 2 days before (A) or 2 days after (B) the pathogen to demonstrate preventive or curative biocontrol, respectively. Treatments included the pathogen plus the AHL degrader *P. fluorescens* P3/pME6863 (Ec+P3/pME6863) or the non-degrading control P3/pME6000 (Ec+P3/pME6000). *P. chlororaphis* PCL1391 relies on AHL-mediated phenazine biosynthesis for plant disease suppression, and was inoculated with the pathogen (Ec+PCL1391) as a comparison of antibiosis vs. AHL degradation as biocontrol mechanisms. Slices were incubated for 4 days following inoculation with *E. carotovora*. Weight of rotted tissue was quantified as in Fig. 3. Values represent the mean of trials with eight treatment replications per trial. Bars indicate standard deviation of the mean.

3.4. *Biocontrol of* A. tumefaciens *on tomato by AHL-degrading bacteria*

Using crown gall as a second disease model, we confirmed the broad-spectrum biocontrol activity of AHL-degrading bacteria. When tomato plants were inoculated with *A. tumefaciens* alone, numerous tumor-like galls could be recovered from the roots. Co-inoculation of tomato with the pathogen and *P. fluorescens* P3/pME6863 significantly reduced the amount and weight of galls, and in fact offered nearly total protection to the plants against this disease (Fig. 6). The wild-type strain *Bacillus* sp. A24 provided the same level of disease protection as P3/pME6863 when it was co-inoculated together with the pathogen. The control treatment with *A. tumefaciens* and *P. fluorescens* P3/pME6000 showed a very limited level of protection, as gall formation was not significantly different from that on plants inoculated with the pathogen alone (Fig. 6). This indicates that the protection observed with P3/pME6863 and *Bacillus* sp. A24 was primarily due to AHL degradation and that pathogen growth inhibition or competition played only a minor role.

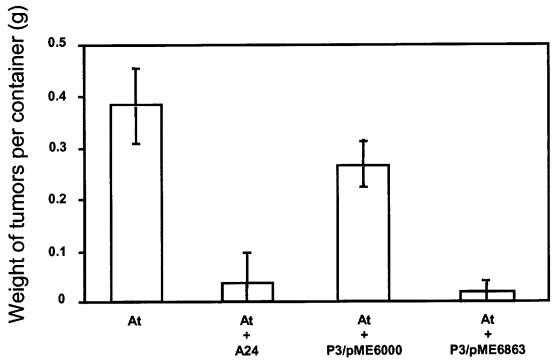

Fig. 6. Biocontrol activity of AHL-degrading bacteria against crown gall on tomato caused by *A. tumefaciens*. Tomato plants were inoculated by dipping alone in solutions containing 10^6 CFU ml^{-1} of *A. tumefaciens* (At), or by dipping in solutions with the pathogen followed by dipping in solutions containing 10^6 CFU ml^{-1} of the AHL-degrading strains *Bacillus* sp. A24 (At+A24), *P. fluorescens* P3/pME6863 (At+P3/pME6863), or the non-AHL-degrading strain P3/pME6000 (At+P3/pME6000). Plants were grown in a hydroponics system (eight plants per container), and crown gall tumors were harvested from roots after 3 weeks. Values represent the mean weight of tumors per container from three trials with three replicate containers per treatment. Bars indicate the standard deviation of the mean.

3.5. *Non-target impact of AHL-degrading bacteria on the biocontrol activity of* P. chlororaphis *strain PCL1391 applied as strain mixtures*

Inoculation of tomato plants with phenazine-producing *P. chlororaphis* PCL1391 provided substantial protection against vascular wilt caused by *F. oxysporum* f. sp. *lycopersici* (Fig. 7). In contrast, neither the wild-type A24 nor the genetically engineered P3/pME6863 AHL-degrading bacteria reduced this fungal disease when they were inoculated individually with the pathogen (Fig. 7). In fact, rather than offering disease protection, these bacteria abolished the protective activity of PCL1391 when they were applied together in strain mixtures. The non-AHL-degrading control P3/pME6000 had no disease-suppressive activity when it was applied alone with the fungal pathogen, and it did not significantly reduce the biocontrol activity of PCL1391 when applied as a strain mixture. This indicates that the interference of A24 and P3/pME6863 with disease control by PCL1391 was due to expression of the *aiiA* AHL-degrading gene in these bacteria.

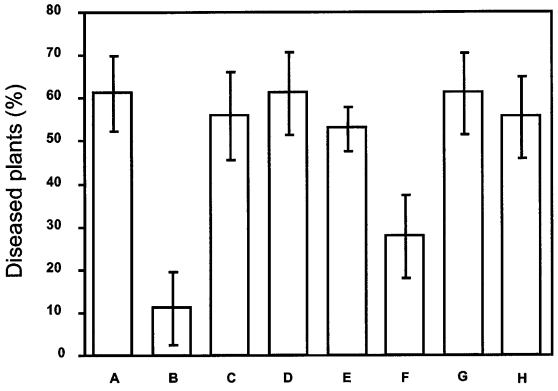

Fig. 7. Non-target impact of AHL-degrading bacteria on biocontrol activity of *P. chlororaphis* PCL1391, which relies on AHL-mediated antibiotic biosynthesis for plant disease suppression. Tomato plants were inoculated with the vascular wilt fungal pathogen *F. oxysporum* f. sp. *lycopersici* alone (A) or together with one of the following bacterial treatments: PCL1391 (panel B), *Bacillus* sp. A24 (panel C), PCL1391 plus A24 (panel D), *P. fluorescens* P3/pME6000 (panel E), PCL1391 plus P3/pME6000 (panel F), *P. fluorescens* P3/pME6863 (panel G), or PCL1391 plus P3/pME6863 (panel H). Plants were grown in a hydroponics system (eight plants per container), and the percentage of plants exhibiting wilt symptoms was assessed after 25 days. Values represent the mean percent diseased plants per container from three trials with three replicate containers per treatment. Bars indicate the standard deviation of the mean.

4. Discussion

We have demonstrated for the first time the efficacy of using a wild-type soil bacterium with AHL-degrading capability for the biocontrol of plant diseases. *Bacillus* sp. strain A24 is able to degrade AHLs produced by plant pathogenic *E. carotovora* and *A. tumefaciens*, and it exhibited broad-spectrum activity by significantly reducing diseases of potato and tomato caused by these phytopathogenic bacteria. By comparing our strains with the antibiotic-producing strains *P. chlororaphis* PCL1391, *Pseudomonas* sp. PITR2 and *Pseudomonas* sp. Q2-87 we showed that AHL degradation was a mechanism for controlling bacterial plant diseases as effective as or better than phenazine and 2,4-diacetylphloroglucinol antibiotic production. Our finding of AHL degradation as a novel mechanism of plant disease suppression opens the door for targeted selection of new wild-type strains that may be even more effective at degrading pathogen autoinducers, much as earlier work demonstrating the important role of antibiotic production in biocontrol has led to designing molecular probes used to streamline the screening process for new biocontrol agents [44]. The *aiiA* gene responsible for AHL degradation in *Bacillus* sp. A24 was cloned [27] and it appears to be widely distributed among *Bacillus* strains [25,26,41]. We were able to confirm the role of AHL degradation in biocontrol using a model where the *aiiA* gene was heterologously expressed in a plant root-colonizing bacterium *P. fluorescens* strain P3 that has no prior documented biocontrol activity [45]. The derivative P3/pME6863 carrying *aiiA* on a plasmid was able to degrade AHLs and to reduce disease caused by both pathogens, whereas the derivative P3/pME6000 carrying the vector plasmid but no *aiiA* gene was ineffective. Using this model we demonstrated the role of AHL degradation in biocontrol, avoiding the effects of other compounds that may be produced by *Bacillus* sp. [46]. Our results using antagonists with the *aiiA* gene echo those reported by Dong et al. [41] showing that heterologous expression in *E. carotovora* of an *aiiA* gene cloned from *Bacillus* sp. strain 240B1 decreased the pathogen's own virulence

gene expression (i.e. pectinolytic enzyme encoding genes). Our results also support work showing that heterologous expression of AHL-degrading genes in plants confer significant disease protection [24]. Deploying wild-type bacteria to degrade pathogen AHLs as we have done, however, has the distinct advantage of avoiding prevailing public objections to the cultivation and use of genetically modified plants in Europe and elsewhere.

Our studies also demonstrate that AHL degradation has curative as well as preventive biocontrol activity. By staggering the application of pathogenic *E. carotovora* and AHL-degrading bacteria, we found that even when the pathogen was given a 2-day head start to establish and initiate disease symptoms, subsequent application of an AHL-degrading strain stopped further disease development. This is one of the rare examples of curative disease control, and has important practical implications for bacterial diseases that typically incubate before any outward symptoms manifest themselves. In such cases, it may be possible to implement treatment measures after disease has already set in.

The broad-spectrum activity of AHL degradation that we observed against bacterial pathogens, which rely on quorum sensing for disease development [30], had its limits when applied for control of a fungal pathogen that does not utilize AHL signals. Our AHL-degrading strains, wild-type *Bacillus* sp. A24 and the model *P. fluorescens* P3/pME6863, were ineffective at controlling Fusarium wilt of tomato. In this system, the antibiotic-producing biocontrol agent *P. chlororaphis* PCL1391 offered a high level of disease protection. Phenazine antibiotic biosynthesis is tightly regulated via AHLs that are structurally similar to those produced by phytopathogenic bacteria, and antibiotic production is a primary mechanism of biocontrol activity in this strain [47–49]. AHL-degrading bacteria severely impaired the ability of PCL1391 to protect tomato plants against *F. oxysporum* f. sp. *lycopersici*. Again, comparing our model *P. fluorescens* P3/pME6863 with the AHL non-degrading companion P3/pME6000 we showed that the major factor behind this interference was indeed AHL degradation. This supports the findings of Pierson et al. [31], who reported that the biocontrol activity of another phenazine-producing strain, *P. aureofaciens* strain 30-84, was severely impaired when its *N*-acyl homoserine lacton synthase gene *phzI* gene was disrupted. In their wheat root system, the biocontrol activity of 30-84 could be restored by co-inoculation with AHL-producing strains or with AHLs contributed by co-existing root-colonizing bacteria [31]. Our results indicate that cross-talk between biocontrol strains that rely on AHLs [47,50] and co-existing bacteria can also have the opposite impact on disease suppression by degrading essential signal molecules and thus blocking biosynthesis of critical antimicrobial metabolites. This highlights the care that needs to be taken in designing effective strain mixtures [51,52]. It also provides an explanation as to why certain strain mixtures may prove ineffective despite an absence of growth inhibition which has been a standard for mixture selection [53,54].

Environmental conditions, abiotic and biotic, influence the expression of a variety of key biocontrol genes in antagonistic bacteria [35,39]. The AHL-degrading activity observed in vitro with *Bacillus* sp. A24 was similar to that of our model P3/pME6863, where this gene was under control of a constitutive promoter, but activity levels varied slightly depending on the medium conditions. In plant tests, the wild-type strain *Bacillus* sp. A24, which did not inhibit the growth of *E. carotovora*, was superior to the transgenic at controlling soft rot disease of potato tubers. These results indicate a degree of environmental control over *aiiA* expression. Whether this is with regard to the biosynthesis of the degrading enzyme in *Bacillus* or the sensitivity of the pathogen is uncertain. Our study provides a foundation for understanding the conditions that are critical to AHL degradation. Such information will enhance efforts to optimize the level and reliability of biocontrol using wild-type bacteria for degradation of pathogen autoinducer compounds.

Acknowledgements

The authors thank D. Schneider for assistance with plant experiments. This work was supported by the European Union ECO-SAFE Project (QLK3-CT-2000-31759/OFES 00.0164-2), the Bonizzi-Theler Foundation and the Jubiläumsstiftung Rentenanstalt Swiss Life.

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