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Involvement of Pyochelin and Pyoverdin in Suppression of *Pythium*-Induced Damping-Off of Tomato by *Pseudomonas aeruginosa* 7NSK2

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The plant growth-promoting rhizobacterium *Pseudomonas aeruginosa* 7NSK2 produces three siderophores when iron is limited: the yellow-green fluorescent pyoverdin, the salicylate derivative pyochelin, and salicylic acid. This *Pseudomonas* strain was shown to be an efficient antagonist of *Pythium*-induced damping-off. The role of pyoverdin and pyochelin in the suppression of *Pythium splendens* was investigated by using various siderophore-deficient mutants derived from *P. aeruginosa* 7NSK2 in a bioassay with tomato (*Lycopersicon esculentum*). To provide more insight into the role of pyochelin in antagonism, mutant KMPCH, deficient in the production of pyoverdin and pyochelin, was complemented for pyochelin production. The complementing clone was further characterized by subcloning and transposon mutagenesis and used to generate a pyochelin-negative, pyoverdin-positive mutant by marker exchange. All mutants were able to reduce *Pythium*-induced preemergence damping-off to some extent. Production of either pyoverdin or pyochelin proved to be necessary to achieve wild-type levels of protection against *Pythium*-induced postemergence damping-off. Mutant KMPCH inhibited *P. splendens* but was less active than the parental strain. This residual protection could be due to the production of salicylic acid. Since pyoverdin and pyochelin are both siderophores, siderophore-mediated iron competition could explain the observed antagonism and the apparent interchangeability of the two compounds. We cannot, however, exclude the possibility that both siderophores act in an indirect way.

Fluorescent pseudomonads are characterized by the production of yellow-green pigments (38), called pyoverdins or pseudobactins, that fluoresce under UV light and function as siderophores. These fluorescent siderophores are low-molecular-weight compounds that are produced under iron-limiting conditions. They chelate the ferric ion and serve as vehicles for the transport of Fe(III) into bacterial cells (43).

Rhizosphere-inhabiting fluorescent pseudomonads play an important role in the biological control of soilborne fungal plant pathogens. In a number of cases, this effect has been attributed to siderophore production (4, 13, 27, 33, 34). In other cases, it was demonstrated that siderophore production was not involved (1, 30, 46). Therefore, other mechanisms have been proposed to explain disease suppression. Pseudomonads may produce antibiotics or HCN, compete with a pathogen for niches and nutrients, or induce systemic acquired resistance in the plant after successful colonization of the roots (3, 45).

Pythium spp. are the causal agents of pre- and postemergence damping-off and root rot of many crops. Seeds rot before or shortly after germination (preemergence damping-off) and newly emerged seedlings collapse (postemergence damping-off) (51).

Pseudomonas aeruginosa 7NSK2, an isolate from the rhizosphere of barley, improves the growth of several crops (20). Under iron limitation, it produces three siderophores: salicylic acid (thin-layer chromatographic [TLC] analysis [this work]); pyochelin (18), a salicylic acid derivative (2); and the fluorescent pyoverdin (18). We found that this strain is an effective antagonist of *Pythium*-induced damping-off of tomato (*Lycopersicon esculentum*). Pyoverdin production has proven to be important in the biological control of *Pythium*-induced damping-off of cotton by *Pseudomonas fluorescens* 3551 (34) and *Pythium*-induced root rot of wheat by different fluorescent *Pseudomonas* strains (4). No role for pyoverdin production could be demonstrated in the biocontrol of *Pythium*-induced damping-off of cucumber by *Pseudomonas putida* N1R (46) and *P. fluorescens* Pf-5 (30). The effect of pyochelin and salicylic acid production on *Pythium*-induced damping-off has to our knowledge not yet been investigated. Preliminary results have indicated a role for pyochelin in the antagonism of *P. aeruginosa* 7NSK2 toward *Pythium* spp. (17). In this work, the importance of pyoverdin and pyochelin production by *P. aeruginosa* 7NSK2 in the biological control of *Pythium splendens* was assessed. Mutants deficient in the production of either or both of these compounds were compared with the parental strain for the ability to control damping-off of tomato. Furthermore, a complementation study using a pyochelin- and pyoverdin-negative mutant was conducted to provide more insight into the role of pyochelin in the antagonism of *P. aeruginosa* 7NSK2 toward *Pythium*-induced damping-off.

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MATERIALS AND METHODS

Strains, plasmids, and culture conditions. The bacterial strains and plasmids used are summarized in Table 1.

Pseudomonads were cultured at 28 or 37°C on modified King's B (MKB) medium (19), on Luria-Bertani (LB) medium (47), or on minimal medium (47) with 4 g of glucose liter^{-1}. *Escherichia coli* strains were grown at 37°C on LB medium.

TABLE 1. Bacterial strains and plasmids used

Strain or plasmid	Relevant characteristics[a]	Reference or source
Strains		
Pseudomonas		
7NSK2	Wild type; Pvd^+ Pch^+ SA^+	22
MPFM1	Pvd^- Pch^+ SA^+ Km^r; obtained by Tn*5* mutagenesis of 7NSK2	20
KMPCH	Pvd^- Pch^- SA^+ Km^r; chemical mutant of MPFM1	18
KMPCH(K5)	Pvd^- Pch^+ SA^+ Km^r; KMPCH complemented for pyochelin production	This work
SPCN1	Pvd^+ Pch^- SA^+ Cb^r	This work
LS128-3	*pchB*; replacement mutant of LS128 (Pvd^-, chemical mutant of *P. aeruginosa* PAO1)	49
LS128-6	*pchA*; replacement mutant of LS128 (Pvd^-, chemical mutant of *P. aeruginosa* PAO1)	49
E. coli		
HB101	*recA hsdB hsdM pro leu thi*; Sm^r	6
C2210	*polA*; Nal^r	41
Plasmids		
pLAFR1	Broad-host-range cosmid cloning vector, RK290 replicon; IncP Tc^r	16
pRK2013	Tra^+ Mob^+ Km^r; ColE1 replicon	15
pRK415	Broad-host-range cloning vector, RK404 replicon; Tc^r	26
pHoHo1	Contains *lacZYA*; pME8 replicon; Cb^r	53
pSShe	Contains *tnpA*; pACYC184 replicon; Cm^r	53
K5	pLAFR1 carrying a 27.8-kb *Eco*RI fragment of *P. aeruginosa* 7NSK2 chromosomal DNA; restores pyochelin biosynthesis in KMPCH; Tc^r	This work
pME3300	pLAFR3 carrying a 25-kb *Hin*dIII-*Eco*RI fragment of *P. aeruginosa* PAO1 chromosomal DNA, containing structural genes for salicylate and pyochelin biosynthesis	49
pBBR1MCS	Broad-host-range cloning vector; Mob^+ Cm^r	29
pSB1	pRK415 carrying a 4.4-kb *Kpn*I fragment derived from K5; Tc^r	This work
pSB2	pRK415 carrying a 7.5-kb *Kpn*I fragment derived from K5; Tc^r	This work
pSB3	pRK415 carrying a 7-kb *Eco*RI fragment derived from K5; Tc^r	This work
pSB4	pRK415 carrying an 11-kb *Bam*HI fragment derived from K5; Tc^r	This work
pSB5	K5 with a 4.4-kb *Sst*I deletion; Tc^r	This work
pSB6	pSB5 with an additional 4.4-kb *Kpn*I deletion; Tc^r	This work

[a] Abbreviations: Pvd, pyoverdin; Pch, pyochelin; SA, salicylic acid; Km^r, Cb^r, Sm^r, Tc^r, and Cm^r, resistant to kanamycin, carbenicillin, streptomycin, tetracycline, and chloramphenicol, respectively.

For pseudomonads, the following antibiotics were used at a concentration of 200 mg $liter^{-1}$: kanamycin, carbenicillin, and tetracycline.

For *E. coli*, 50 mg of kanamycin $liter^{-1}$, 50 mg of carbenicillin $liter^{-1}$, 20 mg of tetracycline $liter^{-1}$, 50 mg of chloramphenicol $liter^{-1}$, and 50 mg of nalidixic acid $liter^{-1}$ were employed.

Construction of a gene library, cloning of pyochelin biosynthetic genes, and complementation of mutant KMPCH for pyochelin production. The broad-host-range cosmid pLAFR1 (Table 1) was used to construct a genomic library. Preparation of chromosomal DNA of *P. aeruginosa* 7NSK2, digestions with restriction enzymes (*Eco*RI), agarose gel electrophoresis, and ligation with T4 DNA ligase were performed as described by Sambrook et al. (47). In vitro packaging and transduction of the ligated DNA were performed according to the manufacturer's instructions (Boehringer Mannheim Biochemicals).

The pLAFR gene bank in *E. coli* HB101 was mobilized en masse into mutant KMPCH with the use of helper pRK2013 (12). Individual transconjugants were screened for pyochelin and pyoverdin production. Pyoverdin production was confirmed by fluorescence on MKB medium. Nonfluorescent transconjugants were checked for pyochelin production on Casamino Acid medium (Casamino Acids [Difco] at 5 g $liter^{-1}$ with 400 μM $MgCl_2$ added after autoclaving) with chrome azurol S (48). Pyochelin-producing strains form orange halos on this medium.

Plasmid DNA was isolated by the alkaline lysis method (5) either directly from the *Pseudomonas* strains with restored pyochelin production or after the clones that restored pyochelin production in mutant KMPCH had been conjugated to *E. coli* HB101 by triparental mating (12). The ability of these cosmids to complement mutant KMPCH for pyochelin production was confirmed by transferring the cosmids from *E. coli* HB101 back to *P. aeruginosa* KMPCH through conjugation.

The gene(s) necessary for pyochelin production was localized by cosmid deletion analysis and subcloning in pRK415 (47).

Construction of a pyoverdin-positive, pyochelin-negative mutant, SPCN1, by transposon mutagenesis and marker exchange. Insertions in the pyochelin clone K5 were obtained by Tn*3*-HoHo1 mutagenesis (53). Tn*3*-HoHo1 provides a useful system for the random generation of *lacZ* gene fusions. In these fusions, the production of β-galactosidase, the *lacZ* gene product, is placed under the control of the gene into which Tn*3*-HoHo1 has been inserted. Consequently, an insertion of Tn*3*-HoHo1 in the right direction into the pyochelin gene will result in a pyochelin-negative, β-galactosidase-positive phenotype. Tn*3*-HoHo1 carries a nonfunctional transposase gene and can thus be transposed only if transposase activity is supplied in *trans* by pSShe. It is stable in the absence of this activity. The pyochelin clone K5 was transformed into *E. coli* HB101(pHoHo1, pSShe), and the resulting transformants were mated en masse into nalidixic acid-resistant (Nal^r) *E. coli* C2210. Transconjugants resistant to nalidixic acid, carbenicillin, and tetracycline were pooled. These transconjugants contained K5 (tetracycline resistance) with a Tn*3*-HoHo1 insertion (carbenicillin resistance). To select for insertions in the pyochelin gene, the transconjugants were conjugated to *P. aeruginosa* KMPCH; conjugation was followed by selection on minimal medium with glucose containing tetracycline, carbenicillin, and X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) (20 mg $liter^{-1}$, chromogenic substrate to detect β-galactosidase activity). Blue clones, unable to produce pyochelin, were identified by the absence of an orange halo on Casamino Acid medium with chrome azurol S. Subsequently, these clones were used to obtain genomic pyochelin mutants in a wild-type background by marker exchange. Gene replacement was promoted by cold treatment (28).

The fidelity of the marker exchange events was confirmed by probing Southern blots of restriction enzyme digests of the mutated chromosomal DNAs with *lacZ* (47). The *lacZ* probe was digoxigenin labelled according to the manufacturer's instructions (Boehringer Mannheim Biochemicals).

Characterization of mutants. Survival in the rhizosphere of tomato plants and root colonization of the introduced strains were assessed by in vivo assays. Approximately 0.5 g of rinsed roots was vigorously vortexed in 4.5 ml of 10 mM $MgSO_4$ buffer for 30 s. Appropriate dilutions to obtain approximately 10 colonies were plated as 10-μl drops on MKB or LB medium with antibiotics. After 12 to 20 h of incubation at 37°C, colonies were counted.

Pyoverdin production (fluorescence) was observed under UV light (λ = 302 nm) after 1 day of growth on MKB medium at 28°C.

TLC was performed to assess pyochelin and salicylic acid production (10, 54). Pyochelin and salicylic acid were isolated by ethyl acetate extractions of acidified supernatants obtained by centrifugation (2,800 × *g*, 15 min) of 30-h bacterial cultures in Casamino Acid medium. After evaporation of the ethyl acetate phase

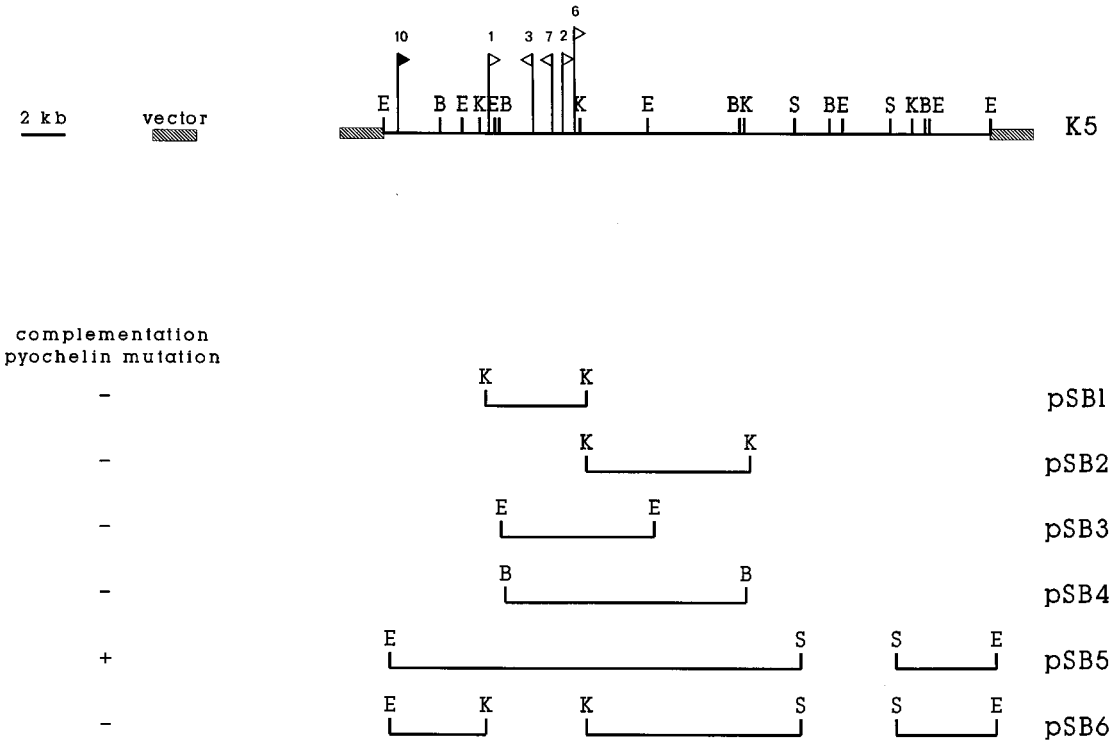

FIG. 1. Restriction map of K5. Numbered flags refer to different Tn*3*-HoHo1 insertions and indicate the orientation of transcription. Open flags indicate the inability and the solid flag indicates the ability of the transposon-mutagenized fragment to complement *P. aeruginosa* KMPCH for pyochelin production. Subclones constructed in pRK415 (pSB1 to -4) and deletion mutants of K5 (pSB5 and -6) that complement or do not complement the pyochelin mutation in the pyochelin- and pyoverdin-negative *P. aeruginosa* mutant KMPCH are shown. Abbreviations for restriction endonucleases: E, *Eco*RI; B, *Bam*HI; K, *Kpn*I; and S, *Sst*I.

under vacuum, the dry residue was resuspended in a small volume of methanol and applied to Silicagel 60F254 TLC plates (Merck), which were developed in chloroform-acetic acid-ethanol (90:5:2.5, vol/vol/vol). Pyochelin was fluorescent under UV (λ = 302 nm) and red-brown when sprayed with 0.1 M $FeCl_3$ in 0.1 N HCl and had an R_f of 0.4. Salicylic acid was blue fluorescent under UV (λ = 302 nm), turned red-violet when sprayed with 0.1 M $FeCl_3$ in 0.1 N HCl, and had an R_f of 0.6.

In vivo *Pythium* control assay. Disease suppression by *P. aeruginosa* 7NSK2 and its derivatives was tested in a model system with tomato. *P. splendens* 706, an isolate from *Monstera* roots (Swiss cheese plant), was maintained on corn meal agar (Difco) and cultured on V-8 broth (40). After 1 week of incubation at 24°C, the fungus produced abundant hyphal swellings. These hyphal swellings were used for artificial infection at a concentration of 150 hyphal swellings per g of soil (potting mixture, Triomf 17 [pH 6.5; electroconductivity, 530 $\mu S\ cm^{-1}$]; TRIO BV, Westerhaar, The Netherlands).

Tomato seeds (cv. Marmande; Somers BV, Mechelen, Belgium) were soaked for 5 min in sterile water (control) or in a suspension of washed bacterial cells of a 1-day-old culture in MKB medium (28°C, 180 rpm) prior to sowing. Each treatment included 40 seeds and was replicated at least four times.

Pots were watered daily to their starting weight to keep the soil moisture content constant at 70%. Plants were kept in a greenhouse at 24 ± 4°C.

Pythium infection was assessed by determining emergence (mostly 1 week after sowing) and the number of plants remaining healthy during the experiment. Plants that did not collapse and showed no symptoms of root rot at the end of the experiment were considered healthy.

Data analysis. All experiments were set up in a randomized block design. Trials with SPCN1 were repeated four times, whereas trials with KMPCH(K5) were repeated seven times. Emergence and infection data were transformed to their respective arcsine of square root values before analysis of variance (52). Data from experiments with a common design were pooled for analysis. In this case, repeated experiments were treated as blocks in time. As analysis of variance revealed no significant interaction between strain and time of scoring for a P of 0.05, data for all evaluation points were taken together for analysis. Means were compared by using Fisher's least-significant-difference test.

RESULTS

Complementation of a pyoverdin- and pyochelin-negative mutant (KMPCH) for pyochelin production and characterization of the pyochelin clone. Preliminary work indicated a role for pyochelin production in the protection of tomato plants by *P. aeruginosa* 7NSK2 against *Pythium*-induced damping-off (17). A mutant deficient in the production of pyochelin and pyoverdin, KMPCH, was less effective in controlling *Pythium*-induced postemergence damping-off than were the wild-type strain, 7NSK2, and a pyoverdin-negative mutant, MPFM1. Both mutants, KMPCH and MPFM1, were unable to grow on MKB medium amended with 20 μg of EDDHA [ethylenediamine di(*o*-hydroxyphenylacetic acid)] ml^{-1}. Mutant KMPCH still produced salicylic acid, as was demonstrated by TLC analysis (data not shown). Since in *P. aeruginosa*, pyochelin is produced from chorismic acid with salicylic acid as an intermediate (2, 49), the pyochelin mutation in mutant KMPCH is downstream of salicylic acid. Although salicylic acid acts as a siderophore in *P. aeruginosa* (54), it is unable to remove the ferric ion from the blue Fe(III)-CAS complex. To more precisely define the role of pyochelin in the biological control of *Pythium*-induced damping-off, mutant KMPCH was complemented for pyochelin production.

A gene region that has a role in pyochelin synthesis was isolated by mobilizing an *Eco*RI gene bank of *P. aeruginosa* 7NSK2 in mutant KMPCH. Five of the 3,519 tested clones in this gene bank were able to restore pyochelin production in mutant KMPCH, as visualized by an orange halo on Casamino Acid medium with chrome azurol S and blue fluorescence under UV on MKB medium. Restriction fragment analysis of the five clones that restored pyochelin production in mutant KMPCH revealed that all clones were identical. One clone (K5), containing 27.8 kb of insert DNA, was used for further study (Fig. 1). Analysis by TLC confirmed that the complemented mutant KMPCH(K5) produced both salicylic acid and pyochelin.

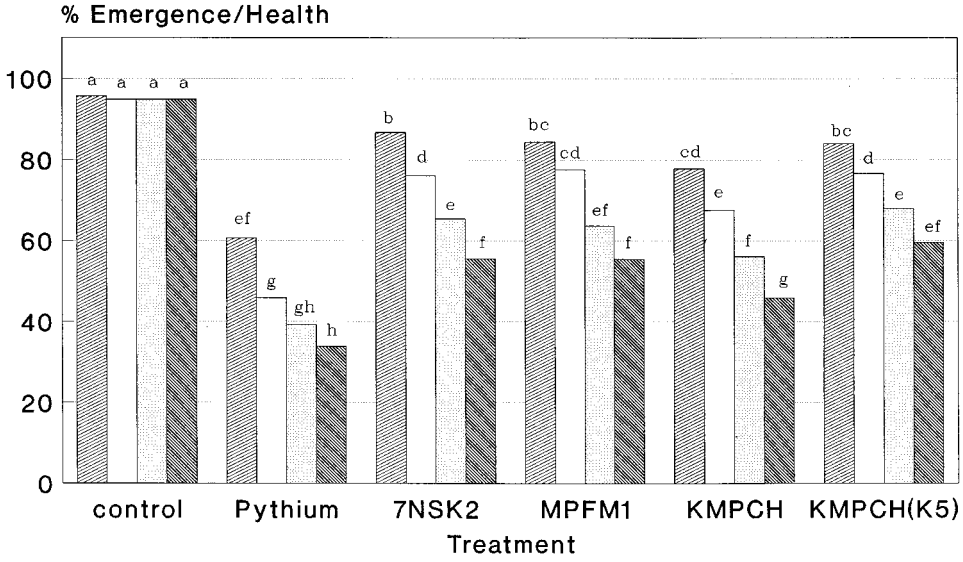

FIG. 2. Influence of *P. aeruginosa* 7NSK2 and its pyoverdin- and/or pyochelin-negative derivatives on emergence of tomato seeds (hatched bars) and health of tomato seedlings 1, 4, and 9 days after emergence (open, dotted, and finely hatched bars, respectively) in a *Pythium* biocontrol experiment. Values are the means of seven separate experiments, with every treatment replicated at least four times. Data represented by bars with a common letter do not differ significantly according to Fisher's least-significant-difference test ($P = 0.05$).

The location of the pyochelin biosynthesis genes was determined by subcloning and deletion analysis of the 27.8-kb insert and by Tn*3*-HoHo1 mutagenesis (53). Only pSB5 contained all the genetic information required for restoration of pyochelin production in mutant KMPCH (Fig. 1). Insertions causing a loss of complementation (Fig. 1) were all mapped in a 4.5-kb *Kpn*I fragment of clone K5. However, this fragment, cloned in two directions behind the *lacZ* promoter in vector pBBR1MCS, did not complement the pyochelin mutation in mutant KMPCH. It therefore seems that a larger fragment is necessary to complement the pyochelin mutation in mutant KMPCH.

Transferring clone K5 to a pyoverdin- and pyochelin-negative *P. fluorescens* strain did not result in pyochelin production by this strain (data not shown). This indicated that not all the genetic information necessary for pyochelin production was present on clone K5.

Clone K5 was also not able to complement *P. aeruginosa* PAO1 mutants mutated in either *pchA* (LS128-6) or *pchB* (LS128-3), indicating that genetic information necessary for salicylate production from chorismic acid is lacking on clone K5. Transfer of clone pME3300 (49), containing most of the genes involved in salicylic acid and pyochelin biosynthesis by *P. aeruginosa* PAO1, to mutant KMPCH gave complementation of the pyochelin mutation (data not shown).

Role of pyochelin in in vivo antagonism of *P. aeruginosa* 7NSK2 toward *P. splendens* in tomato. All bacterial treatments significantly reduced preemergence damping-off (Fig. 2) compared with the untreated *Pythium*-infested control (61% emergence). Bacterial treatments in the absence of the fungus had no significant effect on emergence and growth of the tomato plants (data not shown). There was no difference in preemergence protection against *P. splendens* among the wild-type strain, 7NSK2 (83% emergence), the pyoverdin-negative mutant MPFM1 (82% emergence), and the pyoverdin- and pyochelin-negative mutant complemented for pyochelin production, KMPCH(K5) (84% emergence). The pyoverdin- and pyochelin-negative mutant, KMPCH, also reduced *Pythium*-induced damping-off (76% emergence) but was less effective than the parental strain.

The best protection against postemergence damping-off was obtained by treating the tomato seeds with the pyochelin-producing strains 7NSK2, MPFM1, and KMPCH(K5) (Fig. 2; see data for 1, 4, and 9 days postemergence, respectively). These inoculation treatments increased the number of healthy plants 9 days after emergence from 34% in the untreated *Pythium*-infested control to more than 55%. Strain KMPCH showed a reduced ability to suppress *Pythium*-induced postemergence damping-off (46% healthy seedlings 9 days after emergence) compared with the parental strain and mutants MPFM1 and KMPCH(K5) (Fig. 2). The reduced protection of tomato seedlings against *P. splendens* by mutant KMPCH was not due to impaired root colonization ability. Rhizosphere population densities 9 days after emergence in the bioassay were not significantly different for the different bacterial strains (5×10^5 to 1×10^6 CFU g^{-1} of root). Enumeration of KMPCH(K5) on media with and without tetracycline revealed that the cosmid was stably maintained throughout the course of the experiment.

Complementation of mutant KMPCH for pyochelin production resulted in restoration of its antagonism toward *Pythium*-induced damping-off to a level comparable to that of the wild type.

In conclusion, pyoverdin production was not required for biological control in a pyochelin-producing strain. When neither pyochelin nor pyoverdin was produced (*P. aeruginosa* KMPCH), significantly less-effective control of *Pythium*-induced damping-off than that shown by the wild-type strain was obtained. These results suggest that pyochelin could be responsible for the biocontrol ability of *P. aeruginosa* 7NSK2.

Construction of a pyoverdin-positive, pyochelin-negative mutant, SPCN1. To further investigate the role of pyochelin in the antagonism toward *P. splendens*, a pyoverdin-positive, pyochelin-negative mutant was constructed. Complementation of mutant KMPCH for pyoverdin production was attempted. None of approximately 3,500 clones of an *Eco*RI genomic library of *P. aeruginosa* 7NSK2 constructed in pLAFR1 was able to restore pyoverdin production after mobilization into mutant KMPCH. Therefore, we decided to construct a pyover-

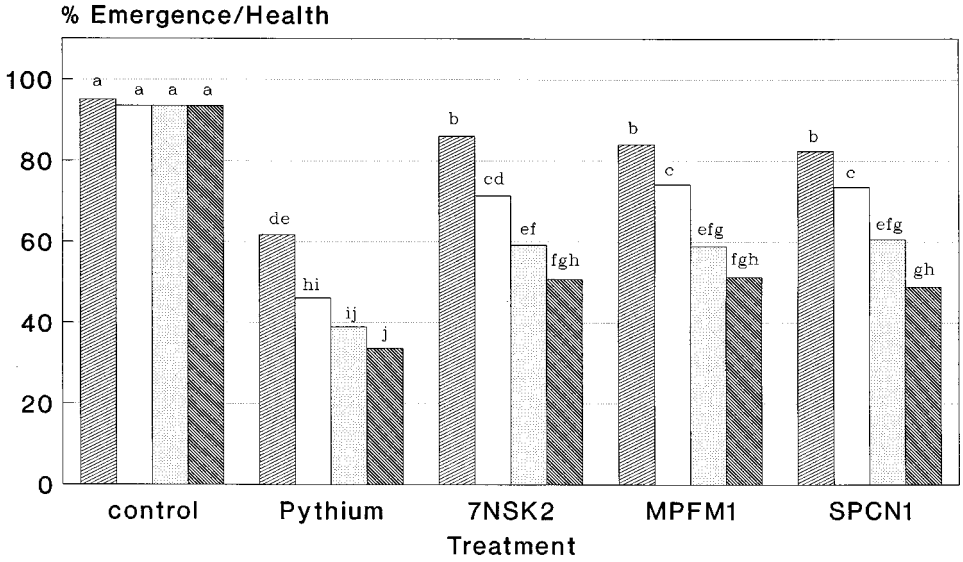

FIG. 3. Influence of *P. aeruginosa* 7NSK2 and its pyoverdin- or pyochelin-negative derivatives on emergence of tomato seeds (hatched bars) and health of tomato seedlings 1, 4, and 9 days after emergence (open, dotted, and finely hatched bars, respectively) in a *Pythium* biocontrol experiment. Values are the means of four separate experiments, with every treatment replicated at least four times. Data represented by bars with a common letter do not differ significantly according to Fisher's least-significant-difference test ($P = 0.05$).

din-positive, pyochelin-negative mutant by using the pyochelin clone K5.

By Tn*3*-HoHo1 mutagenesis of K5, clones that did not restore pyochelin production after conjugation to mutant KMPCH were obtained. These clones were conjugated to the wild-type strain, 7NSK2.

Only inserts at site 1 (Fig. 1) could be stably integrated into the chromosome of strain 7NSK2. The resulting mutant, SPCN1, had a pyoverdin-positive, pyochelin-negative phenotype as confirmed by TLC analysis and was resistant to carbenicillin (marker of Tn*3*-HoHo1) and sensitive to tetracycline (marker of pLAFR1) (data not shown). The fidelity of the marker exchange event was confirmed by Southern analysis. Mutant SPCN1 was able to grow on MKB medium amended with 200 μg of EDDHA ml^{-1}.

Inserts at other places resulted in a pyochelin-positive and tetracycline-resistant phenotype, meaning that the cosmid was still present and no recombination had occurred.

Suppression of *Pythium*-induced damping-off by mutant SPCN1. The pyoverdin-positive, pyochelin-negative mutant, SPCN1, was as effective as the parental strain, 7NSK2, and the pyoverdin-negative mutant MPFM1 in suppressing *Pythium*-induced damping-off of tomato (Fig. 3).

Mutant SPCN1 reduced preemergence damping-off, resulting in an increase in emergence from 61% in the *Pythium*-infested control to 82%. Also, significant protection against postemergence damping-off was obtained 1, 4, and 9 days after emergence by seed inoculation with the pyochelin-negative mutant SPCN1. This protection was comparable to that obtained by the parental strain and the pyoverdin-negative mutant MPFM1. All bacterial treatments resulted in approximately 50% healthy seedlings (49 to 54% according to the strain), whereas only 34% healthy seedlings were counted in the untreated *Pythium*-infested control 9 days after emergence. Survival and colonization of tomato roots by mutant SPCN1 were comparable to those of the wild type and the pyoverdin-negative mutant (5×10^5 CFU g^{-1} of root, 9 days after emergence).

These observations indicate that pyochelin production is not required for biological control of *Pythium*-induced damping-off by *P. aeruginosa* 7NSK2 in a pyoverdin-producing strain.

DISCUSSION

P. aeruginosa 7NSK2 produces three siderophores under iron limitation: pyoverdin (18), pyochelin (18), and salicylic acid (this work). This *Pseudomonas* strain controls *Pythium*-induced damping-off of tomato. The production of siderophores has been linked to the disease suppression ability of certain fluorescent *Pseudomonas* spp. (35). We compared the wild-type strain with mutants deficient in the production of pyochelin and/or pyoverdin to further characterize the role of these siderophores in the protection mechanism.

All mutant strains retained at least to some extent the ability to protect germinating tomato seeds against *Pythium*-induced seed and radicle rot (preemergence damping-off), possibly by competing with the pathogen for seed exudates (14). Seed exudates are required for the germination of *Pythium* sporangia and play a role in the attraction of the pathogen to the germinating seed (44).

The production of either pyochelin or pyoverdin proved to be necessary to achieve high levels of protection against *Pythium*-induced postemergence damping-off. The actions of pyoverdin and pyochelin seemed to be mutually exchangeable because a mutant producing only pyochelin and not pyoverdin was equally antagonistic as a mutant producing only pyoverdin and not pyochelin. Moreover, with both mutants, wild-type levels of protection were obtained.

As pyochelin and pyoverdin are both siderophores, iron competition with the pathogen could explain the observed disease suppression and their interchangeable effects. Mycelial growth (34, 39) but not sporangial germination (46) of *Pythium* spp. is inhibited by iron starvation. Despite its low iron-binding coefficient (5×10^5 at acidic pH [10]), pyochelin may act as a strong iron chelator in vivo. In clinical work, it has been shown that pyochelin is very active in iron transport (8) and growth stimulation in media containing transferrin (9).

The role of pyoverdin, the fluorescent siderophore produced

by fluorescent pseudomonads, in the reduction of *Pythium*-induced damping-off is ambiguous as described in the literature. Pyoverdin production seems not to be important for the biocontrol of *Pythium*-induced preemergence damping-off of cucumber. Paulitz and Loper (46) and Kraus and Loper (30) could not detect a difference in activity against *Pythium*-induced reduction in emergence of cucumber between *P. putida* N1R and *P. fluorescens* Pf-5, respectively, and their pyoverdin-negative derivates. On the contrary, in other systems fluorescent siderophores do play a role in antagonism toward *Pythium* spp. Pyoverdin production by *P. fluorescens* 3551 is important for the biocontrol of *Pythium*-induced damping-off of cotton (34). Siderophore production by fluorescent pseudomonads has a role in the increased growth response of wheat which is attributed to the control of *Pythium* spp. (4).

Well-characterized mutants that are deficient in siderophore production are valuable tools for assessing the potential role of siderophores in biocontrol, but this approach has some limitations (35). For instance, the biosynthesis of metabolites other than siderophores may be altered in such mutants. Moreover, this approach may lead to a wrong conclusion, especially when more than one factor is involved in the biocontrol. From our experiments it can be concluded that pyoverdin production is not essential in antagonism if a mutant still produces pyochelin and that pyoverdin plays a role when a mutant is deficient in pyochelin production.

It cannot be ruled out that pyoverdin and pyochelin act in an indirect way. For example, the internal iron pool of mutant KMPCH could be depleted, resulting in a reduced level of biosynthesis of secondary metabolites important for the antagonism toward *Pythium* spp.

However, the fact that salicylic acid has been reported to exhibit siderophore activity (54) may render the hypothesis of internally depleted iron pools less likely. In addition, *P. aeruginosa* 7NSK2 does not produce 2,4-diacetylphloroglucinol or pyoluteorin (23). These antibiotics have a demonstrated effect against *Pythium* spp. and other fungal pathogens (21, 25, 36, 50). Their production is either not regulated by iron (2,4-diacetylphloroglucinol) (50) or stimulated by low iron concentrations (pyoluteorin) (21, 36). Strain 7NSK2 is also not able to produce hydrogen cyanide (data not shown), a metabolite responsible for the control of plant pathogens (55). Pseudomonads need sufficient iron to produce HCN (24). Moreover, we did not observe inhibition zones in dual cultures of *P. aeruginosa* and *P. splendens* on different media such as LB medium and potato dextrose agar (iron-rich media) or MKB medium (iron-limited medium), indicating that no other metabolites that directly affect the growth of the fungus are produced on these media. On these media, strain 7NSK2 does not produce pyocyanin, a phenazine compound produced by most *P. aeruginosa* strains (32). *P. aeruginosa* 7NSK2 makes pyocyanin only on Pseudomonas P agar (Difco), which is low in phosphate (data not shown). We did not test whether pyocyanin has a direct antifungal effect on *P. splendens.*

An alternative mode of action for pyochelin may lie in its interaction with pyocyanin. Pyocyanin has the capacity to undergo redox cycling under aerobic conditions, resulting in the generation of superoxide and hydrogen peroxide. Pyochelin, particularly when iron is bound to it (ferripyochelin), has been found to catalyze the formation of hydroxyl free radicals from superoxide and hydrogen peroxide. Exposure of mammalian endothelial cells to ferripyochelin and pyocyanin produced by *P. aeruginosa* spp. resulted in cell injury. This suggests that pyochelin and pyocyanin act synergistically by the generation of hydroxyl free radicals to damage local tissues at sites of *Pseudomonas* infection (7). Superoxide, hydrogen peroxide, and hydroxyl free radicals represent active oxygen species that are thought to be involved in induction of disease resistance in plants (37). It is attractive to hypothesize that active oxygen species generated by the pyochelin-pyocyanin interaction induce resistance in tomato plants, which results in an enhanced protection against *Pythium*-induced damping-off. It remains to be shown whether or not this phenomenon can occur on plant roots. To our knowledge, only pyochelin is reported to be involved in free-radical formation. In fact, most iron chelators, including pyoverdin (42), appeared to have free-radical scavenging properties. Viewed from that perspective, the observed antagonism of *P. aeruginosa* 7NSK2 toward *P. splendens* could be explained by pyoverdin-mediated iron competition and induction of resistance by pyochelin.

Salicylic acid is known to induce systemic acquired resistance in plants (11). A rise in the level of salicylic acid precedes development of systemic acquired resistance and the induction of resistance-associated peroxidase activity. It is therefore interesting that mutant KMPCH, which is impaired in pyoverdin and pyochelin production but still makes salicylic acid, retained some ability to suppress *Pythium*-induced damping-off (Fig. 2). Salicylic acid produced by rhizobacteria might be taken up by plants, thereby inducing resistance systemically. Leeman (31) showed that exogenously applied salicylic acid suppressed fusarium wilt of radish in an induced-resistance bioassay at a concentration as low as 100 fg per root system. Further research will reveal the role of salicylic acid in the control of *Pythium*-induced postemergence damping-off.

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