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Rhizoxin analogs, orfamide A and chitinase production contribute to the toxicity of *Pseudomonas protegens* strain Pf-5 to *Drosophila melanogaster*

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Summary

***Pseudomonas protegens* strain Pf-5 is a soil bacterium that was first described for its capacity to suppress plant diseases and has since been shown to be lethal to certain insects. Among these is the common fruit fly *Drosophila melanogaster*, a well-established model organism for studies evaluating the molecular and cellular basis of the immune response to bacterial challenge. Pf-5 produces the insect toxin FitD, but a Δ*fitD* mutant of Pf-5 retained full toxicity against *D. melanogaster* in a noninvasive feeding assay, indicating that FitD is not a major determinant of Pf-5's oral toxicity against this insect. Pf-5 also produces a broad spectrum of exoenzymes and natural products with antibiotic activity, whereas a mutant with a deletion in the global regulatory gene *gacA* produces none of these exoproducts and also lacks toxicity to *D. melanogaster*. In this study, we made use of a panel of Pf-5 mutants having single or multiple mutations in the biosynthetic gene clusters for seven natural products and two exoenzymes that are produced by the bacterium under the control of *gacA*. Our results demonstrate that the production of rhizoxin analogs, orfamide A, and chitinase are required for full oral toxicity of Pf-5 against *D. melanogaster*, with rhizoxins being the primary determinant.**

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Introduction

Pseudomonas fluorescens is a diverse group of Gammaproteobacteria composed of more than 55 named species. Several strains within the *P. fluorescens* group are lethal to insects (Castrillo *et al.*, 2000; Commare *et al.*, 2002; Otsu *et al.*, 2004) including *P. protegens* strains Pf-5 and CHA0, which are known to exhibit both injectable and oral toxicity against several insect species (Péchy-Tarr *et al.*, 2008; Devi and Kothamasi, 2009; Olcott *et al.*, 2010; Kupferschmied *et al.*, 2013; Ruffner *et al.*, 2013; Chen *et al.*, 2014; Flury *et al.*, 2016). *Pseudomonas protegens* Pf-5 and CHA0 are best known for their capacities to produce a broad spectrum of natural products with antibiotic properties, colonize seeds or roots, and protect plants from infection by plant-pathogenic fungi and oomycetes (Haas and Défago, 2005; Loper *et al.*, 2007). These bacteria also produce the insect toxin FitD (Péchy-Tarr *et al.*, 2008), which is closely related to the insecticidal Mcf ('makes caterpillars floppy') toxin of *Photorhabdus luminescens* (Daborn *et al.*, 2002). Production of the FitD toxin contributes to the toxicity of strains Pf-5 and CHA0 when injected to *Manduca sexta*, the tobacco hornworm (Péchy-Tarr *et al.*, 2013), as well as the toxicity of strain CHA0 when ingested by several lepidopteran insect pests (Ruffner *et al.*, 2013). In addition to FitD, other unknown factors contribute to insect toxicity of *P. protegens* Pf-5 and CHA0, as Δ*fitD* mutants of these strains continue to exhibit significant levels of insect toxicity, albeit not to the levels of wildtype strains (Ruffner *et al.*, 2013). For example, hydrogen cyanide produced by *P. protegens* CHA0 contributes to its capacity to kill termites, *Odontotermes obesus* (Isoptera) (Devi and Kothamasi, 2009). For the most part, however, the mechanisms of action for insect lethality of bacteria in the *P. fluorescens* species complex have yet to be revealed.

Our studies focus on the soil bacterium *Pseudomonas protegens* Pf-5, which is known for its capacity to suppress plant diseases and produce a large spectrum of metabolites with antibiotic activity (Loper *et al*., 2007; Gross and Loper, 2009). The antibiotics produced by Pf-5 include pyrrolnitrin (Howell and Stipanovic, 1979); pyoluteorin (Howell and Stipanovic, 1980); analogs of rhizoxin (Brendel *et al*., 2007; Loper *et al*., 2008); hydrogen cyanide (Kraus and Loper, 1992); 2,4-diacetylphloroglucinol (DAPG) (Nowak-Thompson *et al*., 1994); monoacetylphloroglucinol (MAPG) (Kidarsa *et al*., 2011), an intermediate in the DAPG biosynthetic pathway (Shanahan *et al*., 1993; Bangera and Thomashow, 1999); the lipopeptide orfamide A (Gross *et al*., 2007); and toxoflavin (Philmus *et al*., 2015). In *Pseudomonas* spp., the production of antibiotics and other exoproducts requires the Gac/Rsm system, which controls the expression of target genes through a complex signal transduction pathway involving regulatory RNAs and translational repression (Lapouge *et al*., 2008). Due to the preeminent role of the response regulator GacA in this pathway, Δ*gacA* mutants of Pf-5 do not produce any of the antibiotics produced by wild type Pf-5 (Whistler *et al*., 1998; Gross *et al*., 2007; Loper *et al*., 2008; Hassan *et al*., 2010; Kidarsa *et al*., 2013; Philmus *et al*., 2015). Consequently, when compared to wild type strains, Δ*gacA* mutants can reveal the combined contributions of antibiotics and other exoproducts to a range of biological activities of *Pseudomonas* spp.

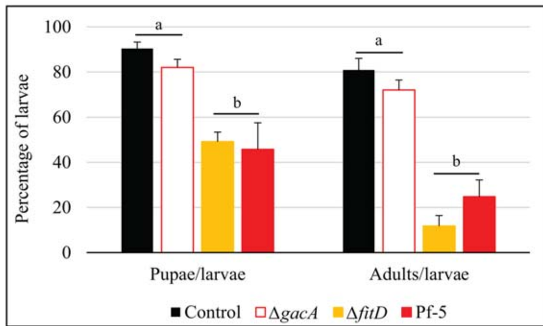
In a previous study, we observed that *P. protegens* Pf-5 exhibited a dose-dependent oral toxicity to larvae of *Drosophila melanogaster* (Olcott *et al*., 2010). Furthermore, larvae that survived feeding with Pf-5 were delayed in the onset of metamorphosis and commonly developed into adults with morphological defects. Bacteria that exhibit oral toxicity to insects can be valuable components of pest management strategies in agriculture and forestry (Melo *et al*., 2016), so we set out to identify the traits of *P. protegens* Pf-5 contributing to this toxicity. Previously, we showed that a functional *gacA* gene was required for the oral toxicity of wild-type Pf-5: a Δ*gacA* mutant of Pf-5 caused no significant death of *D. melanogaste*r (Olcott *et al*., 2010). These results suggested that a natural product or exoenzyme produced by Pf-5 under the positive control of the Gac/Rsm signal transduction system is responsible for the oral toxicity to *D. melanogaster*. In this study, we built upon the well-characterized effects of a Δ*gacA* mutation on the phenotypes and transcriptome of Pf-5 (Hassan *et al*., 2010; Kidarsa *et al*., 2013) and employed an extant mutant set with single or multiple knockouts in two exoenzymes, a type VI secretion system, and all seven of the known antibiotics regulated by *gacA* (i.e. pyrrolnitrin, pyoluteorin, analogs of rhizoxin, hydrogen cyanide, DAPG and its intermediate MAPG, orfamide A and toxoflavin) (Quecine *et al*., 2016). Our study revealed no evidence for a role of the FitD toxin in oral toxicity of Pf-5 to *D. melanogaster*. Instead, the capacity to produce rhizoxin analogs, compounds known for phytotoxicity and anti-fungal properties, was a major determinant of oral toxicity, with the lipopeptide orfamide A and chitinase also having detectable roles.

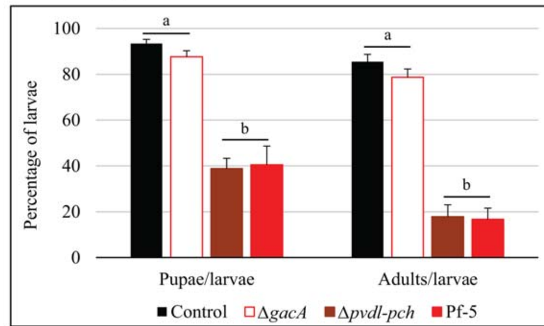
Results and discussion

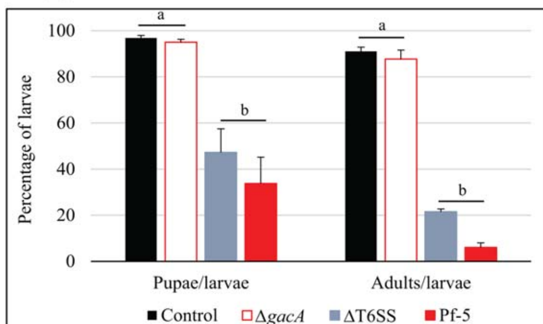
No detectable contribution of FitD or siderophores to oral toxicity of P. protegens *Pf-5 against* D. melanogaster

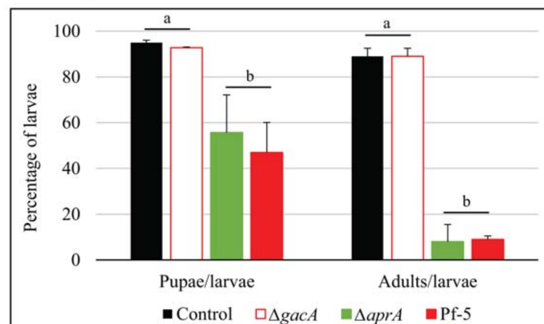
Due to the known role of FitD in insect toxicity, we first set out to determine if FitD contributes to the oral toxicity of Pf-5 to *D. melanogaster* using a previously developed non-invasive assay (Olcott *et al*., 2010). Averaging the results from three replicated experiments, 81% of the larvae developed into adult flies in the control treatment, in which eggs of *D. melanogaster* were fed with water-treated yeast (Fig. 1A). In contrast, only 12% or 25% of the larvae fed with Δ*fitD*- or wildtype Pf-5-inoculated yeast developed into adults respectively. Based on the toxicity of the Δ*fitD* mutant in the three replicated experiments, we conclude that FitD did not contribute detectably to oral toxicity of Pf-5 against *D. melanogaster*. These data contrast with the findings of Ruffner *et al*. (Ruffner *et al*., 2013) who reported that FitD contributes to the oral toxicity of *P. protegens* CHA0 against larvae of three Lepidopteran insect species, *Spodoptera littoralis*, *Heliothis virescens* and *Plutella xylostella* (Ruffner *et al*., 2013). Strain CHA0 is closely related to Pf-5 and both strains exhibit injectable toxicity against the tobacco hornworm *M. sexta*, due in large part to FitD, which is produced by both strains (Péchy-Tarr *et al*., 2008). Possible explanations for the varied role of FitD in oral toxicity to *D. melanogaster* versus the three insects evaluated by Ruffner *et al*. include differential expression of FitD by *P. protegens* in different insect species and differential sensitivities of the Lepidopteran and Dipteran insects to the toxin. *fitD* is not expressed by *P. protegens* CHA0 in culture or on plant roots, but is expressed by bacterial cells in larvae of the wax moth *Galleria mellonella* or the Egyptian cotton leaf worm *S. littoralis* (Kupferschmied *et al*., 2014). It is possible that *fitD* is not expressed by *P. protegens* in all insect hosts, although evaluating the expression of *fitD* in *D. melanogaster* was beyond the scope of this study.

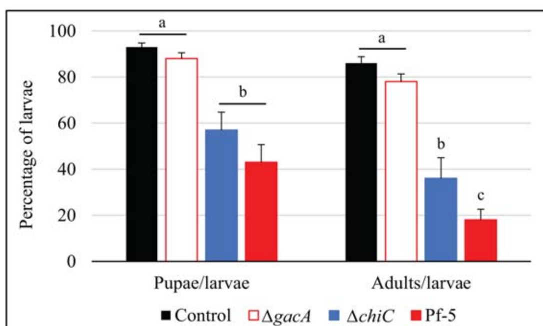
In a previous study evaluating the infection of larvae of wax worms (*Galleria mellonella*) by *Pseudomonas aeruginosa*, the production of a pyoverdine siderophore influenced the rate at which the bacterium killed the larvae (Harrison *et al*., 2006). *P. protegens* Pf-5 produces two siderophores: a pyoverdine (Hartney *et al*., 2013) and enantio-pyochelin (Youard *et al*., 2007). We compared mutants deficient in the production of both siderophores to











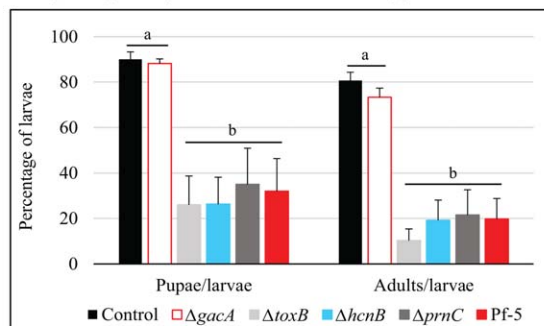


Fig. 1. Influence of varied mutations on oral toxicity of Pf-5. The proportion of larvae that pupariated (Pupae/larvae) or emerged as adults (Adults/larvae) were determined for larvae fed with yeast inoculated with water (black), wildtype Pf-5 (red), a Δ*gacA* mutant of Pf-5 (white with red border) or other mutants of Pf-5 having deletions in: A. *fitD*, which encodes an insect toxin (yellow), B. *pvdI* and *pchC or pchA*, which encode enzymes required for the biosynthesis of the siderophores pyoverdine and enantio-pyochelin (brown), C. PFL_6086 to PFL_6089, which encode components of a Type VI secretion system (blue gray), D. *aprA*, which encodes an extracellular protease (green), E. *chiC*, which encodes a secreted chitinase (blue), F. *toxB* (light gray), *hcnB* (turquoise), and *prnC* (dark grey), which encode enzymes required for the biosynthesis of toxoflavin, hydrogen cyanide or pyrrolnitrin. Values represent the mean and standard errors from one (D, F), two (C), three (A) or five (E, B) experiments, with each experiment having three replicates per treatment, and each replicate evaluating 30 eggs. Treatments denoted with a common letter do not differ significantly ($P = 0.05$) as determined by Fisher's least standard difference. For panels showing the means of multiple experiments (A, B, C, E), there were significant treatment effects, no significant experiment effect, and no significant experiment *X* treatment effect in the analyses considering combined experiments. For panels in which the results of a single experiment are depicted (D, F), the same treatment effects were observed in a repeated experiment.

the wildtype strain Pf-5 in five independent replicated experiments, but observed no significant difference between the mutant and wildtype strains in causing death of *D. melanogaster* (Fig. 1B). Therefore, our experiments revealed no detectable role of siderophore production in the insect lethality caused by Pf-5.

Lack of a detectable role for a type VI secretion system in oral toxicity of P. protegens *Pf-5 against* D. melanogaster

Types III and VI secretion systems that deliver effectors into target eukaryotic cells are important determinants of virulence in *P. aeruginosa* (Filloux, 2011). *Pseudomonas*

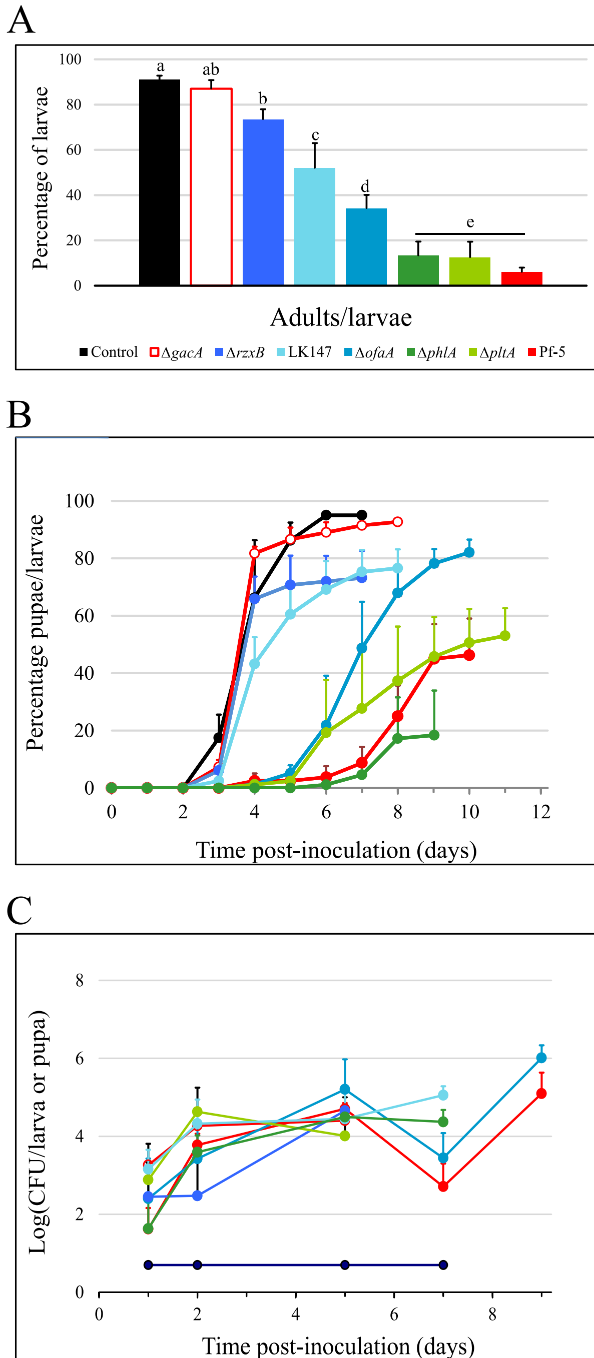


Fig. 2. Influence of mutations in secondary metabolite genes on the oral toxicity of Pf-5. The proportion of larvae that pupariated (Pupae/larvae) or emerged as adults (Adults/larvae) were determined for experiments in which second instar larvae were fed with yeast inoculated with wildtype Pf-5, derivative strains or water.
A. Values represent the mean and standard errors from two experiments, with each experiment having three replicates per treatment, and each replicate evaluating 30 eggs. Neither the experiment ($P > 0.78$) nor experiment X treatment ($P > 0.11$) effect was significant, so the means of the replicates of the two experiments were pooled for statistical analysis. Treatments denoted with a common letter do not differ significantly ($P = 0.05$) as determined by Fisher's least significant difference.
B. Values represent the mean and standard errors from a single experiment, in which pupae were counted daily for approximately 11 days after inoculation. Treatments are: Pf-5 (red circle), Δ*gacA* (open circle with red border), Δ*rzxB* (royal blue), *ofaA* (dark teal), *phlA* (dark green) and *pltA* (light green) mutants, and LK147 (turquoise, Δ*phlD*-Δ*rzxB*-Δ*prnC*-Δ*hcnB*-Δ*pltA*-Δ*ofaA*-Δ*toxB*).

protegens Pf-5 lacks a type III secretion system but has a full complement of genes encoding a type VI secretion system, which are expressed under the control of GacA (Hassan *et al*., 2010). We generated a mutant of Pf-5 having a deletion spanning four genes essential to the Type VI secretion system (*tssA*, *tssB*, *tssC* and *hcp*) and compared it to Pf-5 for virulence to *D. melanogaster*. We observed no significant difference in survival or development of the insects inoculated with wildtype Pf-5 versus the T6SS mutant in two independent replicated experiments (Fig. 1C). These results are consistent with the current view that a primary function of Type VI secretion systems is in bacterial competition and communication (Russell *et al.*, 2014). Indeed, one Type VI effector (Tge2, PFL_3037) has a demonstrated role in competition of Pf-5 with *P. putida* (Whitney *et al.*, 2013).

Chitinase has a detectable role in oral toxicity of P. protegens *Pf-5 against* D. melanogaster

The Δ*gacA* mutant of Pf-5 had no significant toxicity against *D. melanogaster* in any of the experiments of this study (Figs 1 and 2) or a previous study (Olcott *et al.*, 2010). These results confirm that gene(s) expressed under the positive control of the Gac/Rsm signal transduction system are responsible for much of the toxicity of Pf-5 against *D. melanogaster*. Among the many genes regulated by GacA are those encoding for the exoenzymes chitinase and alkaline protease (Hassan *et al.*, 2010; Kidarsa *et al.*, 2013). Proteases produced by bacteria can function as insect toxins or in bacterial defense against antimicrobial peptides produced by insects (Harrison and Bonning, 2010) and the exoprotease AprA is an important virulence factor that protects *Pseudomonas entomophila* from antibacterial peptides in *D. melanogaster* (Liehl *et al.*, 2006). We detected no significant role for AprA in insect toxicity of *P. protegens* Pf-5, however, as a Δ*aprA* mutant was similar to wildtype Pf-5 in causing lethality of *D. melanogaster* (Fig. 1D). Similar to Pf-5, *P. aeruginosa* PA14 also does not require AprA for oral toxicity towards *D. melanogaster* (Limmer *et al.*, 2011). In contrast, a Δ*chiC* mutant of Pf-5, which lacks chitinase production (Loper *et al.*, 2012), caused significantly less lethality than the wildtype strain, assessed at the adult stage (Fig. 1E). These results are in line with those from a recent report that a *chiC* mutant of P. protegens strain CHA0 shows

reduced oral toxicity to larvae of the lepidopteran insects *G. mellonella* and *P. xylostella* (Flury *et al*., 2016). Chitinases produced by entomopathogens can degrade the peritrophic membrane, a chitin-based matrix in the insect mid-gut that functions in protection against mechanical and chemical damage and serves as a barrier to infection by pathogens (Lehane, 1997). For example, the ChiAII endochitinase of *Serratia marcescens* can cause mortality or act synergistically to enhance the toxicity of the Bt CrylC protein (Regev *et al*., 1996) and a chitin-degrading protein is a virulence factor for foulbrood of honey bees caused by the entomopathogen *Paenibacillus larvae* (Garcia-Gonzalez *et al*., 2014). The peritrophic matrix is also known to have an important role in protection of *D. melanogaster* from infection by *P. entomophila* (Kuraishi *et al*., 2011). We speculate that the chitinase produced by Pf-5 could contribute to oral toxicity by enhancing ingress of bacterial metabolites or the bacterium itself from the mid-gut into other tissues or hemolymph of *D. melanogaster*.

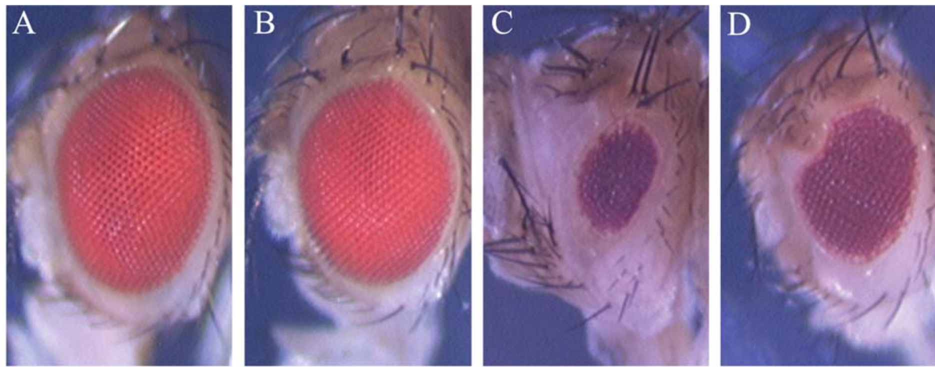
Rhizoxin analogs and orfamide A contribute to oral toxicity of P. protegens *Pf-5 against* D. melanogaster

To further explore the roles of Pf-5's GacA-controlled phenotypes on insect toxicity, we next evaluated a panel of mutants representing all seven known antibiotic biosynthetic gene clusters expressed by Pf-5 under the positive control of GacA (Hassan *et al*., 2010; Kidarsa *et al*., 2013). Experiments evaluating Δ*prnC*, Δ*hcnB*, Δ*toxB*, Δ*pltA* and Δ*phlA* mutants provided no evidence for a role of pyrrolnitrin, HCN, toxoflavin, pyoluteorin or 2,4-diacetylphloroglucinol in oral toxicity of Pf-5 against *D. melanogaster* (Figs 1F and 2). We are not aware of previous studies evaluating pyrrolnitrin, toxoflavin, pyoluteorin or 2,4-diacetylphloroglucinol in microbe-arthropod interactions, but HCN production contributes to lethality of *D. melanogaster* following injection of adult flies with *P. aeruginosa* PAO1 (Broderick *et al*., 2008). Our study differs from the study of Broderick *et al*. (2008) in many respects, including the species of *Pseudomonas* evaluated and inoculation through feeding versus injection. These differences likely explain why a role for HCN production was observed in *P. aeruginosa* PAO1 but not in Pf-5. Because cyanide is a potent toxin in *D. melanogaster* (Broderick *et al*., 2008), the lack of a detectable role for HCN production in our study is probably due to low production of HCN by Pf-5 in our assay or to pleiotropic effects of the *hcnB* mutation. The Δ*hcnB* mutant of Pf-5 overproduces several secondary metabolites, including 2,4-diacetylphloroglucinol, rhizoxin analogs and orfamide A (Quecine *et al*., 2016). It may not be possible to detect the influence of HCN on oral toxicity by comparing an *hcnB* mutant to Pf-5 because the mutant overproduces secondary metabolites toxic to *D. melanogaster*, such as orfamide A and rhizoxin analogs, as described below. In contrast to the Δ*hcnB* mutant, the Δ*prnC*, Δ*pltA* and Δ*phlA* mutations have relatively small effects on the production of other secondary metabolites by Pf-5 (Quecine *et al*., 2016).

The Δ*ofaA* mutant caused less lethality than wildtype Pf-5, indicating that the production of the lipopeptide orfamide A had a significant role in oral toxicity of Pf-5 to *D. melanogaster* (Fig. 2). In an earlier study, purified orfamide A was shown to have insecticidal activity when applied topically to nymphs of the green peach aphid (*Myzus persicae*) (Jang *et al*., 2013). Similarly, topical application of the lipopeptide viscosin causes mortality of several aphids (Hashimoto, 2002). Our observation that a Δ*ofaA* mutant exhibits diminished oral toxicity to *D. melanogaster* provides indirect evidence that orfamide A is produced by Pf-5 in concentrations needed to kill the larvae and pupae.

Of all the biosynthesis mutants evaluated in this study, the Δ*rzxB* mutant, which lacks production of rhizoxin analogs, was the most diminished in insect toxicity. In two independent replicated experiments, only 6% of the larvae fed with wildtype Pf-5 developed into adults whereas 74% of those fed with the Δ*rzxB* mutant developed into adults (Fig. 2A). Also, larvae fed wildtype Pf-5 were delayed in development into pupae whereas larvae fed the Δ*rzxB* mutant or the Δ*gacA* mutant showed less developmental delay (Chi square analysis, $P < 001$) (Fig. 2B). Rhizoxin is a 16-member macrolide that exhibits phytotoxic, antifungal and antitumor activities by binding to β-tubulin, thereby interfering with microtubule dynamics during mitosis (Gupta and Bhattacharyya, 2003). Pf-5 synthesizes five derivatives of rhizoxin, with the primary form being WF-1360F, which is inhibitory to at least some Ascomycetes and Stramenopiles, including plant pathogens such as *Botrytis cinerea*, *Fusarium* spp. and *Phytophthora* spp. (Brendel *et al*., 2007; Loper *et al*., 2008; Takeuchi *et al*., 2015; Quecine *et al*., 2016). WF-1360F also inhibits human tumor cell lines and exhibits phytotoxicity, albeit not to the level of rhizoxin itself (Loper *et al*., 2008). Due to the broad toxicity of rhizoxin against eukaryotes, it could be expected that rhizoxin is toxic to insects. Nevertheless, we are unaware of previous reports demonstrating a role of rhizoxin or its analogs in bacteria–insect interactions.

Surprisingly, the sevenfold mutant of Pf-5 (LK147), which does not produce rhizoxin analogs, orfamide A or five other secondary metabolites, was slightly more toxic to *D. melanogaster* than the Δ*rzxB* mutant. This could be caused by the pleiotropic influence of one of the seven mutations on an unknown factor contributing to insect toxicity by Pf-5. For example, as stated above, the *hcnB* mutant overproduces several known secondary metabolites. Accordingly, the presence of this mutation in LK147 could result in the overproduction of an unknown factor(s) contributing to insect toxicity. Although the Δ*rzxB* and Δ*gacA* mutants did not differ significantly in their toxicities to *D. melanogaster* in the



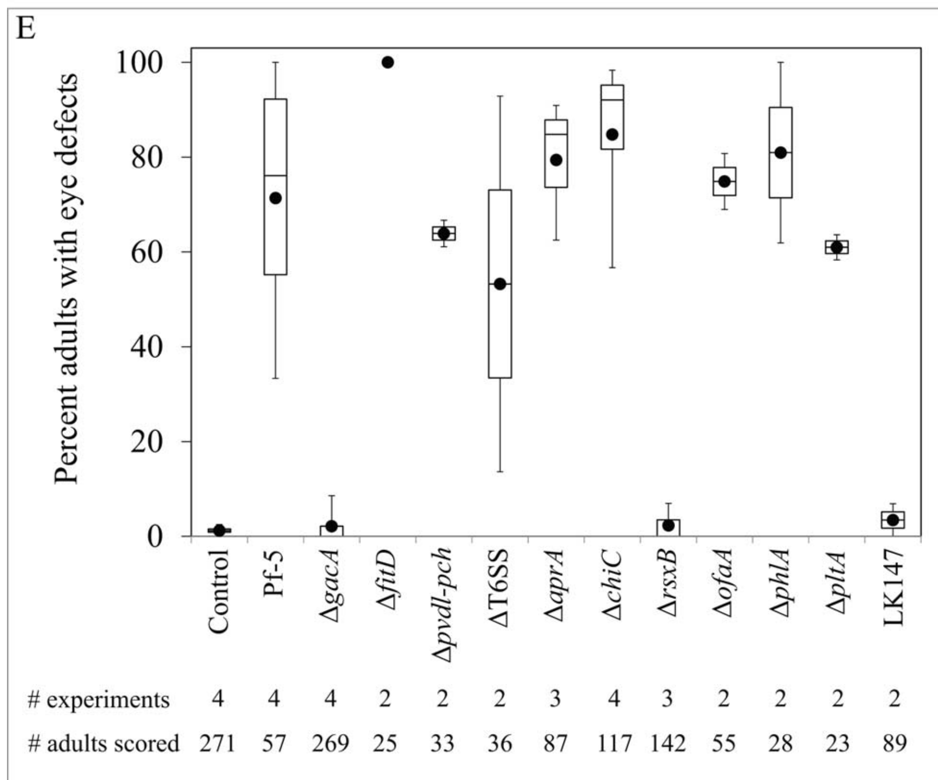


Fig. 3. Proportion of adult survivors with eye defects. Adults who survived following ingestion of Pf-5 or derivative strains were examined for eye defects, including small eyes or the presence of eye nicks.
A and B. Examples of normal eyes, seen in adult flies that developed from larvae of the water control, Δ*gacA* or Δ*rzxB* treatments. C. A small eye and (D) an eye with a nick, seen in adult flies that developed from larvae of the Pf-5 treatment.
E. The proportion of adult flies that had an eye defect (i.e., small and/or nicked eye). The black circle in the box plot shows the mean value and the horizontal line represents the median from two to four experiments. The number of experiments evaluated for each strain is shown in parentheses. The upper box is the third quartile and the lower box is the first quartile. The vertical line extends from the minimum to maximum value across all experiments.

two replicated experiments of this study, it is possible that there is a difference in toxicities of the two mutants that would be revealed in additional replicated experiments. We are confident that the production of rhizoxin analogs, chitinase, and orfamide A contributes to insect toxicity of Pf-5, but do not exclude the possibility that additional factors also contribute.

Production of rhizoxin analogs by P. protegens *Pf-5 causes eye defects in adult flies*

On average, 71% of adults that survived following ingestion of *P. protegens* Pf-5 had eye defects, including abnormally small eye size or the presence of nicks in the eye (Fig. 3C and D), as observed in our previous study (Olcott *et al.*, 2010). The Δ*gacA* and Δ*rzxB* mutants of Pf-5 caused virtually no eye defects: the proportion of adult flies with eye defects did not differ significantly between the Δ*gacA*, Δ*rzxB* or water control treatments (Fig. 3E). Similarly, LK147, which has a deletion in *rzxB* and six other genes, caused very few eye defects. The other mutants evaluated in this study caused eye defects in an average of 53% to 100% of adults (Fig. 3E). Therefore, the production of rhizoxin analogs was a primary factor responsible for the eye defects caused by oral ingestion of Pf-5 by *D. melanogaster*. Because rhizoxin is known to interfere with microtubule assembly and function (Prota *et al.*, 2014), we speculate that the eye defects are due to disruptions of cell division leading to cell death during development.

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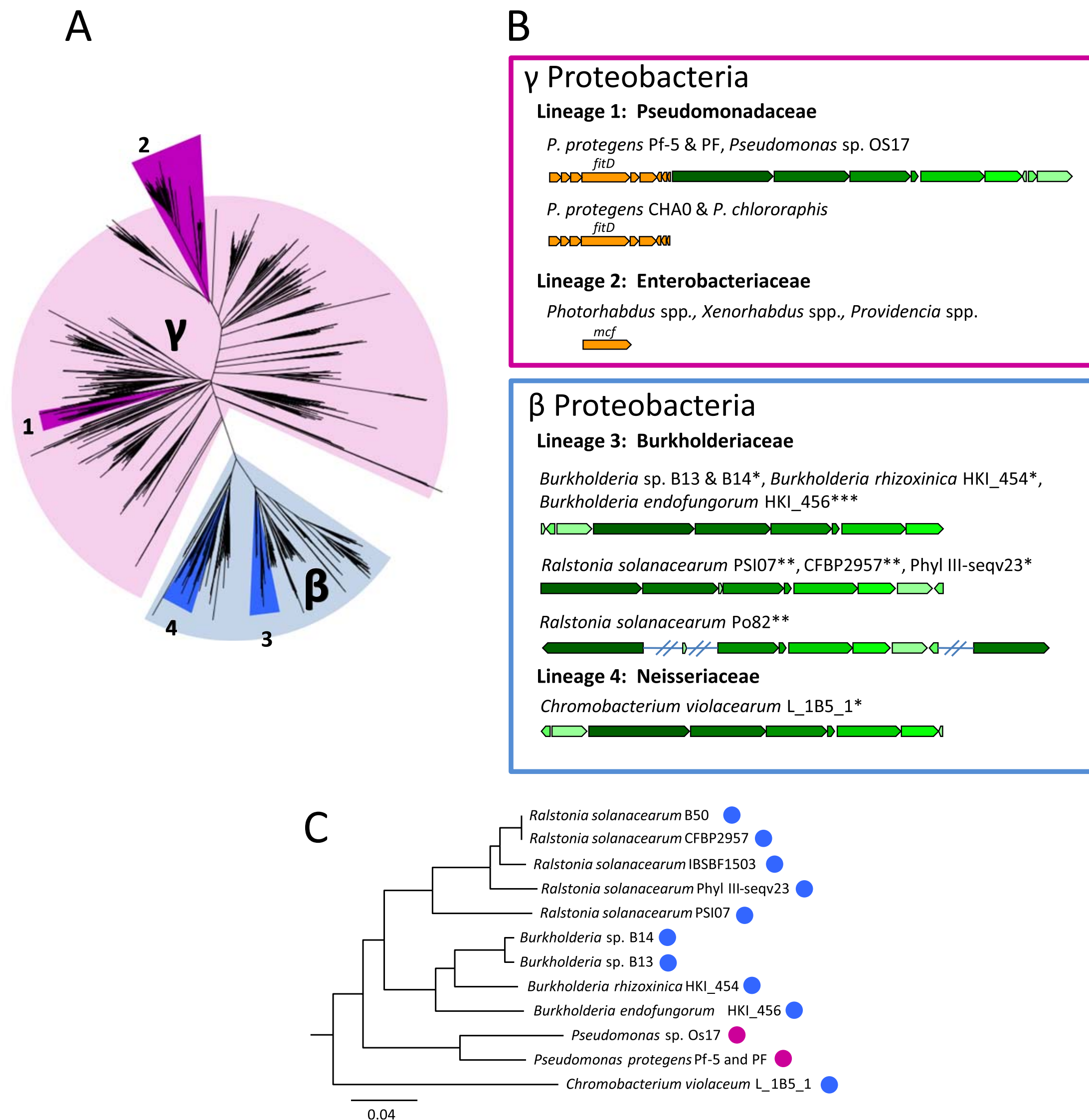

Fig. 4. Distribution of the rhizoxin biosynthesis gene cluster and genes encoding the FitD/Mcf insect toxin in the Proteobacteria.
A. An unrooted distance-based phylogeny of the 2158 type species of the Gammaproteobacteria and Betaproteobacteria is shown in the left panel. Lineages of the Gammaproteobacteria are highlighted pink and lineages of the Betaproteobacteria are highlighted blue. Darker shading denotes the four lineages having strains with FitD/Mcf or rhizoxin gene clusters.
B. Maps of the *fit* or *mcf* clusters (orange) and the rhizoxin biosynthesis cluster (green) in genomes of Gammaproteobacteria (pink box) or Betaproteobacteria (blue box). A single asterisk denotes a genome in which the rhizoxin gene cluster is in a putative genomic island, as determined by Alien_Hunter, IslandPick, SIGI-HMM or IslandPath-DIMOB software packages. Double asterisks denote genomes in which the rhizoxin gene clusters reside on megaplasmids. The triple asterisk for *B. endofungorum* HKI_456 indicates that the genomic sequence is not available, so the presence of the rhizoxin cluster on a plasmid or genomic island is not known. *Ralstonia solanacearum* Po82 is shown as a representative of many *R. solanacearum* strains (e.g. UW179, UW181, IBSBF1503 and B50) in which the rhizoxin gene cluster is fragmented and dispersed throughout the genome, although the organization shown for strain Po82 differs from those in other genomes.
C. Subclade of the larger S-adenosine-dependent methytransferase phylogenetic tree (full tree in Supporting Information Figure S1) containing RzxI/RhiI sequences from genera of the Gammaproteobacteria (pink) and Betaproteobacteria (blue).

Linkage of two loci for insect toxicity in the Pf-5 genome

The rhizoxin biosynthesis gene cluster is adjacent to the gene cluster encoding for production of the FitD insect toxin in the genome of Pf-5 (Péchy-Tarr *et al.*, 2008). Gene clusters for rhizoxin biosynthesis are also present in two families within the Betaproteobacteria: a strain of *Chromobacterium violacearum*, and many, but not all, strains of *Ralstonia solanacearum* (Remenant *et al.*, 2010) and *Burkholderia* spp. (Partida-Martinez and Hertweck, 2007) have complete rhizoxin biosynthesis gene clusters (Fig. 4). Homologs of *fitD* are present in two families of

Gammaproteobacteria. Within the Pseudomonadaceae, *fitD* is present in genomes of *P. chlororaphis* and *P. protegens* (Loper *et al.*, 2012; Ruffner *et al.*, 2015). Within the Enterobacteriaceae, genomes of insect pathogens in the genera *Heterorhabdus* and *Photorhabdus*, and certain *Providencia* spp. have *mcf*, a homolog of *fitD*. To date, only *P. protegens* strains Pf-5 (Loper *et al.*, 2012) and PF (Ruffner *et al.*, 2015) and the related strain *Pseudomonas* sp. OS17 (Takeuchi *et al.*, 2015) are known to have both *fit* and rhizoxin gene clusters (Fig. 4), which are linked (i.e. the *fit-rzx* cluster) in all three genomes.

The patchy distribution of rhizoxin gene clusters in a small fraction of strains in three distantly related bacterial families is typical of genes that are inherited via horizontal gene transfer (HGT). To test the hypothesis that the rhizoxin gene cluster has been disseminated through HGT, we constructed phylogenetic trees for four genes in the cluster. In all cases, homologous rhizoxin genes from the four bacterial genera form a well-defined clade (Supporting Information Figs S1–S4). For example, RxzI encodes an S-adenosine-dependent methytransferase, a large protein family that is prevalent in the Gamma and Beta Proteobacteria and other organisms. Our phylogenetic analysis shows that RzxI clusters with homologs in the rhizoxin clusters from species of *Ralstonia*, *Burkholderia* and *Chromobacterium* rather than with other P450 monooxygenases present in genomes of *Pseudomonas* species (Fig. 3C and Supporting Information Fig. S1). The same pattern is observed for RzxA (polyketide synthease/nonribosomal synthetase hybrid), RzxG (acyl transferase) and RzxH (cytochrome P450 monooxygenase), all of which cluster with homologs in rhizoxin gene clusters present in distantly-related bacterial taxa (Supporting Information Figs S2–S4) despite the prevalence of genes encoding these protein families in *Pseudomonas* genomes. Furthermore, the topology of the phylogenetic clades are similar for all rhizoxin genes, indicating that *Chromobacterium violaceum* contains the earliest diverging form of the rhizoxin cluster currently available for analysis. As expected for a gene inherited via HGT, the phylogeny of the bacteria (Fig. 3A) differs from that of the rhizoxin genes in the corresponding bacterial genomes (Fig. 3B), with the rhizoxin genes from *P. protegens* flanked by those from the Betaproteobacteria. Certain *Ralstonia* genomes have fragmented rhizoxin gene clusters. Others have only a partial set of rhizoxin genes, suggesting that certain genes have been lost following acquisition of the cluster by these strains.

To further explore the possibility that rhizoxin was acquired through HGT, we evaluated eight genomes having a rhizoxin gene cluster for the presence of genomic islands. The rhizoxin biosynthesis gene clusters in *C. violacearum* strain L-1B5_1, *R. solanacearum* Phyl III-seqv23, and some strains of *Burkholderia* spp. and *R. solanacearum* fall into genomic islands detected by Alien_hunter, IslandPick, SIGI-HMM or IslandPath-DIMOB software packages (Fig. 4). In *R. solanacearum* strains PSI07, CFBP2957 and Po82, the rhizoxin genes are on megaplasmids. The genomic regions with the *fit-rzx* gene clusters of *P. protegens* Pf-5 and *Pseudomonas* sp. OS17 were not identified as genomic islands by the same software but we cannot exclude the possibility that these regions were also acquired via HGT in the distant past and have since conformed to the characteristics of the genome as a whole. Over time, the compositional variation between the core genome and genomic islands lessens, which makes it difficult to detect more ancient HGT events with confidence. The distribution of repetitive extragenic palindromic (REP) elements, which are short nucleotide sequences, typically 20–60 nt long, provides another criterion for the identification of recently acquired genomic regions in *Pseudomonas* genomes (Paulsen *et al.*, 2005). REP elements appear to accumulate in *Pseudomonas* genomes over time, as they are not randomly distributed throughout the genome but instead are typically distributed in intergenic spaces within the core genome (Loper *et al.*, 2012). Therefore, the lack of REP elements is characteristic of genomic regions termed REP deserts, which have been acquired recently and have not yet incorporated these elements. The *fit-rzx* cluster is located in a REP desert of the Pf-5 genome (Paulsen *et al.*, 2005), which suggests it was acquired via HGT. Furthermore, the *fit-rzx* cluster is located near phage elements and regions of atypical G+C content (Paulsen *et al.*, 2005), which was highlighted in a recent analysis of the evolution of the *fit* cluster (Ruffner *et al.*, 2015). Taken together, bioinformatic analyses suggest that the *fit-rzx* cluster has a complex evolutionary history that includes HGT. Our results demonstrating that the production of rhizoxin analogs is a major determinant of the oral toxicity of Pf-5 to *D. melanogaster* shows that the *fit-rzx* cluster confers oral and injectable toxicity to a broader set of insects than either the *fit* or *rzx* clusters alone.

Conclusions

The results of this study demonstrate that several genes contribute to oral insect toxicity of *P. protegens* Pf-5. Here, we employed a set of well-characterized mutants of Pf-5 with deficiencies in a variety of extracellular enzymes, natural products and the FitD insect toxin to identify genes key to Pf-5's oral toxicity to the common fruit fly *D. melanogaster*. We discovered that an extracellular chitinase, rhizoxin analogs, and orfamide A contribute to this oral toxicity. Due to the known role of the FitD toxin in both oral and injectable insect toxicity of *P. protegens* (Péchy-Tarr *et al.*, 2008; Kupferschmied *et al.*, 2013; Ruffner *et al.*, 2013; Kupferschmied *et al.*, 2014; Ruffner *et al.*, 2015), it was surprising that a Δ*fitD* mutant of Pf-5 exhibited

Table 1. *Pseudomonas protegens* Pf-5 and derivative strains used in this study.

Strain	Designation	Description[a]	Reference
Pf-5	JL4585	Soil isolate. Ofa$^+$, Prn$^+$, Plt$^+$, HCN$^+$, MAPG$^+$, DAPG$^+$, Rzx$^+$, Tox$^+$, FitD$^+$, Chi$^+$, Apr$^+$, T6SS$^+$	Howell and Stipanovic (1979); Paulsen *et al.* (2005)
Mutants of Pf-5:			
Δ*gacA*	JL4577	Insertion of *aphI* at the site of a 626 bp (nt 1–626) deletion in *gacA*; Altered in the many phenotypes regulated by GacA; Km^r	Hassan *et al.* (2010)
Δ*gacA*	JL4975	612 bp deletion in *gacA*; Altered in the many phenotypes regulated by GacA	Henkels *et al.* (2014)
Δ*fitD*	JL4896	9,061 bp deletion in *fitD*. FitD$^-$	This study
Δ*pvdI*-Δ*pchC*	JL4900	527 bp deletion in *pvdI*, 86 bp insertion of FRT in *pchC*. Pvd$^-$, Pch$^-$	Hartney *et al.* (2011)
Δ*pvdI*-Δ*pchC*-Δ*pchA*	LK035	1,138 bp deletion in *pchA*. Pvd$^-$, Pch$^-$	Hartney *et al.* (2011)
ΔT6SS	JL4894	3,698 bp deletion in T6SS	This study
Δ*aprA*	JL4921	1,137 bp deletion in *aprA*. Apr$^-$	Loper *et al.* (2012)
Δ*chiC*	JL4801	*chiC*::Tn5. Chi$^-$, Km^r	D. Kobayashi
Δ*chiC*	JL4944	1,359-bp deletion in *chiC*. Chi$^-$	Loper *et al.* (2012)
Δ*ofaA*	JL4807	1,143-bp deletion in *ofaA*. Ofa$^-$	Hassan *et al.* (2010)
Δ*phlA*	LK023	639-bp deletion of *Bgl*II fragment in *phlA*; DAPG$^-$, MAPG$^-$	Kidarsa *et al.* (2011)
Δ*pltA*	JL4805	275 bp deletion in *pltA*. Plt$^-$	Henkels *et al.* (2014)
Δ*prnC*	JL4793	86 bp insertion of FRT site in *prnC*; Prn$^-$	Henkels *et al.* (2014)
Δ*rzxB*	JL4808	1,342 bp deletion in Δ*rzxB*. Rzx$^-$	Henkels *et al.* (2014)
Δ*hcnB*	JL4909	239 bp deletion in *hcnB*. HCN$^-$	Loper *et al.* (2012)
Δ*toxB*	JL4832	41 bp deletion in *toxB*. Tox$^-$	Quecine *et al.* (2016)
Δ*phlA*-Δ*rzxB*-Δ*prnC*-Δ*hcnB*-Δ*pltA*-Δ*ofaA*	LK147	Prn$^-$, DAPG$^-$, MAPG$^-$, Plt$^-$, Rzx$^-$, HCN$^-$, Ofa$^-$	This study

a. Phenotype abbreviations: GacA, Global activator; FitD, Fluorescens insect toxin; Pvd, pyoverdine; Pch, enantio-pyochelin; T6SS, Type VI secretion system; Apr, alkaline protease; Chi, chitinase; Ofa, orfamide A; DAPG, 2,4-diacetylphloroglucinol; MAPG, monoacetylphloroglucinol; Plt, pyoluteorin; Prn, pyrrolnitrin; Rzx, rhizoxin analogs; HCN, hydrogen cyanide; Tox, toxoflavin. Mutants of Pf-5 containing deletions in *fitD*, *pvdI*, *pchC*, T6SS, *ofaA*, *pltA*, *prnC*, *rzxB*, *hcnB* or *toxB* have FRT scars (85–86 bp fragment recognition target sequences) in those genes. In-frame deletions were generated in *gacA*, *phlA, aprA, chiC* and *pchA* and the deleted genes do not have inserted FRT sequences.

wildtype levels of oral toxicity to *D. melanogaster* in this study. Clearly, a single trait is not responsible for all insect toxicity exhibited by *P. protegens* Pf-5, as *fitD* is a major determinant of the strain's injectable toxicity to the tobacco hornworm *M. sexta* (Péchy-Tarr *et al.*, 2008) but had no detectable role in oral toxicity to *D. melanogaster* in this study. Rhizoxin, a macrolide that binds to β-tubulin, thereby interfering with microtubule dynamics during mitosis (Gupta and Bhattacharyya, 2003), is known to inhibit a broad spectrum of organisms, including plants and fungi, and the production of rhizoxin analogs was a major contributor to oral insect toxicity by *P. protegens* Pf-5 in this study. The toxicity of rhizoxin to insects has not been reported previously, to our knowledge. Intriguingly, the rhizoxin and *fit* gene clusters are adjacent to one another in the genome of Pf-5 and a few other strains of *P. protegens*, but are present in mutually-exclusive taxa of bacteria outside of this species. *Mcf*, a homolog of *fitD*, is present in strains of *Photorhabdus* spp. and *Xenorhabdus* spp. that live as endosymbionts in entomopathogenic nematodes. The rhizoxin gene cluster is present in the genomes of certain strains of *Burkholderia* spp. and *Ralstonia* spp., Betaproteobacteria that are distantly related to *Pseudomonas* or other Gammaproteobacteria that harbor the *fit* cluster. Taken together with earlier studies demonstrating a role of the fit cluster in toxicity of *Pseudomonas* spp. to insects in the order Lepidoptera (Péchy-Tarr *et al.*, 2013; Ruffner *et al.*, 2013), the findings of this study highlight the *fit-rzx* cluster as a unique chimera that confers toxicity to at least two major orders of insects.

Material and methods

Bacterial strains and culture conditions

Pseudomonas protegens Pf-5 was isolated from soil in Texas, USA (Howell and Stipanovic, 1979). Derivatives of Pf-5 having mutations in genes influencing antibiotic or exoenzyme production were derived previously (Table 1). Each of the mutants has been confirmed to lack production of the corresponding antibiotic or exoenzyme by HPLC analysis (Quecine *et al.*, 2016) or enzyme assay (Loper *et al.*, 2012; Henkels *et al.*, 2014) respectively. We also tested each mutant for exoprotease activity on litmus milk agar (Becton, Dickinson and Company, Sparks, MD, USA) to ensure that we did not inadvertently select mutants in the Gac/Rsm signal transduction pathway during mutagenesis process. With the exception of the Δ*gacA* and Δ*aprA* mutants, all mutants evaluated in this study produced zones on litmus milk agar, indicating that they have a functional Gac/Rsm regulatory system, which is required for exoprotease production (Whistler *et al.*, 1998).

Derivatives of Pf-5 with mutations in *fitD* or a type VI secretion system (T6SS) were constructed using an overlap-extension PCR method (Choi and Schweizer, 2005) followed by a mating and selection process described previously (Brazelton *et al.*, 2008; Hassan *et al.*, 2010; Henkels *et al.*, 2014). A Flp recombinase was used in this process, resulting in *fitD* and T6SS deletions flanked by 85–86 bp Flp recombinase target (FRT) sites (Choi and Schweizer, 2005). The sequences flanking deletions in the genomes of all mutants were confirmed to be as expected by performing PCR across the deletion sites and sequencing the resultant products. Primers used to construct and confirm the deletions in the *fitD* and T6SS mutants are shown in Supporting Information Table S1.

Inoculum for larval feeding experiments was obtained by culturing strains of *P. protegens* on King's Medium B (KMB) (King *et al.*, 1954) and incubating plates overnight at 27°C. Strains were then inoculated into culture tubes containing 5 ml of KMB broth and incubated with shaking (200 r.p.m.) overnight prior to harvest by centrifugation. Cells were washed once, resuspended in sterile deionized water and diluted to an OD_{600} of 0.1. The initial number of colony forming units for each experiment was determined by spreading samples from serial dilutions on KMB.

Insect toxicity assay

To test the toxicity of *P. protegens* to *D. melanogaster*, we used a noninvasive assay described previously (Olcott *et al.*, 2010). *Drosophila melanogaster* line Canton S A was maintained at 25°C, in a 12 h light–dark cycle, on standard cornmeal, dextrose, yeast and agar media with Nipagin (*p*-hydroxybenzoic acid methyl ester, Sigma Aldrich, St. Louis, MO, USA). Adult flies were transferred to Petri plates containing Apple agar (http://cshprotocols.cshlp.org/content/2011/9/pdb.rec065672.short) prepared without Nipagin and supplemented by killed yeast grains (20 mg). After a 4 h egg lay period at 25°C, 30 eggs were transferred aseptically to the surface of non-nutritive agar (2% wt/vol agar in water) having 2–3 mg killed yeast grains distributed on the agar surface in a 35 mm Petri plate. Plates were incubated at 25°C. The number of first instar larvae per plate was determined on Days −2 and −1 by counting the number of empty egg cases. The hatch rate averaged 87% among the six experiments of this study. On Day 0, 200 μl of a yeast suspension was added to the middle of the plate to serve as a food source for second instar larvae. The yeast suspension was prepared by dissolving 0.2 g killed yeast in 1.2 ml of sterile water or a bacterial suspension, prepared as described above. Initial bacterial populations on plates used in the six experiments of this study are shown in Supporting Information Table S2. The plates were transferred to 22°C and, starting at Day 2, larvae were fed with 100 μl of a yeast suspension (0.2 mg yeast/1.2 ml sterile water) at 48-h intervals as long as live larvae were observed in the dish. Three replica plates were established for each treatment group. The number of larvae and pupae were counted at 24-h intervals until adult emergence. Means of percent survival, calculated for each replication from the number of pupae or adults per first instar larvae, were analyzed statistically with SAS software version 9.3 (SAS Institute, Cary, NC, USA) with the Fisher's least significant difference ($P < 0.05$). Experiments were analyzed separately. To provide a more comprehensive representation of treatment effects, replicate means for multiple experiments were pooled when significant treatment *X* experiment effects were not observed.

The duration of larval development was calculated for control and treatment groups as described previously (Olcott *et al.*, 2010) as the interval (hours) between inoculation and the time when pupariation had reached 50% of the total number of pupae ($t_{1/2}$). Chi-square analysis was performed on two replicate experiments to compare the larval duration of experimental treatments to the larval duration of control or Pf-5 fed larvae. Larvae fed Δ*gacA* developed into pupae at the same rate as control larvae ($X^2 = 0.06$, df 1, $P > 0.05$). Larval development was documented through images captured by a digital camera mounted on a dissecting scope with images adjusted only for contrast and brightness in Photoshop (Adobe Systems, San Jose, CA, USA) as needed.

Morphological defect analysis

Adults that emerged from two experiments were fixed in ethanol and then examined through a dissecting scope for eye defects, which included small eye size and nicks in the anterior edge. Digital images of eyes were captured directly into a camera mounted onto a dissecting scope. Final images were only adjusted slightly for contrast and brightness.

Phylogenetic analyses

Using the Ribosomal Database Project (rdp.cme.msu.edu) hierarchy browser, all 16S rDNA sequences for type strain isolates of Betaproteobacteria and Gammaproteobacteria were retrieved ($n = 2158$). Nucleotide sequences were aligned using the Clustal Omega method in MegAlign Pro (LaserGene Core Suite). Evolutionary relationships were estimated using a distance-based metric and the tree was built using the BioNJ algorithm (Gascuel, 1997). An unrooted radial phylogenetic tree was produced using the FigTree v1.3.1 graphical viewer (Rambaut, 2009).

For phylogenetic analysis of the rhizoxin genes, amino acid sequences were gathered using the top 100 BLASTP hits for selected *P. protegens* Pf-5 rhizoxin genes (RzxA PKS/NRPS: AAY92269; RzxG acyl transferase: AAY92268; RzxH P450 monoxygenase: AAY92264; RzxI SAM dependent methyltransferase: AAY92267). Sequences were aligned using the MAFFT method in MegAlignPro (LaserGene Core Suite). Evolutionary relationships were estimated using a distance-based metric and the tree was built using the BioNJ algorithm (Gascuel, 1997). A midpoint rooted phylogenetic tree was produced using the FigTree v1.3.1 graphical viewer (Rambaut, 2009). Outside of the single clade in each tree containing the rhizoxin genes, clades with more than two species from the same genus were collapsed.

Genomic island analyses

Genome sequences of *P. protegens* Pf-5, *Pseudomonas sp.* OS17, *Burkholderia rhizoxinica* HKI_454, *Burkholderia sp.* B13 & B14, *Ralstonia solanacearum* Phyl III-seqv23 and *Chromobacterium violacearum* L_1B5_1 were scanned for regions of genomic islands, putative signatures of HGT. The

software package Alien_Hunter (Vernikos and Parkhill, 2006) was run on a local computer with optimized prediction boundaries. IslandPick (Langille *et al*., 2008), SIGI-HMM (Waack *et al*., 2006) and IslandPath-DIMOB (Hsiao *et al*., 2003) results were obtained from the IslandViewer3 website (Dhillon *et al*., 2015) using precomputed genome analyses or uploading most recent genomic assemblies where precomputed results were not available.

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References

Bangera, M.G., and Thomashow, L.S. (1999) Identification and characterization of a gene cluster for synthesis of the polyketide antibiotic 2,4-diacetylphloroglucinol from *Pseudomonas fluorescens* Q2-87. *J Bacteriol* **181:** 3155–3163.

Brazelton, J.N., Pfeufer, E.E., Sweat, T.A., McSpadden Gardener, B.B., and Coenen, C. (2008) 2,4-diacetylphloroglucinol alters plant root development. *Mol Plant Microbe Interact* **21:** 1349–1358.

Brendel, N., Partida-Martinez, L.P., Scherlach, K., and Hertweck, C. (2007) A cryptic PKS-NRPS gene locus in the plant commensal *Pseudomonas fluorescens* Pf-5 codes for the biosynthesis of an antimitotic rhizoxin complex. *Org Biomol Chem* **5:** 2211–2213.

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

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

Chen, W.-J., Hsieh, F.-C., Hsu, F.-C., Tasy, Y.-F., Liu, J.-R., and Shih, M.-C. (2014) Characterization of an insecticidal toxin and pathogenicity of *Pseudomonas taiwanensis* against insects. *PLoS Pathog* **10:** e1004288.

Choi, K.-H., and Schweizer, H.P. (2005) An improved method for rapid generation of unmarked *Pseudomonas aeruginosa* deletion mutants. *BMC Microbiol* **5:** 30–30.

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

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

Devi, K.K., and Kothamasi, D. (2009) *Pseudomonas fluorescens* CHA0 can kill subterranean termite *Odontotermes obesus* by inhibiting Cytochrome C Oxidase of the termite respiratory chain. *FEMS Microbiol Lett* **301:** 147–147.

Dhillon, B.K., Laird, M.R., Shay, J.A., Winsor, G.L., Lo, R., Nizam, F., *et al*. (2015) IslandViewer 3: more flexible, interactive genomic island discovery, visualization and analysis. *Nucleic Acids Res* **43:** W104–W108.

Filloux, A. (2011) Protein secretion systems in *Pseudomonas aeruginosa*: an essay on diversity, evolution and function. *Front Microbiol* **2:** 155.

Flury, P., Aellen, N., Ruffner, B., Pechy-Tarr, M., Fataar, S., Metla, Z., *et al*. (2016) Insect pathogenicity in plant-beneficial pseudomonads: phylogenetic distribution and comparative genomics. *ISME J.*

Garcia-Gonzalez, E., Poppinga, L., Fünfhaus, A., Hertlein, G., Hedtke, K., Jakubowska, A., and Genersch, E. (2014) *Paenibacillus larvae* chitin-degrading protein *Pl*CBP49 is a key virulence factor in American foulbrood of honey bees. *PLoS Pathog* **10:** e1004284.

Gascuel, O. (1997) BIONJ: an improved version of the NJ algorithm based on a simple model of sequence data. *Mol Biol Evol* **14:** 685–695.

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

Gross, H., Stockwell, V.O., Henkels, M.D., Nowak-Thompson, B., Loper, J.E., and Gerwick, W.H. (2007) The genomisotopic approach: a systematic method to isolate products of orphan biosynthetic gene clusters. *Chem Biol* **14:** 53–63.

Gupta, S., and Bhattacharyya, B. (2003) Antimicrotubular drugs binding to vinca domain of tubulin. *Mol Cell Biochem* **253:** 41–47.

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

Harrison, R.L., and Bonning, B.C. (2010) Proteases as insecticidal agents. *Toxins* **2:** 935–953.

Harrison, F., Browning, L., Vos, M., and Buckling, A. (2006) Cooperation and virulence in acute *Pseudomonas aeruginosa* infections. *BMC Biol* **4:** 21.

Hartney, S.L., Mazurier, S., Kidarsa, T.A., Quecine, M.C., Lemanceau, P., and Loper, J.L. (2011) TonB-dependent outer-membrane proteins and siderophore utilization in *Pseudomonas fluorescens* Pf-5. *BioMetals* **24:** 193–213.

Hartney, S.L., Mazurier, S., Girard, M.K., Mehnaz, S., Davis, E.W., 2nd, Gross, H., *et al*. (2013) Ferric-pyoverdine recognition by Fpv outer-membrane proteins of *Pseudomonas protegens* Pf-5. *J Bacteriol* **195:** 765–776.

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

Hassan, K.A., Johnson, A., Shaffer, B.T., Ren, Q., Kidarsa, T.A., Elbourne, L.D.H., *et al*. (2010) Inactivation of the GacA response regulator in *Pseudomonas fluorescens* Pf-5 has far-reaching transcriptomic consequences. *Environ Microbiol* **12:** 899–915.

Henkels, M.D., Kidarsa, T.A., Shaffer, B.T., Goebel, N.C., Burlinson, P., Mavrodi, D.V., *et al*. (2014) *Pseudomonas*

protegens Pf-5 causes discoloration and pitting of mushroom caps due to the production of antifungal metabolites. *Mol Plant Microbe Interact* **27:** 733–746.

Howell, C.R., and Stipanovic, R.D. (1979) Control of *Rhizoctonia solani* in cotton seedlings with *Pseudomonas fluorescens* and with an antibiotic produced by the bacterium. *Phytopathology* **69:** 480–482.

Howell, C.R., and Stipanovic, R.D. (1980) Suppression of *Pythium ultimum*-induced damping-off of cotton seedlings by *Pseudomonas fluorescens* and its antibiotic, pyoluteorin. *Phytopathology* **70:** 712–715.

Hsiao, W., Wan, I., Jones, S.J., and Brinkman, F.S.L. (2003) IslandPath: aiding detection of genomic islands in prokaryotes. *Bioinformatics* **19:** 418–420.

Jang, J.Y., Yang, S.Y., Kim, Y.C., Lee, C.W., Park, M.S., Kim, J.C., and Kim, I.S. (2013) Identification of Orfamide A as an insecticidal metabolite produced by *Pseudomonas protegens* F6. *J Agric Food Chem* **61:** 6786–6791.

Kidarsa, T.A., Goebel, N.C., Zabriskie, T.M., and Loper, J.E. (2011) Phloroglucinol mediates crosstalk between the pyoluteorin and 2,4-diacetylphloroglucinol biosynthetic pathways in *Pseudomonas fluorescens* Pf-5. *Mol Microbiol* **81:** 395–414.

Kidarsa, T.A., Shaffer, B.T., Goebel, N.C., Roberts, D.P., Buyer, J.S., Johnson, A., *et al.* (2013) Genes expressed by the biological control bacterium *Pseudomonas protegens* Pf-5 on seed surfaces under the control of the global regulators GacA and RpoS. *Environ Microbiol* **15:** 716–735.

King, E.O., Ward, M.K., and Raney, D.E. (1954) Two simple media for the demonstration of pyocyanin and fluorescin. *J Lab Clin Med* **44:** 301–307.

Kraus, J., and Loper, J.E. (1992) Lack of evidence for a role of antifungal metabolite production by *Pseudomonas fluorescens* Pf-5 in biological control of Pythium damping-off of cucumber. *Phytopathology* **82:** 264–271.

Kupferschmied, P., Maurhofer, M., and Keel, C. (2013) Promise for plant pest control: root-associated pseudomonads with insecticidal activities. *Front Plant Sci* **4:**287. doi: 10.3389/fpls.2013.00287.

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

Kuraishi, T., Binggeli, O., Opota, O., Buchon, N., and Lemaitre, B. (2011) Genetic evidence for a protective role of the peritrophic matrix against intestinal bacterial infection in *Drosophila melanogaster*. *Proc Natl Acad Sci USA* **108:** 15966–15971.

Langille, M.G.I., Hsiao, W.W.L., and Brinkman, F.S.L. (2008) Evaluation of genomic island predictors using a comparative genomics approach. *BMC Bioinformatics* **9:** 329–329.

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

Lehane, M.J. (1997) Peritrophic matrix structure and function. *Ann Rev Entomol* **42:** 525–550.

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

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

Loper, J.E., Kobayashi, D.Y., and Paulsen, I.T. (2007) The genomic sequence of *Pseudomonas fluorescens* Pf-5: insights into biological control. *Phytopathology* **97:** 233–238.

Loper, J.E., Henkels, M.D., Shaffer, B.T., Valeriote, F.A., and Gross, H. (2008) Isolation and identification of rhizoxin analogs from *Pseudomonas fluorescens* Pf-5 by using a genomic mining strategy. *Appl Environ Microbiol* **74:** 3085–3093.

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

Melo, A.L.D., Soccol, V.T., and Soccol, C.R. (2016) *Bacillus thuringiensis*: mechanism of action, resistance, and new applications: a review. *Crit Rev Biotechnol* **36:** 317–326.

Nowak-Thompson, B., Gould, S.J., Kraus, J., and Loper, J.E. (1994) Production of 2,4-diacetylphloroglucinol by the biocontrol agent *Pseudomonas fluorescens* Pf-5. *Can J Microbiol* **40:** 1064–1066.

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

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

Partida-Martinez, L.P., and Hertweck, C. (2007) A gene cluster encoding rhizoxin biosynthesis in "*Burkholderia rhizoxina*", the bacterial endosymbiont of the fungus *Rhizopus microsporus*. *Chembiochem* **8:** 41–45.

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

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

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

Philmus, B.J., Shaffer, B.T., Kidarsa, T.A., Yan, Q., Raaijmakers, J.M., Begley, T.P., and Loper, J.E. (2015) Investigations into the biosynthesis, regulation and self-resistance of toxoflavin in *Pseudomonas protegens* Pf-5. *ChemBioChem* **16:** 1782–1790.

Prota, A.E., Bargsten, K., Diaz, J.F., Marsh, M., Cuevas, C., Liniger, M., *et al.* (2014) A new tubulin-binding site and pharmacophore for microtubule-destabilizing anticancer drugs. *Proc Natl Acad Sci USA* **111:** 13817–13821.

Quecine, M.C., Kidarsa, T.A., Goebel, N.C., Shaffer, B.T., Henkels, M.D., Zabriskie, T.M., and Loper, J.E. (2016) An Interspecies signaling system mediated by fusaric acid has parallel effects on antifungal metabolite production by *Pseudomonas protegens* Pf-5 and antibiosis of *Fusarium* spp. *Appl Environ Microbiol* **82:** 1372–1382.

Rambaut, A. (2009) FigTree v.1.3.1 Tree figure drawing tool. In *Institute of Evolutionary Biology.* UK: University of Edinburgh.

Regev, A., Keller, M., Strizhov, N., Sneh, B., Prudovsky, E., Chet, I., *et al*. (1996) Synergistic activity of a *Bacillus thuringiensis* delta-endotoxin and a bacterial endochitinase against *Spodoptera littoralis* larvae. *Appl Environ Microbiol* **62:** 3581–3586.

Remenant, B., Coupat-Goutaland, B., Guidot, A., Cellier, G., Wicker, E., Allen, C., *et al*. (2010) Genomes of three tomato pathogens within the *Ralstonia solanacearum* species complex reveal significant evolutionary divergence. *BMC Genomics* **11:** 379.

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

Ruffner, B., Péchy-Tarr, M., Höfte, M., Bloemberg, G., Grunder, J., Keel, C., and Maurhofer, M. (2015) Evolutionary patchwork of an insecticidal toxin shared between plant-associated pseudomonads and the insect pathogens *Photorhabdus* and *Xenorhabdus*. *BMC Genomics* **16:** 609.

Russell, A.B., Peterson, S.B., and Mougous, J.D. (2014) Type VI secretion system effectors: poisons with a purpose. *Nat Rev Microbiol* **12:** 137–148.

Shanahan, P., Glennon, J.D., Crowley, J., Donnelly, D., and O'Gara, F. (1993) Liquid chromatographic assay of microbially derived phloroglucinol antibiotics for establishing the biosynthetic route to production, and the factors affecting their regulation. *Anal Chim Acta* **272:** 271–277.

Takeuchi, K., Noda, N., Katayose, Y., Mukai, Y., Numa, H., Yamada, K., and Someya, N. (2015) Rhizoxin analogs contribute to the biocontrol activity of newly isolated *Pseudomonas* strain. *Mol Plant Microbe Interact* **28:** 333–342.

Vernikos, G.S., and Parkhill, J. (2006) Interpolated variable order motifs for identification of horizontally acquired DNA: revisiting the *Salmonella* pathogenicity islands. *Bioinformatics* **22:** 2196–2203.

Waack, S., Keller, O., Asper, R., Brodag, T., Damm, C., Fricke, W., *et al*. (2006) Score-based prediction of genomic islands in prokaryotic genomes using hidden Markov models. *BMC Bioinformatics* **7:** 1–12.

Whistler, C.A., Corbell, N.A., Sarniguet, A., Ream, W., and Loper, J.E. (1998) The two-component regulators GacS and GacA influence accumulation of the stationary-phase sigma factor and the stress response in *Pseudomonas fluorescens* Pf-5. *J Bacteriol* **180:** 6635–6641.

Whitney, J.C., Chou, S., Russell, A.B., Biboy, J., Gardiner, T.E., Ferrin, M.A., *et al*. (2013) Identification, structure, and function of a novel type VI secretion peptidoglycan glycoside hydrolase effector-immunity pair. *J Biol Chem* **288:** 26616–26624.

Youard, Z.A., Mislin, G.L., Majcherczyk, P.A., Schalk, I.J., and Reimmann, C. (2007) *Pseudomonas fluorescens* CHA0 produces enantio-pyochelin, the optical antipode of the *Pseudomonas aeruginosa* siderophore pyochelin. *J Biol Chem* **282:** 35546–35553.

Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1. Mid-point rooted distance-based phylogeny of proteins selected as the top 100 BLASTP hits against the *Pseudomonas protegens* Pf-5 RzxI sequence (accession number: AAY92267). The predicted amino acid sequences of *rzxI* (also annotated as *rhiI*) from rhizoxin biosynthesis gene clusters of different bacteria all fall in the clade boxed in red. Some of the accession numbers shown correspond to more than one strain: CAL69886.1, *Burkholderia rhizoxinica* HKI_454 and B1; WP_043944229.1, *Ralstonia solanacearum* B50 and IBSBF1900; AMP76341.1, *Ralstonia solanacearum* IBSBF1503 and CFBP6783.

Fig. S2. Mid-point rooted distance-based phylogeny of polyketide synthases selected as the top 100 BLASTP hits against the *Pseudomonas protegens* Pf-5 RzxA sequence (accession number: AAY92269). The predicted amino acid sequences of *rzxA* (also annotated as *rhiA*) from rhizoxin biosynthesis gene clusters of different bacteria all fall in the clade boxed in red. Some of the accession numbers shown correspond to more than one strain: WP_013435483.1, *Burkholderia rhizoxinica* HKI_454 and B1; WP_042570250.1, *Ralstonia solanacearum* IBSBF1503 and UW163.

Fig. S3. Mid-point rooted, distance-based phylogeny of proteins selected as the top 100 BLASTP hits against the *Pseudomonas protegens* Pf-5 RzxG sequence (accession number: AAY92268). The predicted amino acid sequences of *rzxG* (also annotated as *rhiG*) from rhizoxin biosynthesis gene clusters of different bacteria all fall in the clade boxed in red. Some of the accession numbers shown correspond to more than one strain: WP_014619438.1, *Ralstonia solanacearum* Po82, CFBP6783, IBSBF1503, UW179, UW163, CFBP7014, P673; WP_013435484.1, *Burkholderia rhizoxinica* HKI_454 and B1.

Fig. S4. Mid-point rooted, distance-based phylogeny of proteins selected as the top 100 BLASTP hits against the *Pseudomonas protegens* Pf-5 RzxH sequence (accession number: AAY92264). The predicted amino acid sequences of *rzxH* (also annotated as *rhiH*) from rhizoxin biosynthesis gene clusters of different bacteria all fall in the clade boxed in red. Some of the accession numbers shown correspond to more than one strain: WP_013435479.1, *Burkholderia rhizoxinica* HKI_454 and B1; WP_052307027.1, *Ralstonia solanacearum* Po82 and CFBP6783, IBSBF1503, UW179, UW163, CFBP7014; WP_052477600.1, *Ralstonia solanacearum* B50 and IBSBF1900.

Table S1. Primers used in mutant construction or confirmation.

Table S2. Population size of inoculated strains tested in six experiments evaluating lethality of *P. protegens* Pf-5 and derivative strains.