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Molecular analysis of a novel gene cluster encoding an insect toxin in plant-associated strains of *Pseudomonas fluorescens*

Maria Péchy-Tarr,[1] Denny J. Bruck,[2] Monika Maurhofer,[3] Esther Fischer,[4] Christelle Vogne,[1] Marcella D. Henkels,[2] Kelly M. Donahue,[2] Jürg Grunder,[4] Joyce E. Loper[2] and Christoph Keel[1]*

[1]*Department of Fundamental Microbiology, University of Lausanne, CH-1015 Lausanne, Switzerland.*
[2]*US Department of Agriculture, Agricultural Research Service, Horticultural Crops Research Laboratory, Corvallis, OR 97330, USA.*
[3]*Plant Pathology, Institute of Integrative Biology, Swiss Federal Institute of Technology (ETH), CH-8092 Zürich, Switzerland.*
[4]*Natural Resources Sciences, University of Applied Sciences ZHAW, CH-8820 Wädenswil, Switzerland.*

Summary

***Pseudomonas fluorescens* CHA0 and the related strain Pf-5 are well-characterized representatives of rhizosphere bacteria that have the capacity to protect crop plants from fungal root diseases, mainly by releasing a variety of exoproducts that are toxic to plant pathogenic fungi. Here, we report that the two plant-beneficial pseudomonads also exhibit potent insecticidal activity. Anti-insect activity is linked to a novel genomic locus encoding a large protein toxin termed Fit (for *P. fluorescens* insecticidal toxin) that is related to the insect toxin Mcf (Makes caterpillars floppy) of the entomopathogen *Photorhabdus luminescens*, a mutualist of insect-invading nematodes. When injected into the haemocoel, even low doses of *P. fluorescens* CHA0 or Pf-5 killed larvae of the tobacco hornworm *Manduca sexta* and the greater wax moth *Galleria mellonella*. In contrast, mutants of CHA0 or Pf-5 with deletions in the Fit toxin gene were significantly less virulent to the larvae. When expressed from an inducible promoter in a non-toxic *Escherichia coli* host, the Fit toxin gene was sufficient to render the bacterium toxic to both insect hosts. Our findings establish the Fit gene products of *P. fluorescens* CHA0 and Pf-5 as potent insect toxins that define previously unappreciated anti-insect properties of these plant-colonizing bacteria.**

Received 29 January, 2008; accepted 12 April, 2008. *For correspondence. E-mail christoph.keel@unil.ch; Tel. (+41) 216925638; Fax (+41) 216925605.

Introduction

Root diseases and pests are a serious problem in agricultural crops, causing every year important yield losses. Fungal pathogens and insect pests that attack below-ground plant parts are difficult to control and limited efficacy and concerns over environmental and consumer safety have restricted the use of chemical pesticides. Biological disease and pest control with microbial agents applied to soil or plant material is a promising alternative concept in the overall trend towards greater sustainability in crop production. Two groups of microbial agents have received particular attention for use in biological crop protection: (i) plant-beneficial bacteria, notably root-associated pseudomonads that produce potent antifungal compounds for disease control (Haas and Keel, 2003; Haas and Défago, 2005; Mercado-Blanco and Bakker, 2007), and (ii) entomopathogenic bacteria, mainly *Bacillus thuringiensis* and insect-invading nematode-associated *Photorhabdus* and *Xenorhabdus* that produce potent insecticidal toxins for pest control (Bowen *et al*., 1998; De Maagd *et al*., 2001; ffrench-Constant *et al*., 2003; 2007; Moar, 2003; Bravo *et al*., 2007; Goodrich-Blair and Clarke, 2007).

Plant-beneficial fluorescent pseudomonads that operate in the rhizosphere are well characterized for their capacity to protect plants from root diseases caused by soil-borne pathogenic fungi. Biocontrol efficacy depends on multiple traits that allow these bacteria to effectively colonize the rhizosphere, compete for nutrients and niches, and suppress pathogens directly or indirectly through antibiosis, competition or induction of plant defence mechanisms (Lugtenberg *et al*., 2001; Haas and Keel, 2003; Haas and Défago, 2005; Mercado-Blanco and Bakker, 2007; Loper and Gross, 2007). Plant-beneficial pseudomonads release a remarkable diversity of biologically active exoproducts with antimicrobial, metal-chelating, lytic and phytohormonal activities, and their production of potent antimicrobial compounds is

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considered to be a primary mechanism by which they suppress root diseases (Haas and Keel, 2003; Haas and Défago, 2005; Mercado-Blanco and Bakker, 2007; Loper and Gross, 2007). Among the antimicrobial compounds, 2,4-diacetylphloroglucinol (DAPG), pyoluteorin (PLT), pyrrolnitrin, phenazines, hydrogen cyanide (HCN) and cyclic lipopeptides have received particular attention for their major contribution to the control of fungal and oomycete pathogens causing agronomically important root diseases (Haas and Keel, 2003; Mavrodi *et al*., 2006; Raaijmakers *et al*., 2006; Loper and Gross, 2007; Weller *et al*., 2007). DAPG has been established as a key biocontrol factor of indigenous populations of rhizosphere pseudomonads in soils that are naturally suppressive to root diseases such as take-all of wheat (Weller *et al*., 2007). Most effective biocontrol pseudomonads produce at least one of the above-mentioned toxic compounds. The majority of these compounds display broad-spectrum toxicity against fungi, bacteria, protozoa and nematodes (Haas and Keel, 2003; Raaijmakers *et al*., 2006; Dubuis *et al*., 2007), and their primary ecological function may be to help a biocontrol pseudomonad defend itself and its rhizosphere habitat against competing, predating and plant host-pathogenic organisms attracted by the same niche.

Pseudomonas fluorescens strains CHA0 and Pf-5, the subjects of this study, are prominent, well-characterized representatives of a genetically distinct subgroup of biocontrol pseudomonads that excrete multiple antimicrobial compounds, notably DAPG, PLT, pyrrolnitrin and HCN (Keel *et al*., 1996; Haas and Keel, 2003; Loper and Gross, 2007). Strain CHA0 was isolated from the roots of tobacco grown in a naturally disease-suppressive soil in western Switzerland (Stutz *et al*., 1986). Strain Pf-5 was isolated from a field soil in Texas, USA (Howell and Stipanovic, 1979). Both strains are highly competitive root colonizers and display a remarkably wide spectrum of biocontrol activity against damping-off diseases, root rots and wilts caused by major fungal and oomycete pathogens, including *Gaeummanomyces*, *Thielaviopsis*, *Fusarium*, *Rhizoctonia* and *Pythium*, on various crop plants (Keel *et al*., 1996; Sharifi-Tehrani *et al*., 1998; Haas and Keel, 2003; Loper and Gross, 2007). The gene clusters specifying the biosynthesis of DAPG, PLT, pyrrolnitrin and HCN have been identified in both strains and their regulation and relative importance for plant protection studied in some detail (Voisard *et al*., 1989; Keel *et al*., 1992; Schnider-Keel *et al*., 2000; Haas and Keel, 2003; Baehler *et al*., 2005; 2006; Brodhagen *et al*., 2005; Paulsen *et al*., 2005; Péchy-Tarr *et al*., 2005; Loper and Gross, 2007). Production of the toxic exoproducts is controlled by a complex network of pathway-specific and global regulators that respond to environmental and density-dependent signals, presumably allowing the two biocontrol pseudomonads to coordinate antifungal activity with root colonization, nutrition acquisition and stress tolerance capacities in the rhizosphere environment (Haas and Keel, 2003; Haas and Défago, 2005; Dubuis *et al*., 2007). With the recent decipherment of the genomic sequence of *P. fluorescens* Pf-5, the first complete genome of a biocontrol pseudomonad has become available (Paulsen *et al*., 2005), opening the door for the discovery of additional plant-beneficial compounds. Indeed, in a genomic mining approach, the cyclic lipopeptide orfamide A and derivatives of the macrolide rhizoxin were identified as novel Pf-5 metabolites exhibiting significant toxicity to phytopathogenic oomycetes and fungi (Brendel *et al*., 2007; Gross *et al*., 2007; Loper and Gross, 2007; Loper *et al*., 2008).

Microbial control of insect pests involves the use of entomopathogenic bacteria. The most successful microbial agent commercially is *Bacillus thuringiensis*, which produces the crystal (Cry) proteins, a family of potent insecticidal toxins exhibiting a defined spectrum of activity that usually is restricted to specific groups of lepidopteran, coleopteran, dipteran and hymenopteran insects (De Maagd *et al*., 2001; Bravo *et al*., 2007). *B. thuringiensis* toxins have a long history of use as biological insecticides in form of powders or sprays containing spore–crystal toxin mixtures and, for the past decade, insect-resistant transgenic maize and cotton containing specific Cry toxin genes have been grown widely (Moar, 2003). There is great interest in the discovery of additional bacterial toxins with insecticidal activity. In recent years, *Photorhabdus* and *Xenorhabdus* spp. that live as mutualists in the intestines of entomophagous nematodes have been recognized as a source of novel insecticidal proteins for crop protection (Bowen *et al*., 1998; ffrench-Constant *et al*., 2003; 2007; Goodrich-Blair and Clarke, 2007). Nematodes with these bacterial mutualists actively seek out susceptible insect larvae, penetrate the cuticle, invade the blood system and regurgitate the insecticidal bacteria. The bacteria release a range of toxins that kill the insect host and convert the cadaver into a bacterial and nematodal food source and breeding ground (ffrench-Constant *et al*., 2003; Goodrich-Blair and Clarke, 2007). The recent sequencing of the *Photorhabdus luminescens* genome confirmed that this entomopathogen is equipped to produce at least three classes of insect toxins (Duchaud *et al*., 2003). The first class consists of several toxin complexes (Tc) that are large orally active toxins requiring three components (ABC) for full toxicity (Bowen *et al*., 1998; ffrench-Constant *et al*., 2007). Tc toxin components have been successfully used to create insect-resistant plants (Liu *et al*., 2003). A second class comprises the potent 'Makes caterpillars floppy' Mcf1 and Mcf2 toxins that are active upon injection (Daborn *et al*., 2002; Waterfield *et al*., 2003). Mcf2 is an N-terminally truncated variant of Mcf1 with similar toxicity to insect larvae. The

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Mcf1 toxin exhibits pro-apoptotic activity (Dowling *et al*., 2004; 2007). A recently discovered third class contains the '*Photorhabdus* insect-related' (PirAB) binary toxins that have both oral and injectable activities in some insects (ffrench-Constant *et al*., 2007).

A few fascinating, very recent genomic observations indicate that the genomes of certain soil-inhabiting and plant-associated members of the genus *Pseudomonas* may harbour specific loci possibly endowing them with anti-insect activity (ffrench-Constant *et al*., 2007). A prominent example is the entomopathogenic soil bacterium *Pseudomonas entomophila*, which kills *Drosophila* and some lepidopteran insects after oral infection, i.e. without need of a vector such as a nematode (Vodovar *et al*., 2005). The exact basis of insect virulence is yet to be determined, but genomic regions encoding gut-active insect toxins resembling the *Photorhabdus* Tc components and other exoproducts that may harm insects are present in this bacterium (Duchaud *et al*., 2003; Vodovar *et al*., 2006). Strikingly, loci related to the *Photorhabdus* Tc genes can also be spotted in the genomes of the leaf pathogen *Pseudomonas syringae* and the soil saprophyte *P. fluorescens* Pf0-1, i.e. both bacteria with no known insect association (Guttman *et al*., 2002; ffrench-Constant *et al*., 2007).

Here, we report on the first identification and analysis of a proteinaceous insect toxin from plant-associated pseudomonads. We demonstrate that the root-colonizing disease-suppressive agents *P. fluorescens* CHA0 and Pf-5 exhibit potent insecticidal activity. We show that anti-insect action is linked to a genomic locus encoding a novel protein toxin that is related to the potent insect toxin Mcf1 of the entomopathogen *P. luminescens* (ffrench-Constant *et al*., 2007). In support of this finding, we present data illustrating that toxin-deficient mutants of the two pseudomonads are markedly impaired in anti-insect activity and that transgenic expression of the toxin in non-toxic *Escherichia coli* is sufficient to render this bacterium lethal to insects.

Results

Identification of a novel insect toxin locus in P. fluorescens *biocontrol strains CHA0 and Pf-5*

Examination of the complete genomic sequence of *P. fluorescens* Pf-5 (Paulsen *et al*., 2005) revealed the presence of an open reading frame (ORF) (locus tag PFL_2983, GenBank Accession No. CP000076) encoding a large protein that is related to the insecticidal toxin Mcf of *P. luminescens* (Daborn *et al*., 2002). To determine whether a corresponding locus is present in the closely related *P. fluorescens* CHA0, a chromosomal cosmid library of the strain was screened with a polymerase chain reaction (PCR)-based approach using primers directed against PFL_2983 (see *Experimental procedures*). A positive cosmid clone, pME9006, carrying a 24 kb chromosomal DNA insert (Fig. 1) was selected. Sequence analysis confirmed the presence of the *mcf* homologue in the genome of *P. fluorescens* CHA0 and the deduced product was found to be highly similar (98% identity at the amino acid sequence level) to its Pf-5 counterpart. Based on the anti-insect activity demonstrated in this study, we termed the novel insect toxin Fit for *P. fluorescens* insecticidal toxin.

Sequence analysis indicated that the toxin gene is part of a conserved locus in strains CHA0 and Pf-5 that also contains genes predicted to function in toxin transport and regulation of toxin production (Fig. 1). The locus comprises eight ORFs designated *fitA*, *fitB*, *fitC*, *fitD*, *fitE*, *fitF*, *fitG* and *fitH* that correspond to ORFs PFL_2980 through PFL_2987 of *P. fluorescens* Pf-5 (Paulsen *et al*., 2005). As the chromosomal DNA insert in pME9006 did not cover the entire CHA0 toxin region, a 3.8 kb fragment comprising the missing part, i.e. *fitA* with its upstream region and the 5′ part of *fitB*, was PCR-amplified from CHA0 DNA and used to generate pME9007 (Fig. 1). Comparison of the deduced amino acid sequences of the corresponding *fit* genes in *P. fluorescens* CHA0 and Pf-5 revealed identities of 97–99% (data not shown). In the following, sequence comparisons with other microbial genomes are therefore detailed only for strain CHA0.

The deduced product of the Fit toxin gene *fitD* (3004 amino acids, 327.3 kDa) is 72% identical to the insecticidal protein Mcf1 of the *P. luminescens* strains W14 (AAM88787) and TTO1 (NP_931332, locus tag Plu4142) and 67% identical in an about 2100-amino-acid overlap to a second N-terminally truncated Mcf protein termed Mcf2 (AAR21118; Plu3128) produced by these strains (Daborn *et al*., 2002; Waterfield *et al*., 2003; ffrench-Constant *et al*., 2007). *fitD* is flanked upstream by *fitABC* and downstream by *fitE* (Fig. 1), which are predicted to encode components of a type I protein secretion system. The deduced products of *fitA* (713 amino acids, 79.1 kDa), *fitB* (462 amino acids, 51.5 kDa) and *fitC* (719 amino acids, 78.9 kDa) show similarities to the putative ABC-type toxin transporters MtpB, MtpD and MptE of *P. luminescens* strain W14 (AY445665; 71%, 63% and 72% identity respectively) and Plu3125, Plu3126, and Plu3127 of *P. luminescens* strain TTO1 (68%, 64% and 73% identity) that are associated with the *mcf2* locus (Waterfield *et al*., 2003). FitA, FitB and FitC share also at least 46% identity with RTX toxin transporters of *Vibrionaceae* (Boardman and Satchell, 2004). *fitE* encodes a protein (489 amino acids, 54.4 kDa) that is 55–61% identical to type I secretion outer membrane efflux proteins of the TolC family of *P. fluorescens* strain PfO-1 (CP000094, locus tag Pfl01_0491) and other *Pseudomonas* species (data not

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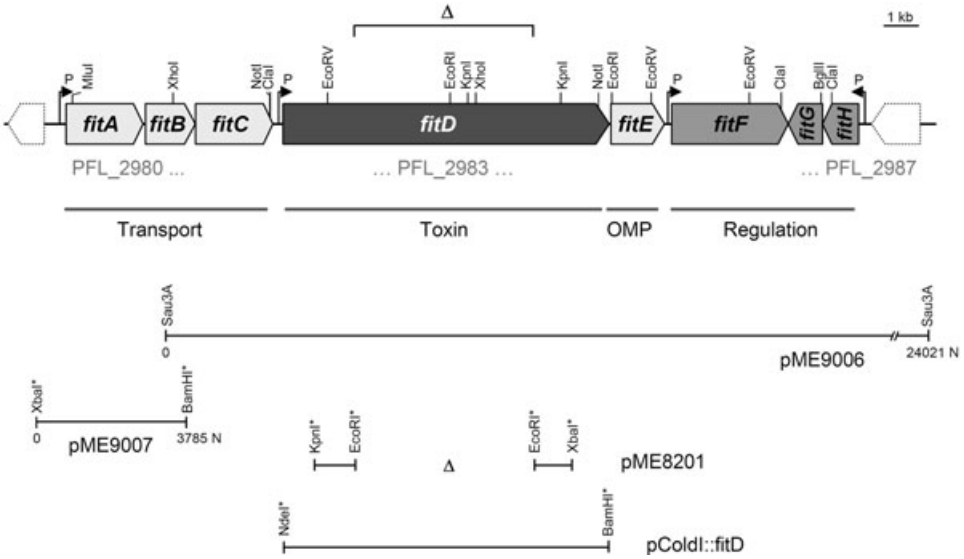

Fig. 1. Organization of the insect toxin locus in *P. fluorescens* strains CHA0 and Pf-5. The locus was termed *fit* (for *P. fluorescens* insecticidal toxin). Putative functions encoded by the locus are indicated below the shaded arrows representing the *fit* genes. The representation is based on sequence data of strain CHA0 obtained by sequencing the chromosomal fragments inserted in pME9006 and pME9007. The *fitABCDEFGH* genes correspond to open reading frames designated with locus tags PFL_2980 through PFL_2987 in the complete annotated sequence of strain Pf-5 (Paulsen *et al*., 2005). Δ, region deleted in the *fitD* mutants CHA1151 and Pf-5-1151, and in plasmid pME8201. The horizontal bars designate the DNA fragments of CHA0 cloned into cosmid pRG930-Cm to give pME9006, into pUC18 to give pME9007, into suicide plasmid pME3087 to give pME8201 and into the expression vector pColdI to give pColdI::*fitD*. Artificial restriction sites are marked with an asterisk. OMP, outer membrane protein; P, putative promoter.

shown). *fitE* is followed by *fitF*, *fitG* and *fitH* (Fig. 1), which encode putative regulatory functions. The deduced product of *fitF* (1056 amino acids, 116.1 kDa) is predicted to code for a hybrid histidine kinase-response regulator and shows 41% identity and 57% similarity in a 558-amino-acid overlap with a sensor hybrid histidine kinase of *Dechloromonas aromatica* RCB (CP000089, locus tag Daro_1896) and 34% identity and 54% similarity in a 661-amino-acid overlap with a hybrid sensory kinase of *Synechocystis* sp. PCC 6803 (BA000022, locus tag slr2104). The protein encoded by *fitG* (305 amino acids, 34 kDa) is 45% and 42% identical (63% and 59% similar) to LysR-type transcriptional regulators of *P. fluorescens* PfO-1 (Pfl01_2914) and *Pseudomonas aeruginosa* PAO1 (AE004091, locus tag PA3565) respectively. The predicted *fitH* product (326 amino acids, 35.1 kDa) displays 53% and 52% identity in a 120-amino-acid overlap with response regulators of *Synechocystis* sp. PCC 6803 (slr2100) and *D. aromatica* RCB (Daro_3458) respectively.

In *P. fluorescens* Pf-5, the *fit* locus is linked, downstream, to a 79 kb gene cluster (PFL_2989 through PFL_2997) encoding the biosynthesis of several rhizoxin derivatives that are toxic against phytopathogenic fungi (Brendel *et al*., 2007; Loper *et al*., 2008). Remarkably, sequence analysis of the region downstream of *fitH* on the pME9006 insert (Fig. 1) indicates that the rhizoxin cluster is not present in *P. fluorescens* CHA0. In this bacterium, *fitH* is followed by ORFs that are 99% identical to PFL_2998, PFL_2999, PFL_3000, PFL_3001 and PFL_3002 of strain Pf-5, and homologues of these ORFs can also be found in other pseudomonads (data not shown). The absence of the rhizoxin cluster from the genome of strain CHA0 was confirmed by PCR and Southern hybridization analyses of chromosomal DNA using primers and probes, respectively, directed against the *rzx* genes of strain Pf-5 (data not shown).

We were not able to detect sequences with appreciable similarities to the Fit toxin genes in any of the sequenced strains of the *Pseudomonadaceae* family, including the entomopathogen *P. entomophila*, the opportunistic human pathogen *P. aeruginosa*, different pathovars of the plant pathogen *P. syringae*, and the soil and plant saprophytes *Pseudomonas putida*, *P. fluorescens* and *P. stutzeri*. The only notable exception is a limited similarity of the *P. fluorescens* Fit toxins to two predicted proteins of 2380 and 2397 amino acids, respectively, encoded by the related ORFs Pfl01_0608 and Pfl01_0609 of the soil bacterium *P. fluorescens* PfO-1 (GenBank Accession No. CP000094). Pfl01_0608 of strain PfO-1 and the Fit toxins of strains CHA0 and Pf-5 share 28% identity and 44% similarity in a 1679-amino-acid overlap towards their C-termini and similarity levels with Pfl01_0609 are slightly lower. A predicted protein of a *P. fluorescens* strain with a similar distant relatedness to the Mcf1 and Mcf2 toxins of *P. lumine-*

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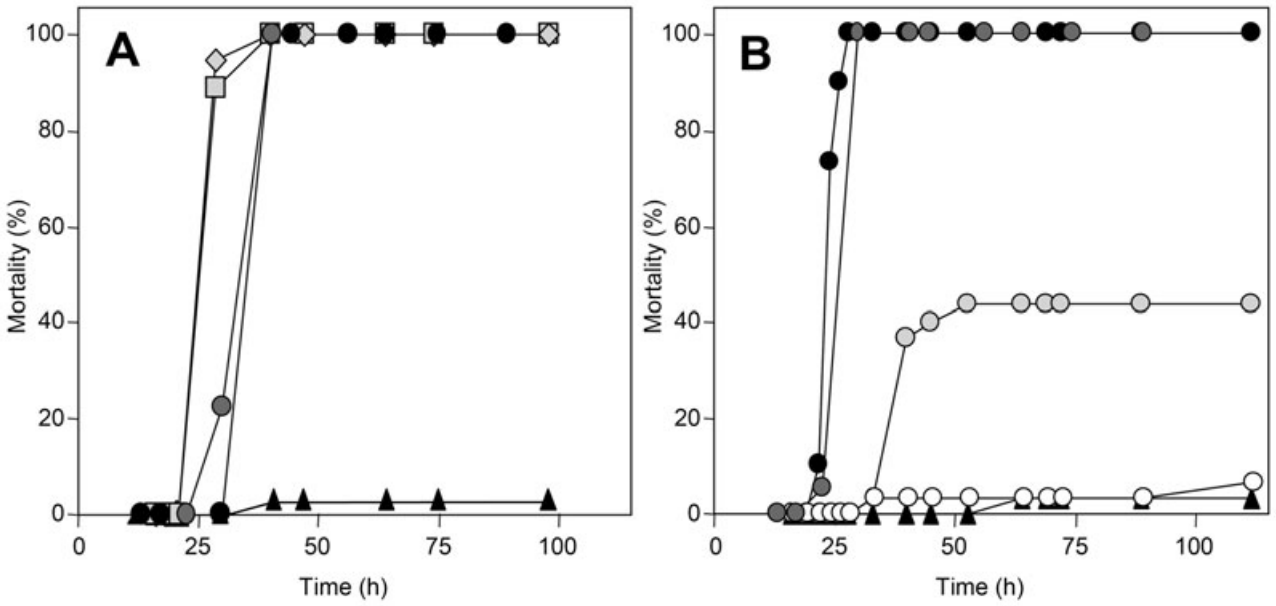

Fig. 2. Relative toxicity of *Pseudomonas fluorescens* strains CHA0 and Pf-5 to larvae of *Galleria mellonella* as compared with (A) known entomopathogens and (B) root-colonizing pseudomonads that do not contain the *fit* locus. Mortality is shown for larvae treated with CHA0 (dark circles, ●), Pf-5 (dark grey circles, ●), *Photorhabdus luminescens* (light grey rhombi, ◆), *Xenorhabdus bovienii* (light grey squares, ■), *Pseudomonas fluorescens* Q2-87 (light grey circles, ●) and *Pseudomonas fluorescens* P3 (open circles, ○). Bacteria were injected at (A) 3×10^2 and (B) 3×10^4 cells per larva in 5 µl of sterile 0.9% NaCl solution. Controls (dark triangles, ▲) received 5 µl of sterile NaCl solution. Eighteen to 30 larvae per treatment were tested.

scens has been mentioned previously (Waterfield *et al*., 2003; ffrench-Constant *et al*., 2007). These proteins have not been characterized so far and it remains to be seen whether they constitute yet another class of Mcf-related proteins with insecticidal properties.

In summary, the genomes of the root-associated, plant-beneficial pseudomonads CHA0 and Pf-5 harbour a unique locus encoding a protein related to a potent insect toxin of the nematode-associated entomopathogen *P. luminescens* and putative toxin transport and regulatory functions. This finding suggested that these pseudomonads may exhibit anti-insect activity in addition to their potent antifungal activity.

P. fluorescens *strains CHA0 and Pf-5 have potent insecticidal activity*

Prompted by the presence of an insect toxin gene homologue in the genome, we tested *P. fluorescens* CHA0 and Pf-5 for toxicity against the greater wax moth *Galleria mellonella* and the tobacco hornworm *Manduca sexta*. When injected into to the haemocoel of ultimate-instar *G. mellonella* at 3×10^2 cells per larva, both CHA0 and Pf-5 killed most of the insects within about 40 h (Fig. 2A). The insecticidal activity of the two pseudomonads was similar to that of the known entomopathogens *P. luminescens* and *Xenorhabdus bovienii* (Goodrich-Blair and Clarke, 2007), which were injected at the same dose, although the two insect pathogens killed the larvae more quickly (Fig. 2A). We then compared anti-insect activities of strains CHA0 and Pf-5 to those of root-associated pseudomonads that do not carry the *fit* locus, as verified by PCR and Southern hybridization analyses (data not shown). Two representative strains were tested, i.e. *P. fluorescens* Q2-87, an effective biocontrol agent that produces the toxic exoproducts DAPG and HCN (Keel *et al*., 1996; Bangera and Thomashow, 1999; Maurhofer *et al*., 2004), and *P. fluorescens* P3, a strain that does not produce the biocontrol-relevant toxic exoproducts DAPG, PLT or HCN, and lacks biocontrol activity (Voisard *et al*., 1989; Maurhofer *et al*., 1998). Injection of CHA0 or Pf-5 at 3×10^4 cells per *Galleria* larva killed all insects within 24 h whereas about 60% and 95% of the larvae survived exposure to an identical cell concentration of the naturally Fit-deficient strains Q2-87 and P3 respectively (Fig. 2B). Strains CHA0 and Pf-5 also exhibited considerable toxicity when injected into larvae of *M. sexta*. When injected with 3×10^4 cells of these bacteria per insect, most *M. sexta* larvae were killed within about 50 h (see Fig. 3C and D). Again, the naturally Fit-negative *P. fluorescens* strain P3 had no negative effect on larvae of this insect host (data not shown).

In summary, these experiments illustrate that *P. fluorescens* strains CHA0 and Pf-5 have potent insecticidal activity which is comparable to that of known

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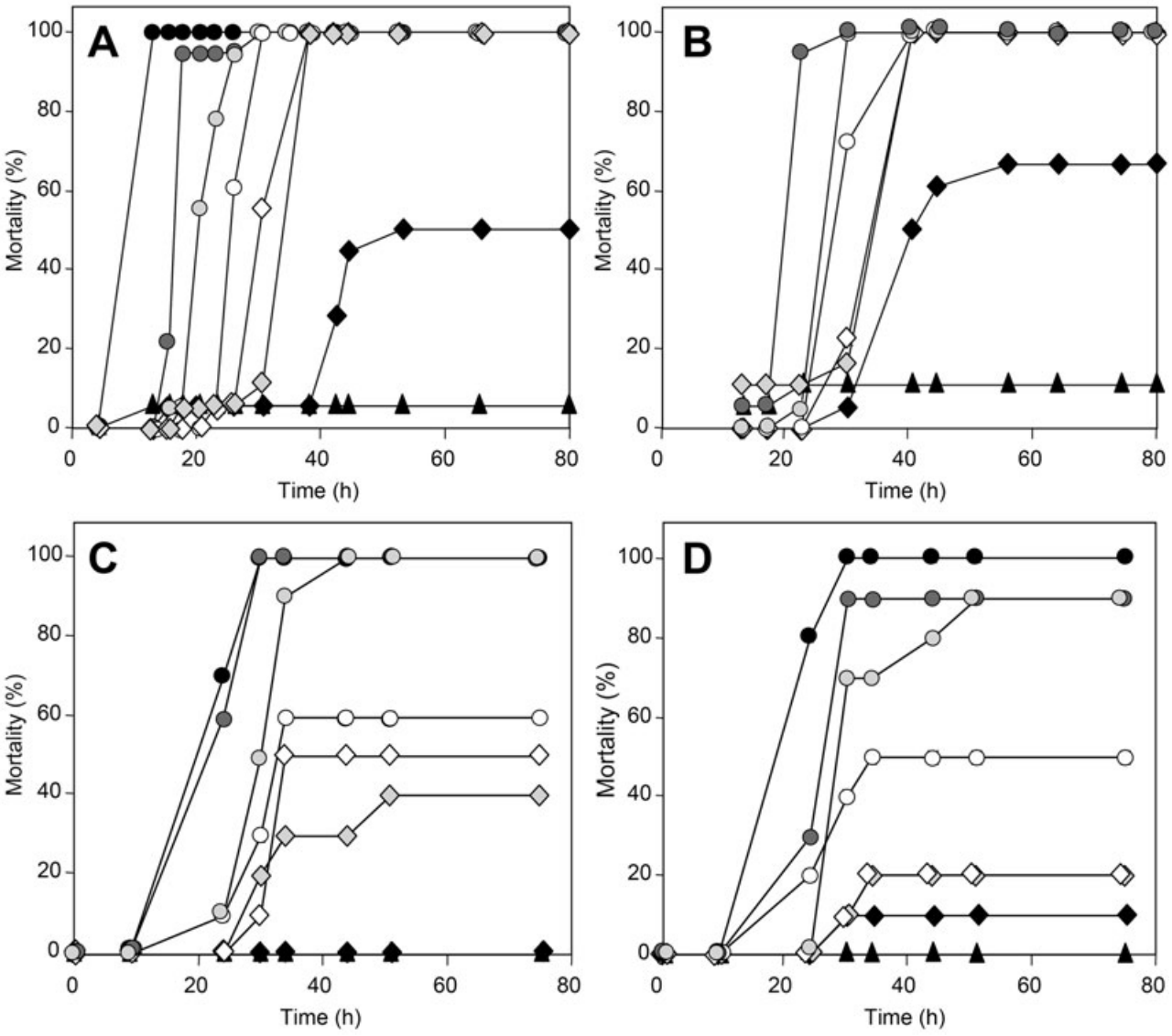

Fig. 3. Dose–response curves for the insecticidal activity of *Pseudomonas fluorescens* strains CHA0 and Pf-5 to larvae of *Galleria mellonella* and *Manduca sexta*: (A) CHA0 on *Galleria*; (B) Pf-5 on *Galleria*; (C) CHA0 on *Manduca*; and (D) Pf-5 on *Manduca*. Bacteria were injected at a concentration of 3×10^6 (dark circles, ●), 3×10^5 (dark grey circles, ●), 3×10^4 (light grey circles, ●), 3×10^3 (open circles ○), 3×10^2 (open rhombi, ◇), 3×10^1 (light grey rhombi, ◇) or 3×10^0 (dark rhombi, ◆) cells in 5 μl of sterile 0.9% NaCl solution per larva for *Galleria* and in 10 μl of sterile H_2O per larva for *Manduca*. Controls (dark triangles, ▲) received the same quantities of sterile NaCl solution or H_2O respectively. Ten larvae of *M. sexta* and at least 18 larvae of *G. mellonella* were inoculated for each treatment. The experiment was repeated once with highly similar results.

entomopathogens. In the root-associated pseudomonads tested, the presence of the *fit* locus was correlated with insecticidal activity. The limited insect toxicity exhibited by the Fit-deficient, but DAPG- and HCN-positive *P. fluorescens* strain Q2-87 suggests that other toxic products may contribute to anti-insect activity. It should also be noted here that the insecticidal activity appears to be associated with the bacterial cells, as injection of culture supernatants of strains CHA0 or Pf-5 had no effect on insect larvae (data not shown).

Dose–response relationships for insect toxicity of P. fluorescens *CHA0 and Pf-5*

To learn more about the insecticidal potency of *P. fluorescens* CHA0 and Pf-5, we then monitored dose–response relationships for injection of the two pseudomonads into larvae of *G. mellonella* and *M. sexta*. To this end, increasing bacterial inocula ranging from 3×10^0 to 3×10^6 washed cells per larva were injected and insect mortality was recorded over a period of 80 h.

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Table 1. Lethal time (LT_{50}) and lethal dose (LD_{50}) for *Pseudomonas fluorescens* strains CHA0 and Pf-5 following injection into larvae of *Galleria mellonella* and *Manduca sexta*.

	Galleria larvae[a]		*Manduca* larvae[a]	
	CHA0	Pf-5	CHA0	Pf-5
LT_{50} (h post infection)[b]				
3×10^1 cells	38 (38; 38)	40 (40; 40)	NC	NC
3×10^2 cells	30 (30; 38)	40 (40; 40)	NC	NC
3×10^3 cells	26 (26; 30)	30 (30; 40)	NC	NC
3×10^4 cells	20 (20; 23)	30 (30; 30)	30 (30; 34)	30 (30; 51)
3×10^5 cells	17 (17; 17)	22 (22; 22)	24 (24; 30)	30 (24; 30)
3×10^6 cells	13 (13; 13)	ND	24 (24; 30)	24 (24; 30)
LD_{50} (bacterial cells per larva) at 30 h post infection[c]	183 (135; 286)	980 (190; 2080)	4360 (303; 112 564)	3534 (912; 15 237)

a. Bacteria were injected at the shown concentrations in 5 μl of sterile 0.9% NaCl solution per larva for *G. mellonella* and in 10 μl of sterile H_2O per larva for *M. sexta*. Ten larvae of *M. sexta* and at least 18 larvae of *G. mellonella* were inoculated for each treatment. Controls were injected with the same quantities of sterile NaCl solution or H_2O respectively.
b. Estimates of LT_{50} values at each concentration were made using survivorship analysis (Kaplan–Meier, Statistix 8). Values are medians with the lower and upper confidence intervals shown in brackets. NC, LT_{50} values could not be calculated at the lower concentrations because the 50% mortality was not hit at 30 h. ND, not determined.
c. Estimates of LD_{50} values for mortality 30 h post injection were performed using probit analysis (PriProbit, Polo Plus) and values were compared on the basis of overlapping 95% confidence intervals (shown in brackets). Control mortality was less than 15% in all assays.

For *G. mellonella*, injection of as few as 30 cells of CHA0 or Pf-5 per larva was sufficient to cause 100% mortality within 40 h post infection (Fig. 3A and B). At higher doses, 100% mortality was reached after even shorter incubation times. At a concentration of three cells injected per larva, a significant proportion of the larvae, i.e. about 50% for CHA0 (Fig. 3A) and more than 30% for Pf-5 (Fig. 3B), was still alive at the end of the experiment. For *M. sexta*, bacterial concentrations of at least 3×10^4 cells per larva and incubation times of up to 50 h were required to reach a mortality rate of 90% or higher (Fig. 3C and D). With bacterial inocula of 3×10^3 cells per larva and lower, at least 40% of the larvae survived exposure to the two pseudomonads until the end of the experiment (Fig. 3C and D).

Based on the data obtained, we estimated the average time required to kill 50% of the injected insect larvae (LT_{50}). For *G. mellonella*, median LT_{50} values ranged from 13 to 38 h when infected with a high dose (3×10^6 cells) and a low dose (3×10^1 cells), respectively, of CHA0; and from 22 h (3×10^5 cells) to 40 h (3×10^1 cells) when infected with Pf-5 (Table 1). For *M. sexta*, LT_{50} values were 24–30 h for bacterial concentrations ranging from 3×10^4 to 3×10^6 cells per larva (Table 1). We also estimated the lethal dose causing 50% mortality of the larvae (LD_{50}) at 30 h after bacterial infection. LD_{50} values for injection into *G. mellonella* were estimated to be 1.8×10^2 cells for CHA0 and 9.8×10^2 cells for Pf-5 (Table 1). Estimated LD_{50} values were markedly higher for *M. sexta*, corresponding to about 4×10^3 cells for the two pseudomonads (Table 1).

Taken together, these data highlight that *P. fluorescens* CHA0 and Pf-5 exhibit considerable toxicity to both insect hosts when injected at remarkably low cell concentrations. Data indicate that both bacteria are capable of killing *G. mellonella* at lower doses and more quickly than they kill *M. sexta*.

Contribution of the Fit toxin to insecticidal activity of P. fluorescens

To determine the role of the novel Fit toxin in insecticidal activity of *P. fluorescens*, we created in-frame deletion mutations in the *fitD* genes of strains CHA0 and Pf-5 (Fig. 1), and compared the resulting *fitD* mutants (CHA1151 and Pf-5-1151) with the respective parental strain for toxicity towards the two insect hosts. Injection of 3×10^6 cells of wild-type CHA0 or Pf-5 into *M. sexta* killed 89% and 65% of the larvae, respectively, within 24 h, whereas a significantly lower percentage of the larvae survived exposure to their respective toxin-deficient mutants, i.e. 35% for CHA1151 (Fig. 4A) and 22% for Pf-5-1151 (Fig. 4B). At 48 h post infection, almost all of the *Manduca* larvae infected with wild-type strains CHA0 and Pf-5 were dead, whereas 20–40% of larvae treated with the *fitD* mutants survived (Fig. 4A and B). The high mortality of the larvae exposed to the *fitD* mutants at this time point suggests that additional mechanisms could be involved in the toxicity of the two pseudomonads. Figure 5A depicts the typical two types of symptoms exhibited by *M. sexta* larvae following injection of wild-type CHA0 or Pf-5. The floppy phenotype, indicative of massive loss of body turgor, was observed in almost all dead larvae by 24 or 48 h after inoculation, with the exception of those few larvae that died in the water-injected controls. The melanized phenotype was also commonly observed in larvae injected with the two *P. fluorescens* strains. Although seen most often (but not

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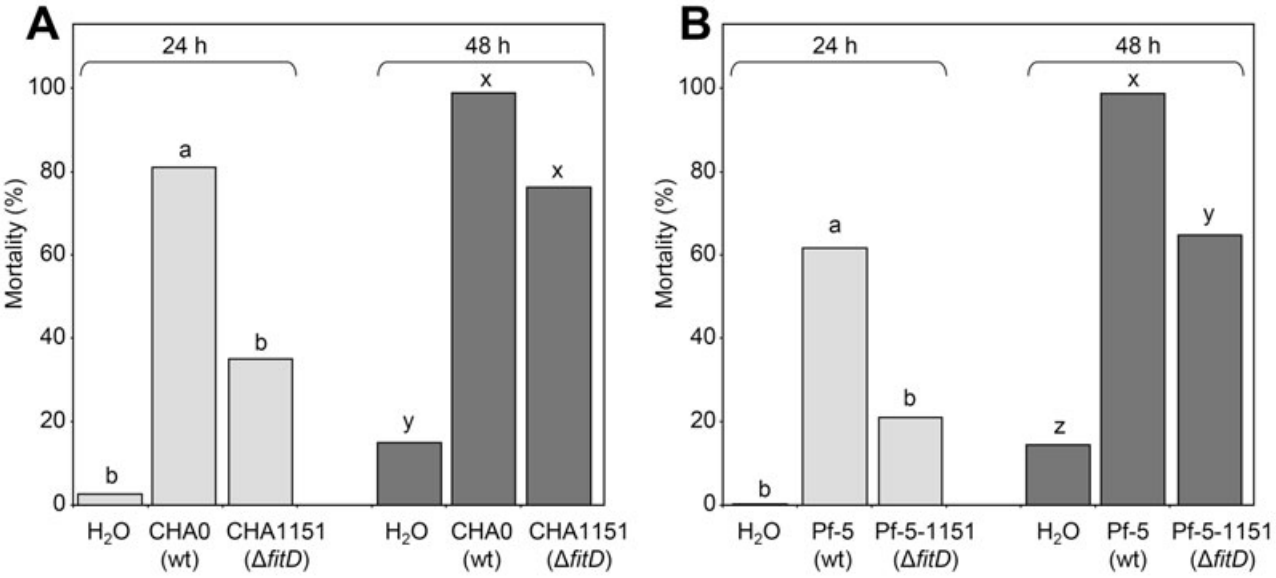

Fig. 4. Contribution of *fitD* to toxicity of *Pseudomonas fluorescens* strains to *Manduca sexta*. Mortality of *M. sexta* larvae at 24 h and 48 h after injection with 3×10^6 cells per larva of (A) strain CHA0 or (B) strain Pf-5 and the corresponding Δ*fitD* mutants. Larvae injected with sterile H_2O were negative controls. Values are means from four (Pf-5) and five (CHA0) individual experiments, each with 15 replicates (larvae) per treatment. Data were analysed by ANOVA followed by Fisher's protected least significant difference (LSD) test. Columns for the same time point marked with different letters are significantly different ($P \leq 0.05$) from each other.

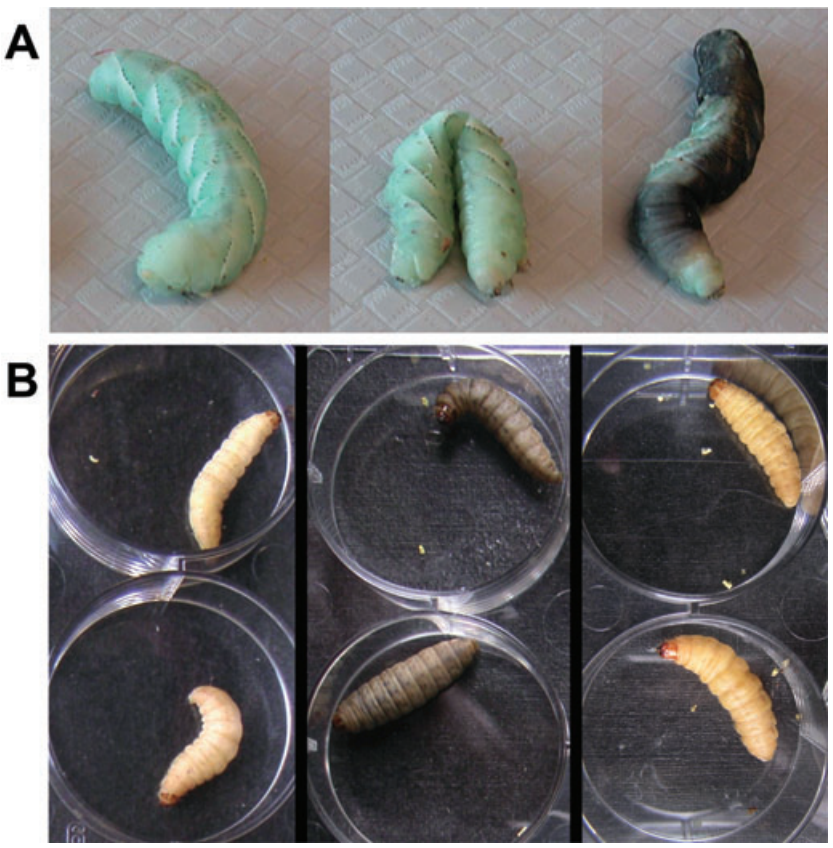

Fig. 5. A. Typical symptoms exhibited by larvae of *Manduca sexta* at 24 h after inoculation with 3×10^6 cells of *Pseudomonas fluorescens* CHA0 or Pf-5. Left: healthy non-inoculated control larva; centre and right: inoculated larvae exhibiting the floppy phenotype associated with loss of body turgor or the melanization phenotype, respectively. B. Typical symptoms exhibited by larvae of *Galleria mellonella* at 21.5 h following injection of 3×10^4 cells of *P. fluorescens* CHA0 and Pf-5 and their Δ*fitD* mutants. Left: non-inoculated healthy control larvae; centre: strongly melanized larvae injected with wild-type CHA0; right: significantly less melanized larvae inoculated with the Δ*fitD* mutant CHA1151.

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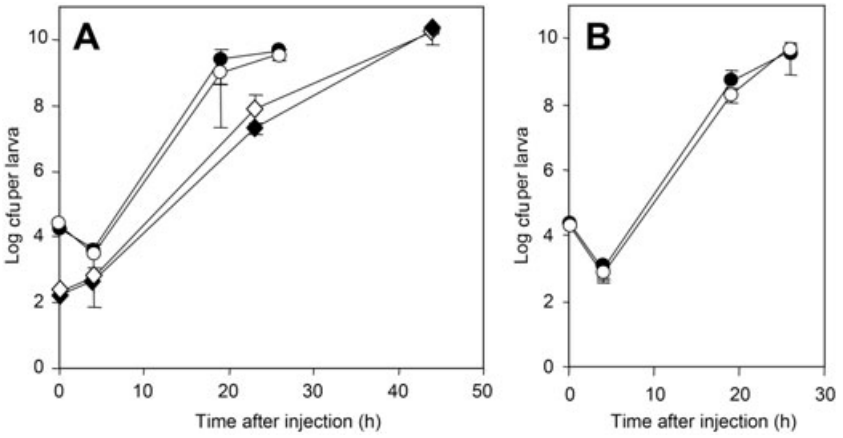

Fig. 6. Colonization of larvae of *Galleria mellonella* by *Pseudomonas fluorescens* strains (A) CHA0 and (B) Pf-5 and their *fitD* mutants. Wild-type strains (dark circles, ●) and *fitD* mutants (open circles, ○) were injected at 3×10^4 cells per larva. A lower injection dose of 3×10^2 cells was only tested for strain CHA0 (dark rhombi, ◆) and its *fitD* mutant (open rhombi, ◇). At each time point and concentration, *P. fluorescens* population sizes were determined for three individual larvae by selective plating. No pseudomonads could be reisolated from control larvae that were injected with sterile NaCl solution. Background bacterial populations in the controls varied between 2.1×10^5 and 6.2×10^5 cfu per larva.

always) on the dead larvae at 48 h, some larvae had already developed the blackened tissue by 24 h after injection.

The contribution of FitD to insecticidal activity was less readily visible with the more sensitive insect host *G. mellonella*. This is illustrated by the following representative assay in which *G. mellonella* (30 larvae per treatment) were injected with 3×10^4 cells of *P. fluorescens* CHA0 or Pf-5 and their respective *fitD* mutants. Differences between wild-type strains and *fitD* mutants were only visible in a relatively narrow time window, as is evident from the results obtained. At 19 h post infection, all larvae were alive and exhibited no signs of melanization. At 21.5 h post infection, 40.2 ± 24.6% of the larvae infected with wild-type CHA0 were melanized (Fig. 5B) whereas only 6.7 ± 4.1% of the larvae treated with the *fitD* mutant CHA1151 exhibited the melanized phenotype. At this time point, insect mortality was still below 10% for CHA0 and its *fitD* mutant. At 24 h, all of the larvae inoculated with CHA0 were melanized and over 73% were dead. At 26 h, all inoculated larvae were dead. Compared with the wild-type CHA0, melanization at the time of death was less pronounced for *G. mellonella* larvae inoculated with the *fitD* mutant (Fig. 5B), with these larvae often exhibiting a dorsal bowing phenotype. In contrast, a floppy phenotype, as apparent from a softening of the larval body, was typically observed in larvae injected with the wild-type strain. Similar observations were made for strain Pf-5 and its *fitD* mutant Pf-5-1151 (data not shown). All non-inoculated control larvae remained non-melanized and alive during the evaluation period.

Insect colonization by P. fluorescens

As doses as low as 30 cells of *P. fluorescens* CHA0 or Pf-5 per larva were sufficient to cause 100% mortality in *Galleria* (Fig. 3A and B), it was of interest to determine whether the two bacteria multiply in the insect host and whether the FitD toxin is required for insect colonization. When injected at a dose of 3×10^4 cells, populations of both strains CHA0 and Pf-5 increased by more than five log units to a level of about 5×10^9 colony-forming units (cfu) per larva within a period of 26 h (Fig. 6A and B). Population levels of the *fitD* mutants CHA1151 (Fig. 6A) and Pf-5-1151 (Fig. 6B) did not differ significantly from those of their parental strains, indicating that the toxin is not a prerequisite for bacterial multiplication in the insect host. In larvae injected with a lower dose of only 3×10^2 cells of CHA0 and its *fitD* mutant, the bacterial strains still multiplied to a level of 10^{10} cfu per larva within 44 h (Fig. 6A). This finding further underscores the proliferation potential of these pseudomonads in the insect host.

Pf-5 and CHA0 were also capable of multiplying in *M. sexta* larvae. During a 24 h period, bacterial populations increased by almost five log units (to about 10^8 cfu per larva) in the majority of the inoculated larvae (Fig. 7). In a fraction of the inoculated larvae, bacterial populations did not increase appreciably, as was observed in two of six larvae inoculated with Pf-5 in one experiment (Fig. 7). There was no visible difference between sampled larvae, and determining an explanation for the variability in bacterial population sizes among larvae was beyond the scope of this study.

In conclusion, our findings demonstrate that the FitD toxin makes a significant contribution to insecticidal activities of *P. fluorescens* strains CHA0 and Pf-5, but may not be crucial for the capacity of these bacteria to multiply in the infected host. The fact that *fitD* mutants still retained a certain level of toxicity indicates that additional factors may contribute to anti-insect activity.

Controlled expression of the P. fluorescens *Fit toxin in a non-toxic* E. coli *host*

To confirm the insecticidal activity of the FitD protein in the absence of possible effects of other toxic *P. fluorescens* products, the protein was overexpressed in *E. coli* BL21. For this purpose, the *fitD* gene of strain CHA0 was cloned

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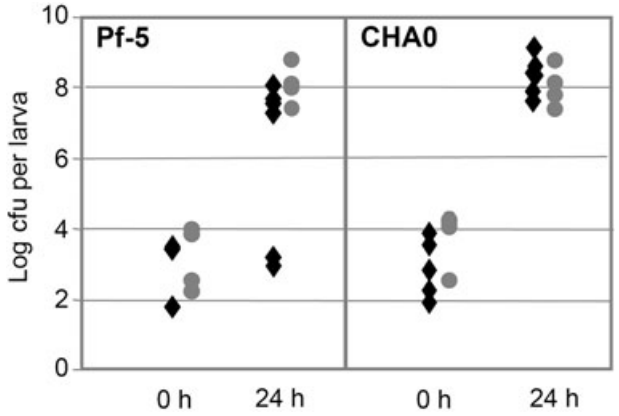

Fig. 7. Colonization of larvae of *Manduca sexta* by *Pseudomonas fluorescens*. Each point represents the population size of CHA0 or Pf-5 in an individual larva at 0 h or 24 h after inoculation with 3×10^4 cells per larva. Bacterial population sizes were estimated from six larvae at each time point, and the results from two experiments (experiment 1, dark rhombi, ◆; experiment 2, dark grey circles, ●) are shown. Background bacterial populations in the water-injected controls were typically below the limit of detection (1×10^2 cfu) with a maximum of 9×10^2 cfu per larva at the 0 h time point, and were below the limit of detection (1×10^3 cfu) in all larvae at the 24 h time point.

under the control of the cold shock-inducible *cspA* promoter in the pColdI vector. Following cold shock and isopropyl-β-D-thiogalactoside (IPTG) induction of *E. coli* cells carrying the resulting construct pColdI::*fitD* (Fig. 1), a protein band corresponding to the predicted molecular mass of 327.3 kDa of the FitD protein could be detected by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis (Fig. 8A and B, lanes 2 and 3). The protein band could not be detected in cell extracts of *E. coli* carrying the empty pColdI vector control (data not shown). The additional bands in Fig. 8B may be indicative of some degradation of the FitD protein. Additional analyses of crude cells extracts of *fitD*-expressing transgenic *E. coli* revealed that a major fraction of the toxin was localized in the cell debris, but was not detectable in the cytosol or in culture supernatants, suggesting that the toxin largely remains cell wall-associated in this strain (data not shown).

When injected into larvae of both *G. mellonella* and *M. sexta* following induction of FitD toxin expression, transgenic *E. coli* cells exhibited marked insecticidal activity in both insect hosts (Table 2). At a dose of 3×10^6 cells of *fitD*-expressing *E. coli* BL21/pColdI::*fitD*, more than 70% of the *G. mellonella* larvae were killed within 48 h and a similar mortality rate was observed for larvae of *M. sexta* when they were injected with a dose of 5×10^7 cells of the bacterium (Table 2). Injection of *E. coli* carrying the empty vector control caused a basal lethality among the larvae that was not significantly different from the non-inoculated controls (Table 2).

Dose–response curves for the effect of injection of FitD toxin-expressing *E. coli* were established for *G. mellonella* (Fig. 9). A dose of at least 3×10^5 cells was required to cause 100% mortality among the larvae within 92 h post infection; a dose of 3×10^4 cells was sufficient to cause more than 50% mortality within the same period (Fig. 9). At lower bacterial concentrations, mortality levels were not significantly different from those of larvae inoculated with *E. coli* carrying the empty vector or uninoculated (Fig. 9). Larvae of *G. mellonella* and *M. sexta* injected with *fitD*-expressing transgenic *E. coli* exhibited the melanized and floppy phenotypes described above.

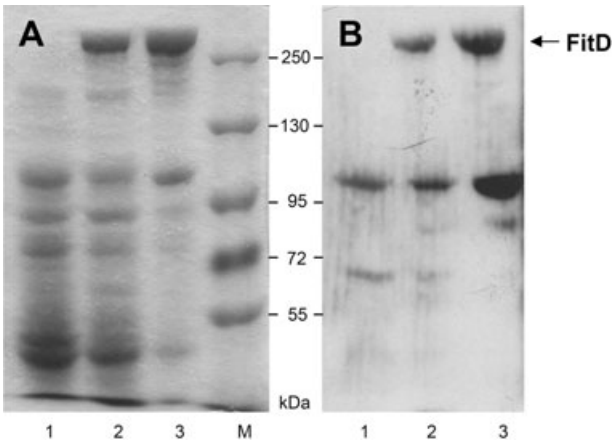

Fig. 8. (A) SDS-PAGE and (B) Western blot analysis of FitD expression from a cold shock-inducible promoter in *Escherichia coli*. Lanes: 1, no induction, 37°C, 24 h incubation; 2, cold shock at 15°C, 30 min incubation; 3, cold shock at 15°C and addition of 0.1 mM IPTG, 24 h incubation; M, molecular mass standards. Samples were prepared from cells of *E. coli* BL21/pColdI::*fitD*. The arrow indicates the FitD protein which was tagged with His6. The SDS-6% PAGE gel was stained with Coomassie brilliant blue.

Table 2. Mortality of larvae of *Galleria mellonella* and *Manduca sexta* following injection of *Escherichia coli* expressing the Fit toxin of *P. fluorescens* CHA0 from pColdI.

	Mortality (%) of *Galleria* larvae		Mortality (%) of *Manduca* larvae	
Treatment[a]	24 h	48 h	24 h	48 h
No bacteria	0.0 a	9.3 a	0.0 a	0.0 a
E. coli BL21/pColdI	4.8 a	15.9 a	0.0 a	0.0 a
E. coli BL21/pColdI::*fitD*	8.5 a	76.3 b	68.9 b	73.3 b

a. Fit toxin expression was induced by growing bacteria at 15°C in the presence of 0.1 mM IPTG for 24 h. Induced bacteria were injected at a concentration of 3×10^6 cells in 5 µl of sterile 0.9% NaCl solution per *Galleria* larva and at 5×10^7 cells in 10 µl of sterile H_2O per *Manduca* larva. *Escherichia coli* carrying the empty vector control injected at the same cell concentration and sterile NaCl solution (or sterile water) served as negative controls. Values are means from three individual experiments, each experiment with at least 15 replicates (larvae) per treatment. Data were analysed by ANOVA followed by Fisher's protected least significant difference (LSD) test. Data in the same column marked with different letters are significantly different ($P \leq 0.05$) from each other.

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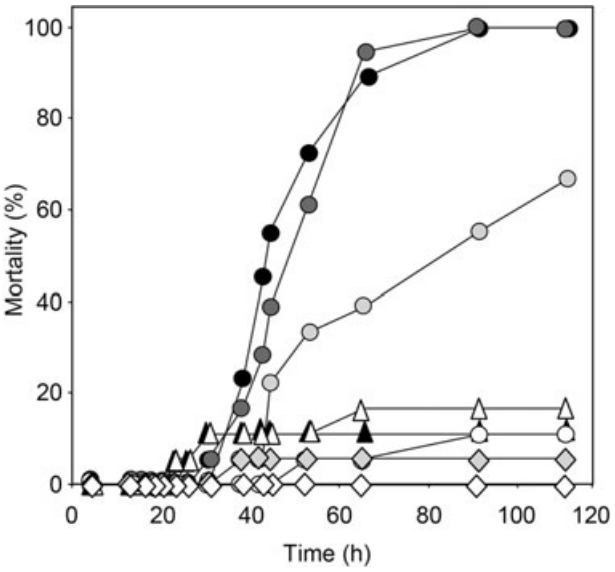

Fig. 9. Dose–response curves for toxicity of *Escherichia coli* expressing the FitD toxin of *Pseudomonas fluorescens* CHA0 from a cold shock-inducible promoter on pColdI to larvae of *Galleria mellonella*. Fit expression was induced by incubating bacteria at 15°C in the presence of 0.1 mM IPTG for 24 h. Bacteria were then injected at a concentration of 3×10^6 (dark circles, ●), 3×10^5 (dark grey circles, ●), 3×10^4 (light grey circles, ●), 3×10^3 (open circles, ○), 3×10^2 (open rhombi, ◇) or 3×10^1 (light grey rhombi, ◇) cells per larva in 5 µl of sterile 0.9% NaCl solution. For *E. coli* carrying the empty vector control (open triangles, △), only the highest bacterial concentration (3×10^6 cells) injected is shown. Larvae injected with sterile NaCl solution (dark triangles, ▲) served as negative control. Eighteen to 30 larvae per treatment were tested. The experiment was repeated twice with similar results.

Together, these data demonstrate that the *Pseudomonas* insect toxin FitD expressed in *E. coli* is sufficient to allow this non-toxic host to kill larvae of two insect species, thus corroborating the anti-insect potency of the novel toxin.

Discussion

The plant growth-promoting *P. fluorescens* strains CHA0 and Pf-5 are known as efficient root colonizers and biocontrol agents of fungal plant diseases and over the past years we have gathered in-depth knowledge about relevant traits and mechanisms that contribute to disease-suppressive activity (Haas and Keel, 2003; Loper and Gross, 2007). In this study, we present the first evidence that the two root-associated bacteria also exhibit potent insecticidal activity and kill larvae of *M. sexta* and *G. mellonella* at cell concentrations and within time frames that were comparable to those of the known insect pathogens *Photorhabdus* and *Xenorhabdus* (Figs 2A and 3). Previously, injected doses of 20 cells or less of the entomopathogens were reported to be sufficient to kill a variety of insect larvae within 48–72 h (ffrench-Constant *et al*., 2003; Goodrich-Blair and Clarke, 2007). By comparison, injection of 30 cells of CHA0 or Pf-5 was sufficient to kill *Galleria* larvae within 40 h, and a 100-fold higher dose was required to kill larvae of *Manduca* in the present study. To date, only a few studies point to the possibility that some soil- and plant-associated pseudomonads harm insects. Commare and colleagues (2002) found that treatment of rice leaves with two plant growth-promoting *P. fluorescens* strains altered the feeding behaviour and increased the mortality of leaf-folder larvae (*Cnaphalocrocis medinalis*). Similarly, treatment of tomato plants with an epiphytic *P. fluorescens* strain caused significant mortality among leaf-feeding larvae of the ladybird beetle *Epilachna vigintioctopunctata* (Otsu *et al*., 2004). A recent study of Chapuisat and colleagues (2007) demonstrated that a *P. fluorescens* isolate from nest material was detrimental to larvae and adult workers of wood ants (*Formica paralugubris*) and that the insects use resin to protect themselves against the bacterium. Finally, a *P. fluorescens* soil isolate was reported to produce unidentified toxic exoproducts that are lethal to larvae and pupae of mosquitoes (Prabakaran *et al*., 2003). However, the molecular basis of anti-insect action of *P. fluorescens* was not established in these studies.

Our study is the first report of an insect toxin in plant-associated pseudomonads. Several lines of evidence support the important role of the Fit toxin in the insecticidal activity of *P. fluorescens* strains CHA0 and Pf-5: (i) Fit toxin-deficient mutants were significantly impaired in insecticidal activity (Figs 4 and 5B), (ii) heterologous expression of the Fit toxin gene in *E. coli* was sufficient to render the non-toxic bacterium capable of killing insect larvae (Table 2; Fig. 9), and (iii) naturally Fit toxin-negative rhizosphere pseudomonads had no or only limited insecticidal activity (Fig. 2B). The Fit toxin produced by the two plant colonizers is most closely related to the insect toxin Mcf1 produced by the animal colonizer *P. luminescens* (Daborn *et al*., 2002). Similar to the Fit toxin, the single Mcf1 toxin and its truncated variant Mcf2 are also sufficient to kill larvae of *Manduca* and *Galleria* when expressed in *E. coli* (Daborn *et al*., 2002; Waterfield *et al*., 2003). The relatedness of the *Pseudomonas* Fit toxin to the *Photorhabdus* Mcf toxin is further underscored by the fact that injection of Fit toxin-expressing cells of *P. fluorescens* or *E. coli* caused to die the larvae with a typical floppy phenotype (Fig. 5A), similar to that associated with loss of larval body turgor induced by Mcf1 and Mcf2 of *P. luminescens* (Daborn *et al*., 2002; Waterfield *et al*., 2003). In our experiments, the floppy phenotype was often accompanied by a strong melanization of the larval body.

In silico analysis of available sequence data from microbial genomes indicates that the Mcf/Fit toxin gene

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family is present in *P. luminescens* and a subset of *P. fluorescens* strains represented by CHA0 and Pf-5 respectively. At this stage, nothing is known on how frequently Fit toxin loci occur in plant-associated pseudomonads and whether they are linked to specific genomic/ecological groups or particular abilities (e.g. biocontrol activity) of these organisms. *Pseudomonas fluorescens* strains CHA0 and Pf-5 are representatives of genotypically and phenotypically related root-associated pseudomonads that produce multiple antifungal compounds (e.g. DAPG, PLT, pyrrolnitrin and HCN) and cluster in the same amplified 16S ribosomal DNA restriction analysis (ARDRA), randomly amplified polymorphic DNA (RAPD) and BIOLOG (carbon source utilization profiles) groups (Keel *et al*., 1996; Wang *et al*., 2001). A preliminary hybridization experiment in which a *fitD* probe of CHA0 was tested on a strain selection indicated that the Fit toxin locus might be present in specific subgroups of these plant-associated pseudomonads (C. Keel and M. Péchy-Tarr, unpubl. data). In future experiments, it may be worthwhile to investigate the frequency and diversity of Fit toxin-producing strains among natural *Pseudomonas* populations in the rhizosphere. This may help to determine whether *Pseudomonas* Fit proteins are conserved or divergent. In *Photorhabdus*, two Mcf toxin variants (Mcf1 and Mcf2) that share a similar central domain but differ in their N- and C-termini have been described so far (Waterfield *et al*., 2003; Dowling *et al*., 2007).

Toxin-negative *fitD* mutants of *P. fluorescens* CHA0 and Pf-5 exhibited a strongly reduced insecticidal activity, which was most evident with *M. sexta* as the insect host. Differences between the *fitD* mutants and their parental strains were less pronounced with *G. mellonella*. Typically, *G. mellonella* was more sensitive than *M. sexta* to inoculation with the insecticidal pseudomonads (see Fig. 3), so variability in the defence response of the two insect hosts (Kavanagh and Reeves, 2004; Eleftherianos *et al*., 2007) may have accounted for the observed discrepancy. Moreover, a recent study with *P. luminescens* highlights that small nuances in the age and developmental stage of insect larvae can strongly influence the extent of the immune response to the insect pathogen (Eleftherianos *et al*., 2008). In our insect toxicity assays, *Galleria* larvae were in the non-feeding stage prior to pupation at the time of bacterial inoculation whereas *Manduca* larvae were feeding and still actively growing. It is possible that these discrepancies in the host developmental stage and additional experimental variables (e.g. temperature) that differed between the two assays systems may have influenced the responsiveness of the test insects to the *P. fluorescens* wild-type and *fitD* mutant strains.

Besides the Fit toxin, accessory factors may contribute to anti-insect activity in *P. fluorescens* CHA0 and Pf-5 as toxin-deficient mutants exhibited residual insect toxicity. Both pseudomonads are known to release a wide array of biologically active exoproducts including several broad-spectrum antimicrobial metabolites (DAPG, PLT, pyrrolnitrin and HCN) and an extracellular protease controlled by the GacS/GacA two-component regulatory system (Haas and Keel, 2003; Siddiqui *et al*., 2005; Dubuis *et al*., 2007; Loper and Gross, 2007). It is tempting to speculate that some of these exoproducts might act as accessory factors sustaining anti-insect activity of the Fit toxin. A few studies in which several of these GacS/GacA-controlled exoproducts were proposed to contribute to toxicity of strain CHA0 towards other non-mammalian hosts, i.e. nematodes and protozoa, point to this possibility (Schlimme *et al*., 1999; Siddiqui *et al*., 2005; Jousset *et al*., 2006; L. Bjørnlund, C. Keel, M. Maurhofer and O. Nybroe, unpublished). In *P. entomophila*, a natural entomopathogen of *Drosophila*, an excreted metalloprotease, functions as an important virulence factor and a number of additional GacS/GacA-controlled exoproducts are considered to be candidates contributing to pathogenicity and defence against the insect's immune response (Liehl *et al*., 2006). Likewise, multiple virulence factors depending on GacS/GacA, quorum-sensing regulators or a functional type III secretion system are thought to be required for virulence of the opportunistic human pathogen *P. aeruginosa* to *Drosophila* and *Galleria*, which have served as non-mammalian model hosts for studies into virulence mechanisms of the bacterium (D'Argenio *et al*., 2001; Fauvarque *et al*., 2002; Miyata *et al*., 2003). Hydrogen cyanide has been identified as a major factor responsible for killing of *Caenorhabditis elegans* by this bacterium (Gallagher and Manoil, 2001). In the case of *P. fluorescens* CHA0 and Pf-5, it remains to be seen which combinations of toxic or lytic exoproducts best sustain Fit action and at which stage they are implicated in anti-insect activity. As CHA0 and Pf-5 were capable of proliferating to high population densities in both insect hosts and, at least in *Galleria*, the Fit toxin was not a major factor required for insect colonization (Figs 6 and 7), it may be interesting to investigate whether the above-mentioned accessory exoproducts constitute the crucial determinants allowing these bacteria to establish in the insect host and cope with host defence reactions. In *P. luminescens*, insect virulence is multifactorial involving multiple toxins and exoenzymes (Duchaud *et al*., 2003; ffrench-Constant *et al*., 2007; Goodrich-Blair and Clarke, 2007) and a fascinating recent report illustrates that a hydroxystilbene compound with broad-spectrum antimicrobial activity produced by the bacterium can act as a suppressor of host defences (Eleftherianos *et al*., 2007).

At the present stage, it is difficult to speculate about the possible mode of action of the *P. fluorescens* Fit toxin. The nearest relative is the Mcf1 toxin produced by

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P. luminescens, which when expressed in non-toxic *E. coli* destroys both insect gut cells and phagocytes (haemocytes) by promoting their apoptosis (Daborn *et al.*, 2002). Mcf1-mediated destruction of the insect midgut results in a complete loss of body turgor inducing the typical floppy phenotype. Mcf1 also promotes apoptosis in several mammalian cell lines (Dowling *et al.*, 2004). Recent findings suggest that the toxin is internalized and mediates apoptosis from within the host cells via the mitochondrial pathway (Dowling *et al.*, 2007). Two domains that possibly contribute to toxin action have been identified, i.e. the central region of Mcf1 that shows similarities with the putative translocation domain of *Clostridum difficile* toxin B (Jank *et al.*, 2007) and an N-terminal BH3-like domain that is functionally required for the pro-apoptotic activity of the toxin (ffrench-Constant *et al.*, 2007; Dowling *et al.*, 2007). Remarkably, a similar domain structure can be detected in the Mcf homologues of *P. fluorescens* (ffrench-Constant *et al.*, 2007; our unpublished findings). The central region of the CHA0 Fit toxin (residues 1648–2186) shows 22% identity and 43% similarity with the *C. difficile* toxin B translocation domain (AM180355, locus tag CD0660, residues 891–1410) and the N-terminus contains a consensus sequence (LKTGLASLGAGFEPR) showing 73% identity with the BH3-like domain of Mcf1 proposed by Dowling and colleagues (2007). It remains to be seen whether the Mcf1-like domain structure of the *Pseudomonas* Fit toxin translates also in a similar mode of action in insect toxicity.

The occurrence of insecticidal activity in the plant-colonizing pseudomonads CHA0 and Pf-5 is somewhat surprising as these bacteria have no known insect association. Our study is in line with increasing evidence for the occurrence of anti-insect activity (Commare *et al.*, 2002; Otsu *et al.*, 2004; Chapuisat *et al.*, 2007) and insect toxin-like genomic loci (Guttman *et al.*, 2002; ffrench-Constant *et al.*, 2007) in certain environmental isolates of *P. fluorescens* that may be indicative of unrecognized insect interactions or a fundamental role in other ecological interactions. To date, we can only speculate about the possible ecological role of insect toxins in root-associated pseudomonads. The rhizosphere, i.e. the immediate surroundings of the roots, not only provides nutrients and niches for biocontrol pseudomonads but also is teeming with other members of the soil microflora and microfauna. In order to survive and multiply in this ecological niche, root-colonizing pseudomonads have to cope with a variety of organisms that are attracted by the same habitat, among them competing microorganisms (e.g. pathogenic fungi) that colonize, invade and exploit the plant host (Haas and Keel, 2003; Loper and Gross, 2007), root-feeding insects and nematodes (Hunter, 2001; Tian *et al.*, 2007), and bacteria-feeding predators, in particular nematodes, protozoa and microarthropods (Thimm *et al.*, 1998; Bonkowski, 2004; Fountain and Hopkin, 2005). We hypothesize that the Fit toxins may be part of an arsenal of toxic exoproducts that help these pseudomonads improve their ecological competitiveness and defence against predators. Whereas well-characterized antimicrobial compounds such as DAPG, PLT and HCN have a demonstrated role in toxicity towards bacteria, fungi, nematodes and protozoa (Haas and Keel, 2003; Bonkowski, 2004; Jousset *et al.*, 2006; Dubuis *et al.*, 2007; Loper and Gross, 2007), the Fit insect toxins discovered in this study may specifically endow the pseudomonads with the capacity to compete with root-feeding insects that destroy their habitat and food base. Alternatively, they may help the bacteria defend themselves against certain predators, in particular microarthropods that they are likely to encounter in the rhizosphere. Strategies to escape predation and to counter competition may ultimately help these plant-beneficial pseudomonads to maintain root populations densities required for biocontrol efficacy.

The present study highlights the potential of plant-associated pseudomonads to serve as a novel reservoir for biocontrol agents and microbial toxins with anti-insect activity. Insect toxin production by efficient root colonizers and plant disease-suppressive agents implies possibilities to develop novel pathogen and pest control strategies in which antifungal and anti-insect activities in plant-beneficial pseudomonads are combined for improving root health of agricultural crops.

Experimental procedures

Bacterial strains, plasmids and culture conditions

The bacterial strains and plasmids used in the present study are listed in Table 3. *Pseudomonas fluorescens*, *P. luminescens* and *X. bovienii* strains were cultured routinely on nutrient agar (NA), on King's medium B (KMB), in Luria–Bertani broth (LB) and in nutrient broth containing 0.5% (w/v) glycerol (NBGly) (Schnider-Keel *et al.*, 2000; Sambrook and Russell, 2001; Brodhagen *et al.*, 2005; Péchy-Tarr *et al.*, 2005; Baehler *et al.*, 2006). *Escherichia coli* strains were grown on NA and in nutrient yeast broth (NYB) (Sambrook and Russell, 2001). *Escherichia coli* strains carrying the pColdI vector (Qing *et al.*, 2004; Takara Bio) and derivatives thereof were cultured on LB plates and in LB broth. Incubation temperatures were 30°C for pseudomonads and 37°C for *E. coli*, unless otherwise specified. For electrotransformation or in triparental matings with *E. coli*, *P. fluorescens* was grown at 35°C to improve its capacity to accept heterologous DNA. Growth media were amended with the following antibiotics as required: ampicillin sodium salt, 100 μg ml^{-1}; chloramphenicol, 50 μg ml^{-1}; spectinomycin dihydrochloride pentahydrate, 50 μg ml^{-1}; and kanamycin sulfate, 25 μg ml^{-1} for *E. coli*; tetracycline hydrochloride, 25 μg ml^{-1} for *E. coli* and 125 μg ml^{-1} for *P. fluorescens* strains. In mating experiments, chloramphenicol was used at 10 μg ml^{-1} to select *P. fluorescens* and to counterselect *E. coli*.

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Table 3. Bacterial strains, plasmids and oligonucleotides used in this study.

Strain, plasmid or oligonucleotide	Relevant characteristics[a] or sequence (5′→3′)	Reference or source
Pseudomonas fluorescens		
CHA0	Wild type; biocontrol agent; FitD$^+$	Stutz *et al.* (1986); Haas and Défago (2005)
CHA1151	Δ*fitD* in-frame deletion mutant of CHA0	This study
Pf-5	Wild type; biocontrol agent; FitD$^+$	Howell and Stipanovic (1979); Paulsen *et al.* (2005)
Pf-5-1151	Δ*fitD* in-frame deletion mutant of Pf-5	This study
P3	Wild type; poor biocontrol agent; naturally *fit* deficient	Voisard *et al.* (1989)
Q2-87	Wild type; biocontrol agent; naturally *fit* deficient	Bangera and Thomashow (1999)
Photorhabdus luminescens		
TT01	Wild type; entomopathogen; Mcf$^+$	Duchaud *et al.* (2003); Waterfield *et al.* (2003)
Xenorhabdus bovienii		
X15	Wild type; entomopathogen	This study
Escherichia coli		
BL21, DH5a, HB101, XL1-Blue	Laboratory strains	Schnider-Keel *et al.* (2000); Sambrook and Russell (2001); Stratagene
Plasmids		
pColdI	Cold shock-based expression vector; Ap^r	Qing *et al.* (2004); Takara Bio
pColdI::*fitD*	pColdI with the *fitD* gene flanked by an N-terminal His_6 tag under the control of the *cspA* promoter; Ap^r	This study
pME497	Mobilizing plasmid; IncP-1, Tra; RepA(Ts); Ap^r	Schnider-Keel *et al.* (2000)
pME3087	Suicide vector; ColE1 replicon; RK2-Mob; Tc^r	Schnider-Keel *et al.* (2001)
pME8200	pUK21 carrying a 2055 bp KpnI–XbaI insert with a deletion in *fitD*; Km^r	This study
pME8201	pME3087 carrying the 2055 bp KpnI–XbaI fragment of pME8201; Tc^r	This study
pME9006	pRG930-Cm with a 24 kb genomic fragment of *P. fluorescens* CHA0 containing the 3′ end of *fitB* and *fitCDEFGH* with its downstream region; Cm^r	This study
pME9007	pUC18 with a 3.8 kb XbaI–BamHI fragment containing *fitA* of CHA0 with its upstream region and the 5′ part of *fitB*; Ap^r	This study
pRG930-Cm^R	pGV910-derived cosmid; Sm^r, Sp^r, Cm^r	Matthijs *et al.* (2004)
pUC18	pBR322- and M13mp18-derived cloning vector; *lacZ*a; Ap^r	Yanisch-Perron *et al.* (1985)
pUK21	pUC118/pUC119-derived cloning vector; *lacZ*a; Km^r	Vieira and Messing (1991)
Oligonucleotides[b]		
Pcoldmcf1	GGAATTCCATATGGCTTTTATGTCCAAGGACTTCACGCGC, NdeI	This study
Pcoldmcf1E	AGCACACTCATGTGCTCATCGACGAAACGA	This study
Pcoldmcf2E	CTGGCTGAGCACCGACGATGGCACCTT	This study
Pcoldmcf2	CGGGATCCTCAGGTCAGTGAAGGCACC, BamHI	This study
Pmcfecofw	AACACCAGTTGAGCAGCCAGTGGATACCGA	This study
Pmcfecorev	TGGTAGGCCTTGTCCAGGGTGTCGAAGTAA	This study
Pmcf1	GGGGTACCTGAGCGAACTGATCAACCAGAA, KpnI	This study
Pmcf2	GGAATTCCTGGTTCAGCCGGTTGTTGATCA, EcoRI	This study
Pmcf3	GGAATTCGCCCATCAGTTGCACGGCCAATA, EcoRI	This study
Pmcf4	GCTCTAGAACCAGTTACTGTGGGCCTTGAG, XbaI	This study
Pmcf8	GCTCTAGAAGCAGCATCGTCAGGATCAGCG, XbaI	This study
Pmtp3B	CGGGATCCTGACGAAACCGACGTCCTTGTT, BamHI	This study

a. Ap^r, ampicillin; Cm^r, chloramphenicol; Km^r, kanamycin; Sm^r, streptomycin; Sp^r, spectinomycin; and Tc^r, tetracycline resistance, respectively.
b. Specified restriction sites are underlined.

DNA manipulations and sequence analyses

Chromosomal DNA of *P. fluorescens* was isolated as described previously (Schnider-Keel *et al.*, 2000). Small- and large-scale plasmid preparations were performed with the QIAprep Spin Miniprep kit (Qiagen, Hombrechtikon, Switzerland) and the Jetstar 2.0 Plasmid Midiprep kit (Genomed, Basel, Switzerland) respectively. Polymerase chain reactions in general were carried out using the GoTaq DNA polymerase kit (Promega, Dübendorf, Switzerland) following amplification protocols detailed previously (Baehler *et al.*, 2005; Péchy-Tarr *et al.*, 2005). Standard techniques were used for restriction, agarose gel electrophoresis, isolation of DNA fragments from low-melting-point agarose gels and ligation (Schnider-Keel *et al.*, 2000; 2001; Sambrook and Russell, 2001). Restriction fragments were purified from agarose gels using the MinElute Gel Extraction or QIAquick Gel Extraction kits (Qiagen) depending on the size of the fragment. Electrotransformation of bacterial cells with plasmid DNA was performed as described previously (Schnider-Keel *et al.*, 2000). All con-

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structs involving PCR techniques were verified by sequence analysis. Nucleotide sequences were determined by Microsynth (Balgach, Switzerland). Nucleotide and deduced amino acid sequences were analysed with programs of the European Molecular Biology Open Software Suite EMBOSS (http://www.ch.embnet.org/EMBOSS/index.html). For predictions about functionality, the deduced amino acid sequence of each gene of the *fit* locus was examined for patterns, profiles and motifs using databases, tools and software packages accessible via the ExPASy (Expert Protein Analysis System) proteomics server of the Swiss Institute of Bioinformatics (http://expasy.org/).

Identification and cloning of the fit locus in P. fluorescens *CHA0*

For the construction of a genomic library of *P. fluorescens* CHA0, total DNA was partially digested with Sau3A and fragments averaging 15–30 kb were ligated into the BamHI-restricted cosmid vector pRG930-Cm^R (Matthijs *et al*., 2004) in *E. coli* strain XL1-Blue (Stratagene, Amsterdam, the Netherlands). Approximately 4100 colonies were selected, transferred to 96-well microplates and screened by PCR for the presence of the *fitD* toxin gene using primer pair Pmcfecofw and Pmcfecorev (Table 3) in a procedure involving several steps of pooling and subpooling of the clones. To determine the extent of coverage of the entire *fit* locus, positive clones were further analysed by a series of primer pairs (not shown) specific to different regions of the toxin locus and its surroundings. Primers were designed on the basis of genomic data available for the closely related *P. fluorescens* strain Pf-5 (Paulsen *et al*., 2005). One positive clone, pME9006 (Fig. 1), was finally selected and the insert sequenced to confirm the identity of the cloned fragment. The insert in pME9007 (Fig. 1) was generated by PCR amplification of CHA0 DNA using primers Pmcf8 and Pmtp3B (Table 3).

Construction of fitD *in-frame deletion mutants of* P. fluorescens

To construct the Δ*fitD* mutant of *P. fluorescens* CHA0, a 4452 bp fragment was deleted in frame in the central part of the 9015 bp *fitD* gene (Fig. 1) as follows. Using CHA0 DNA as a template, a 1037 bp fragment of the 5′ end of *fitD* and a 1012 bp fragment of the 3′ end of the gene were amplified by PCR with primer pairs Pmcf1-Pmcf2 and Pmcf3-Pmcf4 respectively. The fragments obtained were digested with KpnI and EcoRI and with EcoRI and XbaI, respectively, and cloned by a triple ligation into pUK21 opened with KpnI and XbaI. The 2055 kb KpnI–XbaI insert in the resulting plasmid pME8200 (Table 3) was checked by sequencing, excised and cloned into the suicide plasmid pME3087 digested with the same enzymes, giving pME8201 (Fig. 1). The Δ*fitD* mutant CHA1151 (Fig. 1; Table 3) was then obtained by integrating pME8201 into the chromosome of strain CHA0 by a triparental mating using *E. coli* HB101/pME497 as the mobilizing strain, with selection for tetracycline- and chloramphenicol-resistant recombinants. Excision of the vector by a second crossing-over occurred after enrichment for tetracycline-sensitive cells (Schnider-Keel *et al*., 2000; 2001). The same pME8201-based gene replacement strategy was followed to generate an in-frame deletion in PFL_2983 of *P. fluorescens* strain Pf-5 (Fig. 1), creating the Δ*fitD* mutant Pf-5-1151 (Table 3).

Controlled expression of the Fit toxin in E. coli *and detection by SDS-PAGE and Western blotting*

The *P. fluorescens* CHA0 *fitD* gene was cloned into the expression vector pColdI (Qing *et al*., 2004; Takara Bio) in a two-step cloning procedure as follows. A 5072 bp fragment encompassing the start codon and the unique native EcoRI site located in the central part of *fitD* (Fig. 1) were amplified from CHA0 DNA by PCR with primers Pcoldmcf1 and Pcoldmcf1E (Table 3) using the PrimeSTAR® HS high-fidelity DNA polymerase kit (Takara Bio) according to the recommendations of the manufacturer. In the same manner, the primers Pcoldmcf2E and Pcoldmcf2 (Table 3) were used to amplify a 4012 bp fragment comprising the same EcoRI site and the stop codon of *fitD*. The resulting PCR products were digested with NdeI and EcoRI and with EcoRI and BamHI, respectively, and combined in a triple ligation into the NdeI and BamHI sites of the pColdI vector to create pColdI::*fitD* (Fig. 1). This placed *fitD* under the control of the cold shock-inducible *cspA* promoter and permitted the expression of FitD with an N-terminal His6 tag. The insert was checked by sequencing and the construct was electroporated into *E. coli* strain BL21.

For cold shock-induced expression of FitD, overnight cultures of *E. coli* BL21/pColdI::*fitD* were diluted 1:100 with fresh LB supplemented with 100 μg ml^{-1} ampicillin and grown at 37°C with agitation at 180 r.p.m. to an OD at 600 nm of 0.5. After cooling on ice for 15 min, cultures were incubated at 15°C with rotational shaking at 180 r.p.m. and protein expression was induced by adding IPTG at a final concentration of 0.1 mM. *Escherichia coli* BL21 carrying the empty pColdI vector and treated the same way served as a control.

For FitD-His6 detection by SDS-PAGE and Western blotting, cells were harvested by centrifugation after 24 h of growth and washed twice with cold 50 mM Tris-HCl buffer (pH 7.5). A fraction of the washed cells was saved and another fraction further used for the preparation of crude cell extracts. To this end, cell pellets were re-suspended in lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, 5 mM β-mercaptoethanol; pH 7.5) supplemented with Pefabloc SC (Roche, Cologne, Germany) and lysed by sonication on ice for 20 s five times with a Branson 450 Sonifier set at an amplitude of 60%. The cell debris and the soluble fraction in the crude cell extracts were separated by centrifugation for 15 min at 7000 *g* and 4°C. Proteins in the samples prepared from washed cells and crude cell extracts (soluble fraction and cell debris) were fractionated by SDS-PAGE. The peqGOLD pre-stained Protein Marker V (Peqlab Biotechnologie GMBH, Ehrlangen, Germany) was used as a molecular mass standard. Protein bands were stained with Coomassie brilliant blue G250 (Serva, Catalys, Wallisellen, Switzerland). For Western blot analysis, the separated proteins were electroblotted at 15 V during 20 h onto Amersham Hybond-P membranes (GE Healthcare Europe GmbH, Otelfingen, Switzerland) following the protocol of the supplier. The FitD-

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His6 protein was detected with mouse monoclonal anti-polyhistidine antibodies (H-1029, Sigma-Aldrich) at a dilution of 1:18 000 as the primary antibody and rabbit anti-mouse IgG peroxidase antibodies (A9044, Sigma-Aldrich) at a dilution of 1:24 000 as the secondary antibody. Cross-reactive proteins were detected with the Amersham ECL Plus Western blotting detection system (GE Healthcare) on a Fuji medical X-ray film following the manufacturer's instructions.

Insect toxicity assays

For *G. mellonella* toxicity assays, suspensions of washed bacterial cells were prepared in 0.9% sterile NaCl solution and diluted to the desired concentration. Cell suspensions of *Pseudomonas*, *Photorhabdus* and *Xenorhabdus* strains were prepared from fresh LB cultures grown overnight at 27°C. Cell suspensions of *E. coli* carrying pColdI::*fitD* were prepared from cultures in which FitD expression was induced by cold (15°C) for 24 h as detailed in the previous chapter. An aliquot of each bacterial suspension was retained and used to determine the exact numbers of cells injected into the larvae. Immediately prior to bacterial injection, ultimate-instar *G. mellonella* (Hebeisen Fishing, Zürich, Switzerland) were surface-disinfested in 0.05% sodium-hypochlorite for 5 min and rinsed with sterile distilled water. Aliquots of 5 μl of the bacterial cell suspensions were injected into the haemolymph of individual larvae using a Hamilton microsyringe with a 26-gauge needle. Injection was performed dorsally, towards the tail. Larvae injected with sterile NaCl solution or left untreated served as controls. Injected larvae were placed into Greiner six-well plates (one larva per well) and incubated at 22°C in the dark. For the evaluation of bacterial toxicity, larvae were observed for signs of infection (i.e. blackening, softening) (Fig. 5B) and were scored as live or dead over time. Larvae were considered dead when they no longer reacted to being poked repeatedly.

For *M. sexta* toxicity assays, bacterial cell suspensions were prepared as described above except that strains of *P. fluorescens* were cultured in NBGly medium and cells were suspended in sterile distilled water. Eggs of *M. sexta* (Carolina Biological Supply, Charlotte, NC, USA) were hatched in mass on meridic diet (http://www.entm.purdue.edu/Entomology/outreach/recipe/artificialdiet.pdf, Department of Entomology, Purdue University) at a 16:8 h light to darkness (L:D) regime and 27°C. Five days after hatch, individual larvae were transferred into 50 ml plastic centrifuge tubes (Labcon North America, Model# 3282-345-600) containing 15 ml of meridic diet. Tubes were plugged with cotton to facilitate air exchange and larvae were maintained at 16:8 h (L:D) and 27°C for approximately 2 weeks until they reached the fourth to fifth instar. Prior to injection, each larva was rinsed with tap water and dried with a tissue to remove any diet or frass adhering to the surface of the insect. Larvae were injected between the second and third abdominal segments with 10 μl of each treatment using a Dutky-Fest microinjector mounted with a 1 ml syringe with a 27-gauge needle (Becton Dickinson & CO., Model# 309623) (Klein, 1997). For toxicity assays, larvae were placed back into their original rearing tube, returned to the incubator at 16:8 h (L:D) and 27°C, and assessed for mortality over time.

Bacterial colonization of larvae

To monitor colonization of infected *G. mellonella* larvae by *P. fluorescens* strains CHA0, Pf-5 and their *fitD* mutants, we determined the bacterial concentrations in the larvae at different time points following injection of a dose of 3×10^2 or 3×10^4 cells per larva. Bacterial cell suspensions were prepared as described above. Larvae were extracted after incubation for 4, 19, 23, 26, 44 and 68 h at 22°C. Each larva was surface-disinfested for 30 s in 70% ethanol, rinsed with sterile water and then homogenized in 10 ml of sterile 0.9% NaCl solution in a Polytron PT-DA 2112 blender (Kinematica, Littau, Switzerland) for 30 s. Serial dilutions were prepared from the resulting homogenate and plated onto NA containing 40 μg ml^{-1} ampicillin and 13 μg ml^{-1} chloramphenicol to select for the injected *P. fluorescens* strains. As a control, extracts of larvae injected with sterile 0.9% NaCl solution were homogenized and plated on NA with and without selective antibiotics to determine population levels of the natural bacterial community of the larvae.

To monitor colonization of infected *M. sexta*, larvae with a healthy appearance (Fig. 5) were sampled immediately after inoculation with 10^4 cells per larva and after 24 h incubation at 27°C and 16:8 h (L:D). Each larva sampled was rinsed with tap water, dried with a tissue to remove any diet or frass adhering to the surface of the insect, and then homogenized for 30 s in 10 ml of sterile distilled H_2O using a Tekmar SDT Tissumizer (Cincinnati, OH, USA). The resulting suspension was diluted serially, samples were spread on KMB and plates were incubated for 24 h at 27°C prior to enumeration of bacterial colonies.

Statistical analysis

LD_{50} values for mortality 30 h post injection were estimated using probit analysis (PriProbit, Polo Plus) (Sakuma, 1998). LD_{50} values were compared on the basis of overlapping 95% confidence intervals. Control mortality was less than 15% in all assays. LT_{50} values at each concentration were estimated using survivorship analysis (Kaplan–Meier, Statistix 8) using right censored data for insects surviving beyond the 75 h incubation period (Analytical Software, 2003, Statistix 8, Tallahassee, FL, USA). Probit analysis for correlated data, as is the case with our LT_{50} bioassay data, differs from a standard probit analysis because in addition to their variances, the covariances of the probits are also estimated to account for correlation between observations (Throne *et al.*, 1995; Kleinbaum and Klein, 2005). Estimates of LT_{50} values were compared on the basis of overlapping 95% confidence intervals.

Statistical analyses for data presented in Fig. 4 and Table 2 were performed using the statistic program Systat, version 10.0 (Systat, Evanston, IL, USA). Data from three (Table 2) or four to five (Fig. 4) individual repetitions over time were analysed first by one-way ANOVA followed by Fisher's protected least significant difference (LSD) test.

Nucleotide sequence accession number

Nucleotide and amino acid sequence data reported for *P. fluorescens* CHA0 are available in the GenBank and EMBL databases under Accession No. EU400157.

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