Involvement of Phenazines and Anthranilate in the Antagonism of *Pseudomonas aeruginosa* PNA1 and Tn5 Derivatives Toward *Fusarium* spp. and *Pythium* spp.

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Accepted 12 May 1998.

Pseudomonas aeruginosa PNA1, isolated from the rhizosphere of chickpea in India, suppressed *Fusarium* wilt of chickpea, caused by *Fusarium oxysporum* f. sp. *ciceris*, and *Pythium* damping-off of bean, caused by *Pythium splendens*. When grown in culture, PNA1 produced the phenazine antibiotics phenazine-1-carboxylic acid and oxychloraphene, and inhibited mycelial growth of *F. oxysporum* f. sp. *ciceris*, *P. splendens*, and certain other phytopathogenic fungi. Two mutants (FM29 and FM13) deficient in phenazine production were obtained following transposon mutagenesis of PNA1. The transposon in the genome of FM29 was localized to *phnA*, which is thought to encode a subunit of anthranilate synthase II involved in the phenazine biosynthesis. The FM13 mutation was complemented by *trpC*, which encodes indole glycerol phosphate synthase in the tryptophan biosynthesis pathway; consequently, FM13 could not grow on a minimal medium in the absence of tryptophan. Neither FM29 nor FM13 suppressed *Fusarium* wilt of chickpea to the level achieved by the wild-type strain, indicating that phenazine production contributed to the biocontrol of this disease by *P. aeruginosa* PNA1. FM29 was also less effective than the phenazine-producing parental strain in biological control of *Pythium* damping-off of bean, but FM13 was as effective as the parental strain in suppressing this disease. Anthranilate, an intermediate in the tryptophan biosynthesis pathway, suppressed mycelial growth of *Pythium* spp. in culture and *Pythium* damping-off of bean and lettuce. Anthranilate, excreted by FM13 as a consequence of the *trpC* mutation, may have contributed to the suppression of *Pythium* damping-off by the mutant.

Additional keywords: anthranilate synthase, antifungal compound, biocontrol.

Some members of the fluorescent pseudomonads produce and excrete phenazines, which are pigmented, nitrogen-containing heterocyclic compounds that inhibit mycelial growth of several fungal pathogens of plants (Haynes et al. 1956; Sierra and Veringa 1958; Turner and Messenger 1986). Phenazine-1-carboxylic acid (PCA), produced by the root-colonizing bacteria *Pseudomonas fluorescens* 2-79 and *P. aureofaciens* 30-84, has a dominant role in the control of take-all disease of wheat caused by the fungus *Gaeumannomyces graminis* var. *tritici* (Hamdan et al. 1991; Pierson and Thomashow 1992; Thomashow and Weller 1988). Phenazine-deficient mutants from these two bacteria fail to inhibit mycelial growth of *G. graminis* var. *tritici*, and are significantly reduced in their ability to suppress take-all, whereas mutants genetically restored for the production of PCA coordinately regain antifungal activity in culture and on wheat (Pierson and Thomashow 1992; Thomashow and Weller 1988). The same mutants are impaired in their ability to effectively compete with the indigenous microflora on plant roots (Mazzola et al. 1992; Pierson and Pierson 1996). PCA has been isolated from a wheat rhizosphere colonized by strains 2-79 and 30-84 and its presence correlated with disease suppression (Thomashow et al. 1990). Likewise, pyocyanine, a phenazine produced by *P. aeruginosa*, suppresses wheat blotch caused by *Septoria tritici* (Flaishman et al. 1990).

Phenazines are synthesized from chorismic acid, possibly via anthranilate, which is also an intermediate in the tryptophan biosynthesis pathway (Fig. 1). Essar et al. (1990a) proposed that anthranilate is synthesized from chorismate by two distinct anthranilate synthases in *P. aeruginosa*. Anthranilate synthase I, which is required for tryptophan biosynthesis, is composed of two subunits encoded by *trpG* and *trpE*, whereas anthranilate synthase II, required for phenazine production, is encoded by *phnA* and *phnB*. PhzB, whose deduced amino acid sequence is similar to those of anthranilate synthases, is required for phenazine antibiotic production in *P. aureofaciens* 30-84 (Pierson et al. 1995). Anthranilate itself has antifungal activity: anthranilate inhibits mycelial growth of *G. graminis* var. *tritici*

and, when grown in culture medium, *P. fluorescens* 2-79 produces concentrations of anthranilate adequate for fungal inhibition. Nevertheless, anthranilate had a limited role in suppression of wheat take-all disease by strain 2-79 (Hamdan et al. 1991).

In this paper, we describe *P. aeruginosa* PNA1, a strain isolated from the chickpea rhizosphere in India, that suppresses Fusarium wilt of chickpea, caused by *Fusarium oxysporum* f. sp. *ciceris*, and Pythium damping-off of bean, caused by *Pythium splendens*. PNA1 produces two phenazine antibiotics, PCA and phenazine-1-carboxamide (oxychlororaphine, or OCP), that contribute to biological control of these plant diseases. We also demonstrate that anthranilate inhibits mycelial growth of *Pythium* spp. in culture and suppresses Pythium damping-off of lettuce and bean when added to soil. The excretion of anthranilate by a tryptophan auxotroph of PNA1, which did not produce PCA and OCP, may contribute to the capacity of this mutant to inhibit *Pythium* spp. in culture and in the rhizosphere.

RESULTS

Selection of strain PNA1.

A total of 98 fluorescent pseudomonads were isolated from the rhizosphere of chickpea (*Cicer arietinum* L.) and purified. All strains produced a yellow-green fluorescent pyoverdine but only eight isolates inhibited mycelial growth of *F. oxysporum* f. sp. *ciceris* on an agar medium. Strain PNA1 was selected for further study because it inhibited mycelial growth of all fungal species tested (*F. oxysporum* f. sp. *ciceris*, *Fusarium udum*, *Sclerotium rolsii*, *Macrophomina phaseolina*, *Pythium debaryanum*, and *P. splendens*). Strain PNA1 was classified as *P. aeruginosa* by API 20 NE strips for non-enteric, gram-negative rods.

Detection and identification of antifungal compounds.

Filter-sterilized culture supernatants of *P. aeruginosa* PNA1 had antifungal activity, suggesting that one or more diffusible metabolites were responsible for inhibition of fungi. Two metabolites (PN1 and PN2), isolated via preparative thin layer chromatography (TLC), had UV spectra characteristic of phenazine metabolites produced by other strains of *Pseudomonas* spp. ($UV_{max} = 251$ and 368 nm; Kanner et al. 1978; Turner and Messenger 1986). Photo-diode array analysis of culture extracts following separation by high pressure liquid chromatography (HPLC) confirmed this result (PN1 $t_R = 4.6$ min; PN2 $t_R = 3.8$ min). PN1 and PN2 subsequently were purified from a 1-liter culture by column chromatography, and crystallized from ethanol, and their structures identified by spectroscopic analysis. 1H -nuclear magnetic resonance (NMR), ^{13}C -NMR, high resolution mass spectrometry (HRMS), UV, and infra-red (IR) spectroscopic data for PN1 were identical in every respect to those reported for PCA (Brisbane et al. 1987). Both PN1 and PN2 exhibited nearly identical 1H - and ^{13}C -NMR, suggesting that PN2 was also a 1-substituted phenazine; however, the 1H -NMR spectrum of PN2 exhibited two additional resonances at δ 10.71 and 6.38 that suggested PN2 possessed a heteroatom functionality with two attached protons, one of which forms an intramolecular hydrogen bond. Subsequently, two characteristic IR absorbances (1715 cm^{-1} [strong] and 1669 cm^{-1} [medium]) indicated that PN2 was likely to be a primary amide. The high-

resolution mass spectrum of PN2 contained a molecular ion peak (m/z 223.0742, M^+ , $C_{13}H_9N_3O$) and two fragment ion peaks (m/z 207.0560, M^+-NH_2 , $C_{13}H_7N_2O$; m/z 180.0685, M^+-CHNO , $C_{12}H_8N_2$) that were both consistent for the presence of a primary amide functionality within a 1-substituted phenazine. Therefore, the structure of PN2 was assigned as phenazine-1-carboxamide (oxychlororaphine, OCP).

Transposon mutagenesis of PNA1 and selection of Tn5 mutants.

A total of 2,400 mini Tn5-Tc mutants of PNA1 were screened for loss or reduction of antagonism against *F. oxysporum* f. sp. *ciceris*. Two mutants, FM13 and FM29, selected for lack of antagonism against *F. oxysporum* f. sp. *ciceris*, produced neither PCA nor OCP detectable by TLC. Despite their lack of phenazine production, both mutants inhibited mycelial growth of *P. debaryanum* on GCY medium. Southern analysis indicated that each mutant had a single genomic insertion of mini Tn5-Tc (results not shown).

Characterization of mutant FM29.

The nucleotide sequence of 305 bp of the DNA flanking the mini Tn5-Tc insertion in the genome of mutant FM29 was

Fig. 1. The tryptophan biosynthesis pathway (Manch and Crawford 1982) and anthranilate as a proposed intermediate of phenazine biosynthesis (Essar et al. 1990a; Pierson et al. 1995). in *Pseudomonas* spp.

identical (98% identity) to *phnA* of *P. aeruginosa* PAO1, which is thought to encode the alpha subunit of an anthranilate synthase involved in pyocyanine synthesis (Essar et al. 1990a). Mutant FM29 grew on succinate minimal medium, indicating that a functional *phnA* was not required for tryptophan prototrophy.

Characterization of mutant FM13.

Mutant FM13 did not grow on succinate minimal medium unless the medium was supplemented with indole or tryptophan. The mutant was considered therefore to be an auxotroph, defective in a step prior to indole in the tryptophan biosynthetic pathway (Essar et al. 1990b). FM13 also grew poorly in GCY medium, but addition of tryptophan at 50 $\mu\text{g ml}^{-1}$ restored the growth of FM13 to the level of the wild type

and restored phenazine production by the mutant (Fig. 2). Higher concentrations of tryptophan further enhanced phenazine production by FM13 but were toxic to PNA1 (Fig. 2).

In GCY medium, mutant FM13 excreted a compound identified as anthranilate by three criteria: (i) the UV absorbance spectrum of the unknown purified compound was identical to that of anthranilate; (ii) mass spectrum analysis of the unknown purified compound detected a molecular ion peak at m/z 138.07, which is consistent with the molecular formula of anthranilate ($\text{C}_7\text{H}_7\text{NO}_2$); and (iii) the unknown compound co-migrated with authentic anthranilate on TLC plates. Anthranilate could be quantified (Smith and Yanofsky 1960) in the culture supernatants of FM13. Addition of 50 $\mu\text{g ml}^{-1}$ tryptophan to GCY medium considerably reduced the levels of anthranilate excreted by mutant FM13 while, in the presence of 100 $\mu\text{g ml}^{-1}$ tryptophan, no anthranilate could be detected (Fig. 2). No anthranilate could be detected in the culture supernatants of wild-type PNA1.

Ten cosmids from a library of genomic DNA of PNA1 restored growth of FM13 on succinate minimal medium (tryptophan prototrophy), and, at the same time, antagonism against *F. oxysporum* f. sp. *ciceris* on GCY medium. One complementing cosmid, pTrp1, containing an insert of 25 kb, was selected for further analysis. Plasmid pTrp2, containing a 3.3-kb *Nco*I DNA fragment from pTrp1 (Fig. 3), also complemented FM13 for growth on succinate minimal medium and for antagonism against *F. oxysporum* f. sp. *ciceris* on GCY medium. Further subcloning, followed by complementation, led to the isolation of pTrp3, which contains a 0.8-kb *Pst*I-*Nco*I fragment internal to the insert of pTrp2 (Fig. 3). pTrp3 conferred on FM13 the ability to grow on succinate minimal medium, although growth of the complemented strain was slower than that of PNA1. Both pTrp2 and pTrp3 also complemented PAO 922, a *trpC* mutant of *P. aeruginosa* PAO (Calhoun et al. 1973), for growth on succinate minimal medium.

The nucleotide sequence of 350 bp from the *Pst*I site of the 0.8-kb fragment cloned in pTrp3 is nearly identical to that of *trpC* (98% identity at nucleotide level), the gene encoding indole glycerol phosphate synthase (Essar et al. 1990b), which converts 1(O-carboxyphenyl amino)-1-deoxy-ribulose phos-

Fig. 2. Growth (OD 600 nm; A) and phenazine synthesis (OD 368 nm; B) of PNA1 (solid diamond) and mutant FM13 (solid triangle) with supplement of tryptophan at different concentrations in GCY medium. Solid circles show concentrations of anthranilate in the supernatant of FM13 cultures.

Fig. 3. Physical map of the 3.3-kb *Nco*I fragment of pTrp2 complementing mutant FM13 for tryptophan prototrophy and phenazine synthesis. Arrows represent organization of corresponding *trpGDC* operon in PAO1 (Essar et al. 1990b). Open triangle indicates approximate location of Tn5 insertion as determined by restriction analysis of the mutant DNA and Southern blot hybridization (results not shown).

phate to indole glycerol phosphate (Fig. 1). Therefore, *trpC* was identified as the gene mutagenized in FM13. Furthermore, the positions of the restriction sites in the 3.3-kb *NcoI* fragment presented in Figure 3 correspond perfectly to the sequence of the *trpGDC* locus of PAO1 (Essar et al. 1990b).

Inhibition of *Pythium* spp. by anthranilate.

Because FM13 accumulated anthranilate and inhibited mycelial growth of *P. splendens* and *P. debaryanum* in culture, we evaluated the antifungal activity of anthranilate. In GCY medium, 100 $\mu\text{g ml}^{-1}$ of anthranilate caused total inhibition of mycelial growth of *P. debaryanum* (results not shown) while the same concentration of anthranilate reduced considerably the radial growth of *P. splendens* on PDA medium (Table 1). Anthranilate caused no detectable inhibition of the other root pathogens evaluated with similar concentrations (results not shown).

Suppression of *Pythium* damping-off of lettuce and bean by anthranilate.

Because anthranilate inhibited mycelial growth of *P. debaryanum* and *P. splendens* (given for *P. splendens* in Table 1) in culture, its effect on *Pythium* damping-off of lettuce (*Lactuca sativa*), caused by *P. debaryanum*, and of bean (*Phaseolus vulgaris*), caused by *P. splendens*, was evaluated. When added to potting mix at 50 $\mu\text{g g}^{-1}$, anthranilate significantly reduced damping-off of lettuce (Fig. 4), compared with untreated seeds planted in *P. debaryanum*-infested potting mix. At 50 $\mu\text{g g}^{-1}$ potting mix, anthranilate did not influence germination of lettuce seeds in the absence of *P. debaryanum*, but at 100 or 200 $\mu\text{g g}^{-1}$ of potting mix, anthranilate inhibited seed germination. Low concentrations of anthranilate (25, 12, and 6 $\mu\text{g g}^{-1}$ of potting mix) did not inhibit seed germination, but also did not suppress *Pythium* damping-off of lettuce (data not shown).

At concentrations of 50 and 100 $\mu\text{g g}^{-1}$ of potting mix, anthranilate significantly reduced damping-off of bean (Table 1). In potting mix infested with *P. splendens*, the percentage of healthy seedlings increased from 28% from water-treated seeds to 47 and 70% from seeds treated with 50 and 100 μg of anthranilate per gram of potting mix, respectively.

Suppression of *Pythium* damping-off of bean by PNA1 and derivative strains.

Inoculation of bean seeds with PNA1 or mutant FM13 significantly reduced the incidence of damping-off caused by *P. splendens* (Table 2). In soil infested with *P. splendens*, the av-

erage proportion of healthy seedlings increased from 8.3% in noninoculated seeds to 63.3 and 66.7% for seeds inoculated with PNA1 and FM13, respectively. Inoculation of seeds with FM29 improved seedling emergence, compared with the non-inoculated seeds, but was not as effective as seed inoculation with PNA1 or FM13.

Suppression of *Fusarium* wilt of chickpea by PNA1 and derivative strains.

Inoculation of chickpea seeds with *P. aeruginosa* PNA1 significantly reduced the incidence of *Fusarium* wilt observed 30 days after seeds were sown (Fig. 5). Neither of the mutant strains deficient in phenazine production (FM13 and FM29) suppressed the disease. In soils that were not infested with *F. oxysporum* f. sp. *ciceris*, no significant difference in seed germination or plant growth was observed between non-inoculated and inoculated seeds. Therefore, bacterial inoculation had no detectable adverse effect on the plants.

DISCUSSION

P. aeruginosa strain PNA1, which was isolated from the rhizosphere of chickpea in India, suppressed *Fusarium* wilt of chickpea and *Pythium* damping-off of bean and lettuce. Production of phenazine antibiotics, mainly PCA and minor amounts of OCP, contributed to the capacity of PNA1 to suppress *Fusarium* wilt and *Pythium* damping-off. A derived strain (FM29) with a mini Tn5-Tc insertion in the phenazine biosynthetic gene *phnA* was deficient in the production of

Fig. 4. Protection of lettuce plants against *Pythium debaryanum*-induced damping-off by addition of anthranilate (50 $\mu\text{g g}^{-1}$ of soil) to the potting mix. Values are means of four replications; data were collected 14 days after sowing. Data represented by bars with the same letter do not differ significantly according to Fisher's least significant difference (LSD) test at $P = 0.05$. (Anthr: anthranilate, PD; *P. debaryanum*, Con: control).

Table 1. Influence of anthranilate concentration on growth of *Pythium splendens* on potato dextrose agar, and on the emergence of bean (*Phaseolus vulgaris* cv. Prelude) seedlings in the presence of *P. splendens*

Anthranilate concentration ($\mu\text{g ml}^{-1}$ or $\mu\text{g g}^{-1}$ substrate)	Mean radial growth (mm) ^z	Healthy seedlings (%) ^z
0	42.5 $\pm$ 0.0 a	28.3 $\pm$ 16.1 c
25	39.5 $\pm$ 0.2 a	20.0 $\pm$ 05.0 c
50	28.5 $\pm$ 0.1 b	46.7 $\pm$ 20.8 b
100	12.8 $\pm$ 0.1 c	70.0 $\pm$ 13.2 a

^z Values followed by the same letter are not significantly different according to Fisher's least significant difference (LSD) test at $P = 0.05$. Data of 48 h are reported for in vitro assays with four replications. Values for plant protection assays are means of four replications and data were collected 6 days after sowing.

Table 2. Biocontrol of *Pythium splendens* in beans (*Phaseolus vulgaris* cv. Prelude) with strain PNA1 and its mutants FM13 and FM29

Treatments	Healthy seedling (%) ^z
<i>Pythium splendens</i>	08.3 $\pm$ 07.6 c
FM29 + <i>P. splendens</i>	38.3 $\pm$ 02.9 b
FM13 + <i>P. splendens</i>	66.7 $\pm$ 06.7 a
PNA1 + <i>P. splendens</i>	66.3 $\pm$ 16.1 a

^z Values are means of four replications. Values followed by the same letter are not significantly different according to Fisher's least significant difference (LSD) test at $P = 0.05$.

PCA and OCP. FM29 did not inhibit mycelial growth of *F. oxysporum* f. sp. *ciceris*, and showed reduced growth inhibition of *P. splendens* or *P. debaryanum* in culture; and was less effective than PNA1 in suppression of Fusarium wilt or Pythium damping-off caused by these pathogens. Many strains of *Pseudomonas* spp. produce phenazine antibiotics (Turner and Messenger 1986; Chang and Blackwood 1969; Kanner et al. 1978), and the in situ production of phenazines by *Pseudomonas* spp. inhabiting the rhizosphere is known to suppress plant diseases caused by certain soilborne fungi such as the take-all pathogen of wheat, *G. graminis* var. *tritici* (Pierson and Thomashow 1992; Thomashow and Weller 1988). The present study provides evidence that in situ phenazine production by rhizosphere pseudomonads can suppress Fusarium wilt and Pythium damping-off. Anthranilate inhibited mycelial

growth of *P. debaryanum* and *P. splendens* in culture and, when added to potting mix, suppressed Pythium damping-off of bean, lettuce, and tomato (data not shown). The reason why the growth of *Pythium* spp. is inhibited by anthranilate while the same compound has no effect on the other root pathogens tested is not clear. However, it should be stressed that *Pythium* spp., belonging to the Oomycota, differ from fungi and are more closely related to algae (Bhattacharya and Stickel 1994). Furthermore, FM13, which did not produce PCA or OCP, but released anthranilate, suppressed Pythium damping-off of bean at levels similar to those resulting from use of the wild-type strain PNA1. This result suggests that either anthranilate excreted by this mutant antagonizes *P. splendens* directly or that sufficient quantities of tryptophan are present in the rhizosphere to restore PCA and OCP production by FM13. The availability of tryptophan in the rhizospheres evaluated here was not determined, but tomato root exudates (in gnotobiotic system) do not provide enough tryptophan to sustain growth and colonization by a tryptophan auxotroph of *P. fluorescens* (Simons et al. 1997). Although a definitive role for in situ anthranilate production in biocontrol activity of FM13 was not established, the results of this study suggest that anthranilate can be an effective antifungal metabolite for suppression of Pythium damping-off.

The findings of this study suggest that metabolic flux through the tryptophan biosynthesis pathway influences phenazine production in strain PNA1. The *trpC* mutation in FM13 was associated with accumulation of anthranilate, as described previously for *trpC* mutants of *P. aeruginosa* (Calhoun et al. 1973), and cessation of PCA production. Addition of tryptophan to the culture medium reduced the amount of anthranilate accumulated by FM13 and concurrently stimulated PCA and OCP production. We speculate, therefore, that phenazine production in PNA1 is either negatively regulated by an accumulated intermediate (such as anthranilate) or positively regulated by tryptophan. Alternatively, the addition of tryptophan to cultures of FM13 may simply increase cell density to a point that is sufficient to transcriptionally induce PCA synthesis via a described homoserine lactone-mediated, quorum-sensing mechanism (Pierson et al. 1995; Stead et al. 1996; Latifi et al. 1996).

Fig. 5. Biocontrol of *Fusarium oxysporum* f. sp. *ciceris* on chickpea JG 62 grown in naturally infested soils with strain PNA1 and mutants FM29 and FM13. Values are means of five replications. Data represented by bars with the same letter do not differ significantly according to Fisher's least significant difference (LSD) test at $P = 0.05$.

Table 3. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Reference or source
Strains		
<i>Pseudomonas aeruginosa</i> PNA1	Wild type; Pvd ⁺ Phz ⁺ Trp ⁺ , Ap ^r	Chickpea rhizosphere isolate from Indian soils
FM13	<i>P. aeruginosa</i> PNA1::Tn5-Tc mutant (Trp ⁻ , Phz ⁻)	This work
FM29	<i>P. aeruginosa</i> PNA1::Tn5-Tc mutant (Phz ⁻)	This work
<i>Escherichia coli</i> HB101	<i>recA hsdB hsdM pro leu thi</i> ; Sm ^r	Boyer and Roulland-Dussoix 1969
<i>E. coli</i> SM10 (λ pir)	<i>thi thr leu tonA lacY supE recA</i> ::RP4-2-Tc::Mu, λ pir	De Lorenzo et al. 1990
<i>E. coli</i> DH5 α	<i>supE44</i> Δ <i>lacU169</i> (ϕ 80 <i>lacZ</i> Δ M15) <i>hsdR17 recA1 endA1 gyrA96 thi-1 relA1</i>	Gibco-BRL, Gent, Belgium
Plasmids		
pUT-mini Tn5-Tc	Ap ^r ; Tn5 based delivery plasmid with Tc ^r	De Lorenzo et al. 1990
pRG930	Sp ^r /Sm ^r , Cos ⁺ , pVS1 origin of replication	Van den Eede et al. 1992
pRK2013	Km ^r , ColE1 origin of replication, Tra ⁺ Mob ⁺	Figurski and Helinski 1979
pBS	Ap ^r , ColE1 origin of replication	Stratagene, La Jolla, CA
pVA1	Tn5 flanking sequence of FM29 in pBlueScript, insert size 3 kb	This work
pTrp1	A 25 kb of genomic DNA from PNA1 cloned in pRG930, restoring the growth of FM13 on succinate minimal agar medium	This work
pTrp2	A 3.3-kb <i>Nco</i> I fragment from pTrp1 cloned in pRG930	This work
pTrp3	A 0.8-kb <i>Pst</i> I- <i>Nco</i> I fragment from pTrp2 cloned in pRG930	This work

MATERIALS AND METHODS

Bacteria, plasmids, and culture conditions.

Bacterial strains and plasmids used in this study are listed in Table 3. *P. aeruginosa* PNA1 was grown at 28°C on casamino acids agar medium (CAA) (Cornelis et al. 1992), King's medium B (KB) (Buysens et al. 1996), Luria-Bertani medium (LB) (Sambrook et al. 1989), glucose casamino acid yeast extract (GCY) medium (glucose 15 g l⁻¹, casamino acids 1.5 g l⁻¹, yeast extract 1.0 g l⁻¹, KH₂PO₄ 1.5 g l⁻¹, MgSO₄ 7H₂O 1.0 g l⁻¹), and succinate minimal medium (succinic acid 4 g l⁻¹, K₂HPO₄ 6.0 g l⁻¹, KH₂PO₄ 3.0 g l⁻¹, (NH₄)₂SO₄ 1.0 g l⁻¹, MgSO₄ 7H₂O, 0.2 g l⁻¹, pH 7.0). *Escherichia coli* was grown at 37°C on LB medium. Unless otherwise mentioned, antibiotics were used at the following concentrations (in µg ml⁻¹), for pseudomonads: tetracycline (Tc), 100; streptomycin (Sm), 400; spectinomycin (Sp), 400; ampicillin (Ap), 100; and for *E. coli*: Tc, 20; Sm, 25; Sp, 50; Ap, 100.

Isolation and identification of fluorescent pseudomonads.

Bacteria were isolated from the rhizosphere of chickpea, pigeonpea, and groundnut crops grown in native soils of Indian fields (International Crops Research Institute for Semi-Arid Tropics, ICRISAT), by plating crushed roots on CAA. Colonies that fluoresced under UV light ($\lambda = 366$ nm) were selected and further purified twice on the same medium. The selected strains were identified by API 20 NE strips (Bio Mérieux, Montalieu, France), used for non-enteric, gram-negative rods.

Phytopathogens.

The fungal phytopathogens *Fusarium oxysporum* f. sp. *ciceris*, *Fusarium udum*, *Macrophomina phaseolina*, and *Sclerotium rolfsii* were obtained from ICRISAT, Hyderabad, India. An isolate of *P. debaryanum* from beet roots was obtained from P. Lepoivre (Faculté des Sciences Agronomiques, Gembloux, Belgium). *P. splendens*, an isolate of *Monstera deliciosa* roots (Buysens et al. 1996), was also used. All fungal species were maintained on GCY agar and potato dextrose agar (PDA).

Antagonism against fungi in culture.

Fluorescent pseudomonads were screened for antagonism of *P. debaryanum* on CAA agar and for antagonism of other fungal species on GCY. An agar plug (4 mm in diameter) was cut from an actively growing fungal culture and placed on the surface of fresh agar medium at the center of a petri plate. Simultaneously, bacteria to be tested were streaked at opposite sides toward the edge of a petri plate 3 cm away from the agar plug. Plates inoculated with fungal plugs alone were used as controls. The plates were incubated at 28°C until fungal mycelia covered the agar surface of the control plate. An antagonistic strain was selected based on in vitro antagonism against all fungal species mentioned above.

Detection and isolation of antifungal compounds.

For routine analysis of metabolites produced by *P. aeruginosa* PNA1, the supernatants of 2-day-old, 5-ml cultures grown in GCY medium were acidified to pH 2.0 with 6 M HCl, extracted with ethyl acetate (3×, 1 ml), and the combined organic layers were dried over anhydrous MgSO₄. Following evaporation of the solvent, the residue was dissolved in methanol and analyzed by TLC (silica gel developed in 3:1 [vol/vol] toluene:acetone) or HPLC (0.8 × 10 Waters C₁₈-

NovaPak radial compression cartridge eluted isocratically with 45% H₂O, 30% acetonitrile, and 25% methanol at 1.5 ml/min) equipped with a diode array detector.

Two compounds, named PN1 and PN2, were purified for spectroscopic analysis from the combined supernatants of 7 150-ml PNA1 cultures grown for 2 days in GCY medium. The supernatant was acidified to pH 2.0, extracted with ethyl acetate (4× 100 ml), and dried over anhydrous MgSO₄, and the solvent evaporated under reduced pressure. Half of the crude yellow residue was fractionated by flash chromatography (silica gel, 2.5 × 15 cm, eluted in a 3:1 toluene:acetone mixture) yielding pure PN1 and a mixture of PN1/PN2. After the samples were concentrated, the PN1/PN2 mixture was subsequently dissolved in ethyl acetate and extracted with 10% (wt/vol) aqueous NaHCO₃. The ethyl acetate layer was then dried with MgSO₄ and the solvent evaporated, yielding pure PN2. Both PN1 and PN2 were recrystallized from ethanol.

PN2 (phenazine-1-carboxamide; oxychloraphene): mp 242°C (dec); UV_{max} (MeOH) 248 and 368 nm; IR (CHCl₃) 3028, 2256, 1715, 1669, 1526, 1426, and 1370 cm⁻¹; ¹H NMR (300 MHz; CDCl₃) δ 10.71 (br s, 1H), 9.01 (dd, 1H, J = 1.5 and 7.1 Hz), 8.42 (dd, 1H, J = 1.5 and 8.7 Hz), 8.28 (m, 1H), 8.22 (m, 1H), 7.99 (m, 3H), and 6.38 (br s, 1H); ¹³C NMR (75 MHz; CDCl₃) δ 166.5, 143.4, 143.1, 141.4, 140.7, 135.9, 134.2, 131.7, 131.0, 129.8, 129.7, 129.0, and 128.8; EI HRMS *m/z* M⁺ 223.0742 C₁₃H₉N₃O (calc. exact mass for C₁₃H₉N₃O 223.07456), M⁺-NH₂ 207.0560 C₁₃H₉N₂O (calc. exact mass for C₁₃H₉N₂O 207.05587), and M⁺-CHNO 180.0685 C₁₂H₈N₂ (calc. exact mass for C₁₂H₈N₂ 180.06873).

PN1 (phenazine-1-carboxylic acid; PCA): mp 243°C; UV_{max} (MeOH) 248 and 368 nm; IR (CHCl₃) 3026, 2938, 2855, 1739, 1714, 1522, and 1457 cm⁻¹; ¹H NMR (300 MHz; CDCl₃) δ 10.65 (br s, exchangeable), 8.96 (dd, 1H, J = 1.5 and 7.1 Hz), 8.51 (dd, 1H, J = 1.5 and 8.7 Hz), 8.32 (m, 1H), 8.26 (m, 1H), and 8.00 (m, 3H); ¹³C NMR (75 MHz; CDCl₃) δ 165.8, 144.0, 143.3, 139.9, 139.7, 137.3, 135.0, 133.1, 131.6, 130.2, 130.0, 127.9, and 124.8; EI HRMS *m/z* M⁺-CO₂ 180.0693 C₁₂H₈N₂ (calc. exact mass for C₁₂H₈N₂ 180.06875), and M⁺-CO₂H 179.0607 C₁₂H₇N₂ (calc. exact mass for C₁₂H₇N₂ 179.06092).

One (or more) antifungal compound(s) active against *P. debaryanum*, and produced on GCY medium by the mutant FM13, were extracted into ethyl acetate, dried under vacuum, and tested for in vitro antagonism against *P. debaryanum*. The extract was analyzed by TLC, UV spectroscopy, and mass spectrometry. TLC analysis was performed in a toluene:acetic acid (9:1, vol:vol) solvent system, with commercial anthranilate (Merck, Darmstadt, Germany) as reference. UV spectra analysis for absorption maxima and mass spectra analysis for molecular weight were performed, with anthranilate as a reference, as described above. Anthranilate present in the culture supernatant of FM13 was quantified by measuring the absorbance at 336 nm as described by Smith and Yanofsky (1960).

Transposon mutagenesis of strain PNA1.

The suicide plasmid pUT containing the mini Tn5-Tc, a derivative of Tn5 that carries a tetracycline resistance gene (De Lorenzo et al. 1990), was used to generate transposon insertions in the chromosome of PNA1. Mating was performed by mixing a mid-log-phase culture of SM10 (λ pir), the host of pUT-mini Tn5-Tc, with PNA1 Ap^r in a 1:1 ratio (Cornelis et al. 1992). After incubation at 28°C for 24 h, presumptive

transposon mutants of PNA1 were selected on LB with 100 $\mu\text{g ml}^{-1}$ Tc and 50 $\mu\text{g ml}^{-1}$ Sm.

Screening and selection of mutants.

Transposon mutants of PNA1 were tested for loss or reduction of antagonism against *F. oxysporum* f. sp. *ciceris*. A total of 2,400 mutants were screened in individual wells of 24-well microtiter plates containing GCY agar medium coinoculated with an agar plug (2 mm in diameter) containing mycelia of the fungus. This method facilitated identification of mutants because the diffusion of antifungal metabolites produced by neighboring colonies was avoided. The plates were incubated at 28°C for 72 h. Mutants that did not inhibit *F. oxysporum* f. sp. *ciceris* in wells of microtiter plates were tested for antagonism against all phytopathogenic fungi listed above, on petri plates. Mutants were tested for auxotrophy by evaluating their growth on succinate minimal medium. Phenazine compounds were extracted from the mutants, quantified by absorption at OD 368 nm, and evaluated by TLC as described above.

DNA manipulations and sequencing.

DNA isolations, restriction enzyme digestions, and agarose gel electrophoresis were performed essentially as described by Sambrook et al. (1989). Southern blotting was done as described earlier (Cornelis et al. 1992). The genomic DNA of mini Tn5-Tc mutants of PNA1 was digested with *Xho*I, which does not cut in the transposon, and probed with a 2.0-kb *Eco*RI fragment containing the Tc^r gene labeled with digoxigenin as described by the manufacturer (Boehringer Mannheim, Mannheim, Germany).

Mutant FM29 was characterized by cloning the sequence flanking the transposon. The genomic DNA of the mutant was digested with *Pst*I and *Kpn*I, which do not cut in the transposon, and, after purification, these fragments were ligated into the pBluescript SK⁻ plasmid. Colonies resistant to Tc and Ap were selected. The resulting plasmid, pVA1, was sequenced with a Pharmacia LKB automatic laser fluorescent DNA sequencer and nucleotide sequence data were compared with known sequences with the FASTA algorithm (Genetics Computer Group, Madison, WI).

Construction of a genomic bank and complementation.

A genomic library of strain PNA1 was constructed by partial digestion of genomic DNA with *Pst*I, under conditions giving fragments ranging between 20 and 40 kb, followed by ligation of fragments into the *Pst*I site of cosmid vector pRG930 (Van den Eede et al. 1992). After in vitro lambda packaging and transfection into *E. coli* HB101, clones with Sm and Sp (25 and 50 $\mu\text{g ml}^{-1}$) resistance were selected. Since HB101 is resistant to 10 $\mu\text{g ml}^{-1}$ Sm, the concentration of Sm in the medium was increased to 25 $\mu\text{g ml}^{-1}$. Two thousand recombinant cosmids were mobilized into auxotrophic mutant FM13 by tri-parental conjugation, with pRK2013 as a helper plasmid. Transconjugants of FM13 restored for prototrophic growth on succinate minimal medium were selected. Subcloning of cosmids was done in pRG930 for complementation studies, and in the pBluescript vector (pBS) for sequencing.

Suppression of Pythium damping-off of lettuce by anthranilate.

P. debaryanum was cultured in V8 broth for 1 week of incubation at 24°C on a rotary shaker (Buysens et al. 1996). Myce-

lial fragments of *P. debaryanum* were prepared by disintegrating mycelial pellets with a homogenizer. The fungal fragments of *P. debaryanum* were added to a potting mixture (Triomf 17 [pH 6.5; electroconductivity, 530 $\mu\text{S cm}^{-1}$]; TRIO BV, Westerhaar, The Netherlands) at a concentration of 150 propagules per gram. Anthranilate was diluted in water and added to the pots. Then, 25 lettuce seeds (cv. Gotte gold yellow) were sown in each pot. Four replications were kept for each treatment. Pots were watered daily to keep the soil moisture content constant at 70% (vol:wt), and they were kept in a Phytotron at $24 \pm 2^\circ\text{C}$. Pythium infection was evaluated and data analyzed as stated for Pythium damping-off of bean (below).

Suppression of Pythium damping off of bean.

Inoculum of *P. splendens* was prepared as described by Buysens et al. (1996) and added to potting mixture (Klasmann-Dielmann, dry matter >25%, organic matter >20%, resistance 2,000 ohm/cm, water retention capacity 80% and pH [H₂O] 6.0 to 6.5) to obtain an inoculum density of 200 hyphal swellings per gram. Bean seeds (cv. Prelude) were soaked for 7 min either in sterile water (control) or in a suspension of bacterial cells (10^9 CFU ml^{-1}) of PNA1, FM13, or FM29 prior to sowing. PNA1 and its mutants were grown overnight at 28°C on KB. In another study, anthranilate was applied at the concentrations of 0, 25, 50, and 100 mg g^{-1} of potting mixture.

Each treatment included 20 seeds and had four replications. Trays were watered daily to the initial weight and incubated in the greenhouse at $25 \pm 5^\circ\text{C}$. Pythium damping-off was assessed by determining emergence and the number of plants remaining healthy during the experiment, a period of 2 weeks. Plants that did not collapse and showed no symptoms of root rot at the end of the experiment were considered healthy. Seedling survival data (percent healthy plants) were transformed to arcsine or square root values before analysis of variance. Means were compared with Fisher's least significant difference test. All experiments were repeated in time at least once. Data of representative experiments are shown.

Suppression of Fusarium wilt of chickpea.

Seeds of a chickpea cultivar highly susceptible to Fusarium wilt (cv. JG 62) were surface disinfected with 15% bleach for 2 min and washed thoroughly with sterile water before inoculation with *P. aeruginosa*. One milliliter of each bacterial strain grown overnight in KB broth was spread on KB agar plate and incubated for 48 h at 28°C. Then, 3 ml of 0.5% carboxy methyl cellulose was added to each plate, and the bacterial cells were scraped into suspension. Each bacterial suspension was mixed with seeds in a petri plate, and the seeds were coated (10^8 CFU/seed), then dried for 4 h under sterile air. Seeds treated with carboxy methyl cellulose alone served as controls. The initial population density of bacteria on the seeds was determined by dilution plating on KB agar.

Soil (medium Vertisol, pH 7) naturally infested with *F. oxysporum* f. sp. *ciceris* was collected from fields located at ICRISAT, A.P., India, where Fusarium wilt of chickpea had been severe. *Fusarium* spp. propagules in these disease-conducive soils were estimated to be 1,125 per gram of soil as estimated by plating on Malachite green medium. Pots (15 cm in diameter) were filled with infested soil and eight seeds were sown on the soil surface. After emergence, seedlings were

thinned to four per pot. Experiments were completely randomized with five replicate pots, each containing four seedlings. Pots were kept in the glasshouse at a maximum temperature of $28 \pm 5^\circ\text{C}$, and watered daily. Plants were examined for the wilt symptoms for 30 days. Plants that did not collapse were scored as healthy. The healthy plant stand data were transformed to arcsine or square root values before analysis of variance. Fisher's least significant difference test was used to compare treatment means. The experiment was repeated once.

ACKNOWLEDGMENTS

We wish to thank the Fonds voor Wetenschappelijk Onderzoek (FWO) for its support. V. A. has been supported by a VUBAROS fellowship and acknowledges M. V. Reddy and Jill Lenne for providing the materials and technical support of ICRISAT. J. T. T. is supported by an ABOS fellowship. We also wish to thank D. Tourwe for the mass spectrometry measurements. The precious administrative help of L. Eeckhaut is also appreciated.

NOTE ADDED IN PROOF

Mavrodi et al. (1998) recently proposed an alternative pathway for phenazine-1-carboxylic acid biosynthesis in *P. fluorescens*, in which aminodeoxyisochorismate and 3-hydroxyanthranilate, rather than anthranilate, are intermediates. They propose that *phzE* from *P. fluorescens* 2-79 (a homolog of *phzB* from *P. aureofaciens* 30-84) encodes a bacterial class I glutamine amidotransferase that synthesizes aminodeoxyisochorismate from chorismate. The phenazine biosynthetic pathway proposed by Mavrodi et al. does not involve anthranilate synthase II, which is a component of the biosynthetic schemes proposed previously (Essar et al. 1990b; Pierson et al. 1995).

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