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e-Xtra*

Pseudomonas protegens Pf-5 Causes Discoloration and Pitting of Mushroom Caps Due to the Production of Antifungal Metabolites

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Bacteria in the diverse *Pseudomonas fluorescens* group include rhizosphere inhabitants known for their antifungal metabolite production and biological control of plant disease, such as *Pseudomonas protegens* Pf-5, and mushroom pathogens, such as *Pseudomonas tolaasii.* Here, we report that strain Pf-5 causes brown, sunken lesions on peeled caps of the button mushroom (*Agaricus bisporus*) that resemble brown blotch symptoms caused by *P. tolaasii*. Strain Pf-5 produces six known antifungal metabolites under the control of the GacS/GacA signal transduction system. A *gacA* mutant produces none of these metabolites and did not cause lesions on mushroom caps. Mutants deficient in the biosynthesis of the antifungal metabolites 2,4-diacetylphloroglucinol and pyoluteorin caused less-severe symptoms than wild-type Pf-5 on peeled mushroom caps, whereas mutants deficient in the production of lipopeptide orfamide A caused similar symptoms to wild-type Pf-5. Purified pyoluteorin and 2,4-diacetylphloroglucinol mimicked the symptoms caused by Pf-5. Both compounds were isolated from mushroom tissue inoculated with Pf-5, providing direct evidence for their in situ production by the bacterium. Although the lipopeptide tolaasin is responsible for brown blotch of mushroom caused by *P. tolaasii*, *P. protegens* Pf-5 caused brown blotch–like symptoms on peeled mushroom caps through a lipopeptide-independent mechanism involving the production of 2,4-diacetylphloroglucinol and pyoluteorin.

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Pseudomonas is a diverse genus of *Gammaproteobacteria* with more than 120 type species exhibiting varied lifestyles in a wide range of environments, including soil, water, plant surfaces, and animals (Ramos 2004). Members of the genus are well known for their ubiquity in the natural world, capacity to utilize a striking variety of organic compounds as energy sources, and resistance to a wide range of medically and agriculturally important antimicrobial compounds. Within the genus is the large, heterogeneous *P. fluorescens* group, composed of more than 50 type species that fall into at least nine subgroups (Mulet et al., 2010). Bacteria within the *P. fluorescens* group have diverse ecological roles, including as plant epiphytes, endophytes, and rhizosphere inhabitants.

Certain members of the *P. fluorescens* group cause disease of the button mushroom *Agaricus bisporus*, which is commonly visualized as a discoloration of the pileus (i.e., mushroom cap). The best-characterized mushroom pathogen in the *P. fluorescens* group is *P. tolaasii*, which causes brown blotch on mushroom caps during cultivation and postharvest (Rainey et al. 1992; Tolaas 1915; Wong and Preece 1979). *P. tolaasii* produces the lipopeptide tolaasin, which is responsible for the dark brown, pitted lesions caused by the pathogen on mushroom caps (Rainey et al. 1991, 1992; Soler-Rivas et al. 1999). The effect of tolaasin on mushroom caps is attributed to its capacity to disrupt cell membrane function by forming transmembrane pores (Brodey et al. 1991; Hutchison and Johnstone 1993; Rainey et al. 1991). Lipopeptides (LP) are a class of molecules having both peptide and lipid moieties (Raaijmakers et al. 2010; Roongsawang et al. 2011), and the primary form of tolaasin, tolaasin I, is composed of an 18–amino acid peptide chain linked to a β-hydroxyoctanoyl fatty acid moiety (Bassarello et al. 2004; Nutkins et al. 1991) (Fig. 1).

LP produced by *Pseudomonas* species are structurally diverse due to variations in the lipid moiety and in the composition, number, and configuration of the amino acids in the peptide chain (Gross and Loper 2009; Nybroe and Sorensen 2004; Raaijmakers et al. 2006). The structurally diverse LP have varied roles in the many distinctive habitats occupied by *Pseudo-*

monas species in nature, including toxicity towards other organisms, motility, and attachment to surfaces (Gross and Loper, 2009; Raaijmakers et al. 2006; Reder-Christ et al. 2012). In addition to tolaasin production by *P. tolaasii*, a structurally distinct LP called the white line–inducing principle (WLIP) (Mortishire-Smith et al. 1991) (Fig. 1) is produced by certain other mushroom-associated pseudomonads. WLIP forms a precipitate with tolaasin that can be visualized as a white line formed between colonies of WLIP-producing and tolaasin-producing strains on certain agar media (Wong and Preece 1979). Strains exhibiting this striking white line reaction when co-cultured with *P. tolaasii* have been referred to as '*Pseudomonas reactans*,' a designation lacking taxonomic validity. WLIP-producing strains are taxonomically diverse, falling into at least two major groups of *Pseudomonas*, the *P. fluorescens* and *P. putida* groups (Rokni-Zadeh et al. 2013). Although many strains of *P. reactans* are saprophytic (Mortishire-Smith et al. 1991; Wong and Preece 1979), others are known to cause discoloration of mushroom caps (Wells et al. 1996). It is not clear whether all *P. reactans* strains produce WLIP or if some strains produce other LP that also form a white precipitate with tolaasin (Munsch and Alatossava 2002a).

In addition to *P. tolaasii*, many other members of the *P. fluorescens* group are pathogenic on mushroom, exhibiting a range of symptoms (Wells et al. 1996). For example, *Pseudomonas agarici* causes drippy gill disease (Young 1970). '*P. gingeri*,' a designation lacking taxonomic validity, causes lesions on mushroom caps that are light brown and flat, lacking the pitting caused by *P. tolaasii* (Cutri et al. 1984; Wong et al. 1981). *Pseudomonas costantinii* (Munsch et al. 2002) and *Pseudomonas* sp. strain NZI7 (Godfrey et al. 2001) produce the dark, sunken lesions on mushroom caps that are characteristic of brown blotch disease due to the production of tolaasin (Scherlach et al. 2013) or a tolaasin-like LP (Burlinson et al. 2013). Factors responsible for the discoloration and spoilage caused by other members of the *P. fluorescens* group remain unknown.

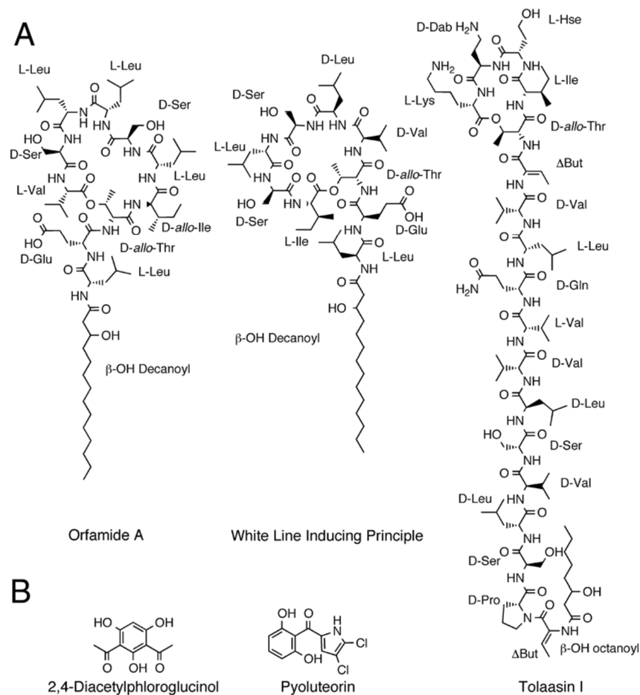


Fig. 1. A, Structures of the lipopeptides orfamide A, the white line inducing principle, and tolaasin I, and **B,** the antibiotics 2,4-diacetylphloroglucinol and pyoluteorin.

An initial goal of this study was to gain insight into the prevalence of mushroom pathogenicity of the *P. fluorescens* group by inoculating peeled caps of the cultivated mushroom (*Agaricus bisporus*) with a collection of nine well-characterized strains representing three lineages of these bacteria (Loper et al. 2012). Of the strains tested, *Pseudomonas protegens* Pf-5, a strain closely related to the mushroom pathogen *Pseudomonas* sp. strain NZI7 (Burlinson et al. 2013) but known for secondary metabolite production and biological control of plant disease (Gross and Loper 2009; Haas and Keel 2003; Loper and Gross 2007; Sonnleitner and Haas 2011), caused the most severe symptoms on peeled mushroom caps. Pf-5 produces many antifungal metabolites including the LP orfamide A (Gross et al. 2007), 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), and monoacetylphloroglucinol (MAPG), an intermediate in the DAPG biosynthetic pathway (Achkar et al. 2005; Bangera and Thomashow 1999; Shanahan et al. 1993). The production of these antibiotics is regulated by the GacS/GacA two-component 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 GacA in this pathway, *gacA* mutants of Pf-5 do not produce any of the other aforementioned metabolites (Gross and Loper 2009; Gross et al. 2007; Hassan et al. 2010; Loper et al. 2008; Whistler et al. 1998). Here, we report that Pf-5 causes brown blotch–like symptoms on peeled mushroom caps by a mechanism dependent on GacA but independent of LP production, as a mutant deficient in orfamide A production exhibited wild-type levels of toxicity against mushrooms. We also report that DAPG and pyoluteorin production contribute to the brown blotch–like symptoms caused by Pf-5 on mushrooms. The results of this study expand knowledge of factors contributing to mushroom toxicity by *Pseudomonas* species.

RESULTS

P. protegens Pf-5 causes brown, sunken lesions on mushroom caps.

To gain insight into the prevalence of mushroom pathogenicity within the *P. fluorescens* group, we tested nine well-characterized strains that were recently evaluated in a comparative genomics study (Loper et al. 2012) by inoculating peeled mushroom caps with each of the strains. Six of the inoculated strains (*P. chlororaphis* strains O6 and 30-84; *P. fluorescens* strains SS101, SBW25, and A506; and *P. synxantha* BG33R) (Table 1) caused no observable discoloration or pitting of mushroom caps (data not shown). Two strains, *P. brassicacearum* Q8r1-96 and *P. fluorescens* Q2-87, caused light-brown discoloration of the peeled mushroom caps and more severe symptoms were caused by *P. protegens* Pf-5, the focus of our study.

We compared the symptoms caused by Pf-5 to those caused by the well-known brown blotch pathogen *P. tolaasii* NCPPB2192, which produces tolaasin (Bassarello et al. 2004; Nutkins et al. 1991). As expected, *P. tolaasii* NCPPB2192 caused severe brown blotch symptoms, manifested as dark-brown, sunken lesions on the peeled mushroom caps. *P. protegens* Pf-5 also caused dark-brown, sunken lesions, but the symptoms were less severe than those caused by *P. tolaasii* (Fig. 2). *Pseudomonas* sp. strain NCPPB387 (also called *P. reactans*), which produces WLIP (Mortishire-Smith et al. 1991),

consistently caused less-severe symptoms than the other two strains (Fig. 2). Lesions caused by NCPPB387 were light brown and flat (versus sunken), as previously described for this strain and certain other strains of *P. reactans* (Wells et al. 1996).

Production of the LP orfamide A has a negligible role in symptoms caused by Pf-5 on mushroom caps.

Because tolaasin, an LP produced by *P. tolaasii*, has a major role in brown blotch disease, we evaluated the role of the LP orfamide A in the symptoms caused by Pf-5 on peeled mushroom caps. The severity of symptoms caused by a mutant of Pf-5 (Pf-5 Δ*ofaA*) that lacks orfamide A production (Gross et al. 2007) was similar to those caused by the wild-type strain (Fig. 2), indicating that the orfamides do not have a major role in the brown blotch–like symptoms caused by Pf-5.

For comparative purposes, we derived a WLIP-deficient mutant of *Pseudomonas* sp. strain NCPPB387 (NCPPB387 WLIP^{-}) and a tolaasin-deficient mutant of *P. tolaasii* NCPPB2192 (*P. tolaasii* Tol^{-}) by screening a collection of random transposon mutants of each strain, using the white line

Table 1. Strains of *Pseudomonas* spp. used in this study

Strain	Description[a]	Reference
P. protegens		
Pf-5	Soil isolate; produces Ofa, Prn, Plt, HCN, MAPG, DAPG, Rzx	Howell and Stipanovic 1979; Paulsen et al. 2005
Pf-5 Δ*gacA*	612-bp deletion in *gacA*; altered in the many phenotypes regulated by GacA	This study
Pf-5 Δ*chiC*	1,359-bp deletion internal to *chiC*; Chi^{-}	Loper et al. 2012
Pf-5 Δ*ofaA*	1,143-bp deletion internal to *ofaA*; has FRT scar; Ofa^{-}	Hassan et al. 2010
Pf-5 Δ*phlA*	639-bp deletion of *Bgl*II fragment internal to *phlA*; DAPG^{-}, MAPG^{-}	Kidarsa et al. 2011
Pf-5 Δ*pltA*	275-bp deletion in *pltA*; has FRT scar; Plt^{-}	This study
Pf-5 Δ*prnC*	87-bp insertion of FRT scar into *prnC*; Prn^{-}	This study
Pf-5 Δ*rzxB*	1,343-bp deletion in *rzxB*; has FRT scar; Rzx^{-}	This study
Pf-5 Δ*hcnB*	239-bp deletion in *hcnB*; has FRT scar; HCN^{-}	Loper et al. 2012
Pf-5 Δ*phlA*-Δ*pltA*	DAPG^{-}, MAPG^{-}, Plt^{-}	This study
Pf-5 Δ*phlA*-Δ*prnC*	DAPG^{-}, MAPG^{-}, Prn^{-}	This study
Pf-5 Δ*phlA*-Δ*rzxB*	DAPG^{-}, MAPG^{-}, Rzx^{-}	This study
Pf-5 Δ*phlA*-Δ*hcnB*	DAPG^{-}, MAPG^{-}, HCN^{-}	This study
Pf-5 Δ*phlA*-Δ*hcnB*-Δ*rzxB*	DAPG^{-}, MAPG^{-}, HCN^{-}, Rzx^{-}	This study
Pf-5 Δ*phlA*-Δ*prnC*-Δ*hcnB*	DAPG^{-}, MAPG^{-}, HCN^{-}, Prn^{-}	This study
Pf-5 Δ*phlA*-Δ*prnC*-Δ*rzxB*	DAPG^{-}, MAPG^{-}, Prn^{-}, Rzx^{-}	This study
Pf-5 Δ*phlA*-Δ*prnC*-Δ*rzxB*-Δ*pltA*-Δ*hcnB*	DAPG^{-}, MAPG^{-}, Prn^{-}, Rzx^{-}, Plt^{-}, HCN^{-}	This study
Pf-5 Δ*rpoS*	927-bp deletion in *rpoS*; overproduces DAPG and Plt and reduced Prn and Rzx production	This study
Pf-5 Δ*rpoS*-Δ*phlA*	Overproduces Plt and reduced Prn and Rzx production; DAPG^{-}, MAPG^{-}	This study
Pf-5 Δ*rpoS*-Δ*pltA*	Overproduces DAPG and reduced Prn and Rzx production; Plt^{-}	This study
Pf-5 Δ*rpoS*-Δ*phlA*-Δ*pltA*	Reduced Prn and Rzx production; DAPG^{-}, MAPG^{-}, Plt^{-}	This study
P. tolaasii		
NCPPB2192	Type strain of *P. tolaasii*, also called LMG2342 and ATCC 33618T; Tol^{+}	Wong and Preece 1979
P. tolaasii Tol^{-}	MiniTn*5::gfp::lux* mutant of NCPPB2192 with an insertion in an NRPS-encoding gene (gb|AJXK01001228.1|), also called LK084 and P1637; Tol^{-}	This study
P. brassicacearum		
Q8r1-96	Isolated from the wheat rhizosphere, Washington, U.S.A.; suppresses take all of wheat; DAPG^{+}, MAPG^{+}	Raaijmakers and Weller 1998
Q8 Phl^{-}	Q8r1-96 *phlD*::mini-Tn*5lacZ*, also called 4C5; DAPG^{-}, MAPG^{-}, PG^{-}	de Souza et al. 2003a
P. chlororaphis		
30-84	Isolated from the rhizosphere of wheat in Washington, U.S.A.	Thomashow et al. 1990
O6	Isolated from soil in Utah, U.S.A.	Tucker et al. 1995
P. fluorescens		
A506	Isolated from the pear phyllosphere in California, U.S.A.	Wilson and Lindow 1993
Pf0-1	Isolated from soil in Massachusetts, U.S.A.	Silby et al. 2009
SBW25	Isolated from the sugar beet phyllosphere, Oxfordshire, U.K.	Silby et al. 2009
SBW25 Δ*viscA*	Mutant of SBW25 with transposon insertion in *viscA*, deficient in viscosin production	de Bruijn et al. 2007
SS101	Isolated from the wheat rhizosphere, The Netherlands	de Souza et al. 2003b
SS101 Δ*massA*	Mutant of SS101 with site-directed mutation in *massA*, deficient in massetolide A production	de Bruijn et al. 2008
Q2-87	Isolated from the wheat rhizosphere, Washington, U.S.A.; suppresses take all of wheat; DAPG^{+}, MAPG^{+}	Vincent et al. 1991
Q2-1 Phl^{-}	Q2-87 *phlD*::Tn5, also called Q2-87 Tn5; DAPG^{-}, MAPG^{-}, PG^{-}	Vincent et al. 1991
Q2-2 Phl^{-}	Q2-87 *phlD*::*lacZ*, also called Q2-87 DZ Lt; DAPG^{-}, MAPG^{-}, PG^{-}	Weller et al. 2012
P. synxantha		
BG33R	Isolated from the rhizosphere of peach, South Carolina, U.S.A.	Kluepfel et al. 1993
Pseudomonas sp.		
NCPPB387	WLIP^{+}, the original *P. reactans* strain used to elucidate the structure of the white line inducing principle (WLIP), also called LMG2338 and ATCC 14340	Mortishire-Smith et al. 1991
NCPPB387 WLIP^{-}	MiniTn*5::gfp::lux* mutant of NCPPB387 with an insertion in a homolog of *wlpA* (AFJ23819.1), also called LK089; WLIP^{-}	This study

[a] Phenotype abbreviations: Chi, chitinase; DAPG, 2,4-diacetylphloroglucinol; HCN, hydrogen cyanide; MAPG, monoacetylphloroglucinol; NRPS, nonribosomal peptide synthetase; Ofa, orfamide A; PG, phloroglucinol; Plt, pyoluteorin; Prn, pyrrolnitrin; Rzx, rhizoxin derivatives; Tol, tolaasin; WLIP, white-line inducing principle. Mutants of Pf-5 containing deletions in *ofaA*, *pltA*, *prnC*, *rzxB*, or *hcnB* have FRT scars (85 to 86 bp fragment length polymorphism recognition target sequences) in those genes. In-frame deletions were generated in *gacA*, *rpoS*, *chiC*, and *phlA*, and the deleted genes do not have inserted FRT sequences. The production of Plt, Prn, Rxz, Ofa, DAPG, and MAPG by Pf-5 and all of the Pf-5 mutants having a single deletion was determined by high-performance liquid chromatography analysis of cultures grown with shaking in nutrient yeast broth for 48 h at 20°C.

assay. *Pseudomonas* sp. strain NCPPB387 is known to produce a white line when grown in proximity to *P. tolaasii*, due to interactions between tolaasin and WLIP (Wong and Preece 1979), which was observed in this study (Fig. 3). NCPPB387 WLIP⁻ did not produce a white line when grown in proximity to *P. tolaasii* NCPPB2192 (Fig. 3A). By sequencing DNA flanking the transposon, we mapped the insertion to *wlpA*, which encodes a nonribosomal peptide synthetase (NRPS) participating in WLIP biosynthesis of *Pseudomonas putida* RW10S2 (Rokni-Zadeh et al. 2012). Conversely, *P. tolaasii* Tol⁻ did not produce a white line when grown in proximity to NCPPB387 (Fig. 3B), as shown previously for tolaasin-deficient mutants of other strains of *P. tolaasii* (Rainey et al. 1993). The DNA flanking the transposon insertion in the Tol⁻ mutant mapped to a gene encoding an NRPS in the genome of NCPPB2192, which is similar to the recently described tolaasin-biosynthesis gene cluster in *P. costantinii* (Scherlach et al. 2013). Based on the sequence data and the tolaasin-deficient phenotype of the mutant, it is very likely that the transposon is in a structural gene for tolaasin biosynthesis in the genome of NCPPB2192.

P. tolaasii Tol⁻ was greatly reduced in virulence compared with the wild-type strain NCPPB2192 (Fig. 2), confirming the known role of this LP in mushroom brown blotch (Hutchison and Johnstone 1993; Rainey et al. 1991, 1992). *Pseudomonas* sp. strain NCPPB387 caused mild brown blotch symptoms compared with the other strains evaluated, and the virulence of NCPPB387 WLIP⁻ did not differ significantly from the wild-type strain (Fig. 2). These results are in line with a previous report that purified WLIP is much less toxic to mushrooms than tolaasin (Lo Cantore et al. 2006). Nevertheless, the results were not expected, given the proposed role of WLIP in mushroom pathogenicity (Lo Cantore et al. 2006). To confirm the results of our mushroom inoculation experiments, we repeated them by testing two bacterial inoculum densities and assessing symptoms for up to 4 days (Supplementary Table S1). The results of these experiments clearly show that different LP have divergent roles in the symptoms caused by *Pseudomonas* species on mushroom caps, with WLIP and orfamide A production being nearly benign relative to the severe pitting and browning associated with tolaasin production.

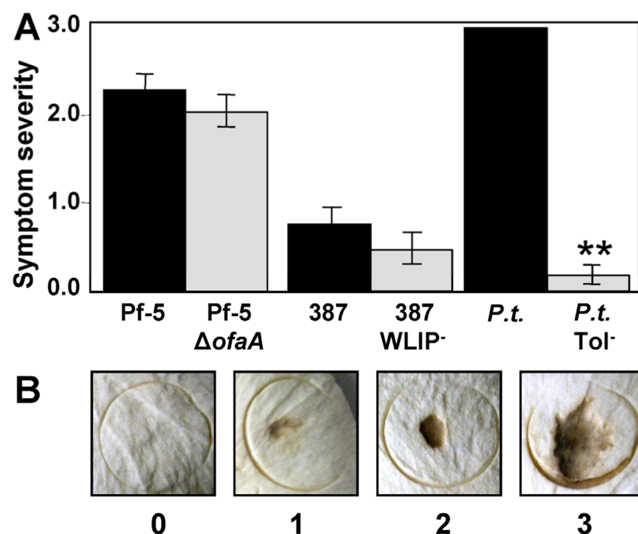


Fig. 2. Severity of symptoms caused by three *Pseudomonas* strains and their lipopeptide-deficient mutants on peeled mushroom caps. **A,** Strains were *P. protegens* Pf-5 and a mutant deficient in orfamide production (Pf-5 Δ*ofaA*), *Pseudomonas* sp. strain NCPPB387 (387) and a mutant deficient in production of the white-line inducing principle (387 WLIP⁻), and *P. tolaasii* NCPPB2192 (*P.t.*) and a mutant deficient in tolaasin production (*P.t.* Tol⁻). For each strain, 10^7 CFU were spotted on the surface of 11 peeled mushroom caps. Severity of symptoms was assessed at 48 h after inoculation, using a numerical 0 to 3 rating scale, with 3 being most severe. Values represent the mean ratings for 11 replicate mushroom caps. A double asterisk indicates that the mean value of the mutant differs significantly from that of the parental strain, as determined by Fisher's protected least significance difference at $P = 0.05$. Error bars represent the standard error of the mean. **B,** Rating scale for symptoms.

LP produced by mushroom-associated strains of *Pseudomonas* species have different functionalities.

Due to the observed differences among the LP with respect to their roles in the symptoms caused by the three strains of *Pseudomonas* species on mushroom caps, we compared the three mutant/wild-type pairs for phenotypes associated with LP production (Fig. 4).

Droplet collapse. Due to their surfactant properties, LP can strongly decrease the surface tension of the culture medium. This decrease in surface tension can be observed by placing a droplet of the spent culture medium on a waxy surface. If a surfactant has been secreted by the bacterium into the culture medium, the droplet collapses and the droplet diameter increases; when no surfactant is present in the culture medium, the droplet maintains its round shape and small diameter (de Bruijn et al. 2007). Culture supernatants of *P. protegens* Pf-5 and *Pseudomonas* sp. strain NCPPB387 exhibited the greatest surfactant activity in the droplet-collapse assay as assessed by the diameter of droplets on the waxy surface (Fig. 4A). The surfactant activity was especially high when cells were grown on King's medium B (KMB) containing glycerol rather than glucose. Pf-5 Δ*ofaA* and NCPPB387 WLIP⁻ were negative in the droplet-collapse assay, which is consistent with earlier re-

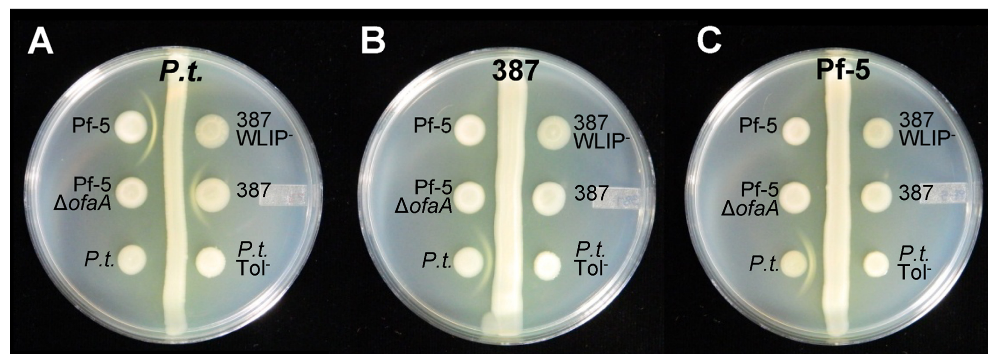


Fig. 3. White line assay. Strains were grown on King's medium B at 27°C for 24 h. *P.t.*, *Pseudomonas tolaasii* NCPPB2192 and *P.t.* Tol⁻, a mutant deficient in tolaasin production; 387, *Pseudomonas* sp. strain NCPPB387 and 387 WLIP⁻, a mutant deficient in production of the white-line inducing principle; Pf-5, *P. protegens* Pf-5 and Pf-5 Δ*ofaA*, a mutant deficient in orfamide production.

ports that orfamide A and WLIP, respectively, are responsible for the surfactant activity of the wild-type strains (Coraiola et al. 2006; Gross et al. 2007). Culture supernatants of *P. tolaasii* exhibited low levels of surfactant activity, and droplet diameters were only slightly smaller in the Tol⁻ mutant than in the wild-type strain (Fig. 4A).

Hemolytic activity. Many LP produced by *Pseudomonas* species have hemolytic activity (Loper et al. 2012; Vallet-Gely et al. 2010), including WLIP (Coraiola et al. 2006; Lo Cantore et al. 2006; Munsch and Alatossava 2002b; Rokni-Zadeh et al. 2012) and tolaasin (Rainey et al. 1991). As expected, the three wild-type strains evaluated here exhibited hemolysis on blood agar plates, whereas the LP-deficient mutants (Pf-5 Δ*ofaA*, *P. tolaasii* Tol⁻, and NCPPB387 WLIP⁻) were not hemolytic. Pf-5 produced the largest diameter zone (Fig. 4B), whereas clearing of blood agar by *P. tolaasii* was observed only directly under the bacterial colony. Purified orfamide A also exhibited hemolysis on blood agar (Supplementary Fig. S1).

Swarming motility. Due to their surfactant properties, many LP facilitate the swarming motility of bacteria across solid surfaces (Andersen et al. 2003; Berti et al. 2007; de Bruijn et al. 2007, 2008; Gross et al. 2007; Kuiper et al. 2004; Roongsawang et al. 2003; Vallet-Gely et al. 2010). Pf-5 and NCPPB387 exhibited swarming motility (Fig. 4C; Supplementary Table S2), whereas Pf-5 Δ*ofaA* and NCPPB387 WLIP⁻ did not swarm, which is consistent with earlier reports that orfamide A (Gross et al. 2007) and WLIP (Rokni-Zadeh et al. 2012, 2013) are necessary for swarming of the producing strains. In contrast to Pf-5 and NCPPB387, *P. tolaasii* NCPPB2192 and the Tol⁻ mutant exhibited little swarming motility under the conditions of this study. Diameters of colonies of *P. tolaasii* Tol⁻ were similar to those of the wild-type strain on the modified KMB swarming medium (Fig. 4C). On standard succinate swarming medium (SSM), diameters of colonies of *P. tolaasii* Tol⁻ were slightly greater than those of the wild type. We did not explore possible explanations for the slightly increased swarming motility of the tolaasin-deficient mutant versus the wild-type strain on that medium, but our results clearly indicate a different role of tolaasin vs. WLIP and orfamide A in swarming motility of the producing strains.

White line precipitate formation. Pf-5 formed a white line precipitate when grown in proximity to P. *tolaasii* on the surface of KMB agar but did not form a precipitate when grown in proximity to NCPPB387 (Fig. 3A and B). Likewise, *P. tolaasii* NCPPB2192 formed a white line when grown in proximity to Pf-5, whereas NCPPB387 did not (Fig. 3C). Neither Pf-5 Δ*ofaA* (Fig. 3A) nor Pf-5 Δ*gacA* (data not shown) produced a white line when co-cultured with *P. tolaasii*, indicating that orfamide A production is necessary for the white line reaction.

Three strains of *P. fluorescens* that caused no discoloration of mushroom caps (SBW25, SS101, and BG33R) are known to produce LP that function as biosurfactants; SBW25 produces viscosin (de Bruijn et al. 2007), SS101 produces massetolide A (de Bruijn et al. 2008), and BG33R produces a chemically uncharacterized LP related to massetolide (Loper et al. 2012). The activities of these LP in the droplet-collapse assay, hemolysis, and swarming motility were reported earlier (de Bruijn et al. 2007, 2008; Gross et al. 2007; Loper et al. 2012). Here, we evaluated each of the strains in a white line assay against the tolaasin-producing strain *P. tolaasii* NCPPB2192 and the WLIP-producing *Pseudomonas* sp. strain NCPPB387. We observed a faint white precipitate directly adjacent to or under the colonies of SS101, SBW25, and BG33R that were co-cultured with the tolaasin-producing strain *P. tolaasii* NCPPB2192 (Supplementary Fig. S2). No white precipitate was associated with a massetolide A-deficient mutant of SS101 or a viscosin-deficient mutant of SBW25 in these tests, indicating that the LP were responsible for the positive reaction with tolaasin in the white line assay. The white line reactions associated with massetolide A and viscosin were not observed until at least 72 h after the inoculation of the medium and, even at 120 h after inoculation, they were subtle compared with the white line reactions observed for the orfamide A–producing strain Pf-5 or

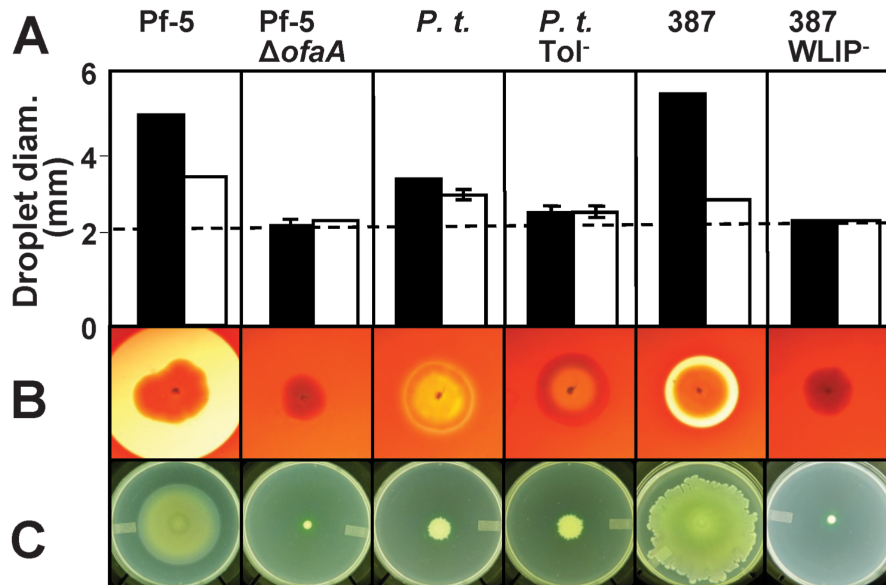


Fig. 4. Phenotypes associated with lipopeptide (LP) production by three *Pseudomonas* species strains: *P. protegens* Pf-5 and a Δ*ofaA* mutant deficient in orfamide production (Pf-5 Δ*ofaA*), *Pseudomonas* sp. strain NCPPB387 (387) and a mutant deficient in production of the white-line inducing principle (387 WLIP⁻), and *P. tolaasii* NCPPB2192 (*P.t.*) and a mutant deficient in tolaasin production (*P.t.* Tol⁻). **A,** Surfactant activity was determined from strains grown on King's medium B (KMB) broth (black bars) or KMB-glucose broth (white bars) using the droplet-collapse assay. Culture supernatants were spotted onto parafilm, and the reduced surface tension caused by certain LP in supernatants resulted in collapse and increased diameter of the droplet. Mean diameters of droplets from three replicate cultures are shown, and error bars represent the standard error of the mean. The dotted line represents the diameter of control droplets (noninoculated media). **B,** Hemolytic activity exhibited by LP-producing strains grown on blood agar. **C,** Swarming motility on modified KMB (0.6% agar) plates incubated at 20°C for 24 h.

the WLIP-producing *Pseudomonas* sp. strain NCPPB387. Nevertheless, these results provide convincing evidence that the LP massetolide A and viscosin, like WLIP and orfamide A, form a white precipitate with tolaasin.

Taken together, the assays highlight differences in the properties of the LP produced by these *Pseudomonas* species. Comparisons between the strains and their respective LP mutants demonstrate that the LP differ in many respects beyond their different roles in mushroom toxicity.

Strain Pf-5 requires the GacS/GacA regulatory system to cause brown blotch–like symptoms.

To narrow down the possible factors that could be responsible for mushroom toxicity, we tested Pf-5 Δ*gacA*, which is deficient in the production of many antifungal metabolites and exoenzymes. Pf-5 Δ*gacA* did not cause browning or pitting symptoms on mushroom (Fig. 5), indicating that one or more of the many genes controlled by GacA is responsible for toxicity.

Lack of a role of chitinase production in symptoms caused by Pf-5 on mushroom caps.

Among the many genes regulated by GacA is *chiC* (Kidarsa et al. 2013), which encodes a chitinase (Loper et al. 2012), one of the factors implicated in brown blotch disease (Burlinson et al. 2008). To evaluate the potential role of chitinase in mushroom symptoms caused by *P. protegens* Pf-5, we first compared Pf-5 with the known mushroom-associated strains *P. tolaasii* NCPPB2192 and *Pseudomonas* sp. strain NCPPB387 for the capacity to degrade three chitin substrates. Neither *P. tolaasii* NCPPB2192 nor *Pseudomonas* sp. strain NCPPB387 exhibited chitinase activity on any of the three substrates (Table 2). In contrast, *P. protegens* Pf-5 degraded 4-methylumbelliferyl N,N'-diacetyl-β-D-chitobioside and 4-methylumbelliferyl β-D-N,N,',N"-triacetylchitotriose, indicative of exochitinase and endochitinase activity, respectively. As reported earlier (Loper et al. 2012), the *chiC* mutant of Pf-5 was deficient in these chitinase activities but did not differ from the wild-type strain in causing brown blotch–like symptoms on peeled mushroom caps (Fig. 5A), indicating that chitinase production did not contribute significantly to mushroom toxicity.

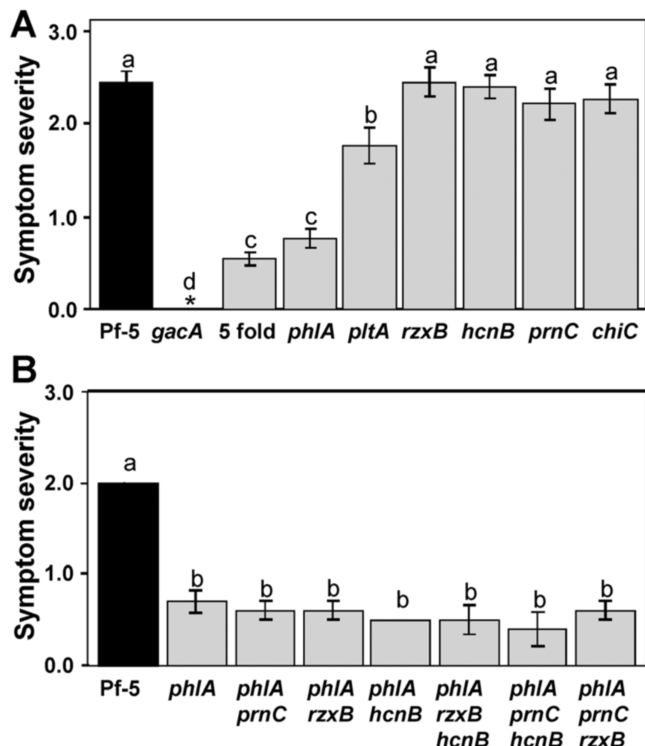


Fig. 5. Severity of symptoms caused by *Pseudomonas protegens* Pf-5 and derivatives lacking the production of secondary metabolites or chitinase. For each strain, 10^7 CFU were spotted on the surface of peeled mushroom caps, and symptoms were assessed at 48 h after inoculation, using a 0 to 3 rating scale, with 3 being most severe. **A,** Symptoms caused by Pf-5 or derivative strains having single deletions in genes for the biosynthesis of 2,4-diacetylphloroglucinol (*phlA*), pyrrolnitrin (*prnC*), rhizoxin (*rzxB*), hydrogen cyanide (*hcnB*) or chitinase (*chiC*) or a fivefold mutant (Δ*phlA*-Δ*prnC*-Δ*rzxB*-Δ*pltA*-Δ*hcnB*). Values represent the mean ratings for 11 replicate mushroom caps. An asterisk designates a disease rating of zero. **B,** Symptoms caused by Pf-5 and derivatives having a mutation in *phlA* as well as one or more genes for the biosynthesis of other secondary metabolites. Values represent the mean ratings for six replicate mushroom caps. Mean values having the same lowercase letter are not significantly different by Fisher's protected least significance difference at P = 0.05, and error bars represent the standard error of the mean.

Role of antifungal metabolites in mushroom toxicity of Pf-5.

In addition to orfamide A, five known secondary metabolites are produced by Pf-5 under the control of the GacS/GacA two-component regulatory system: DAPG, pyoluteorin, pyrrolnitrin, rhizoxin, and hydrogen cyanide. To identify the one or more specific compounds responsible for mushroom toxicity, we generated mutants of Pf-5 having individual deletions in biosynthesis genes for each of these five metabolites. A derivative of Pf-5 harboring all five deletions (Δ*phlA* for DAPG and its intermediate MAPG, Δ*pltA* for pyoluteorin, Δ*prnC* for pyrrolnitrin, Δ*rzxB* for rhizoxin, and Δ*hcnB* for hydrogen cyanide) lacked production of all of the compounds, whereas the five derivative strains with individual deletions lacked production of one metabolite and continued to produce all of the other compounds (data not shown).

The fivefold mutant (Pf-5 Δ*hcnB*-Δ*phlA*-Δ*pltA*-Δ*prnC*-Δ*rzxB)* caused less-severe symptoms on peeled mushroom caps than did wild-type Pf-5 (Fig. 5A), indicating that one or more of the deleted metabolites was involved in mushroom toxicity. Of the five mutants lacking production of an individual antifungal metabolite, Pf-5 Δ*phlA*, which is deficient in DAPG and MAPG production, differed most from the wild-type strain in toxicity to peeled mushroom caps (Fig. 5A). Symptoms caused by Pf-5 Δ*pltA* were less severe than those caused by the wild type but more severe than those caused by Pf-5 Δ*phlA* or the fivefold mutant. Mutants lacking production of the other three metabolites (Pf-5 Δ*hcnB*, Pf-5 Δ*prnC*, and Pf-5 Δ*rzxB*) did not differ significantly from Pf-5 in mushroom toxicity.

The severity of symptoms caused by Pf-5 Δ*phlA* was similar to that of symptoms caused by the fivefold mutant. Because MAPG or DAPG appeared to be a dominant factor contributing to browning of mushroom caps, we recognized that the contributing effects of other antifungal factors may not be evi-

Table 2. Chitinase production by three strains of *Pseudomonas* spp.

Strain	4-Methylumbelliferone released from substrates (ng)[a]: 4-Methylumbelliferyl N,N'-diacetyl-β-D-chitobioside	4-Methylumbelliferyl β-D-N,N',N" triacetylchitotriose
P. protegens Pf-5	608.2 ± 28.2	166.0 ± 5.9
P.tolaasii NCPPB2192	2.1 ± 0.0	2.1 ± 3.0
Pseudomonas sp. NCPPB387	7.4 ± 1.5	6.3 ± 0.0

[a] Values represent the mean determined from three replicate cultures grown in King's medium B broth for 4 days, followed by standard errors. The chitinase activities of Δ*gacA* and Δ*chiC* mutants of Pf-5 were below 15.0 (Loper et al. 2012) for both substrates in these assays. Chitinase activity of all strains was negligible on the 4-methylumbelliferyl N-acetyl-β-D-glucosaminide substrate, so values are not shown in the table.

dent in the single mutants (Pf-5 Δ*hcn*, Pf-5 Δ*prn*, Pf-5 Δ*plt*, or Pf-5 Δ*rxz*) that continued to produce MAPG and DAPG. Therefore, we introduced the deletions in each of these biosynthetic loci into Pf-5 Δ*phlA*, and tested the resulting double and triple mutants for toxicity to mushrooms. Double and triple mutants containing Δ*phlA* as well as Δ*prnC*, Δ*rzxB*, or Δ*hcnB* did not differ significantly from Pf-5 Δ*phlA* in causing brown blotch–like symptoms (Fig. 5B). In contrast, Pf-5 Δ*phlA*-Δ*pltA* was less toxic than Pf-5 Δ*phlA* (Fig. 6A), suggesting that pyoluteorin as well as MAPG or DAPG could play a role in the brown blotch–like symptoms caused by Pf-5.

Antifungal compounds purified from Pf-5 cause symptoms on mushroom caps.

We evaluated the symptoms caused by purified pyoluteorin, pyrrolnitrin, DAPG, and MAPG on peeled mushroom caps and found that all four compounds applied at 5.0 µg caused some symptoms (Fig. 7). Pyoluteorin caused browning and pitting at the lowest concentration tested (0.6 µg), with dark-brown pits resulting from the application of 5.0 µg of pyoluteorin. At 5.0 µg, pyrrolnitrin caused softening of the tissue but relatively little brown discoloration. In contrast, >1.3 µg of 2,4-diacetylphloglucinol produced browning but no observable pitting. Similarly, 5.0 µg of MAPG caused browning but no pitting. When applied in aqueous solution, 0.6 to 5.0 µg of purified orfamide A produced no symptoms on mushroom (data not shown). These levels exceed the tolaasin concentrations needed to cause brown blotch symptoms in previous studies reporting that 0.1 µg of tolaasin I can cause some brown blotch symptoms and 1.25 µg tolaasin I causes severe browning and pitting of mushroom when added directly to mushroom caps (Brodey et al. 1991; Hutchison and Johnstone 1993).

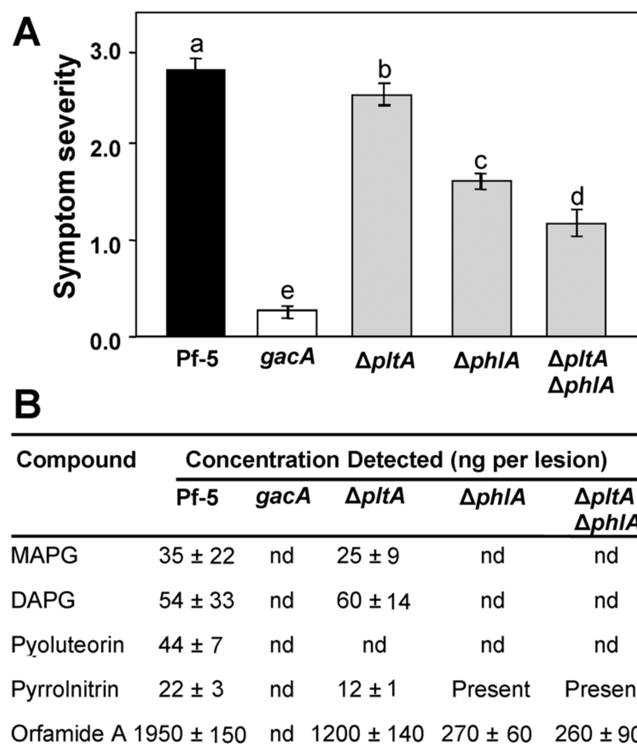
Detection of antifungal metabolites of Pf-5 in mushroom tissue.

To further explore the relative roles of the various antifungal metabolites produced by Pf-5 in mushroom toxicity, we determined the presence of these compounds in peeled mushroom caps inoculated with Pf-5 and Δ*phlA*, Δ*pltA*, or Δ*phlA*-Δ*pltA* derivative strains. At 48 h after inoculation of peeled mushroom caps, the mushroom tissue was macerated and extracted in ethyl acetate, and extracts were analyzed by high-performance liquid chromatography (HPLC). From mushroom tissue inoculated with wild-type Pf-5, we detected DAPG, MAPG, pyoluteorin, pyrrolnitrin, and orfamide A (Fig. 6B) but no rhizoxin WF-1360F (data not shown), which is the major rhizoxin derivative produced by Pf-5 (Loper et al. 2008). None of the compounds was detected in the healthy mushroom-cap tissue that had been inoculated with Pf-5 Δ*gacA*. This result was expected because Pf-5 Δ*gacA* lacks production of all of these compounds in culture (Hassan et al. 2010). Pf-5 Δ*pltA* lacked production of pyoluteorin but produced MAPG, DAPG, pyrrolnitrin, and orfamide A. Pf-5 Δ*phlA* lacked production of MAPG and DAPG and produced orfamide A and pyrrolnitrin, as expected. We detected no pyoluteorin in mushroom tissue inoculated with Pf-5 Δ*phlA*, which was unexpected, because this mutant produces pyoluteorin at approximately wild-type levels under the culture conditions evaluated in this study (Kidarsa et al. 2011).

We also attempted to determine the concentrations of five antifungal metabolites (MAPG, DAPG, pyoluteorin, pyrrolnitrin, and orfamide A) in inoculated mushroom tissue. To estimate recovery rates, we placed solutions containing 5 µg (DAPG, pyoluteorin, or pyrrolnitrin) or 10 µg (MAPG) of purified compound on mushroom caps and extracted mushroom tissue in ethyl acetate 48 h later, which conforms to the duration of the experiments evaluating the toxicity of Pf-5 and derivative strains. Rates of recovery varied markedly between compounds, reflecting both their extraction efficiencies and stabilities in mushroom tissue after 48 h. By adjusting the concentration of each compound in extracts of mushroom tissue (Fig. 6B) using the recovery rates, the estimated concentrations of compounds in mushroom lesions caused by Pf-5 ranged from 0.1 to 87 µg per lesion. For three of the antifungal metabolites (MAPG, DAPG, and pyoluteorin), concentrations estimated from mushroom lesions exceeded the concentrations of purified compounds that were needed to cause symptoms (Fig. 7). In contrast, pyrrolnitrin caused some tissue collapse of mushroom caps when ≥ 0.6 µg of pure compound was applied. Based on our determined recovery rate of 22%, the concentration of pyrrolnitrin in discolored lesions caused by Pf-5 was below this level (0.1 µg per lesion). Pyrrolnitrin did not appear to be produced by Pf-5 on mushroom caps at levels required to cause symptoms. Therefore, the quantitative data support the conclusion from our mutant study that DAPG, MAPG, and pyoluteorin are major factors contributing to mushroom toxicity of Pf-5.

Symptoms caused by *rpoS* mutants of Pf-5 on mushroom caps.

Due to the detected roles of pyoluteorin, MAPG, and DAPG in mushroom toxicity of Pf-5, we tested an *rpoS* mutant of Pf-5 (Pf-5 Δ*rpoS*), which is known to overproduce these metabolites (Kidarsa et al. 2013; Sarniguet et al. 1995), for the capac-



Compound	Concentration Detected (ng per lesion)				
	Pf-5	*gacA*	Δ*pltA*	Δ*phlA*	Δ*pltA* Δ*phlA*
MAPG	35 ± 22	nd	25 ± 9	nd	nd
DAPG	54 ± 33	nd	60 ± 14	nd	nd
Pyoluteorin	44 ± 7	nd	nd	nd	nd
Pyrrolnitrin	22 ± 3	nd	12 ± 1	Present	Present
Orfamide A	1950 ± 150	nd	1200 ± 140	270 ± 60	260 ± 90

Fig. 6. A, Severity of symptoms caused by *Pseudomonas protegens* Pf-5 and derivatives lacking the production of secondary metabolites. For each strain, 10^7 CFU were spotted on the surface of peeled mushroom caps, and symptoms were assessed at 48 h after inoculation, using a 0 to 3 rating scale, with 3 being most severe. Values represent the mean ratings for 27 replicate mushroom caps. Mean values having the same lowercase letter are not significantly different by Fisher's protected least significance difference at P = 0.05, and error bars represent the standard error of the mean. **B,** Concentrations of metabolites extracted from mushroom cap tissue inoculated with each strain ± standard deviation. "Present" indicates that the compound was detected but levels were too low for quantification; "nd" indicates that the compound was not detected.

ity to cause symptoms on peeled mushroom caps. The severity of symptoms caused by Pf-5 Δ*rpoS* or wild-type Pf-5 was similar (Supplementary Fig. S3). The severity of symptoms caused by the Δ*rpoS*-Δ*pltA* double mutant was similar to those caused by Pf-5 Δ*rpoS*. In contrast, the Δ*rpoS*-Δ*phlA* and the Δ*rpoS*-Δ*phlA*-Δ*pltA* mutants caused less-severe symptoms than Pf-5 Δ*rpoS*, Pf-5 Δ*rpoS*-Δ*pltA*, or wild-type Pf-5. These results support the conclusion that DAPG or MAPG are major factors contributing to mushroom toxicity of Pf-5. All of the mutants in the *rpoS* set caused some brown blotch–like symptoms, however, suggesting that unknown factors produced by the *rpoS* mutant but not by the *gacA* mutant also have a role in mushroom toxicity of Pf-5.

Other DAPG-producing *Pseudomonas* strains cause browning of mushroom caps.

Because of the role of DAPG or MAPG production in the brown blotch–like symptoms produced by Pf-5, we evaluated the role of MAPG and DAPG in the browning symptoms produced by two other DAPG-producing strains, Q2-87 and Q8r1-96, on peeled mushroom caps. Browning symptoms caused by these strains were less severe than those caused by Pf-5 and did not include the pitting observed on mushrooms inoculated with Pf-5 (Fig. 8). The *phlD* mutants of Q8r1-96 and Q2-87, which lack DAPG and MAPG production, produced no symptoms on the mushrooms, indicating that DAPG or MAPG also plays a role in browning symptoms caused by these strains.

DISCUSSION

The results of this study demonstrate that the soil bacterium *P. protegens* Pf-5, which is known for its capacity to protect seeds and roots from infection by soilborne plant pathogens (Loper et al. 2007), can cause brown, sunken lesions on peeled caps of the cultivated mushroom *A. bisporus*. Due to the key role of the LP tolaasin in brown blotch caused by *P. tolaasii* (Brodey et al. 1991; Hutchison and Johnstone 1993; Rainey et al. 1991), we evaluated the role of the Pf-5 LP orfamide A in causing the discoloration and pitting observed in response to inoculation with Pf-5. Two lines of evidence indicated that orfamide A has a negligible role: i) purified orfamide A produced no browning or pitting and ii) an orfamide A–deficient mutant of Pf-5 exhibited wild-type levels of toxicity. Therefore, although Pf-5 produced orfamide A in mushroom tissue, this production is not responsible for brown blotch–like symptoms caused by Pf-5 on peeled mushroom caps.

LP are a structurally diverse group of compounds that share certain properties but also have distinct functions in the biology of the producing strains (Raaijmakers and Mazzola 2012; Raaijmakers et al. 2010; Reder-Christ et al. 2012; Roongsawang et al. 2011). All of the LP evaluated in this study exhibited hemolytic activity (Fig. 4; Loper et al. 2012), but only tolaasin played a detectable role in mushroom pathogenicity. Tolaasin I is known to form pores that disrupt the cellular membrane structure in *A. bisporus* (Hutchison and Johnstone 1993), and such pore formation may require specific

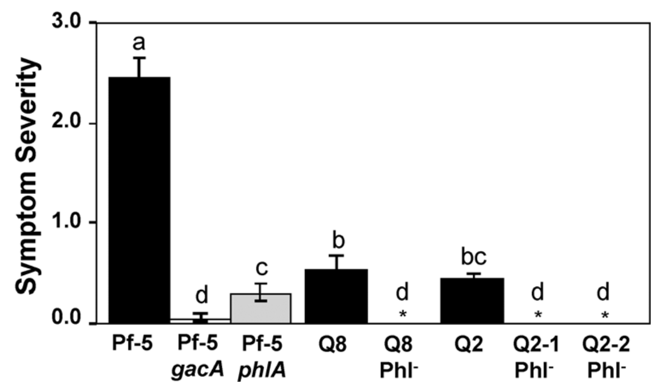


Fig. 8. Severity of symptoms caused by *Pseudomonas protegens* Pf-5, *P. brassicacearum* Q8r1-96 (Q8), *P. fluorescens* Q2-87 (Q2), and derivatives lacking the production of monoacetylphloroglucinol and 2,4-diacetylphloroglucinol. For each strain, 10^7 CFU were spotted on the surface of peeled mushroom caps and brown blotch symptoms were assessed at 48 h after inoculation on a rating scale of 0 to 3, with 3 being most severe. Values represent the mean ratings for 14 replicate mushroom caps. An asterisk designates a disease rating of zero. Mean values having the same lowercase letter are not significantly different by Fisher's protected least significance difference at $P = 0.05$, and error bars represent the standard error of the mean.

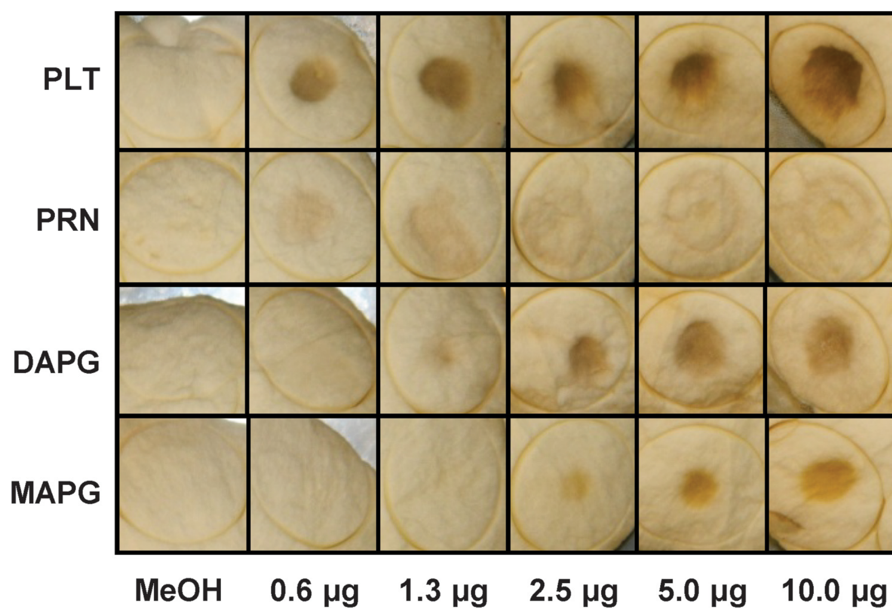


Fig. 7. Symptoms caused by purified metabolites on peeled mushroom caps. Solutions containing the specified amount of compound were placed on peeled mushroom caps and symptoms were photographed 48 h later. PLT, pyoluteorin; PRN, pyrrolnitrin, DAPG, 2,4-diacetylphloroglucinol; MAPG, monoacetylphloroglucinol.

interactions between the LP and components of fungal membranes (Lugones et al. 1996; Wösten 2001). Such specificity has been observed for the LP syringomycin and syringopeptin, which interact with a certain class of fungal lipids, and fungal mutants deficient in these lipids show increased resistance to these toxins (Bensaci et al. 2011; Kaulin et al. 2005). Similar interactions with an individual component of fungal cells may also be critical to the toxicity of tolaasin and, if specific to certain LP, could explain the negligible role of orfamide A in mushroom brown blotch–like symptoms. We also detected no significant difference in mushroom toxicity of *Pseudomonas* sp. strain NCPPB387 versus its WLIP-deficient mutant; both strains caused relatively mild symptoms on peeled mushroom caps. This result was unexpected given the proposed role of WLIP in mushroom disease (Lo Cantore et al. 2006), which was based on the observation that phenotypic variants of *P. reactans* that no longer gave a positive response in the white line assay were avirulent (Iacobellis and Lo Cantore 2003). Because those variants were not fully characterized, it is possible that they had a mutation in a regulatory gene that influenced many phenotypes in addition to LP production, similar to the *gacA* mutation of Pf-5 described herein. Although we detected no contribution of WLIP to the mild symptoms caused by *Pseudomonas* sp. strain NCPPB387 in this study, it remains to be seen if WLIP production contributes to the mushroom toxicity of other strains of *Pseudomonas* species.

The striking white precipitate (i.e., white line) that forms in agar between colonies of *P. tolaasii* and *P. reactans* was once considered to be evidence of a unique interaction of tolaasin with WLIP. Here, we show that several LP produced by members of the *P. fluorescens* group, including orfamide A, massetolide A, and viscosin, also form a white precipitate with tolaasin. Viscosin is nearly identical to WLIP, differing only in the chirality of one amino acid in the peptide chain; Leu5 has a D configuration in WLIP and an L configuration in viscosin. Therefore, it is not surprising that viscosin, like WLIP, formed a white line with tolaasin, although this observation contradicts an earlier report (Rainey et al. 1992). The white lines associated with the viscosin- or massetolide A–producing strains were relatively inconspicuous, with only small amounts of white precipitate directly below the bacterial colony as compared with the striking reactions of the WLIP^{-} or orfamide A–producing strains. The reasons for this visual distinction are unknown but could be due to structural differences among LP that alter diffusion or reactivity or to differences in LP production levels. Based on our observation that orfamide A, massetolide A, and viscosin form a white precipitate with tolaasin, we speculate that some strains previously designated as *P. reactans* may not produce WLIP but, instead, produce an LP that, like orfamide A, forms conspicuous white precipitate when grown in proximity to tolaasin-producing strains.

The results of this study also demonstrate that antifungal metabolite production is a primary determinant of the browning and pitting symptoms caused by Pf-5 on peeled mushroom caps. Three lines of evidence indicate that MAPG or DAPG and pyoluteorin production play a role. i) Symptoms caused by a mutant deficient in the production of MAPG, DAPG, and pyoluteorin were much less severe than those caused by wild-type Pf-5. ii) Pyoluteorin, MAPG, and DAPG were present in lesions caused by Pf-5 on mushroom caps, which provides direct evidence that the compounds were produced in situ by the bacterium. iii) When applied to peeled mushroom caps, purified pyoluteorin, MAPG, and DAPG caused browning and pyoluteorin also caused pitting of mushroom tissue. The mechanisms by which pyoluteorin and MAPG or DAPG led to pitting and browning of mushroom tissue remain unknown. We are not aware of any published studies that shed light on the mode of action of pyoluteorin, but DAPG is known to act as a proton ionophore that disrupts the proton gradient across the mitochondrial membrane and leads to the loss of mitochondrial membrane potential in the yeast *Saccharomyces cerevisiae* and the fungus *Neurospora crassa* (Gleeson et al. 2010; Troppens et al. 2013). DAPG may have similar effects on mitochondria of *A. bisporus*, but the steps by which these effects would be manifested in browning of mushroom cap tissue are unclear. Tolaasin causes both pitting, associated with cell lysis, and browning, due to quinines and melanins formed by the oxidative action of polyphenol oxidases such as tyrosinase (Jolivet et al. 1998; Mayer 2006; Soler-Rivas et al. 1999). The results of this study provide convincing evidence that pyoluteorin and MAPG or DAPG production contribute to the toxicity of Pf-5 to mushroom, but further work is needed to determine the mechanisms by which these metabolites cause brown blotch symptoms. The importance of these antifungal metabolites in mushroom toxicity in other *Pseudomonas* strains is also an open question. For example, *Pseudomonas* sp. strain NZI7 can produce DAPG, yet production of a tolaasin-like LP appears to be the primary mechanism by which strain NZI7 causes brown blotch (Burlinson et al. 2013). It seems likely that strain NZI7 does not produce adequate concentrations of DAPG in mushroom tissue to cause the toxicity associated here with DAPG production by Pf-5, but further research is needed to explore this possibility. Our results expand the spectrum of bacterial metabolites associated with brown blotch symptoms beyond the LP and demonstrate that, in addition to tolaasin (Scherlach et al. 2013), other metabolites produced by *Pseudomonas* species can be present in discolored lesions on edible mushroom caps. The importance of MAPG-, DAPG-, and pyoluteorin-producing strains like Pf-5 as pathogens of mushroom in nature or in commercial production systems remains to be determined.

In this study, we relied on a mutagenesis approach to narrow our search for the factors contributing to brown blotch symptoms. Strain Pf-5 is known to produce a spectrum of antifungal metabolites under the positive control of the global regulator GacA (Hassan et al. 2010; Kidarsa et al. 2013; Whistler et al. 1998). Because a *gacA* mutant of Pf-5 lacked the ability to cause brown blotch symptoms, we focused our search for molecular determinants of mushroom toxicity on GacA-dependent phenotypes, which include exoenzyme and secondary metabolite production. We found that chitinase production had no significant role but obtained conclusive evidence that at least two antifungal compounds, MAPG or DAPG and pyoluteorin, contribute to the brown blotch–like symptoms caused by Pf-5 on mushroom caps. Nevertheless, our results reflect some shortcomings of the mutagenesis approach used to identify the specific metabolites contributing to mushroom toxicity. It is well-known that mutants having specific genetic lesions in a gene cluster for the biosynthesis of one secondary metabolite can also vary in the production of one or more other metabolites (Baehler et al. 2005; Brodhagen et al. 2004; Kidarsa et al. 2011; Schnider-Keel et al. 2000). Although rigorous methods were used to generate site-specific mutants and confirm the exact location of each mutation and its phenotype in culture, the metabolic profile of the mutants in mushroom tissue was not exactly as expected. Most notably, we detected no pyoluteorin and less than wild-type levels of pyrrolnitrin and orfamide A in the lesions caused by the *phlA* mutant of Pf-5, although only MAPG and DAPG production of the *phlA* mutant differed from the wild type under the culture conditions evaluated. We do not know the reason for this discrepancy but suspect that environmental differences between the culture medium and mushroom tissue are one factor. It should also be recognized that the quantification of bacterial metabolites in natural substrates is notoriously difficult (Thomashow et al. 2002) and the

rates of recovery of Pf-5 metabolites from mushroom tissues were extremely low in this study. It is very possible that pyoluteorin was produced by the *phlA* mutant at levels below detection. Overall, the mutagenesis approach provided an expedient route to identify secondary metabolites that contribute to the brown blotch–like symptoms caused by Pf-5. The proposed role of these metabolites was then supported by demonstrating their in situ production by Pf-5 in mushroom tissue and showing that purified compounds mimic symptoms caused by Pf-5. We recognize that pyoluteorin and MAPG or DAPG are not likely to be the sole determinants of mushroom toxicity, as the Δ*pltA*-Δ*phlA* mutant of Pf-5 caused some symptoms. Because the *gacA* mutant caused no symptoms, future studies will focus on other GacA-regulated traits of Pf-5 that could also contribute to mushroom toxicity.

P. tolaasii is the most well-known pathogen of mushrooms, but other bacteria within the large and diverse *P. fluorescens* group have also been known for decades to cause discoloration of mushroom caps. Infection by various *Pseudomonas* species has long been associated with a range of symptoms, with lesions varying in the extent and color of discoloration and by the presence or absence of pitting. Here, we demonstrate that different antifungal compounds produced by *P. protegens* Pf-5 cause distinct symptoms on peeled mushroom caps. Because a range of symptoms have been associated with *Pseudomonas* infection in the past, we speculate that some of the mushroom pathogens identified earlier (Cutri et al. 1984; Wells et al. 1996; Wong et al. 1981) may produce antifungal compounds that cause distinctive symptoms on mushroom caps. Here, we tested nine well-characterized strains representing diverse lineages within the *P. fluorescens* group for their capacity to cause discoloration of peeled mushroom caps and found that only three strains caused discoloration. This result is consistent with the historical literature indicating that mushroom toxicity is far from ubiquitous in *Pseudomonas* species. Nevertheless, strains representing three distinct lineages within the *P. fluorescens* group (Loper et al. 2012) caused discoloration of peeled mushroom caps due to antifungal metabolite production: *P. brassicacearum* Q8r1-96 and *P. fluorescens* Q2-87 caused mild discoloration, *P. protegens* Pf-5 caused browning and pitting, and *P. tolaasii* caused the most severe brown blotch symptoms. The results of our study suggest that certain antifungal metabolites, long associated with the biocontrol capabilities of these bacteria against fungal plant pathogens, can be toxic to *A. bisporus*, manifested as discoloration or pitting of mushroom caps. Here, we identified pyoluteorin and MAPG or DAPG as determinants of mushroom toxicity in Pf-5, but we speculate that other antifungal metabolites produced by *Pseudomonas* species can also cause brown blotch–like symptoms. This work highlights a novel mechanism by which *Pseudomonas* species interact with *A. bisporus* that is likely to extend beyond the individual antifungal metabolites studied herein.

MATERIALS AND METHODS

Bacterial strains and culture conditions.

Bacterial strains used in this study are described in Table 1. *Pseudomonas* species were grown routinely on KMB (King et al. 1954) at 27°C. *Escherichia coli* was grown in Luria Bertani (LB) medium (10 g of Bacto tryptone [Becton, Dickinson and Co., Sparks, MD, U.S.A.], 5 g of Bacto yeast extract [Becton, Dickinson and Co.], and 10 g of NaCl, pH 6.8, per liter) (Atlas 1993) or on solidified LB at 37°C.

The Δ*prnC* mutant of Pf-5 was using a site-directed mutagenesis method based on fragment length polymorphism (FLP) recombinase, which promotes recombination within the FLP recognition target (FRT) sequence (Hoang et al. 1998). The FRT-Gm^r-*gfp*–FRT cassette from pPS858 (Hoang et al. 1998) was cloned into a unique *Hin*dIII site of *prnC*. Next, the 3.4-kb DNA fragment containing the interrupted *prnC* was amplified by polymerase chain reaction (PCR) with the primers prnseq10 and prnmut1 and KOD Hot Start DNA polymerase (EMD Millipore, Billerica, MA, U.S.A.). The amplicon was cloned into pEX18Tc (Hoang et al. 1998), and the resulting plasmid was mobilized into Pf-5 via mating with *E. coli* S-17λ-*pir* (de Lorenzo and Timmis 1994). The *prnC*::FRT-Gm^r-*gfp*–FRT clones were isolated on LB agar supplemented with streptomycin (100 μg/ml) and gentamicin (40 μg/ml) and were screened for double crossovers on LB agar supplemented with gentamicin and 5% sucrose. The FLP recombinase–mediated in vivo excision of the Gm^r-*gfp* cassette was conducted by electroporating the mutants with pFLP2-Km and selecting transformants on sucrose-containing LB agar. pFLP2Km (D. V. Mavrodi, *unpublished*) was constructed by cloning the kanamycin-resistance cassette from pUC4K (Vieira and Messing 1982) into the unique *Sca*I site of pFLP2 (Hoang et al. 1998). The resultant unmarked Δ*prnC* clones were confirmed for the absence of *sacB*, Tc^r and Gm^r markers by PCR with primer sets SAC1 and SAC2, TET_UP and TET_LOW, and GM_UP and GM_LOW (Mavrodi et al. 2001).

Other deletion mutants of Pf-5 were constructed using overlap extension PCR and were cloned into pEX18Tc (Hoang et al. 1998). Some mutations (Δ*ofaA*, Δ*pltA*, Δ*prnC*, Δ*rzxB*, and Δ*hcnB*) were created using a method modified from Choi and Schweizer (2005) as described previously (Hassan et al. 2010) and have an 85- to 86-bp FRT sequence at the site of the deletion. Other mutations (Δ*gacA*, Δ*rpoS*, Δ*chiC*, and Δ*phlA*) were derived by generating in-frame deletions as described previously (Kidarsa et al. 2011). For each mutant, the sequence of the mutated locus was confirmed to be as expected by performing PCR across the deletion site and sequencing the resultant product. Primers used to construct the mutants and confirm the sequences of mutated loci are in Supplementary Tables S3 and S4. Multiple mutants were created by repetition of the mating and selection process.

LP-deficient mutants of *P. tolaasii* NCPPB2192 and *Pseudomonas* sp. strain NCPPB387 were identified from a Tn*5*::*gfp*::*lux* transposon mutant library using the white line assay for tolaasin and WLIP production, as described previously (Burlinson et al. 2013). The *P. tolaasii* NCPPB2192 mutant library was screened against wild-type *Pseudomonas* sp. strain NCPPB387 and the *Pseudomonas* sp. strain NCPPB387 library was screened against wild-type *P. tolaasii* NCPPB2192. The sites of Tn*5*::*gfp*::*lux* insertions were localized by thermal asymmetric interlaced PCR using a set of three nested primers complementary to the transposon sequence (T1, T2, and T3) and a degenerate primer (AD2) (Supplementary Table S5). To preferentially amplify sequences flanking the transposon insertions, annealing temperatures were varied, resulting in low and high stringency cycles spread across three rounds of PCR. Sequences of the resulting PCR products were used as queries in BLASTn searches to identify the target gene.

All mutants of *Pseudomonas* species were tested for exoprotease production on BBL litmus milk agar (Becton, Dickinson and Co.) to ensure that they did not have a spontaneous mutation in *gacA* or *gacS*, because such mutants can accumulate in cultures of *Pseudomonas* species (Duffy and Defago 2000; Grewal et al. 1995). Only the Δ*gacA* mutant of Pf-5 was deficient in exoprotease production.

Evaluating symptoms caused by bacterial strains and purified compounds on peeled mushroom caps.

Mushroom caps of healthy *A. bisporus* were peeled with a single edge razor blade by removing strips of the epidermis

toward the apex. The stalk of the mushroom was removed and the cap was placed in a sterile petri dish. A sterile number 6 cork borer was used to imprint 6 to 8 circles around the perimeter of the cap; these circles served as a reference point marking the inoculation site, which assisted in the assessment of symptoms. The peeled mushroom caps were inoculated by placing a 10-µl droplet of a bacterial suspension or purified compound at the center of each imprinted circle. Bacterial suspensions were tested on at least six mushroom caps, and purified compounds were tested on four mushroom caps. Mushroom caps contained in closed petri dishes were incubated at 27°C for 48 h prior to visually assessing brown blotch symptoms according to a numerical rating scale (Fig. 2C). For statistical analysis of numerical ratings, each mushroom cap served as a replicate, and mean separation was achieved by Fisher's protected least significant difference test at $P = 0.05$, using the analysis of variance procedure of SAS (Statistical Analysis Systems, Cary, NC, U.S.A.). All experiments were done twice with similar results.

Bacterial suspensions for mushroom inoculations were prepared from 5-ml cultures grown for 24 h in KMB broth with shaking at 27°C. Cells were collected and washed by centrifugation, followed by resuspension in sterile deionized water. Cells were adjusted using a spectrophotometer to an optical density at 600 nm (OD_{600}) of 0.5, corresponding to approximately 10^9 CFU/ml.

Pure samples of orfamide A, phloroglucinol, MAPG, DAPG, pyoluteorin, and pyrrolnitrin were evaluated for their effects on peeled mushroom caps. Orfamide A was a gift from H. Gross, University of Tubingen; phloroglucinol was purchased from Sigma-Aldrich Corp., St. Louis; DAPG and MAPG were gifts from C. Keel of the University of Lausanne; and pyrrolnitrin and pyoluteorin were purified from cultures of Pf-5. Compounds were dissolved in methanol and water solutions, and controls were 10 or 20% methanol solutions, corresponding to the percentage of methanol needed to bring the compounds into solution.

Assays to evaluate LP properties.

For all assays, two replicate cultures were evaluated for each strain, and the experiments were done at least two times with similar results.

Droplet collapse. A droplet-collapse assay (de Bruijn et al. 2007) was used to test the surfactant activity of bacterial supernatants. Bacteria were grown in culture tubes containing 5 ml of KMB broth or KMB-glucose broth in which 10 g of glucose per liter was substituted for the 10 g of glycerol per liter in the recipe. Cultures were grown for 1 day with shaking (200 rpm) at 27°C. Cells were removed by centrifugation and 10 µl of supernatant was placed on parafilm (Pechiney Plastic Packaging; Neenah, WI, U.S.A.). There were five replicate droplets for each culture supernatant. The flattening of droplets, measured as an increase in the diameter of the droplet, was assessed from the residue on the parafilm after droplets had been allowed to dry.

Hemolysis. Hemolytic activity of the bacterial strains and pure orfamide A was evaluated on freshly prepared blood agar plates, which contained 1% of BBL blood agar base (Becton, Dickinson and Co.) and 5% sterile defibrinated bovine blood (HemoStat Laboratories, Dixon, CA, U.S.A.). To evaluate the strains, sterile toothpicks were used to transfer overnight cultures from KMB agar to the blood agar plates. For hemolytic activity of orfamide A, small filter disks (6 mm diameter) were placed on the agar surface. Purified orfamide was dissolved in MeOH and was further diluted with water, and solutions were slowly pipetted onto the disks until liquid was absorbed. A 20% MeOH solution was used as a control. Plates were incubated at 27°C, and photographs were taken at 48 h.

White line assay. The white line assay was done on KMB as described by Wong and Preece (1979).

Swarming motility. Swarming motility was evaluated on soft agar (0.6% wt/vol) in three media: a modified KMB, SSM, and SSM amended with 1% (wt/vol) casamino acids (Becton, Dickinson and Co.). Modified KMB consisted of (per liter): 10 g of Bacto proteose peptone no. 3 (Becton, Dickinson and Company), 1.5 g of $MgSO_4 \cdot 7H_2O$, 1.2 g of $K_2HPO_4 \cdot 3H_2O$, and 6.0 g Bacto agar (Becton, Dickinson and Company). SSM and SSM amended with casamino acids were prepared as described by de Bruijn and Raaijmakers (2009). Three-microliter samples of a bacterial suspension (OD_{600} of 0.2) were spotted onto the agar surface at the center of each plate with three replicates per treatment. Plates were incubated for 24 h at room temperature.

Chitinase assay.

To determine chitinase activity present in culture supernatants of the bacterial strains, we used a fluorometric assay to assess the hydrolysis of three chitinase substrates. Bacteria were grown in culture tubes containing 5 ml of KMB broth for 4 days with shaking (200 rpm) at 27°C. Bacterial cells were collected by centrifugation, and supernatants were evaluated for chitinase activity. A fluorometric chitinase assay kit (Sigma) containing the substrates 4-methylumbelliferyl N-acetyl-β-D-glucosaminide (exochitinase activity β-D-glucosaminidase), 4-methylumbelliferyl N,N'-diacetyl-β-D-chitobioside (exochitinase activity-chitobiosidase), and 4-methylumbelliferyl β-D-N,N,',N''-triacetylchitotriose (endochitinase activity) was used per the manufacturer's specifications. Chitinolytic hydrolysis was measured by adding 10 µl of culture supernatant to 90 µl of substrate in 96-well, black, flat-bottomed plates. The reaction was allowed to proceed for 30 min, and the fluorescent compound 4-methylumbelliferone was measured by a fluorometer with a 360 nm excitation and 450 nm emission wavelength. The amount of 4-methylumbelliferone released was calculated using a regression line generated with standard 4-methylumbelliferone.

Quantification of secondary metabolites produced by *P. protegens* Pf-5.

Extraction and HPLC analysis of secondary metabolites from cells and culture supernatants were performed as described previously (Hassan et al. 2010). *P. protegens* Pf-5 or derivative strains were grown at 20°C for 48 h with shaking (200 rpm) in culture tubes (18 mm diameter) containing 5 ml nutrient yeast broth (NYB) amended with 1% glycerol (vol/vol) and 0.35 mM $ZnSO_4$, a medium conducive to the production of all known antibiotics by Pf-5. NYB was composed (per liter) of 8 g Difco nutrient both and 5 g Bacto yeast extract (Becton, Dickinson and Co.). Cultures from two replicate tubes were extracted and analyzed by HPLC.

For quantification of secondary metabolites produced by Pf-5 and derivative strains in mushroom tissue, the mushrooms were inoculated and incubated as described above for the toxicity assays. Metabolites were extracted from lesions excised from the mushroom cap. An equivalent volume of mushroom cap tissue was also excised from sites inoculated by strains that did not cause visible lesions. The excised tissue was macerated in 1 ml of water using a glass rod and was extracted twice in 2.5 ml of ethyl acetate. For each strain, three replicates were evaluated, with each replicate composed of tissue excised from seven to nine lesions or inoculation sites on an individual mushroom. The ethyl acetate extracts of the tissue samples from an individual replicate were pooled, were dried under vacuum, and were resuspended in methanol for analysis by HPLC, which was done as described previously (Hassan et al. 2010).

To determine extraction efficiencies, authentic samples of pyoluteorin, DAPG, MAPG, and pyrrolnitrin were suspended in 10% methanol. A volume needed to deposit 5.0 µg (pyoluteorin, DAPG, and pyrrolnitrin) or 10.0 µg (MAPG) of each compound was spotted at three distinct sites on the surface of a peeled mushroom cap. An equivalent volume of 10% methanol was spotted on the mushroom caps to serve as controls. Mushrooms were incubated for 48 h at 27°C, at which time discoloration was observed at sites treated with some of the compounds. Mushroom cap tissue was excised and extracted by the methods described above, with each inoculation site serving as a replicate. Recovery rates were calculated by dividing the amount of compound extracted by the amount deposited were as follows: MAPG, 0.04%; DAPG, 0.20%; pyoluteorin, 0.07%; and pyrrolnitrin, 22%.

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