Chromosomal Insertion of Phenazine-1-Carboxylic Acid Biosynthetic Pathway Enhances Efficacy of Damping-off Disease Control by *Pseudomonas fluorescens*

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A disarmed Tn5 vector (pUT::Ptac-*phzABCDEFG*) was used to introduce a single copy of the genes responsible for phenazine-1-carboxylic acid (PCA) biosynthesis into the chromosome of a plant-growth-promoting rhizobacterium *Pseudomonas fluorescens*. The PCA gene cluster was modified for expression under a constitutive Ptac promoter and lacked the *phzIR* regulators. PCA-producing variants significantly improved the ability of the wild-type *P. fluorescens* to reduce damping-off disease of pea seedlings caused by *Pythium ultimum*, even under conditions of heavy soil infestation. Under conditions of oxygen limitation that are typical of the rhizosphere, PCA production per cell in vitro was greater than that recorded in fast-growing, nutrient-rich cultures. Similarly, when the in vitro nutrient supply was limited, *P. fluorescens*::*phz* variants that produced the most PCA effectively competed against *P. ultimum* by suppressing mycelial development. Soil-based bioassays confirmed that the level of PCA biosynthesis correlated directly with the efficacy of biological control and the persistence of inocula in soil microcosms. They also showed that soil pretreatment with bacteria provides a suitable method for plant protection by reducing infection, effectively decontaminating the soil. These data demonstrate that the insertion of a single chromosomal copy of the genes for a novel antifungal compound, PCA, enhances the ecological fitness of a natural isolate already adapted to the rhizosphere and capable of suppressing fungal disease.

The infestation of soils and seeds by plant pathogenic *Pythium* spp. causes serious agronomic losses. As a result, considerable effort has been made in the development of natural

isolates for the biological control of damping-off disease. Plant-growth-promoting rhizobacteria (PGPR), particularly soil- and rhizosphere-competent *Pseudomonas* spp., produce antimicrobial metabolites that are effective in disease control by the direct suppression of fungal phytopathogens. Notable variation in the performance of PGPRs has been recorded, particularly in field trials. In order to reduce and control variation, it is necessary to undertake detailed assessments of the mechanistic nature of disease control. These studies have included the description of the genetic basis of the mode of fungal antagonism mediated by suppressive bacteria and the identification of factors that influence colonization and persistence in soils and the rhizosphere.

It is apparent that many of the candidate antagonists produce a range of antifungal compounds that may be either broad spectrum or more specific in their activity. For example, a number of *Pseudomonas* spp. have the capacity to synthesize phenazines. The operon responsible for directing the biosynthesis of these nitrogen-containing heterocyclic compounds has been described for a number of isolates, including *P. fluorescens* 2-79 (Mavrodi et al. 1998) and *P. aureofaciens* 30-84 (Pierson et al. 1995; Pierson and Thomashow 1992).

In vitro and in vivo studies confirm that phenazine-1-carboxylic acid (PCA) biosynthesis by *P. fluorescens* 2-79 is an essential component for effective control of take-all disease of wheat caused by *Gaeumannomyces graminis* var. *tritici* (Thomashow and Weller 1988). PCA has the capacity to inhibit the mycelial growth of several plant fungal pathogens, including *Fusarium* spp. and *Pythium* spp. (Anjaiah et al. 1998). The production of PCA in the rhizosphere also was necessary for effective competition against indigenous microflora and the persistence of *P. fluorescens* 2-79 and *P. fluorescens* 30-84 in soils (Mazzola et al. 1992). More recently, the sequence of the PCA biosynthetic locus of *P. fluorescens* 2-79 has been described and its gene organization determined (Mavrodi et al. 1998). The expression of the seven biosynthetic genes, *phzABCDEFG*, is under control of the regulatory genes *phzI* and *phzR*, components of a density-dependent quorum-sensing circuit. This dependence has been demonstrated

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in the rhizosphere, where N-acyl-homoserine lactone signals produced by an isogenic strain restored the expression of phenazine in a *phzI* deletion mutant of strain 30-84 (Wood et al. 1997).

In previous investigations, we described the characterization of *P. fluorescens* SBW25 isolated from the sugar beet rhizosphere (Bailey et al. 1995). *P. fluorescens* has been shown to survive well in soils and in the rhizosphere and to be effective in the control of a number of diseases caused by fungal phytopathogens, particularly damping-off disease caused by *Pythium* spp. (Ellis et al. 2000). Genetic analysis of *P. fluorescens* SBW25 by transposon mutagenesis produced a representative bank of biocontrol-deficient mutants but no single, specific common locus was identified (Ellis 1998). The bacterium did not produce or have the genetic capacity to express the antifungal metabolites PCA, pyoluteorin, hydrogen cyanide, or 2,4-diacetylphloroglucinol (Ellis et al. 2000). Although no specific antifungal metabolites were identified in this *P. fluorescens* strain, studies demonstrated that its principal modes of action were in the suppression of oospore germination by *P. ultimum* Trow, direct competition for nutrients based on assessment of the Biolog sole carbon utilization index, and to a lesser extent, reduction in hyphal extension (Ellis et al. 1999).

Here we demonstrate that introducing the capacity for constitutive PCA biosynthesis significantly enhances the biological control ability of *P. fluorescens* to persist in soils, suppresses infestation, and controls pea seedling infection by *P. ultimum*. The isolate was modified by the chromosomal insertion of a single copy of *phzABCDEFGHI* under the control of a *Ptac* promoter in the absence of the known regulatory components *phzI* and *phzR*. Previous investigations of complementation or heterologous expression of introduced genes for syn-

thesis of antifungal metabolites typically describe the use of plasmid vectors or the overexpression of native antifungal metabolites (Fenton et al. 1992; Reimann et al. 1997; Sarniguet et al. 1995; Schnider et al. 1995). Here we report that the introduction of a single copy of the genes required for PCA biosynthesis into the chromosome *P. fluorescens* significantly enhances its ability to control damping-off disease in peas.

RESULTS

Isolation, characterization, and biological assessment of PCA-producing transposon mutants, *P. fluorescens*::*phz*.

Following transposon mutagenesis of *P. fluorescens* with pUT::P_{tac}-*phzABCDEFGHI* (mini-Tn5 vector carrying PCA biosynthetic genes), 10 isolates were selected, which produced green crystals of PCA on Luria-Bertani (LB) agar with 1.5% glucose and growth rates comparable to that of the wild type in antibiotic-free nutrient broth (NB) at 16 and 28°C (data not shown). Each isolate had a different site of single chromosomal insertion of the *phzABCDEFGHI*-kanamycin-resistance cassette as confirmed by Southern hybridization. Analysis of the extracted genomic DNA confirmed the absence of plasmid DNA in the wild type and the variants. The amounts of PCA produced by the wild-type *P. fluorescens* and *P. fluorescens* 2-79 and the 10 variants were assessed in stationary phase cultures. Levels of PCA in wild-type *P. fluorescens* were zero when measured at 340_{nm}. *P. fluorescens* 2-79 and the variants expressed different amounts of PCA as indicated by optical density (OD) 340_{nm}.

The amounts of PCA produced under optimal and stressed (presumed oxygen-limiting) growth conditions were also compared for four of the variants, which were inoculated at the same cell density from saturated overnight cultures into LB 1.5% (wt/vol) glucose and incubated at 28°C. Despite a significant decrease in growth rate for stressed cells, greater concentrations of PCA were synthesized per cell at these lower growth rates (Fig. 1). *P. fluorescens*::*phz* inocula, derived from stationary phase cultures, produce relatively greater quantities of PCA per cell than *P. fluorescens* 2-79. Expression levels were maintained under imposed oxygen stress that may be typical of soil habitats, as oxygen can be limiting in the rhizosphere (Dekkers et al. 1998; Hojberg et al. 1999).

PCA production increases competitiveness against *P. ultimum*.

The relative fitness, as determined by estimates of growth rate for each pseudomonad variant, was determined following inoculation of 3×10^6 CFU/ml in 1/10 NB and in 1/10 NB supplemented with 4% (vol/vol) pea seed exudate (PSE) in direct competition with *P. ultimum* (3×10^6 propagules per milliliter) (Table 1) to simulate conditions in the rhizosphere. The mean generation time (*td*) for the bacteria was estimated over the log growth phase and included a minimum of eight independent data points (*n* = 2). A typical sigmoidal growth curve was observed when wild-type *P. fluorescens* or the *P. fluorescens*::*phz* variants were grown in 1/10 NB supplemented with 4% (vol/vol) PSE (*td* = 1.29 h). In 1/10 NB alone, the growth rates for the variants were reduced signifi-

Fig. 1. Comparison of rates of phenazine-1-carboxylic acid (PCA) synthesized per cell with growth rate in liquid culture (Luria-Bertani + 1.5% glucose) under aerated, standard conditions (circles) and under oxygen-limiting, stressed conditions (squares) for *Pseudomonas fluorescens* 2-79; *P. fluorescens*::PHZ, 23.1; ::PHZ, 23.4; ::PHZ, 23.8; and ::PHZ, 23.10. Phenazine production per cell was calculated as 340_{nm}/620_{nm} and growth rate per hour as generations per hour. Data for wild type not shown because no PCA was produced.

cantly ($td = 3.61$ h), but did not differ from that of the wild type under the same conditions. Although the presence of *P. ultimum* did not significantly influence growth in media supplemented with PSE ($td = 1.43$ h), the presence of *P. ultimum* oospores in nutrient-poor conditions (1/10 NB) reduced bacterial growth rate $td = 5.29$ h (95% analysis of variance [ANOVA] confidence interval 4.35 to 5.54). Growth of the variants under nutrient limitation in the presence of *P. ultimum*, however, was significantly greater than that of the unmodified wild-type *P. fluorescens* ($td = 7.87$) or the apparent PCA⁻ mutant 23.1, whose PCA production was almost negligible at OD 340_{nm} ($td = 6.21$). Growth of *P. fluorescens* 2-79, the source of the PCA gene cluster, was not significantly affected in 1/10 NB in competition with *P. ultimum* ($td = 6.06$ versus 5.69). The enhanced growth of the PCA⁺ variants, relative to the parental strain when grown together with *P. ultimum* and the inability of *P. ultimum* to impair growth of 2-79 under the same conditions, implicates PCA as a competitive growth factor. When data for individual variants were compared against the mean value for the collection, growth rates for *P. fluorescens::phz*-23.4 and 23.10 were significantly greater ($P = 0.05$) under nutrient-limiting conditions in competition with *P. ultimum* ($td = 3.63$ and 1.19, respectively) (Table 1). This elevated activity was not as apparent in the presence of PSE where additional nutrients were available (Table 1) and the competitive advantage correlated with high PCA production (Fig. 1). Of particular relevance is the enhanced growth rate of 23.10 ($td = 1.19$) in 1/10 NB in competition with *P. ultimum* when compared with 1/10 NB alone ($td = 2.04$), which is approximately four times the rate observed for other PCA⁺ variants or the wild type.

Enhanced biological activity of PCA-producing *P. fluorescens::phz* strains in the suppression of *P. ultimum* growth and infection of pea seedlings.

PCA⁺ variants and wild-type strains were compared in order to demonstrate their ability to inhibit oospore germination and

hyphal extension of *P. ultimum* in vitro. We calculated the mean inhibition area zone of clearing, which represents inhibition of *P. ultimum* oospore germination and hyphal extension on agar plates (Fig. 2). After 30 h of incubation, all variants containing Ptac-*phz*ABCDEF, with the exception of *phz* 23.2, inhibited oospore germination and hyphal extension to a greater extent than the wild-type strains. When efficacy was measured after 48 h of incubation, only variants *P. fluorescens::phz* 23.4 and 24.10 remained effective in their sustained ability to maintain clear zones free of mycelia around the colonies.

Soil-based microcosm assays were designed to simulate treatment of *P. ultimum*-infested seeds (condition A), pretreatment of uninfested soils to establish biological control activity (condition B), and decontamination of infested soils prior to planting (condition C). All soil-based assessments of disease suppression were undertaken in soils infested with approximately 1×10^6 *P. ultimum* propagules per gram of soil. This quantity represents approximately 100-fold more pathogen inoculum than that in which wild-type *P. fluorescens* controls damping-off disease of peas at 95% efficacy (data not shown). Variation in the performance among individual strains of bacterial inocula was observed under the conditions tested. Some transposon variants (*phz::*23.1 and *phz::*23.8) performed less effectively than the wild-type strain, whereas others (*phz::*23.4 and *phz::*23.10) were more effective. Figure 3 shows the biological control assessment (BCA) scores of these individual variants and wild types under the three assay conditions. Disease suppression was optimal when *P. ultimum*-infested soils were treated with bacteria for up to 42 days prior to planting (Fig. 3). The average semiquantitative biological control score for PCA-producing variants ($n = 10$) was 4.3 in direct control of infection at the time of planting, 6.1 for soil pretreatment, and 8.1 in the treatment of contaminated soils. The effectiveness of pretreatment of infested or *P. ultimum*-free soils (subsequently planted with infected seed) demonstrated that the activity of the bacterial inocula was

Table 1. Fitness effect of phenazine production under nutrient limitation in vitro

Strain	Mean generation time (td) of bacterial culture ^a			
	1/10 nutrient broth (NB)	1/10 NB + <i>Pythium ultimum</i>	1/10 NB + PSE ^b	1/10 NB + PSE ^b + <i>P. ultimum</i>
SBW 25 (wt)	3.59	7.87	1.34	1.36
2-79 (wt)	6.06	5.69	2.73	3.41
<i>Pseudomonas fluorescens::phz</i> mutants				
23.1	2.95	6.21	1.26	1.37
23.2	2.69	6.20	1.26	1.43
23.3	3.59	6.03	1.23	1.35
23.4	2.88	3.63	1.24	1.28
23.5	5.65	5.86	1.21	1.34
23.6	5.51	5.82	1.22	1.32
23.7	3.40	5.98	1.26	1.43
23.8	4.47	6.15	1.63	2.04
23.9	2.87	5.78	1.30	1.38
23.10	2.04	1.19	1.30	1.38
Mean	3.61	5.29	1.29	1.43

^a The td ($n = 2$ replicates $\times$ 10 variants) of *P. fluorescens* and *P. fluorescens::phz* variants in four different growth media: (a) 1/10 NB, (b) 1/10 NB + 3% *Pythium* spores, (c) 1/10 NB + 4% PSE, and (d) 1/10 NB + 4% PSE + 3% *Pythium* spores. By one-way analysis of variance, the growth rates of phenazine-1-carboxylic acid expressing variant bacteria in conditions c and d were not significantly different and formed a homogeneous subset. Growth rates in conditions a and b were significantly different ($P < 0.05$) from each other and conditions containing PSE.

^b Pea seed exudate of 4% in 1/10 NB. td estimates are at 28°C with shaking.

maintained for at least 42 days. The efficacy of individual PCA-producing variants was compared with the response of the variants as a whole with the Kruskal-Wallis test. A significant difference in efficacy was observed among the grouped performance of all variants in condition A when compared with condition C. Biological control in the pretreatment (condition B) was intermediate. The Kruskal-Wallis mean rank of scores was condition A, 10.1; condition B, 16; and condition C, 20.4.

Persistence and efficacy of PCA-producing *P. fluorescens::phz* strains in soils.

Comparisons of estimates of the population density of total and introduced pseudomonads in soil microcosms were made at $t = 0, 11, 25$, and 42 days. Persistence, as determined by estimates of colony-forming units on agar plates, varied from strain to strain after the inoculation of approximately 1×10^8 CFU/g of soil ($t = 0$) and declined gradually ($t = 42$ days) to background levels typical of pseudomonad populations in soil over the duration of the investigation (data not shown). Pathogen-free control soils, untreated soils (no bacterial inocula), and soils inoculated with bacteria (including wild type) 42 days after inoculation supported an average total population density of pseudomonads estimated at 1.74×10^4 CFU/g (coefficient of variation = 10%). To facilitate statistical analysis of the impact of constitutive expression of PCA on host fitness in soils, data from all 10 variants were combined and analyzed as a population. This approach provides a more realistic assessment of the effect of the introduced genes on a bacterium rather than the analysis of a single, optimized isolate. Furthermore, assessment of performance of an individual isolate is validated by such comparisons. A two-sample t test (equal variance) showed that the average estimates of CFU/g

of soil for total pseudomonads present after 42 days were not significantly different in either the presence ($P = 0.7$, 42 days = $4.95 \log_{10}$ CFU/g) or absence ($P = 0.11$, 42 days = $4.80 \log_{10}$ CFU/g) of *P. ultimum* inocula. Bacteria were inoculated at the same densities for $t = 0$ ($8.49 \log_{10}$ CFU/g). Population densities of *P. fluorescens::phz* variants were stable initially, 11 days in the presence of *Pythium* ($8.14 \log_{10}$ CFU/g), and in the absence of *Pythium* ($6.87 \log_{10}$ CFU/g), began to decline after 25 days in the presence ($P = 0.0002$; 25 days = $5.10 \log_{10}$ CFU/g; 42 days = $3.05 \log_{10}$ CFU/g) or absence ($P = 0.0003$; 25 days = $5.28 \log_{10}$ CFU/g; 42 days = $2.89 \log_{10}$ CFU/g) of *P. ultimum* inocula. It was not possible to directly assess performance against wild-type *P. fluorescens* because neutral selection markers were not available, although estimates of the total pseudomonad densities in microcosms inoculated with the wild type did not differ significantly from those inoculated with variant strains. It was assumed, therefore, that the presence of the PCA genes had no deleterious fitness effect in soils. The density of *P. ultimum*, established at approximately 1×10^6 propagules per gram at the time of inoculation, declined over the 42-day study period to approximately 1×10^3 propagules per gram in either the presence or absence of bacterial inocula.

DISCUSSION

To develop microbial inocula for improved biological control, it is necessary to identify those common features that distinguish certain rhizosphere strains with greater activity from other, less-effective fluorescent pseudomonads that are specialized for survival in soil. Indeed the mode of action of biological control agents, including rhizosphere-proficient fluorescent pseudomonads and other bacteria, is generally

Fig. 2. Agar plate assay and zone of clearing estimated after 30 h incubation on *Pseudomonas*-selective agar inoculated with *Pythium ultimum* oospore suspension. $n = 5$. Standard error shown.

multifactorial (Cook et al. 1995; Pierson and Weller 1994). For strains with specific activities, the regulated production of antifungal compounds at the soil root interface has a direct role in plant disease suppression. Further advances in understanding ecosystem function have been made recently through the identification of ecologically significant genes that provide insight into the mechanistic factors that influence fitness and activity of rhizosphere bacteria (Rainey 1999). More direct investigations of community dynamics and interactions between component antagonist populations and phytopathogens demonstrate the importance of competition for seed exudates in the suppression of *Pythium* development (Paulitz 1991) or root establishment (Gamliel and Katan 1992). The similarity in nutrient requirements, as demonstrated by the assessment of sole carbon source utilization patterns, between bacteria and phytopathogens may lead to the niche exclusion of deleterious fungi by resource exploitation (Ellis et al. 1999). The efficient assimilation of iron from soil also has been implicated in biocontrol efficacy (Becker and Cook 1988; Leong 1986; Loper and Buyer 1991). These features do not fully explain the disease-suppression attributes of fluorescent pseudomonads (Thomashow and Weller 1995). The capacity to produce antifungal compounds, the selective advantages that led to the evolution of these pathways, and the absolute function they play in the natural environment still have to be resolved, despite reports of their wide geographical distribution (Keel et al. 1996; Raaijmakers et al. 1997).

Studies also illustrate that the production of antifungal metabolites (Mazzola et al. 1992), cell signaling systems (Pierson and Pierson 1996), and selection and timing of inocula release (Thompson et al. 1995) provide direct advantage under competition. Therefore, rather than screening thousands of potential natural isolates for properties of plant disease suppression, it

may be more appropriate to select bacteria that demonstrate plant-growth promotion and rhizosphere-soil persistence and modify them to express novel functions to improve biological control. We tested this concept and demonstrated that PCA-producing variants of a natural antagonist of *P. ultimum*, *P. fluorescens* SBW25, have an enhanced ability to suppress damping-off disease in peas compared with either the wild type or *P. fluorescens* 2-79, the source of the PCA biosynthetic pathway.

In related studies, enhanced disease suppression has been demonstrated through modifications to improve expression of or by complementation with auxiliary antifungal traits, which are typically carried on plasmids in the recipient host bacteria. Here we report on chromosomal insertion and high-level expression of a single copy of the seven-gene *phzABCDEFG* biosynthetic pathway into a fluorescent pseudomonad isolated from the sugar beet rhizosphere. Chromosomal insertion provides genetic stability and effective gene containment, important factors in minimizing the potential for horizontal gene transfer in the development of genetically modified microorganisms for biological control and field release (Bailey et al. 1995). Transposon mutagenesis is a random process that often results in gene disruption with concomitant effects on cell physiology. To address this issue, a number of variants were evaluated in growth assays and a subpopulation of 10 variants was studied in detail. Variation was observed in the amounts of PCA produced and the rates of cell growth under nutrient-limiting conditions or in competition with the fungal pathogen. The 10 variants (with the exception of *::phz23.2*) in the analyzed population were effective in reducing hyphal extension and oospore germination of *P. ultimum* in vitro and, with the exception of *::phz23.1*, outperformed either wild-type bacteria in the bioassays for disease suppression in vivo. Inocula persistence in soils did not appear to be adversely af-

Fig. 3. Three conditions were examined in the soil bioassays for disease control. Soils inoculated with *Pythium ultimum* oospores (1×10^6 propagules per gram) were mixed with washed *Pseudomonas fluorescens* variants (1×10^8 CFU/g) or water, planted with pea seeds ($n = 15$), and incubated at 20°C to simulate (a) infectivity: inoculated soils planted with seed and incubated at 20°C for 7 days (left columns); (b) soil pretreatment: soils inoculated with washed bacteria and incubated for 42 days and then mixed with *P. ultimum* oospores and pea seeds ($n = 15$) and incubated at 28°C for an additional 7 days (middle columns); and (c) soil decontamination: soils inoculated with *P. ultimum* oospores and washed *Pseudomonas* isolates, incubated for 42 days, and then planted with pea seeds ($n = 15$) and incubated 28°C for 7 days (right columns). The biological control assessment score was a semiquantitative measure of complete disease suppression (10) to seed infection with no germination (1). Data presented as mean summation of response of replicated treatments for wild-type bacteria, water control, and the four variants to express different levels of phenazine. Standard error shown.

fected for the PCA-producing variants compared with the wild type. This supports the findings of Mazzola et al. (1992), who demonstrated loss of fitness for nonphenazine-producing mutants of *P. fluorescens* 2-79.

The data from the three models tested in this study underline the need for careful consideration of the method of treatment and application in the optimization of disease suppression. Pretreatment of soils or treatment of infested soils prior to planting improved disease suppression by almost 50 and 100%, respectively. In vitro studies confirmed the elevated expression of PCA in stationary phase cultures and cultures grown under oxygen- and nutrient-limiting conditions. The enhanced biological activity of inocula varied with respect to the levels of PCA biosynthesis for each variant. These differences were consistent among assays. Variant 23.10 had the shortest *td* in the low-nutrient medium with and without *Pythium* oospores, produced greater amounts of PCA per cell, did not have impaired fitness in the persistence and survival studies, and provided the greatest degree of protection against preemergence damping-off. The large reduction in the generation time of strain 23.10 in reduced-strength NB in the presence of *Pythium* oospores is far greater than that shown by 23.4 and 23.8 (Table 1), which produced similar levels of PCA. This could be a consequence of the site of insertion affecting the growth rate in this particular medium. PCA production in the transgenic variants was under the control of the constitutive *Ptac* promoter (Mavrodi et al. 1998). Expression profiles of these constructs differed from those of *P. fluorescens* 2-79, which contained the fully regulated operon. PCA concentrations increased with time in growth media, although it was not established whether this is a result of accumulation or an increased rate of synthesis during the stationary phase.

The data reported here confirm that the in situ performance of an already effective biological control agent such as *P. fluorescens*, which survives well in soils and is competitive in low-nutrient environments, can be significantly enhanced by the insertion of novel functional traits. The biological control of the strain was enhanced by the insertion of a single copy of the PCA biosynthetic genes into the chromosome. This is relevant because such constructs are genetically stable and fitness and ecological factors are less likely to be affected by a single-copy insertion compared with modifications based on plasmid carriage (Lilley and Bailey 1997). The expression of the *phz* genes following the chromosomal insertion of an unregulated *phzABCDEFG* operon into *P. fluorescens* also increased in vitro growth rates for one mutant, 23.10, under conditions of nutrient limitation in the presence of *P. ultimum*, which may account for its enhanced ability to suppress damping-off disease of pea seeds.

MATERIALS AND METHODS

Microorganisms and growth conditions.

Fluorescent pseudomonad strains were maintained on *Pseudomonas* selection agar (PSA) (Oxoid Ltd., Basingstoke, Hampshire, U.K.) at 28°C and *Escherichia coli* strains on LB agar (Oxoid) at 37°C and supplemented with antibiotics as appropriate. *P. fluorescens* SBW25 is a prototrophic, wild-type strain isolated from the sugar beet phytosphere (Bailey et al. 1995) that is able to control a variety of fungal diseases of seeds and seedlings (Ellis 1998). *P. fluorescens* 2-79, which

produces PCA (Thomashow and Weller 1988), was included as a wild-type control for the bioassays. All bacterial isolates were stored in 70% glycerol (vol/vol) and 0.85% sodium chloride (wt/vol) at -70°C. Isolates were revived by streaking on Tryptic Soy Broth (Difco Laboratories, Detroit, MI, U.S.A.) with 1.2% (wt/vol) agar. Inocula for all assays were produced by growing isolates in 5 ml of LB in 30-ml sterile tubes at 28°C with shaking to a stationary phase for approximately 18 h. Cells were pelleted by centrifugation at 5,000 × *g* and washed twice in sterile double-distilled water (ddH₂O) before resuspending to the original culture volume in ddH₂O.

P. ultimum was stored in the dark at 15°C as infested soil was prepared by drying and sieving spent soil from infected seed trials (Murray 1994). Essentially, *P. ultimum*-infested soil inoculum was prepared by mixing 1 liter of sterilized Mendip loam (Minster Brand Products, Heatherwood Nurseries, Ashington, Winborne, U.K.) with 6 g of oospore inoculum (Zeneca Agrochemical, Jealott's Hill Research Station, Bracknell, Berkshire, U.K.) and 2 g of Beam natural wheat germ (Vitamins Company, Brentford, Middlesex, U.K.). Infested compost (500 g) was placed in seed trays (16 × 21 × 5 cm) and planted with 25 pea seeds that were covered with compost and watered with 100 ml of ddH₂O. Trays were incubated at 15°C in a growth cabinet and watered daily with 100 ml of ddH₂O for 1 week. During the second week, watering was reduced gradually to encourage the production of oospores. The aerial portion of the plants was removed after 2 weeks. The infested compost was air-dried at 15°C for 4 weeks, sieved (2-mm pore), sealed in airtight containers, and stored in the dark up to several months.

A suspension of *P. ultimum* oospores was prepared and used as a semiquantifiable source of fungal material in the plate- and culture-based assays. Oospore suspensions were produced, essentially as described by Johnson (1988). Nine-millimeter plugs from 3-day-old cultures on potato dextrose agar (PDA) (Oxoid) were placed in 20 ml of cornmeal medium (10 g of cornmeal per liter heated to 60°C and filtered before autoclaving) and incubated in the dark at 15°C for 3 to 6 weeks. The oospores were separated from hyphae by mixing the culture with an equal volume of ddH₂O and blended in a Waring (New Hartford, CT, U.S.A.) blender at room temperature for 1 to 2 min. Hyphal mass was removed from the suspension by filtration through a 150-μm sieve and washed in ddH₂O twice by centrifugation. Oospores were resuspended in ddH₂O and stored at 4°C. Air-dried, commercial pea seeds (*Pisum sativum* cv. Bohartyr) (Nickerson Seeds, Lincolnshire, U.K.) were stored in the dark at 15°C until they were required.

Construction of pUT::Ptac-*phzABCDEFG* and transposon mutagenesis of *P. fluorescens*.

The *phzABCDEFG* biosynthesis genes were isolated from *P. fluorescens* 2-79 (Mavrodi et al. 1998) and introduced as a 6.7-kb *HindIII-XbaI* fragment into pALTER-Ex1 (Mavrodi personal communication) downstream of a *tac* promoter. The *Ptac-phzACDEFG* cassette was transferred into the *SfiI* site of the mini-Tn5Km1 vector (de Lorenzo et al. 1990) and mobilized from *E. coli* S17/1 λ_{pir} into *P. fluorescens* under standard mating conditions. Transconjugants were counterselected on PSA supplemented with 100 μg of kanamycin solution per milliliter. Their ability to express PCA was determined on LB and 1.5% glucose where PCA-producing colonies developed green pigmentation.

In vitro screening of *P. fluorescens::phz* strains against oospore germination and hyphal extension.

PSA plates were spread with 100 µl of oospore inoculum (1×10^7 propagules per milliliter), allowed to dry for 15 min, and inoculated with 10 µl droplets of washed overnight culture (1×10^8 CFU/g) of wild-type bacteria or transposon variants. Plates were incubated at 28°C. The diameter of the clear zones was measured after 30 and 48 h to assess the inhibition of hyphal extension.

In vitro competition assay.

Overnight cultures of *P. fluorescens* wild type and PCA-expressing variants were grown in LB at 28°C with shaking (180 rpm) for 18 h. They were then harvested by centrifugation, washed twice, and resuspended in equal volumes of ddH₂O. Bacterial inocula ($n = 2$) (3×10^6 CFU) were inoculated into 350 µl of (a) 1/10 NB, (b) 1/10 NB + 3% (vol/vol) Pythium oospore preparation (3×10^5 propagules per milliliter), (c) 1/10 NB + 4% (vol/vol) PSE (100 g of pea seeds and 250 ml of ddH₂O at 28°C for 10 h and filter sterilized), or (d) 1/10 NB + 4% (vol/vol) PSE + 3% (vol/vol) Pythium oospore preparation. Multiwell plates were incubated at 28°C with constant shaking. The cell density (OD 600nm) was measured at 30-min intervals for 20 h to assay growth rates directly with a Bioscreen C microplate reader (Lab Systems, Altrincham, U.K.).

Assessment of PCA production by *P. fluorescens::phz* mutants.

After assessment of PCA production by the 10 variants on LB agar with 1.5% glucose was confirmed by production of green crystals of PCA in the colonies, production of PCA in cultures was determined. Cultures ($n = 3$) of wild-type bacteria (*P. fluorescens* and *P. fluorescens* 2-79) and 10 *P. fluorescens::phz* transposon variants were grown in LB supplemented with 1.5% glucose at 28°C with shaking (180 rpm) for 18 h. Cell densities (OD 620nm) were determined, and PCA concentrations in the cell-free supernatant were measured at 340 nm. Wild-type *P. fluorescens* produced no PCA; therefore, absorbance measured at 340 nm was zero. Different levels of PCA production in the 10 individual *P. fluorescens::phz* transposon variants were observed. Further investigations were carried out on four variants, which showed a range of PCA production (*P. fluorescens* 23.1 low, 23.4 medium-high, 23.8 medium, and 23.10 high).

Investigations of growth rate and levels of PCA production also were assessed for these selected variants under normal growth conditions and conditions of oxygen-limited stress. Cultures of wild-type bacteria (*P. fluorescens* and *P. fluorescens* 2-79) and the four variants (*P. fluorescens* 23.1, 23.4, 23.8, and 23.10) were grown at 28°C with shaking (180 rpm) for 18 h. Washed bacteria (500 µl) were inoculated into 50 ml of LB + 1.5% glucose and grown in sealed flasks without headroom and compared with cultures grown with a broth to air ratio of 1:5 per culture volume with shaking at 180 rpm and incubation at 28°C ($n = 3$). Cell density (OD 620nm) and PCA levels (OD 340nm) were measured at 1-h intervals. These values were used to calculate *td* and phenazine production rate.

Estimates of *td* and PCA biosynthesis were compared by ANOVA. Significant differences were identified by the Tukey "Honestly Significantly Different" interval method and a 95% significance level.

$$\frac{\ln t_1 - \ln t_0}{\Delta t} = \mu h^{-1} \quad td = \frac{\ln 2}{\mu}$$

in which t_0 = OD at time 0, t_1 = OD at time 1, and μ = specific growth rate and PCA production rate.

Microcosm design.

Microcosms were established in 50 ml polypropylene tubes containing 30 g of commercial sterilized Mendip-loam soil. Treatments included (a) inoculation with 1×10^6 propagules per gram of *P. ultimum* oospores supplemented with 0.2 g of Bemax natural wheat germ; (b) treatment of similar *P. ultimum*-infested soils with approximately 1×10^8 CFU/g of each strain of a washed, overnight culture of bacteria resuspended in ddH₂O; and (c) control treatments of soils inoculated with water only. Bacteria (approximately 3×10^8 CFU/g) were inoculated into microcosms from an overnight culture washed twice in ddH₂O and resuspended in equal volumes of ddH₂O after inoculation. These counts were confirmed by sampling onto PSA. Microcosms were incubated at 20°C, and samples of 1 g of soil were collected at 11, 25, and 42 days and resuspended in 20 ml of ddH₂O by vortex mixing in the presence of approximately 20×5 -mm glass beads for 5 min. Soil suspensions were serially diluted and plated onto PSA (Oxoid CM 559) containing 10 µg of centrimide, 10 µg of fucidin, and 50 µg of cephaloridine (CFC, Oxoid SR103E) per milliliter for total counts and supplemented with 100 µg of kanamycin per ml to enumerate transposon-marked strains. *P. ultimum* propagules were enumerated by spreading soil suspensions on PDA supplemented with 320 µg of aureomycin per milliliter. Plates were incubated at 28°C and counts were expressed as colony-forming units per gram of wet soil.

Assessment of biological control activity.

The persistence and biological activity of bacterial inocula were evaluated under three different conditions. Condition a was designed to assess disease control in infested soils treated with bacteria at the time of sowing. Sterilized Mendip loam soil was mixed with washed bacteria suspended in water (1×10^8 CFU/g of each variant) and *P. ultimum* (1×10^6 oospores/g) and sown with pea seeds. Conditions b and c were designed to evaluate the effectiveness of bacteria inoculated into soil 42 days prior to sowing against *P. ultimum* that was introduced at the time of bacterial inoculation (soil pretreatment, condition b) or at the time of sowing (soil decontamination, condition c). To evaluate biological control activity under each condition, soil (27 g) from each replicate was placed into 85-mm petri dishes containing five Whatman (Clifton, NJ, U.S.A.) filter papers (75-mm diameter), and then pea seeds ($n = 15$) were added to the dishes. Assays were placed in a plant propagator for an 18-h photoperiod at 20°C for 7 days before a semiquantitative BCA of disease suppression was performed. The BCA scale ranged from 100% infection (0) to 100% seed germination (10), and scoring for mycelial development on all of the independent assays was included. The average biological control score was calculated for PCA-producing variants and an analysis of the efficacy of individual variants was compared with the Kruskal-Wallis test, which assesses scores by simple ranking. Under all conditions tested, the pathogen load was (1×10^6 propagules per gram), an inoculum level 100-fold greater than that normally used in the standard assay in which *P. fluorescens* wild type has greater than 95% efficacy for disease control (Ellis et al. 1999).

Statistical methods.

Sample means were tested for significant differences by the pooled variance *t* test. This test requires equal variances, which were tested for by the F-test. Transformed data ($\log_{10}$) had equal variances and were normally distributed.

Doubling times and the $\log_{10}$ counts in the persistence assay were compared by ANOVA. To confirm the suitability of the data, tests for homogeneity of variances (Bartlett's box F test, Cochran's C test, Hartley's $F_{\max}$ test) and tests for normality of residuals (normal probability plots) were calculated by Unistat version 4.5 (Unistat Ltd., London) and Systat version 5 (Systat Inc., Evanston, IL, U.S.A.). For ANOVA, data were transformed by $\log_{10}(\text{CFU/g} + 1)$ to ensure homogeneity of variances.

The BCA score for the in planta assays of models a, b, and c (new infection, pretreatment, and preconditioning) were compared by the Kruskal-Wallis test. This simple, non-parametric test compared the medians of multiple samples derived from the ranking of the BCA score.

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