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ORIGINAL ARTICLE

Insect pathogenicity in plant-beneficial pseudomonads: phylogenetic distribution and comparative genomics

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Bacteria of the genus *Pseudomonas* occupy diverse environments. The *Pseudomonas fluorescens* group is particularly well-known for its plant-beneficial properties including pathogen suppression. Recent observations that some strains of this group also cause lethal infections in insect larvae, however, point to a more versatile ecology of these bacteria. We show that 26 *P. fluorescens* group strains, isolated from three continents and covering three phylogenetically distinct sub-clades, exhibited different activities toward lepidopteran larvae, ranging from lethal to avirulent. All strains of sub-clade 1, which includes *Pseudomonas chlororaphis* and *Pseudomonas protegens*, were highly insecticidal regardless of their origin (animals, plants). Comparative genomics revealed that strains in this sub-clade possess specific traits allowing a switch between plant- and insect-associated lifestyles. We identified 90 genes unique to all highly insecticidal strains (sub-clade 1) and 117 genes common to all strains of sub-clade 1 and present in some moderately insecticidal strains of sub-clade 3. Mutational analysis of selected genes revealed the importance of chitinase C and phospholipase C in insect pathogenicity. The study provides insight into the genetic basis and phylogenetic distribution of traits defining insecticidal activity in plant-beneficial pseudomonads. Strains with potent dual activity against plant pathogens and herbivorous insects have great potential for use in integrated pest management for crops.
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Introduction

Bacteria of the genus *Pseudomonas* occupy diverse terrestrial, aquatic and atmospheric environments, exhibiting a wide variety of ecological behaviors. Some are feared as human or plant pathogens such as *Pseudomonas aeruginosa* or *Pseudomonas syringae;* others are welcome agents for bioremediation of pollutants such as *Pseudomonas putida.* Members of the *Pseudomonas fluorescens* group are well-known for plant-beneficial effects that improve crop health and agricultural production. Many strains of fluorescent pseudomonads isolated from the rhizosphere have been studied for their ability to suppress root diseases, to promote plant growth and to induce systemic resistance (Haas and Défago, 2005; Bakker *et al.*, 2007). They harbor strain-specific arsenals of antifungal metabolites, which enable them to inhibit pathogen growth through direct antibiosis (Haas and Keel, 2003;

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Raaijmakers *et al*., 2010). All these features make fluorescent pseudomonads interesting organisms for use as biofertilizers and biopesticides in sustainable agriculture and several products have been commercialized (Kupferschmied *et al*., 2013). On top of plant-beneficial activity, genomics has revealed unexpected and broader ecological versatility for these bacteria (Paulsen *et al*., 2005; Loper *et al*., 2012). Three of the best-characterized biocontrol strains, *Pseudomonas protegens* strains CHA0 and Pf-5 and *Pseudomonas chlororaphis* PCL1391, were shown to have potent insecticidal activity (Péchy-Tarr *et al*., 2008; Ruffner *et al*., 2013). When injected into the hemocoel of *Galleria mellonella* or *Manduca sexta* larvae, they rapidly multiply and cause larval death within a few hours (Péchy-Tarr *et al*., 2008). Ecologically more relevant, these strains are also able to infect and kill insect larvae, such as *Drosophila melanogaster* and the agricultural pests *Spodoptera littoralis* or *Plutella xylostella*, after oral uptake (Olcott *et al*., 2010; Ruffner *et al*., 2013). Oral insecticidal activity is considered a rare trait amongst bacteria and requires specific mechanisms to cope with host immune responses and to breach the gut epithelium in order to access the hemocoel (Vallet-Gely *et al*., 2008; Opota *et al*., 2011; Herren and Lemaitre, 2012). How *P. protegens* and *P. chlororaphis* overcome these barriers remains unclear. However, an association with insecticidal activity has been demonstrated for a set of genes termed the *fit* genes (*P. fluorescens* insecticidal toxin) (Péchy-Tarr *et al*., 2008; Péchy-Tarr *et al*., 2013; Ruffner *et al*., 2013). The unique virulence cassette harbors the *fitD* gene encoding the proteinaceous Fit toxin as well as regulatory genes and a type I secretion system (Péchy-Tarr *et al*., 2008; Péchy-Tarr *et al*., 2013; Kupferschmied *et al*., 2014). Nevertheless, *fitD* deletion mutants retain substantial toxicity, indicating the existence of additional virulence factors (Péchy-Tarr *et al*., 2008; Ruffner *et al*., 2013). Mutational analyses provide evidence that some of them are regulated by the global regulator GacA (Olcott *et al*., 2010; Ruffner *et al*., 2013).

Insecticidal activity is not universal to the *P. fluorescens* group. A survey by Ruffner *et al*. (2015) revealed that sub-clade 2 strains (Loper *et al*., 2012) neither harbor *fit* genes nor have ability to kill *G. mellonella* larvae. In contrast, all tested *P. protegens* and *P. chlororaphis* strains, which represent the sub-clade 1 (Loper *et al*., 2012), have both the toxin and injectable activity. Accordingly, *Pseudomonas* sp. strains Pf-01 and Q2-87 (formerly called *P. fluorescens* Pf-01 and Q2-87), both belonging to sub-clade 2, have no oral activity against larvae of *D. melanogaster* and several lepidopteran species, respectively (Olcott *et al*., 2010; Ruffner *et al*., 2013). Interestingly, *Pseudomonas* sp. SBW25 (formerly called *P. fluorescens* SBW25) of sub-clade 3, which does not harbor the *fit* genes, was shown to cause mortality and developmental delay in *D. melanogaster* larvae, but to a much lower extent than *P. protegens* Pf-5 (Olcott *et al*., 2010).

The discovery of insecticidal activity in fluorescent pseudomonads raises diverse ecological and agronomic questions. What ecological advantage may be gained by this ability to switch from a plant to an insect environment? Can we use these pseudomonads as double agents to fight both plant disease and insect pests? To date, our understanding of the interaction of plant-associated pseudomonads with insects is still very poor. Although large differences in their ability to infect insects were found between the strains investigated so far, no extensive data on frequency and distribution of insecticidal activity throughout the whole *P. fluorescens* group is available and individual strains with different phylogenetic background have never been compared directly. Moreover, the precise factors beyond the Fit toxin, which enable certain fluorescent pseudomonads to kill insects, and thereby to occupy a habitat alternative to plant roots, are still elusive. As a first step toward understanding the genomic features enabling insect pathogenicity we have taken an approach that combines bioassays with comparative genomics. We investigated 26 strains of fluorescent pseudomonads for their insecticidal activity and their biocontrol activity against root diseases. The strains included in our study are representative of the three phylogenetic sub-clades within the *P. fluorescens* group that harbor most plant-beneficial pseudomonads and were isolated from root but also from non-root habitats. Strong oral activity was found for all strains belonging to the phylogenetic sub-clade 1, which showed potent dual activity against insects and plant pathogens. However, we identified also a second phylogenetic group, sub-clade 3, containing strains with lower insecticidal activity. The strains were sequenced and comparative genomics revealed around 200 genes that are common and unique to the insecticidal strains and we hypothesize that this specific set of genes may represent major evolutionary events toward insect pathogenicity of *Pseudomonas* spp. Finally, we present first results from testing the involvement of some of the newly identified putative virulence factors in insecticidal activity using a mutational approach.

Materials and methods

Bacterial strains

Strain names and origins are listed in Table 1. We use species names only for strains that cluster closely to a species-type strain in the phylogenetic tree we created based on core genomes (Figure 1) and thus can clearly be assigned to a certain species. All other strains are referred to as *Pseudomonas* sp. For sequencing, strains were taken from our long-term strain storage kept at −80 °C, or were obtained from the German Collection of Microorganisms and Cell

Table 1 Strain information

Strain	*Former name*	*Geographic origin*	*Habitat/Host*[a]	*Biocontrol ability*	*Genome sequenced*	*References*
P. protegens CHA0^T	*P. fluorescens* CHA0^T	Switzerland	Tobacco	Cucumber-Pu, Tobacco-Tb, Wheat-Ggt, Tomato-Forl	Jousset *et al.* (2014)	Stutz *et al.* (1986); Keel *et al.* (1996); Haas and Défago (2005); Ramette *et al.* (2011)
P. protegens PGNR1	*P. fluorescens* PGNR1	Ghana	Tobacco	Cucumber-Pu, Tomato-Forl	This study	Keel *et al.* (1996)
P. protegens BRIP		Switzerland	Cyclops	ND	This study	Ruffner *et al.* (2015)
P. protegens K94.41	*P. fluorescens* K94.41	Slovakia	Cucumber	Cucumber-Pu, Tomato-Forl	This study	Wang *et al.* (2001)
P. protegens PF	*P. fluorescens* PF	Oklahoma, USA	Wheat leaves	Wheat-St	This study	Levy *et al.* (1992); Keel *et al.* (1996)
Pseudomonas sp. CMR5c		Cameroon	Red cocoyam	*Cocoyam-Pm*	This study	Perneel *et al.* (2007)
Pseudomonas sp. CMR12a		Cameroon	Red cocoyam	*Cocoyam-Pm, Bean-Rs*	This study	Perneel *et al.* (2007); D'Aes *et al.* (2011)
P. chlororaphis subsp. *piscium* DSM 21509^T		Lake of Neuchâtel, Switzerland	Intestine of European perch	ND	This study	Burr *et al.* (2010)
P. chlororaphis subsp. *piscium* PCL1391		Spain	Tomato	Tomato-Forl	This study	Chin-A-Woeng *et al.* (1998)
P. chlororaphis subsp. *aureofaciens* LMG 1245^T		Netherlands	River Clay	ND	This study	Kluyver (1956); Peix *et al.* (2007)
P. chlororaphis subsp. *aureofaciens* CD		Switzerland	Cyclops (water)	ND	This study	Ruffner *et al.* (2015)
P. chlororaphis subsp. *chlororaphis* LMG 5004^T		—	Contaminated plate	ND	This study	Peix *et al.* (2007)
P. brassicacearum TM1A3	*P. fluorescens* TM1A3	Switzerland	Tomato	Cucumber-Pu, Cotton-Rs	This study	Fuchs and Defago (1991); Keel *et al.* (1996)
P. thivervalensis DSM 13194^T		France	Rapeseed	ND	This study	Achouak *et al.* (2000)
P. thivervalensis PITR2	*P. fluorescens* PITR2	Albenga, Italy	Wheat	Cucumber-Pu, Tomato-Forl	This study	Keel *et al.* (1996)
P. kilonensis P12	*P. fluorescens* P12	Switzerland	Tobacco	Tobacco-Tb	This study	Keel *et al.* (1996)
P. kilonensis DSM 13647^T		Germany	Agricultural soil	ND	This study	Sikorski *et al.* (2001)
Pseudomonas sp. Q12-87	*P. fluorescens* Q12-87	Washington, USA	Wheat	Wheat-Ggt	This study	Keel *et al.* (1996)
Pseudomonas sp. P97.38	*P. fluorescens* P97.38	Switzerland	Cucumber	Cucumber-Pu, Tomato-Forl	This study	Wang *et al.* (2001)
P. corrugata DSM 7228^T		United Kingdom	Tomato stem	ND	This study	Scarlett *et al.* (1978)
Pseudomonas sp. Pf153	*P. fluorescens* Pf153	Switzerland	Tobacco	Cucumber-Pu	This study	Fuchs *et al.* (2000)
Pseudomonas sp. P1.8		Switzerland	Earthworm	ND	This study	Ruffner *et al.* (2015)
Pseudomonas sp. P1.31		Switzerland	Woodlouse (dead)	ND	This study	Ruffner *et al.* (2015)
P. fluorescens DSM 50090^T		United Kingdom	pre-filter tanks	ND	This study	Rhodes (1959)
Pseudomonas sp. MIACH	*P. fluorescens* MIACH	Switzerland	Wheat	ND	This study	Meyer *et al.* (2011)
Pseudomonas sp. SS101	*P. fluorescens* SS101	The Netherlands	Wheat	Cucumber-Pc, Tomato-Pi	Loper *et al.* (2012)	de Souza *et al.* (2003); Mazzola *et al.* (2007); Tran *et al.* (2007); Kruijt *et al.* (2009)

Abbreviations: Forl, *Fusarium oxysporum* f. sp. radicis-lycopersici; Ggt, *Gaeumannomyces graminis* var. tritici; ND, not documented; Pc, *Phytophthora capsici*; Pi, *Phytophthora infestans*; Pm, *Pythium myriotylum*; Ps, *Phomopsis sclerotioides*; Pu, *Pythium ultimum*; Rs, *Rhizoctonia solani*; St, *Septoria tritici*; Tb, *Thielaviopsis basicola*.
[a]Plant hosts: If not otherwise stated strains were isolated from roots or rhizosphere.

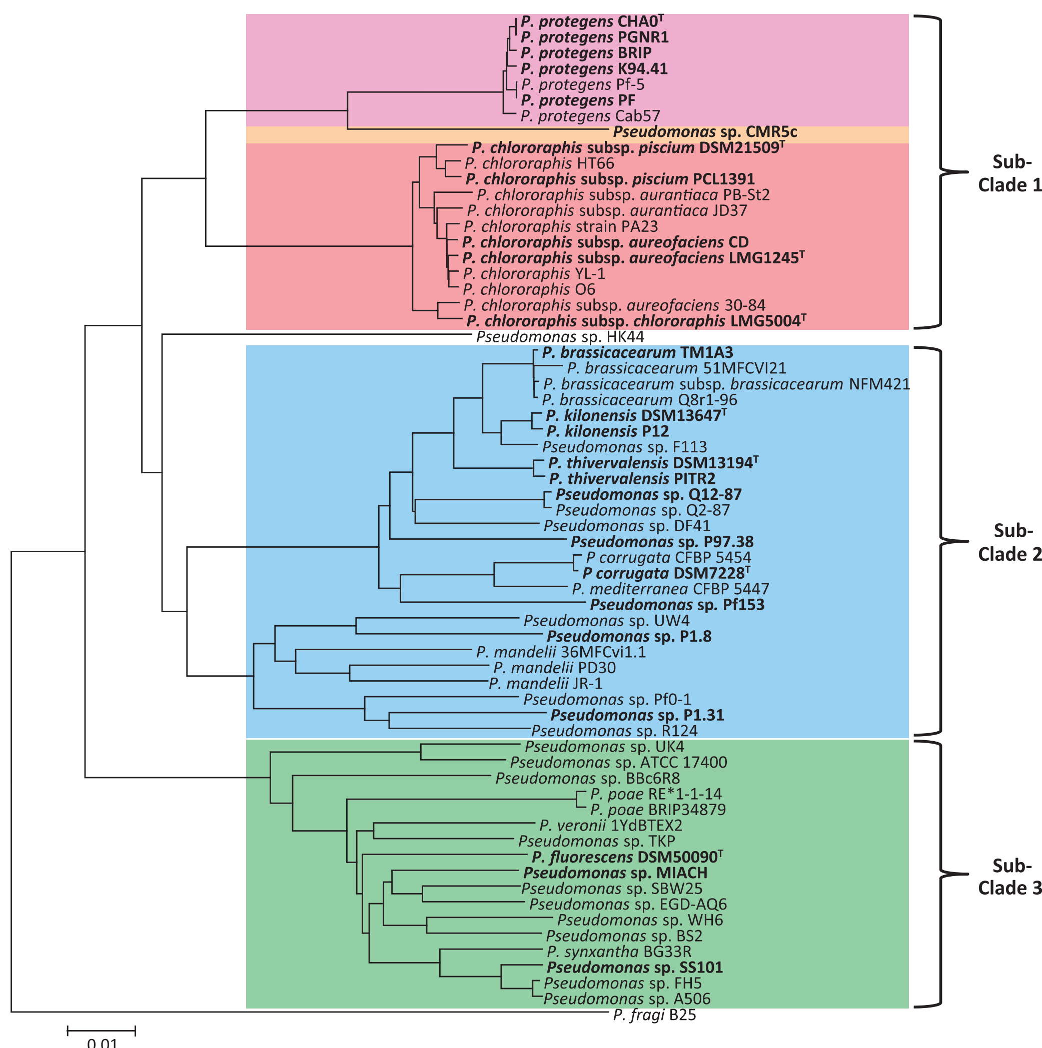

Figure 1 Phylogeny of the *P. fluorescens* group based on the core genome. Genomes sequenced in this study and high-quality genomes that are publicly available by February 2015 were used to generate a core genome tree in EDGAR. Strains investigated in this study are depicted in bold. Sub-clades were defined after Loper *et al*. (2012). Sub-clade 1 corresponds to the *P. chlororaphis* subgroup, sub-clade 3 to the *P. fluorescens* subgroup and sub-clade 2 comprises strains belonging to three different subgroups within the *P. fluorescens* group according to Mulet *et al*. (2012), see also Supplementary Figure 1.

Cultures (DSMZ). If not otherwise stated bacterial cultures for bioassays and sequencing were grown in LB medium (Bertani, 1951) overnight on a rotary shaker (180 r.p.m.) at 24 °C. For bioassays cells were washed in sterile 0.9% NaCl. OD_{600} was measured and cells diluted to the desired concentration, while assuming that a cell suspension with an OD_{600} of 0.125 contains ~10^8 colony forming units (c.f.u.) per ml.

Genome sequencing, assembly and comparative genomics

For sequencing the genomes, DNA was extracted from overnight cultures in LB using the Wizard Genomic DNA Purification Kit (Promega AG, Dübendorf, Switzerland). All genomes apart from *Pseudomonas* sp. CMR5c (Supplementary Methods) were sequenced on an Illumina MiSeq (2 × 300 bp shotgun sequencing) at the Quantitative Genomics Facility (QGF) of the BioSystems Science and Engineering department (BSSE) of ETH Zürich located in Basel, Switzerland. Subsequently, the reads were *de novo* assembled using SeqMan NGen12 (DNASTAR, Madison, WI, USA) and further manually improved *in silico* using different subroutines of the Genomics Package of LASERGENE 12 (DNASTAR, Madison, WI, USA).

All genome sequences generated in this study and several database sequences that do not contain an annotation were automatically annotated in GenDB (Meyer *et al*., 2003). The annotation of the genome of *P. chlororaphis* subsp. *piscium* PCL1391 was manually improved and the whole Genome Shotgun project was deposited at DDBJ/EMBL/GenBank. Genomes of all other sequenced strains were deposited without annotations at DDBJ/EMBL/GenBank. Accession numbers are indicated in Supplementary Table S1.

Comparative genomics was done using EDGAR (Blom *et al.*, 2009). Gene sets common to certain strains, but absent in other strains were calculated with a cut-off of 70% amino-acid identity over 70% of the gene length (Smits *et al.*, 2010). For the phylogenetic tree of the core genomes, annotated assemblies and genomes from the public GenBank database (NCBI) were used. However, for quality reasons only genomes that consisted of <200 contigs and are thus classified as 'high-quality draft genome sequences' by the NCBI, were included.

The phylogeny based on the core genome of all included strains was generated in EDGAR. The phylogenetic tree was created with the neighbor joining algorithm on a Kimura distance matrix as implemented in the PHYLIP package (Blom *et al.*, 2009). Due to the huge size of the core alignment and the long resulting calculation time for a tree, bootstrapping was not performed.

Insect assays
Injection assays with *G. mellonella* were performed with small adaptations as described by Péchy-Tarr *et al.* (2008). More information is placed in Supplementary Methods.

Feeding assays: Eggs of *P. xylostella* were obtained from Syngenta Crop Protection AG (Stein, Switzerland). General growth conditions for larvae before and during the experiments were 26 °C, 60% humidity and a 16-h day, 8-h night cycle. Before experiments, boxes with larvae were placed at 18 °C in the dark for 48 h. For virulence assays, 1-week-old larvae were exposed to 10 µl washed bacterial cells adjusted to the desired concentration or 0.9% NaCl (controls) on a pellet of modified insect diet (Gupta *et al.*, 2005; Ruffner *et al.*, 2013). To prevent injuries each larva was kept separately in 128-cell bioassay trays (Frontier Agricultural Sciences, Delaware, USA). Each treatment was tested on four replicates of eight larvae. Mortality was defined as the inability to react to poking.

Construction of deletion mutants of P. protegens CHA0
The *chiC*, *aprX* and *plcN* genes and the *rebB1-3* cluster of *P. protegens* CHA0 were deleted by an allelic replacement technique using the I-SceI system with the suicide vector pEMG (Martinez-Garcia and de Lorenzo, 2011) as detailed in previous work (Kupferschmied *et al.*, 2014). To construct the pEMG-based plasmids, the 600–700-bp upstream and downstream regions flanking the genomic region to be deleted were amplified by PCR using the primer pairs specified in Supplementary Table S2. The obtained fragments were digested with the relevant restriction enzymes (Supplementary Table S2) and cloned into pEMG via triple ligation. Constructs were verified by sequencing. The obtained suicide plasmids served then to generate the deletion mutants CHA5099 (Δ*chiC*), CHA5222 (Δ*aprX*), CHA5223 (Δ*plcN*) and CHA5221 (Δ*rebB1-3*) (Supplementary Table S2), using the I-SceI system with the expression plasmid pSW-2.

Chitinase activity assay
Chitinase activity was measured in supernatants of cultures grown for 48 h in LB shaking with a methylumbelliferone-based chitinase assay kit (Sigma, St Louis, MO, USA) according to the manufacturer's instructions.

Statistics
Data analysis was performed in R version 3.1.1. (http://www.r-project.org). Mortality rates of the insect toxicity tests with wild-type strains were analyzed by multiple comparisons using Kruskal–Wallis adjusted by Bonferroni–Holm. Lethal time 50 (LT_{50}) values were estimated based on the generalized linear model using the MASS package in R (Venables and Ripley, 2002). To test for significant differences between *P. protegens* CHA0 and its mutant strains the Log-Rank test of the Survival package of R and the Student's *t*-test were used in insect toxicity test and chitinase activity assays, respectively.

Results and discussion

Strain selection
To obtain an extensive overview of the occurrence of insecticidal activity within the *P. fluorescens* group, we selected 26 strains (Table 1). Many strains were isolated from roots and are well-known for their activity against plant pathogens, others were recently isolated from completely different habitats such as perch intestine and cyclops, for example, strains *P. chlororaphis* subsp. *piscium* DSM 21509^{T} and *P. protegens* BRIP, respectively. Type strains were included in the study when considerable indications were present for close relationships of non-assigned strains to existing species.

The included strains, isolated on three different continents, belong to five subgroups within the *P. fluorescens* group (Supplementary Figure S1; Mulet *et al.*, 2012; Gomila *et al.*, 2015), that are covered in the three sub-clades defined by Loper *et al.* (2012) (Figure 1): 12 strains representing sub-clade 1, 11 strains representing 4 known and 5 new species in sub-clade 2 (including the *P. corrugata, P. koreensis* and *P. jessenii* subgroups) and 3 strains in sub-clade 3 (Figure 1 and Supplementary Figure S1). A detailed overview of the phylogeny of the included strains is given in Supplementary Results, Supplementary Figure S1 and Supplementary Table S3.

Insecticidal activity and presence of the Fit toxin
Functions encoded by the Fit gene cluster were demonstrated to contribute to insecticidal activity of

the strains *P. protegens* strains CHA0 and Pf-5 and *P. chlororaphis* PCL1391 (Péchy-Tarr *et al.*, 2008; Ruffner *et al.*, 2013; Kupferschmied *et al.*, 2014). Searching the genomes of the selected strains revealed that the gene cluster is present in all strains of sub-clade 1, but neither in sub-clade 2 nor sub-clade 3 (Figure 2), which is in line with results obtained by Ruffner *et al.* (2015).

All 26 strains were tested for their injectable and oral activity against insect larvae. A summary of the results is given in Figure 2. All strains of sub-clade 1 exhibited strong injectable and oral insecticidal activity whereas no strain of sub-clade 2 had an effect on larval survival in any of the test systems. However, the presence of the *fit* cluster, although indicative of strong insecticidal activity, does not seem to be the sole factor associated with the ability to kill insects, as also the tested strains of sub-clade 3, which do not contain the *fit* cluster caused some mortality, but to a much lower extent than strains of sub-clade 1. Insecticidal activity was associated with specific phylogenetic subgroups, but did not correlate with the origin of the isolate (that is, root or non-root habitat).

To mimic a systemic infection, bacteria were injected into the hemocoel of *G. mellonella* larvae (Figure 3a). All Fit-producing strains, that is, the entire sub-clade 1, were able to cause 100% mortality within the first 48 h, confirming and extending results of an earlier study demonstrating

			Insecticidal activity								Pathogen suppression							
	Species	Strain	Oral	Injection	*fit* [ab]	*chiC* [ab]	*plcN* [ab]	*aprX* [a]	*psl* [a]	*rebB* [a]	In vivo	In vitro	DAPG [c]	Phz [c]	HCN [c]	Prn [c]	Plt [c]	HPR [c]
Sub-clade 1	*P. protegens*	CHA0^{T}	high	high	present	present	present	present	present	present	high *	high	present	absent	present	present	present	absent
Sub-clade 1	*P. protegens*	PGNR1	high	high	present	present	present	present	present	present	high *	high	present	absent	present	present	present	absent
Sub-clade 1	*P. protegens*	BRIP	high	high	present	present	present	present	present	present	high	high	present	absent	present	present	present	absent
Sub-clade 1	*P. protegens*	K94.41	high	high	present	present	present	present	present	present	high *	high	present	absent	present	present	present	absent
Sub-clade 1	*P. protegens*	PF	high	high	present	present	present	present	present	present	high *	high	present	absent	present	present	present	absent
Sub-clade 1	*Pseudomonas* sp.	CMR5c	high	high	present	present	present	present	present	present	no *		present	present	present	present	present	absent
Sub-clade 1	*Pseudomonas* sp.	CMR12a	high	high	present	present	present	present	present	present	medium *		absent	present	present	absent	absent	absent
Sub-clade 1	*P. chlororaphis*	*piscium* DSM21509^{T}	high	high	present	present	present	present	present	present	medium		absent	present	present	absent	absent	absent
Sub-clade 1	*P. chlororaphis*	*piscium* PCL1391	high	high	present	present	present	present	present	present	high *	medium	absent	present	present	absent	absent	absent
Sub-clade 1	*P. chlororaphis*	*aureofaciens* LMG1245^{T}	high	high	present	present	present	present	present	present	no		absent	present	present	present	absent	present
Sub-clade 1	*P. chlororaphis*	*aureofaciens* CD	high	high	present	present	present	present	present	present	high	medium	absent	present	present	present	absent	present
Sub-clade 1	*P. chlororaphis*	*chlororaphis* LMG5004^{T}	high	high	present	present	present	present	present	present	no		absent	present	present	present	absent	present
Sub-clade 2	*P. brassicacearum*	TM1A3	no	no	absent	absent	absent	absent	absent	absent	medium *	high	present	absent	present	absent	absent	absent
Sub-clade 2	*P. kilonensis*	DSM 13647^{T}	no	no	absent	absent	absent	absent	absent	absent	no		present	absent	present	absent	absent	absent
Sub-clade 2	*P. kilonensis*	P12	no	no	absent	absent	absent	absent	absent	absent	no *	high	present	absent	present	absent	absent	absent
Sub-clade 2	*P. thivervalensis*	DSM13194^{T}	no	no	absent	absent	absent	absent	absent	absent	no		present	absent	present	absent	absent	absent
Sub-clade 2	*P. thivervalensis*	PITR2	no	no	absent	absent	absent	absent	absent	absent	high *	high	present	absent	present	absent	absent	absent
Sub-clade 2	*Pseudomonas* sp.	Q12-87	no	no	absent	absent	absent	absent	absent	absent	high *	high	present	absent	present	absent	absent	absent
Sub-clade 2	*Pseudomonas* sp.	P97.38	no	no	absent	absent	absent	absent	absent	absent	high *	high	present	absent	present	absent	absent	absent
Sub-clade 2	*P. corrugata*	DSM7228^{T}	no	no	absent	absent	absent	absent	absent	absent	no		absent	absent	present	absent	absent	absent
Sub-clade 2	*Pseudomonas* sp.	Pf153	no	no	absent	absent	absent	absent	absent	absent	medium *	medium	absent	absent	present	absent	absent	absent
Sub-clade 2	*Pseudomonas* sp.	P1.8	no	no	absent	absent	absent	absent	absent	absent	no	no	absent	absent	absent	absent	absent	absent
Sub-clade 2	*Pseudomonas* sp.	P1.31	no	no	absent	absent	absent	absent	absent	absent	medium	medium	absent	absent	present	absent	absent	absent
Sub-clade 3	*P. fluorescens*	DSM50090^{T}	medium	medium	absent	absent	absent	absent	partially present	absent	no		absent	absent	absent	absent	absent	absent
Sub-clade 3	*Pseudomonas* sp.	SS101	medium	medium	absent	absent	absent	absent	partially present	partially present	medium *		absent	absent	absent	absent	absent	absent
Sub-clade 3	*Pseudomonas* sp.	MIACH	medium	no	absent	absent	absent	absent	partially present	absent	medium		absent	absent	absent	absent	absent	absent

Figure 2 Overview on insecticidal activity, pathogen suppression and presence of associated gene clusters in 26 strains of the *P. fluorescens* group. Colored boxes represent activity against insects and plant pathogens as assessed within this study: ■ high activity, ■ medium activity, ■ no activity. Insecticidal activity was assessed in injection assays against *G. mellonella* larvae and feeding assays against *P. xylostella* and *S. littoralis* larvae, and depicted activities are based on the results presented in Figure 3,Table 2, Supplementary Figure S2 and Supplementary Table S4. Disease suppression was assessed in a cucumber-*Pythium ultimum* assay and activities are based on the data depicted in Supplementary Table S5. Strains indicated by an asterisk were reported to have biocontrol activity against plant diseases in earlier studies (Table 1). *In vitro* inhibition of mycelial growth was assessed on two media against *P. ultimum* and *Fusarium oxysporum* f. sp. *radicis-lycopersici* and activities are based on the results shown in Supplementary Figure S3. Gray boxes represent presence of selected genes/gene clusters that were found to be associated with insecticidal strains (this study) or that are required for the production of the indicated antifungal metabolites. ■ present, □ partially present, ■ absent. Exact loci, which were checked for presence/absence, are indicated in Supplementary Table S1. There, additional genes as well as all additional strains are presented. [a]Selected genes that were identified by comparative genomics to be specific for strains that show insecticidal activity. A complete list is presented in Supplementary Table S6. *P. fluorescens* insecticidal toxin-cluster (*fit*), chitinase C (*chiC*), phospholipase C (*plcN*), metallopeptidase AprX (*aprX*), *rebB*-cluster (*rebB*), *psl*-cluster (*psl*). [b]Genes that were shown to contribute to insecticidal activity in this study (*chiC* and *plcN*) or elsewhere (*fit*) (Péchy-Tarr *et al.*, 2008; Ruffner *et al.*, 2013). [c]Presence/absence of gene clusters required for the production of the indicated antifungal metabolites. DAPG, 2,4-diacetylphloroglucinol; Phz, phenazine; HCN, hydrogen cyanide; Prn, pyrrolnitrin; Plt, pyoluteorin; HPR, 2-hexyl-5-propyl-alkylresorcinol.

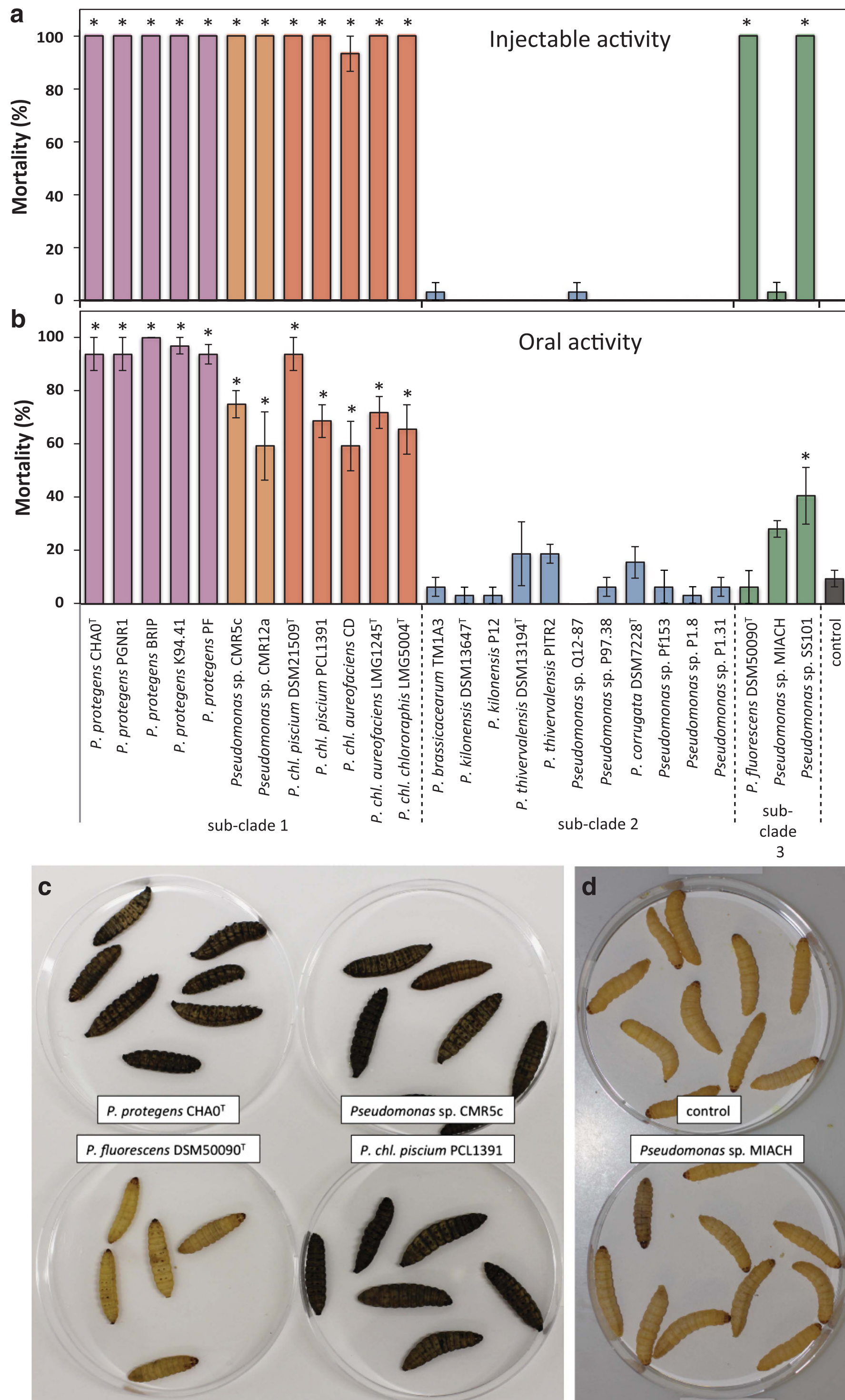

Figure 3 Oral and systemic insecticidal activity is restricted to strains of specific phylogenetic subgroups within the *P. fluorescens* group. (**a**) Systemic activity against *G. mellonella*. Larvae were injected with 4×10^4 bacterial cells. Bars show average mortality of three replicates with 10 larvae after 48 h. The experiment was repeated twice and highly similar results were obtained. (**b**) Oral activity against *P. xylostella*. Larvae were exposed to artificial diet covered with 8×10^7 bacterial cells. Bars show average mortality of four replicates with eight larvae after 3 days. The experiment was repeated and similar results were obtained (Supplementary Table S4). Error bars show s.e.m. Asterisks indicate strains that were significantly different from control larvae treated with 0.9% NaCl based on multiple comparisons by Kruskal–Wallis adjusted by Bonferroni–Holm ($P \leqslant 0.05$). (**c**) Typical melanization symptoms observed after 32 h in infections with *P. protegens* CHA0^T, *P. chlororaphis* subsp. *piscium* PCL1391, *Pseudomonas* sp. CMR5c, but not with *P. fluorescens* DSM 50090^T. (**d**) Although larvae injected with *Pseudomonas* sp. MIACH do not die, they become slightly melanized compared with control larvae. *P. chl.*, *Pseudomonas chlororaphis*.

sub-clade 1 strains to cause 100% mortality when injected into *G. mellonella* (Ruffner *et al*., 2015). Although all strains in sub-clade 1 are highly insecticidal, strain-specific differences for killing rate were observed. The two strains *Pseudomonas* sp. CMR5c and CMR12a, that probably represent a new species within sub-clade 1, were killing more rapidly than *P. protegens* and *P. chlororaphis* as indicated by significantly shorter times to reach 50% larval mortality (LT_{50}; Table 2). *P. chlororaphis* subsp. *aureofaciens* strains CD and LMG 1245^{T} have higher LT_{50} values compared with strains of the *P. chlororaphis* subspecies *piscium* and *chlororaphis* (Table 2). Thus, the kill-time reflects phylogenetic relationships, which may be explained by the presumably multifactorial nature of insecticidal activity of fluorescent pseudomonads. Beside a common arsenal of contributing factors harbored by all insecticidal strains, some specific factors may exist, which enable certain strains or closely related groups of strains to kill more efficiently than others. No injectable activity was found for strains of sub-clade 2 (Figure 3a). In contrast, two strains of sub-clade 3, namely *P. fluorescens* DSM 50090^{T} and *Pseudomonas* sp. SS101, both lacking the *fit* genes, caused lethal infections in *G. mellonella*. However, mortality caused by these strains was delayed compared with infections with most strains of sub-clade 1 (Table 2) and larvae lack the strong melanization response and the floppy phenotype observed after infection by sub-clade 1 (Figure 3c). These symptoms might be attributed to the Fit toxin as larvae injected with a *fitD* deletion mutant of *P. protegens* CHA0 lack these phenotypes (Péchy-Tarr *et al*., 2008) similarly to larvae injected with SS101 or DSM 50090^{T}. Although injectable insecticidal activity seems to be universal to strains of sub-clade 1 this is not the case for sub-clade 3. The third tested strain of this sub-clade, *Pseudomonas* sp. MIACH, was not able to kill insect larvae upon injection (Figure 3a), although larvae started to slightly melanize at 1-day post infection (Figure 3d).

In natural infections, a bacterium first has to breach several barriers to reach the hemocoel. Therefore, the selected strains were further tested for oral activity against *P. xylostella* larvae. All strains that carry the *fit* genes were able to cause high mortality within 3 days (Figure 3b; Supplementary Table S4). In contrast to the injection assays, here *P. protegens* were the most efficient

Table 2 Lethal time 50 (LT_{50}) values for *G. mellonella* and *P. xylostella* larvae upon injection or oral uptake, respectively, of *Pseudomonas* strains

Sub-clade	*Strain*	*Injection, LT_{50} (h)*	*Oral, LT_{50} (d)*
Sub-clade 1	*P. protegens* CHA0^{T}	26.3 (25.9; 26.6)[b]	1.65 (1.44; 1.86)[a]
	P. protegens CHA0^{T}	26.9 (26.5; 27.4)[†γ]	
	P. protegens PGNR1	29.3 (28.9; 29.8)[de]	1.74 (1.56; 1.92)[ab]
	P. protegens BRIP	29.0 (28.6; 29.4)[d]	1.58 (1.40; 1.76)[a]
	P. protegens K94.41	26.3 (25.9; 26.7)[b]	1.58 (1.40; 1.76)[a]
	P. protegens PF	34.4 (33.3; 35.6)[g]	1.63 (1.43; 1.83)[a]
	Pseudomonas sp. CMR5c	24.5 (24.2; 24.9)[†β]	2.24 (1.98; 2.49)[c]
	Pseudomonas sp. CMR12a	22.0 (21.5; 22.6)[†α]	2.63 (2.31; 2.95)[c]
	P. chl. piscium DSM 21509^{T}	27.2 (26.7; 27.6)[c]	1.66 (1.46; 1.86)[a]
	P. chl. piscium PCL1391	24.9 (24.5; 25.3)[a]	2.19 (1.87; 2.51)[bc]
	P. chl. aureofaciens LMG 1245^{T}	30.1 (29.6; 30.6)[e]	2.56 (2.24; 2.89)[c]
	P. chl. aureofaciens CD	33.7 (32.8; 34.7)[g]	2.08 (1.76; 2.39)[abc]
	P. chl. chlororaphis LMG 5004^{T}	26.7 (26.2; 27.1)[bc]	2.33 (1.97; 2.68)[c]
Sub-clade 2	*P. brassicacearum* TM1A3	NA	NA
	P. kilonensis DSM 13647^{T}	NA	NA
	P. kilonensis P12	NA	NA
	P. thivervalensis DSM 13194^{T}	NA	NA
	P. thivervalensis PITR2	NA	NA
	Pseudomonas sp. Q12-87	NA	NA
	Pseudomonas sp. P97.38	NA	NA
	P. corrugata DSM 7228^{T}	NA	NA
	Pseudomonas sp. Pf153	NA	NA
	Pseudomonas sp. P1.8	NA	NA
	Pseudomonas sp. P1.31	NA	NA
Sub-clade 3	*P. fluorescens* DSM 50090^{T}	32.0 (31.4; 32.7)[f]	NA
	Pseudomonas sp. MIACH	NA	NA
	Pseudomonas sp. SS101	37.9 (36.9; 38.8)[†δ]	NA
Control	0.9% NaCl	NA	NA

Abbreviations: NA, no LT_{50} value was calculated, because end mortality was $<50\%$; *P. chl.*, *Pseudomonas chlororaphis*.
G. mellonella larvae were injected with 4×10^{4} washed bacterial cells of the indicated strains. *P. xylostella* larvae were exposed to food pellets inoculated with 8×10^{7} bacterial cells. LT_{50} values are estimates based on the generalized linear model using the MASS package in R (Venables and Ripley, 2002). LT_{50} estimates were calculated from three replicates with 10 larvae per replicate for *G. mellonella* and from four replicates with eight larvae per replicate for *P. xylostella*. Numbers in brackets depict 95% confidence intervals for LT_{50} and significantly different values ($P\leqslant0.05$) within the same column are followed by different letters.
[†]The strains for which significance is indicated by greek letters were tested in a separate experiment.

insect killers in terms of extent and pace (Table 2). None of the strains of sub-clade 2 caused higher mortality than observed for control larvae (Figure 3b). This result was confirmed in a second oral test system where a selection of 15 strains of sub-clades 1 and 2 was fed to *S. littoralis* larvae (Supplementary Methods). No sub-clade 2 strain was able to kill the larvae, whereas all Fit-producing strains showed strong insecticidal activity (Supplementary Figure S2A). Thus, sub-clade 2 strains lack crucial traits enabling them to kill lepidopteran larvae. However, the lack of killing potential does not necessarily mean that these strains might not be able to persist in the insect gut. Persistence without killing could be a clever strategy to use the insect as a means of dispersal as a living insect will transport the bacteria further than a dead one. Monitoring bacterial cells revealed that all strains of sub-clade 1 were able to multiply within the *S. littoralis* larvae (data only shown for CHA0, Supplementary Figure S2B) and to reach about 10^8 c.f.u. per larva whereas large differences were observed for strains in sub-clade 2. Several strains, namely *Pseudomonas* sp. P97.38, Q12-87 and P1.31, were indeed able to persist at levels of 10^6 to 10^7 c.f.u. per larva, whereas others such as *P. thivervalensis* PITR2, *P. kilonensis* P12 or *Pseudomonas* sp. P1.8 underwent a 1000-fold population decline within a few days (Supplementary Figure S2B) indicating that they were cleared from the gut. Thus, although not having the ability to kill insect larvae, some strains of sub-clade 2 seem to possess features allowing certain persistence in insects.

In contrast to sub-clade 2 strains, sub-clade 3 strains were found to cause lethal oral infections in *P. xylostella*, which is to our knowledge the first report for strains of this sub-clade to orally kill lepidopteran insect larvae. However, similar to the results of the injection assay, strains of sub-clade 3 appeared to have strongly reduced oral activity compared with strains of sub-clade 1. This is in line with observations of Olcott *et al*. (2010), who described that oral infections of *D. melanogaster* with *Pseudomonas* sp. SBW25 (sub-clade 3) were less detrimental than infections with *P. protegens* Pf-5 (sub-clade 1). Strain *Pseudomonas* sp. SS101 had significant oral insecticidal activity in all repetitions of the experiment (Figure 3b; Supplementary Table S4). However, killing occurred slower and to a lower extent than it was the case for infections with strains of sub-clade 1. *P. fluorescens* DSM 50090^T which had injectable activity against *G. mellonella* showed either no or weak insecticidal activity when fed to *P. xylostella*. We hypothesize that this strain faces difficulties to breach the gut barrier on its own, but can act as an opportunistic pathogen taking the chance when an insect gets injured or weakened by other factors. More puzzling, is the outcome for *Pseudomonas* sp. MIACH, which, in spite of not killing larvae in injection experiments, seems to have slight oral activity. Strain MIACH caused mortality rates of 30–53% though the effect was significant only in one of the two experiments (Figure 3b; Supplementary Table S4). We hypothesize that this strain is able to do some damage to the insect gut, without killing the insect itself, thereby promoting a secondary infection by other microbes that invade the hemocoel and lead to larval death. Another explanation would be that this strain is less of a generalist and causes lethal infections only in certain insect species. As we tested injectable and oral activity in different insect species, we cannot exclude this possibility. Although some strain-specific differences exist, we conclude that strains in sub-clade 3 mostly possess some insecticidal activity but that it is by far less distinct than in Fit-producing strains of sub-clade 1. To date no factor contributing to pathogenicity of sub-clade 3 strains has been identified, but it was suggested that so-called toxin complexes (Tc), first discovered in the entomopathogen *Photorhabdus luminescens*, could have a role (Loper *et al*., 2012). In accordance to the study of Loper *et al*. (2012) different Tc-related genes could be identified in the genomes of the strains included in this study, but they were not restricted to the strains with insecticidal activity (data not shown). Hence, they might have a rather subtle role in *Pseudomonas* insect associations.

Plant-beneficial effects are phylogenetically less predictable than insecticidal activity

Although biocontrol activity against root pathogens has been demonstrated for many strains of the *P. fluorescens* group, most of the species-type strains have never been investigated. The lack of knowledge for these strains and for the new strains from non-root habitats led us to test all 26 strains investigated for insecticidal activity also for their biocontrol activity against the oomycete pathogen *P. ultimum* on cucumber roots and a subset of strains also for their *in vitro* inhibition of *P. ultimum* and *F. oxysporum* f. sp. *radicis-lycopersici*. Biocontrol activity appeared to be phylogenetically less predictable than insecticidal activity, as effective as well as poor biocontrol strains were found throughout all the three sub-clades (Supplementary Results; Figure 2; Supplementary Table S5; Supplementary Figure S3). Similar to the results on insecticidal activity, no connection between the original habitat and the degree of plant protection was observed. Together, the bioassays with insects and pathogens identified several strains of sub-clade 1, which exhibit potent dual activity against plant pests and diseases and therefore could be of interest for implementation in integrated crop protection strategies.

Comparative genomics to identify potential factors associated to insecticidal activity

Draft genomes of all selected strains were generated with exception of the strains *P. protegens* CHA0 and

Pseudomonas sp. SS101, for which the genomes were already available (Loper *et al.*, 2012; Jousset *et al.*, 2014), and *Pseudomonas* sp. CMR12a, for which the genome description will be released elsewhere and which was therefore not included in the comparative genomics analysis. The average number of contigs per genome was 32 (Supplementary Table S6). The obtained genome sizes range between 6.06 and 7.07 Mbp, which is in accordance to genome sizes obtained for other fluorescent pseudomonads (Loper *et al.*, 2012).

The next step was to search for genes that are common and unique to insecticidal strains, encoding candidate factors potentially involved during the infection of insect larvae. Using EDGAR (Blom *et al.*, 2009), we identified 90 genes that are present in all highly insecticidal strains (sub-clade 1), but neither in moderately insecticidal strains (sub-clade 3) nor in non-insecticidal strains (sub-clade 2; Table 3). We further identified 117 genes that are present in all strains of sub-clade 1 as well as in one or several of the strains in sub-clade 3, but again in none of the strains of sub-clade 2 (Table 3). A full list of all identified genes can be found in Supplementary Table S7. It comprises about 28 putative transporters, 21 putative regulatory genes and over 100 enzymes and hypothetical proteins that are unique to insecticidal strains (Supplementary Table S7). Amongst the identified transporters, there are several putative amino-acid transporters. Insects are very rich in amino acids (Rumpold and Schluter, 2013) and thus these transporters might help to exploit the insect as a source of nutrients. The Fit toxin is specifically expressed in insects but not on plant roots (Péchy-Tarr *et al.*, 2013). This could also be the case for other virulence factors and might involve some of the many regulatory genes that were found to be specific to insecticidal strains. However, besides the Fit toxin (Péchy-Tarr *et al.*, 2008; Péchy-Tarr *et al.*, 2013; Ruffner *et al.*, 2013), no other insecticidal toxin was identified. For most of the 207 genes unique to insecticidal strains, a prediction on the biological function of the encoded product as well as on a possible role during the infection of insects would be very speculative at the present stage. Nevertheless, the comparative genomics also revealed several genes encoding proteins with homology to known virulence factors of other bacteria and that are of interest in terms of a possible association with insecticidal activity. Presence of those genes, which are discussed below, is indicated for our selection of strains in Figure 2 and for all strains included in the phylogeny of Figure 1 in Supplementary Table S1.

Table 3 Genes associated with insecticidal activity

Sub-clade 1	*Sub-clade 2*	*Sub-clade 3*			*Number of Genes*
		SS101	*DSM 50090^T*	*MIACH*	
+	−	−	−	−	90
+	−	+	+	+	57
+	−	+	+	−	20
+	−	+	−	+	7
+	−	+	−	−	20
+	−	−	+	+	13
+	−	−	+	−	0
+	−	−	−	+	0

Numbers of genes that are specific to insecticidal strains.
Presence of genes was defined as 70% amino-acid pairwise identity over at least 70% of gene length for the pairwise comparisons.
Only genes that were common to all strains of sub-clade 1 (highly insecticidal strains), but not found in any strain of sub-clade 2 (non-insecticidal strains) were considered.
+ Indicates genes present in all these strains.
− Indicates genes absent in all these strains.

Upon ingestion of pathogenic bacteria, insects produce reactive oxygen species, antimicrobial peptides and lysozymes to rapidly eliminate infesting bacteria (Lemaitre and Hoffmann, 2007). One mechanism to counter this first line of insect immunity is to produce enzymes degrading antimicrobial peptides. Exoproteases such as the Zn-dependent metallopeptidase AprX, also called serralysin and the AprA alkaline protease were suggested to have a role during the early phase of bacterial infections (Liehl *et al.*, 2006). The gene *aprA* is present in all 25 genomes, except that of strain P1.8, whereas *aprX* was only detected in the genomes of strains belonging to sub-clade 1 (Supplementary Table S1). AprA and AprX belong to the M10 family that includes serralysin, aeruginolysin and other related exopeptidases that cause tissue damage and anaphylactic responses (Park and Ming, 2002). In *Pseudomonas entomophila*, an *aprA* mutant was shown to be slightly less virulent and to have a reduced persistence in *D. melanogaster* (Liehl *et al.*, 2006). Serralysin of *Serratia marcescens* was shown to promote hemolymph bleeding in the silkworm (*Bombyx mori*) (Ishii *et al.*, 2014).

If bacteria persist within the insect gut, living cells or their toxins must breach the peritrophic membrane, a gut-delimiting chitinous matrix, to access the hemocoel (Vallet-Gely *et al.*, 2008). Chitinases affecting the peritrophic matrix are therefore potential virulence factors of entomopathogenic bacteria. For instance chitinases of *B. thuringiensis* subsp. *israelensis* IPS68 and *B. thuringiensis* subsp. *aizawai* HD133 were shown to contribute to insecticidal activity toward *Culicuoides nubeculosus* and *S. littoralis,* respectively (Sampson and Gooday, 1998). In insecticidal strains of the *P. fluorescens* group, we identified two chitinase genes. The chitinase gene *chiC* encoded next to a chitin-binding protein is present exclusively in genomes of sub-clade 1 strains, whereas the second chitinase is present in nearly all *P. chlororaphis* strains and some sub-clade 3 strains (Supplementary Table S1).

PCL1391_2966 encodes for a phosphocholine-specific phospholipase C. This gene, *plcN*, was detected only in sub-clade 1 strains. Phospholipases

are recognized as major virulence determinants in a number of bacterial species, including human, animal and several invertebrate pathogens (Songer, 1997; Farn *et al*., 2001; Yang *et al*., 2012). The *ymt* gene encoding for a phospholipase D in *Yersinia pestis*, for example, is needed for persistence in the flea midgut (Hinnebusch *et al*., 2002). Phospholipase C produced by *Mycobacterium abscessus* is crucial for survival in amoeba and is suggested to cause damage to mouse macrophages presumably by hydrolysis of membrane phospholipids (N'goma *et al*., 2015).

Three small genes with homology to *reb* genes were found to be present in all strains of sub-clade 1 and in *Pseudomonas* sp. SS101, the strain with the highest insecticidal activity of sub-clade 3. Such *reb* genes have been mainly studied in *Caedibacter taenospiralis,* a *Paramecium* endosymbiont. They encode R-bodies, highly insoluble protein ribbons that are typically coiled into cylindrical structures but can unroll under certain conditions (Pond *et al*., 1989) and are associated with the killing trait toward sensitive *Paramecia* (Dilts and Quackenbush, 1986). Orthologs of *reb* were found to be present in many free-living bacteria, but their function remains unclear to date (Raymann *et al*., 2013).

A whole cluster of genes specific to insecticidal strains (loci PCL1391_4983 to PCL1391_4994) has high percentage of sequence identity to the *psl* gene cluster of *P. aeruginosa* which specifies the production of the extracellular polysaccharide Psl (Franklin *et al*., 2011). Psl was shown contributing to biofilm production, tolerance to oxidizing agents and host defensive processes (Friedman and Kolter, 2004; Jackson *et al*., 2004; Mishra *et al*., 2012), that is, traits likely useful in insect interactions.

Other factors, which still have to be kept in mind, are the antimicrobial compounds, such as 2,4-diacetylphloroglucinol, phenazine, pyoluteorin, pyrrolnitrin and hydrogen cyanide that are crucial for biocontrol activity against fungal diseases, although none is shared by all or unique to insecticidal strains (Figure 2; Supplementary Table S1; Haas and Défago, 2005; Mercado-Blanco and Bakker, 2007; Lugtenberg and Kamilova, 2009). However, some have activity against a broad spectrum of organisms including plants, nematodes, arthropods and even mammalian cells (Maurhofer *et al*., 1992; Devi and Kothamasi, 2009; Kwak *et al*., 2011; Neidig *et al*., 2011; Nisr *et al*., 2011; Jang *et al*., 2013) and thus could contribute to *Pseudomonas*-derived insecticidal activity.

Chitinase ChiC and phospholipase PlcN contribute to oral insecticidal activity

In order to verify that our combination of bioassays and comparative genomics indeed led to the identification of valuable candidate genes associated with bacterial virulence toward insects, we generated, in model strain *P. protegens* CHA0, in-frame deletion mutants for selected genes: *plcN*, *chiC*, *aprX* and the cluster encoding homologs of *rebB*. None of the mutants differed in activity from the wild type when injected directly into the hemocoel of *G. mellonella* (Figure 4a; Supplementary Figures S4A and C). However, the *chiC* mutant was always significantly delayed in killing *P. xylostella* larvae upon ingestion (Figure 4b; Supplementary Figure S5A). We therefore conclude that the chitinase C, encoded by *chiC*, a gene common and unique to highly insecticidal strains, contributes to oral insect pathogenicity of *P. protegens* CHA0. Chitinase C was found to be responsible for chitobiosidase as well as endochitinase activity of *P. protegens* CHA0 as the *chiC* mutant exhibited no residual chitinase activity (Figure 4c). As also a *gacA* mutant completely lost chitinase activity (Figure 4c) we believe that we identified with the *chiC* one of the hitherto unknown Gac-regulated virulence factors involved in oral insecticidal activity. Furthermore, the mutant deficient for phopholipase C (*plcN)* also showed reduced oral activity against *P. xylostella*. Although less distinct than for the *chiC* mutant, a delay in killing was always observed for the *plcN* mutant but the effect was not significant in all experiments (Supplementary Figures S4B and S5B).

In contrast, no difference to the wild-type strain CHA0 was found for the *rebB1-3* and *aprX* mutants (Supplementary Figure S4D). However, these results do not exclude a role of factors encoded by these genes under different conditions or in an interaction with other insect species. Accordingly, the impact of the well-characterized Fit toxin on virulence toward insects also varies between insect species. Thus, a *fitD* mutant compared to the wild-type *P. protegens* CHA0 is more strongly reduced in virulence toward *S. littoralis* than toward *P. xylostella* (data not shown).

The mutational analysis performed in this study gives only a first insight into the possible contribution of interesting candidate genes identified in the comparative genomics approach to insecticidal activity. An in-depth analysis of the role of chitinase C and phospholipase C would include the complementation of these mutants and will be subject to further studies.

Conclusions

We provide the first extensive overview on insecticidal activity in the *P. fluorescens* group. Whilst biocontrol activity against fungal pathogens occurs throughout all studied sub-clades, insecticidal activity is unique to sub-clades 1 and 3. Only strains of sub-clade 1 display strong oral insecticidal activity and only they produce the Fit toxin. Intriguingly, Fit seems to contribute to the floppy and melanized phenotype associated with infections by highly pathogenic strains, however, the toxin is clearly not the major killing factor upon oral ingestion. Mutants

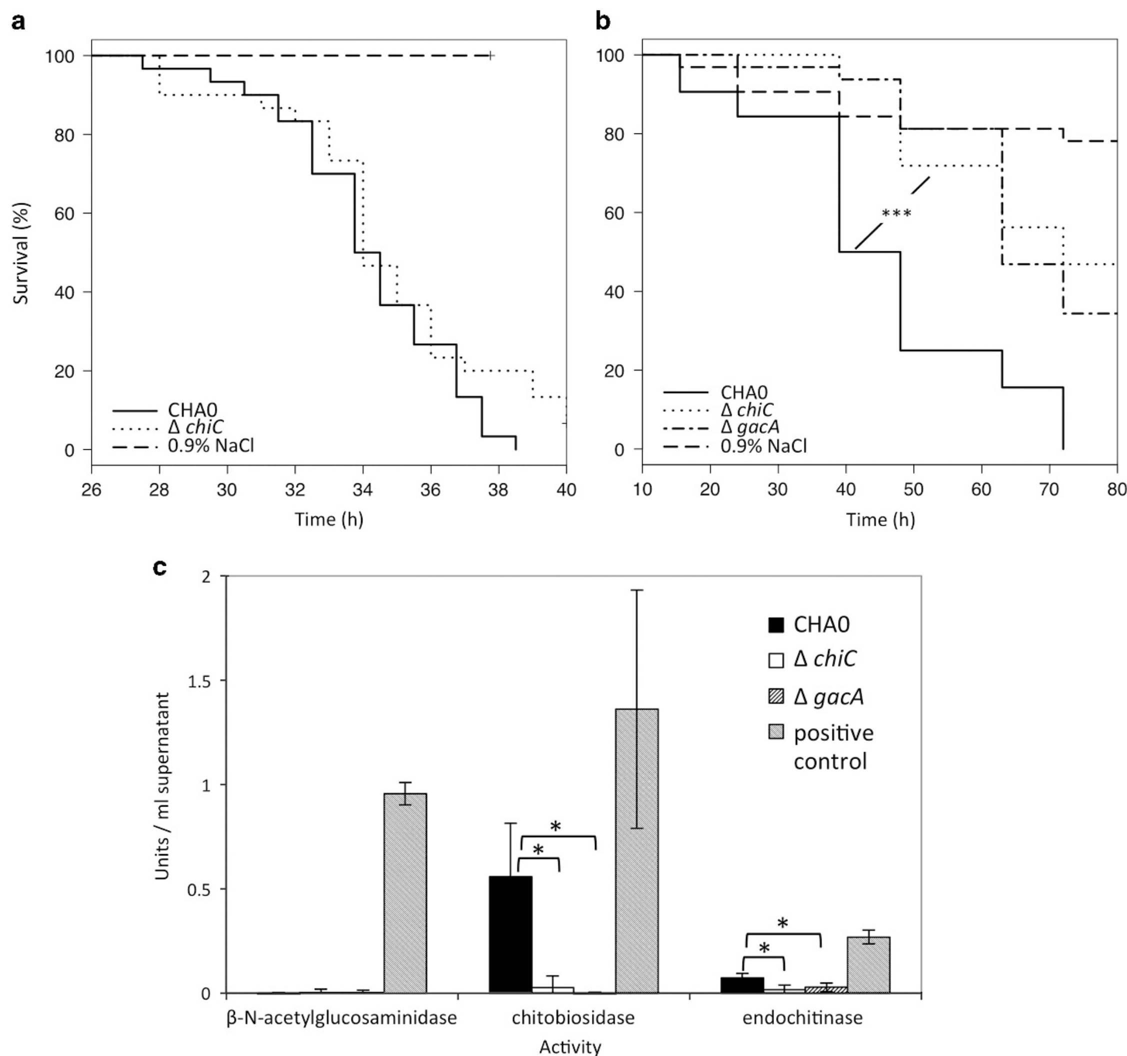

Figure 4 A derivative of *P. protegens* CHA0 deficient for a specific chitinase is reduced in oral, but not in injectable activity against insect larvae. (**a**) Systemic activity against *G. mellonella*. Thirty larvae per treatment were injected with 2×10^3 bacterial cells and survival was recorded hourly. (**b**) Oral activity against *P. xylostella*. Larvae were exposed to artificial diet inoculated with 4×10^6 bacterial cells. Significant differences according to a Log-Rank test (Survival Package in R) between treatments with the wild-type CHA0 and the chitinase C-negative mutant (Δ*chiC*) are indicated with $^{***}P<0.0001$. Each mutant was tested at least three times with similar results. A repetition of the feeding assay is depicted in Supplementary Figure S5. (**c**) Chitinase activity of wild-type CHA0 and its *chiC* mutant was assessed using a chitinase assay kit (Sigma, St Louis, MO, USA). Three different substrates were used to test for exo- (β-N-acetylglucosaminidase and chitobiosidase) and endochitinase activity. Treatments indicated by an asterisk are significantly different based on a *t*-test ($P \leqslant 0.05$). CHA0, wild type; Δ*chiC*, chitinase C-negative mutant; Δ*gacA*, GacA-negative mutant; 0.9% NaCl served as negative control in the virulence assay; a positive control for chitinase activity was provided by the chitinase assay kit.

of strains CHA0 and PCL1391 lacking the *fit* genes cause delayed, but still substantial mortality in *S. littoralis* (Ruffner *et al.*, 2013) when acquired via the oral infection route. By comparative genomics we now identified several candidate genes that might contribute to insecticidal activity and we demonstrated that the absence of two of these genes, encoding a specific chitinase and a phospholipase, negatively affects insecticidal activity. We hypothesize that especially the chitinase C might be involved during the gut stage of the infection process, causing damage to the peritrophic membrane. However, to understand the exact mode of action of these pathogenicity factors during the infection process, further investigations are needed. Nevertheless, the presented data highly increases the knowledge on the genetic basis of insecticidal activity of fluorescent pseudomonads and points to a multifactorial nature of this trait.

Although we provide evidence that many strains of the *P. fluorescens* group can be insect pathogenic and others might persist in insects as commensals, the ecological relevance of insects as a host for these bacteria is still elusive and an intriguing field for future research. The fact that certain pseudomonads, to date considered to be plant-associated, perform very well in a completely different habitat such as an insect raises the question whether these bacteria are

indeed mainly plant-associated. Insecticidal as well as biocontrol activity against plant diseases was found to be independent of the original habitat of a strain. For example, closely related strains can be isolated from fish or cyclops and behave similarly well on roots as root isolates. This observation is in line with other studies that found the isolation source of a bacterial strain not to be predictive for its performance in another habitat (Alonso *et al.*, 1999; Grosso-Becerra *et al.*, 2014). For instance, Hilker and colleagues found no correlation between original habitat and virulence in different test systems for clinical and environmental clones of *P. aeruginosa* (Hilker *et al.*, 2015). In general, fluorescent pseudomonads might be quite ubiquitous and probably possess an arsenal of traits allowing them to easily switch niches and to conquer the habitat they encounter. Insects could be especially useful as a means of dispersal, a phenomenon documented for diverse plant-pathogenic bacteria (Nadarasah and Stavrinides, 2011). Some *Pseudomonas syringae* strains for instance can use the pea aphid as alternative primary host where they replicate to high numbers and can be deposited onto a new plant host via excreted honeydew (Stavrinides *et al.*, 2009). Similarly, the rhizobacterium *P. chlororaphis* strain L11 was found to be transmittable from one plant to another by *Diabrotica undecimpunctata* subsp. *howardi* feeding on colonized plants (Snyder *et al.*, 1998). In contrast to considering the insect as an alternative host, one could even speculate that the plant is not the primary host for species like *P. protegens* and *P. chlororaphis,* but rather a transient host on which they endure until they encounter the next insect host. As research to date is very much biased toward plant-association, future studies especially on strains actually isolated from insects will be required to gain a better understanding of the importance insects have as hosts for strains of the *P. fluorescens* group.

Besides its ecological relevance, insecticidal activity might be of great agronomical interest. Our bioassays revealed several strains, especially of the species *P. protegens*, that display potent dual activity, killing insect larvae and protecting plants against pathogens. Fluorescent pseudomonads are already commercially used for the biological control of plant diseases (Stockwell and Stack, 2007; Berg, 2009). Our discovery of strains with the capacity to control insect pests on top of fungal pathogens renders these bacteria highly interesting for a new field of application and an additional market.

Conflict of Interest

The authors declare no conflict of interest.

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