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Secondary Metabolite- and Endochitinase-Dependent Antagonism toward Plant-Pathogenic Microfungi of *Pseudomonas fluorescens* Isolates from Sugar Beet Rhizosphere

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Forty-seven isolates representing all biovars of *Pseudomonas fluorescens* (biovars I to VI) were collected from the rhizosphere of field-grown sugar beet plants to select candidate strains for biological control of preemergence damping-off disease. The isolates were tested for in vitro antagonism toward the plant-pathogenic microfungi *Pythium ultimum* and *Rhizoctonia solani* in three different plate test media. Mechanisms of fungal inhibition were elucidated by tracing secondary-metabolite production and cell wall-degrading enzyme activity in the same media. Most biovars expressed a specific mechanism of antagonism, as represented by a unique antibiotic or enzyme production in the media. A lipopeptide antibiotic, viscosinamide, was produced independently of medium composition by *P. fluorescens* bv. I, whereas the antibiotic 2,4-diacetylphloroglucinol was observed only in glucose-rich medium and only in *P. fluorescens* bv. II/IV. Both pathogens were inhibited by the two antibiotics. Finally, in low-glucose medium, a cell wall-degrading endochitinase activity in *P. fluorescens* bv. I, III, and VI was the apparent mechanism of antagonism toward *R. solani*. The viscosinamide-producing DR54 isolate (bv. I) was shown to be an effective candidate for biological control, as tested in a pot experiment with sugar beet seedlings infested with *Pythium ultimum*. The assignment of different patterns of fungal antagonism to the biovars of *P. fluorescens* is discussed in relation to an improved selection protocol for candidate strains to be used in biological control.

Fluorescent *Pseudomonas* spp. are known to inhibit plant-pathogenic fungi in the sugar beet rhizosphere (34, 42, 46). Members of both the *Pseudomonas fluorescens-Pseudomonas putida* species complex and *Pseudomonas chlororaphis* (including *Pseudomonas aureofaciens*) have demonstrated in vitro antagonism toward several soil microfungi, but with great variability among the strains (7). Production of secondary metabolites, e.g., antibiotics, Fe-chelating siderophores, and cyanide, is most often associated with fungal suppression by fluorescent pseudomonads in the rhizosphere of sugar beets (27, 42) and other crops (13, 14, 48, 53). In addition, cellulytic activity (10) and chitinolytic (25) activity have occasionally been reported among fluorescent pseudomonads, but compared to the extensive work on these enzymes in other bacteria and microfungi (5, 11, 39, 40, 49, 50, 54), very little work has addressed their role in antagonistic *Pseudomonas* spp. Studies of in vitro antagonism have often demonstrated that medium composition strongly affects production of secondary metabolites and hence affects the inhibition pattern exhibited by specific *Pseudomonas* strains (16, 33, 42). Production of cell wall-degrading enzymes, if operative in *Pseudomonas* spp., is also likely to be medium dependent and variable among strains.

In selecting potential candidates among *Pseudomonas* isolates for in situ biological control of plant-pathogenic fungi, several criteria should be met by the bacteria. These include (i) ecological fitness, including rhizosphere competence, to maintain an effective population size in situ; (ii) rapid root colonization to antagonize fast-growing pathogens, e.g., *Pythium ultimum* (34); and (iii) stable production of antifungal agents under variable growth conditions to sustain antagonism during root development. Unfortunately, discrepancies exist between the antagonistic effect under in vitro conditions and the corresponding in situ efficacy (8, 37, 55), although some studies have demonstrated that production of specific antibiotics in vitro is indeed correlated with their production and antagonism in situ (15, 18, 48). An improved approach for selection of candidate biological control agents may be based on identification of strains showing a constant, medium-independent production of antifungal compounds (31). Further improvement of such a selection protocol may be obtained if the mechanism of antagonism can be related to specific phenotypic traits used for subgrouping, e.g., biovar typing, that are common among the fluorescent *Pseudomonas* spp.

In this study, our aim was to provide a selection protocol for natural *Pseudomonas* strains that demonstrates a stable, medium-independent antagonism toward two widely different fungal pathogens. Hence, we compared a large collection of fluorescent pseudomonads isolated from the sugar beet rhizosphere for in vitro antagonism in three different media toward *Pythium ultimum* (an oomycete with a cellulose-containing cell wall) and *Rhizoctonia solani* (a basidiomycete with a chitin-containing cell wall) to determine if there are distinct functional groups within *P. fluorescens* which correlate with biological activity. Possible mechanisms of in vitro antagonism were

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elucidated by determining secondary-metabolite (antibiotics, siderophores, cyanide, and others) production and cell wall-degrading enzyme (cellulase, hemicellulase, and endochitinase) activity in the three test media. A *P. fluorescens* isolate, DR54, showing medium-independent antibiotic and endochitinase production and in vitro antagonism to both of the plant-pathogenic microfungi was the most promising candidate identified by our selection protocol. The ability of the DR54 isolate to reduce the incidence of damping-off disease in sugar beet seedlings in a pot experiment further supported the application of this approach to identify effective biological control agents.

MATERIALS AND METHODS

***Pseudomonas* isolates from sugar beet rhizosphere.** Bacteria were isolated in 1994 from the rhizosphere of sugar beets on the experimental field of Danisco Seed, Holeby, Denmark. One set of isolates was collected from 1-month-old plants (DR isolates), and another set was collected from 4-month-old plants (PS isolates). The plant roots were carefully shaken to remove excess soil, and five complete root systems were washed in 200 ml of Winogradsky salt solution [0.025% K_2HPO_4, 0.0125% $MgSO_4$, 0.0125% NaCl, 0.0025% $Fe_2(SO_4)_3$, 0.0025% $MnSO_4$ (pH 6.7)] on a shaker (Struers, Copenhagen, Denmark). After 10-fold dilutions were performed, 100-μl aliquots were plated on 10% tryptic soy agar (TSA; Difco, Detroit, Mich.) (DR isolates) and Gould's S1 agar (12) selective for fluorescent pseudomonads (PS isolates). The plates were incubated at 30°C for 2 days before colonies were isolated. A total of 47 *Pseudomonas* sp. isolates showing growth and UV fluorescence on Gould's S1 medium, and further shown to be gram negative and catalase and oxidase positive, were selected for further study. Isolates were maintained in glycerol at −80°C.

For identification to the subspecies (biovar) level of *Pseudomonas* as described by Palleroni (35), the following traits were examined: denitrification by using inverted Durham tubes in tryptic soy broth (TSB; Difco), production of levan exopolysaccharide in 5.0% (wt/vol) sucrose, and growth in 0.4% (wt/vol) mannitol, *meso*-inositol, or sorbitol in minimal medium [0.1% NH_4Cl, 0.2% KH_2PO_4, 0.2% $K_2HPO_4 \cdot 3H_2O$, 0.01% $MgSO_4$, 0.001% $Fe_2(SO_4)_3$, 1 μM concentrations of $CuCl_2$, $ZnSO_4$, $MnSO_4$, $CoCl_2$, $NiCl_2 \cdot 6H_2O$, and $NaMoO_4$].

To group the isolates in clusters of highly similar strains, whole-cell extracts were analyzed for lipopolysaccharide (LPS) profiles by a slight modification of the method of Sørensen et al. (43). The gels were silver stained by incubating in fixation buffer (40% ethanol, 5% glacial acetic acid) for 3 h and subsequently oxidized in fixation buffer containing 0.7% periodic acid for 10 min. The gels were washed three times for 10 min each time in water before being stained in 200 ml of 0.2% $AgNO_3$ with 150 μl of 37% formaldehyde for 20 min. The gels were then washed three times, 20 s each, and then developed in a mixture containing 6% $NaCO_3$, 4 mg of $Na_2S_2O_3$ per liter, and 500 μl of 37% formaldehyde per liter. Finally, the gels were transferred to fixation buffer containing 1% glycerol. The profiles were compared visually, and isolates were grouped in clusters that are referred to by numbers below.

Test fungi. *Pythium ultimum* (strain 92001; Danisco Seed) was originally isolated from diseased cucumber plants (57) and is highly pathogenic to sugar beets. *R. solani* (strain 92009; Danisco Seed) was isolated from sugar beets exhibiting symptoms of damping-off disease. Both fungal strains were maintained at 4°C and subcultured every second month, with cultures alternating between cornmeal agar (Difco) and oatmeal agar (Difco).

In vitro tests of fungal antagonism. The plate test screening for in vitro antagonism against the two plant-pathogenic fungi was performed by placing a 1-cm-diameter agar plug with mycelium in the center of an agar plate and spotting the *Pseudomonas* isolates on the plate at four locations, approximately 3 cm from the center. The plates were incubated at 30°C and checked for zones of inhibition of mycelial growth after approximately 2 days, when the fungal mycelium had reached the edge of the plate. All in vitro tests of antagonism were performed twice, with new coinoculations used each time. The in vitro antagonism was assessed on potato dextrose agar (PDA; 20% peeled potato, 2% glucose, 2% agar), potato agar (PA; PDA without glucose addition [16]), and seed extract agar (SEA). The SEA medium was prepared from nonsterilized sugar beet seeds (cultivar Magnat), essentially as reported by Stephens et al. (45); In our study, the solution was filtered through cheesecloth before centrifugation and subsequently passed through a coarse glass microfiber filter (Whatman, Maidstone, United Kingdom) and a 0.2-μm-pore-size membrane filter (Sartorius, Göttingen, Germany), using a vacuum pump for the last filtration step. The glucose contents in PDA, PA, and SEA media were approximately 2.6, 0.6, and 0.01% (wt/vol), respectively, as determined by the method of Jørgensen and Jensen (17) and reported by James and Gutterson (16). The antagonistic effect of culture extracts from bacteria grown on the test media was evaluated in filter tests similar to the in vitro assay with living cells. Extraction of antagonistic compounds was performed after 5 days of plate incubation at 26°C. Twenty 5-mm-diameter agar plugs were cut from a plate with dense bacterial growth and sonicated for 10 min in 2 ml of dichloromethane-methanol-ethyl acetate (2:1:3) with 1% formic acid by using an ultrasonic bath (Metason 200; Struers). The extract was transferred to a new sample vial and dried under vacuum. The residue was subsequently redissolved in 50 μl of a solvent of methanol and sterile (Milli-Q) water (97:3) with 1% formic acid and a 0.1% concentration of HCl, which provided a 40-fold concentration factor. Extract (20 μl) was spotted onto a 5-mm-diameter coarse glass microfiber filter (Whatman) and dried by evaporation. The redissolution solvent was used as a blank. The filters were placed approximately 1 cm from the edge of the agar plug with fungal mycelium.

Production of secondary metabolites. Visible pigment production by bacteria grown in the test media was recorded up to a week after inoculation, since this could indicate production of chromophore-containing antibiotics (2, 47). High-performance liquid chromatography (HPLC) analysis of culture extracts was also performed to study the production of secondary metabolites. Fourteen representative isolates of all *P. fluorescens* biovars (see below) were grown on agar plates for 4 days at 26°C; medium blanks without bacteria were included. The entire volume of medium from an agar plate (10 to 20 ml) with dense bacterial growth was transferred to a plastic centrifuge bottle. After addition of 30 ml of ethyl acetate, the sample was homogenized in a blender (Ultraturrax TP18-10; Jahnke and Kunkel GmbH IKA-Werk, Staufen, Germany) and extracted for 1 h on a rotary shaker at 200 rpm (Certomat M; B. Braun Melsungen AG, Melsungen, Germany). After centrifugation for 20 min at 3,000 × *g*, the upper organic phase was dried by evaporation in a Speedvac concentrator (Savant A290; Trio-Lab, Copenhagen, Denmark). The residue was redissolved in 3 ml of methanol, which provided a 10-fold concentration factor. HPLC analysis was performed with a Hewlett-Packard 1090M series II chromatograph with a diode array detector. A Novogram column (4.0 mm [inside diameter] by 60 mm [length]) was used in combination with a precolumn (4.0 mm [inside diameter] by 5 mm [length]), both containing C_{18} reverse-phase material (Grom, Herrenberg, Germany). The samples were run with gradient elution from 100% solvent A (0.1% aqueous orthophosphoric acid) to 100% solvent B (acetonitrile, HPLC grade) in 6 min. Chromatograms were analyzed with Hewlett-Packard HPLC 3D Chemstation software (DOS series). Cell extract from the reference strain *P. fluorescens* Pf-5 (provided by Joyce E. Loper, USDA-ARS Horticultural Research Laboratory, Corvallis, Oreg.), which is known to produce several antibiotics, including 2,4-diacetylphloroglucinol (DAPG), pyoluteorin, and pyrrolnitrin (33), was used concomitantly with purified antibiotics (DAPG, pyoluteorin, and pyrrolnitrin) for identification of a similar metabolite(s) produced by our isolates.

Production of siderophores was determined by the method of Schwyn and Neilands (41), using the CAS reagent (chrome azurol S; Fluka Chemika, Buchs, Switzerland). Isolates were grown on CAS agar plates supplemented with 2% glucose, 0.5% L-glutamic acid (neutralized), and 5 ppm (+)-biotin. The presence of orange haloes was recorded up to 7 days after incubation.

Detection of HCN production was performed by the method of Bakker and Schippers (1). Production of cyanide was determined by a color shift from yellow to orange in the filter paper (3).

Production of cell wall-degrading enzymes. Endochitinase activity (hydrolytic splitting within chitin polymer) was tested on 1/10 TSA medium (Difco) containing 1.5 mg of a chromogenic substrate, CM-chitin-RBV (Loewe, Sauerlach, Germany) (56), per ml. Detailed analysis of endochitinase production was further performed on the three test media used for in vitro antagonism (PA, PDA, and SEA), supplemented with this chromogenic substrate. Cellulase, mannanase, xylanase, and 1,3-β-D-glucanase production were tested on 10% TSA, containing 1 mg of specific chromogenic (azurine-dyed, cross-linked; AZCL) substrate per ml (Megazyme, Sydney, Australia), for each enzyme reaction. All enzyme tests were performed twice, and zones of enzyme activity surrounding the bacterial colonies were recorded up to 7 days after inoculation.

Inhibition of damping-off disease in sugar beets. One strain, DR54, was tested for fungal antagonism toward *Pythium ultimum* on sugar beet seedlings in a pot experiment. Cells were grown aerobically overnight in 50 ml of Luria broth (1% Bacto Tryptone, 0.5% yeast extract, 1% NaCl, 0.01% glucose [pH 7.2]) on a rotary shaker at room temperature, and *Pythium ultimum* was grown on PDA (Difco). Sugar beet seeds were planted in 11-cm-diameter pots (0.5 liter) with moist peat soil (1:1 [vol/vol] mixture of TKS2 Instans [Floragard, Oldenburg, Germany] and medium peat [Stenrøgelse, Kjellerup, Denmark]). Twelve seeds were planted per pot, and each pot was infested with 1-cm-diameter agar plugs (8 pieces per pot) taken from the edge of growing *P. ultimum* mycelia. Seeds were inoculated by dripping a 50-μl suspension of bacterial cells (10^8 cells/ml) onto the seeds before covering them with peat. The pots were incubated in a growth chamber (15°C; cycles of 16 h of light and 8 h of darkness) for 14 days. The number of healthy seedlings was recorded. Seeds treated with demineralized water served as a negative control, and other seeds treated with a fungicide (Thiram; KVK Agro, Køge, Denmark) served as a positive control. Each of two separate experiments had three replicates. Data were evaluated by logistic regression analysis in accordance with a binomial distribution (Logit, Proc Genmod, SAS Institute). The analysis provides an estimate of the relative probability (odds ratio) of getting healthy plants by the treatments compared to that by an untreated control.

RESULTS

***Pseudomonas* characterization.** All 47 isolates were identified as *P. fluorescens* by their biochemical traits. As shown in

TABLE 1. LPS clustering and biovar determination for *P. fluorescens* isolates from sugar beet rhizosphere

Isolate	Cluster[a]	Characteristic[b]					Biovar
		Lev	Den	Ino	Sor	Man	
DR54	1	+		+	+	+	I
PS12	2	+	+	+	+	+	II/IV
DR50	3	+	+	+	+	+	II/IV
PS8	4	+	+	+	+	+	II/IV
PS29	4	+	+	+	+	+	II/IV
PS24	4	+	+	+	+	+	II/IV
PS31	4	+	+	+	+	+	II/IV
PS11	5	+	+	+	+	+	II/IV
PS15	5	+	+	+	+	+	II/IV
PS16	5	+	+	+	+	+	II/IV
PS18	5	+	+	+	+	+	II/IV
PS20	5	+	+	+	+	+	II/IV
PS30	5	+	+	+	+	+	II/IV
DR1	6		+	+	+	+	III
DR5	6		+	+	+	+	III
DR4	7		+	+	+	+	III
DR47	7		+	+	+	+	III
DR34	8		+	+	+	+	III
DR46	9		+	+	+	+	III
DR40	10		+	+	+	+	III
DR52	10		+	+	+	+	III
DR57	10		+	+	+	+	III
PS14	11		+	+	+	+	III
PS3	12		+	+	+	+	III
DR17	13		+	+	+	+	III
DR36	13		+	+	+	+	III
DR3	14		+	+	+	+	III
DR8	14		+	+	+	+	III
DR2	15		+	+	+	+	III
DR7	15		+	+	+	+	III
PS1	16		+	+	+	+	III
PS21	17		+	+	+	+	III
DR41	18			+	+	+	V
DR56	19			+		+	V
DR12	20			+		+	V
DR20	21					+	VI
DR48	22					+	VI
PS2	23					+	VI
PS4	23					+	VI
PS7	23					+	VI
PS9	23					+	VI
PS10	23					+	VI
PS13	23					+	VI
PS17	23					+	VI
PS23	23					+	VI
PS25	23					+	VI
PS28	23					+	VI

[a] Cluster number refers to LPS pattern.

[b] Isolates were examined for levan formation (Lev), denitrification (Den), and growth on *meso*-inositol (Ino), sorbitol (Sor), and mannitol (Man). +, positive for indicated characteristic. Blanks indicate negative results.

Table 1, the isolates could be assigned to biovars I, II/IV, III, V, and VI (35) and thus represented all known biovars of *P. fluorescens*. No distinction was made between biovars II and IV. The 47 isolates were further grouped into 23 clusters by the LPS profiles. Fourteen clusters consisted of only one isolate, while other clusters contained several isolates (Table 1). Isolates within a cluster were anticipated to represent a single strain or closely related strains.

In vitro antagonism. Table 2 shows the results of in vitro tests of antagonism toward the plant pathogens *Pythium ultimum* and *R. solani*. Inhibition was clearly discerned by very limited growth or the complete absence of fungal mycelium in the inhibition zone (up to 8 mm) surrounding a bacterial colony. Variability within clusters resulted mainly from variation between isolates.

(i) *Pythium ultimum*. On PDA medium, growth of *Pythium ultimum* was excellent, but more than two-thirds of the *P. fluorescens* clusters showed antagonism toward the hyphal growth (Table 2). While most clusters gave rise to only small inhibition zones, cluster 1 (*P. fluorescens* bv. I) and clusters 4 and 5 (biovar II/IV) resulted in significantly larger inhibition zones. Fungal growth on PA medium was similar to that on PDA, but inhibition zones generally were small and were observed only for cluster 1 (biovar I) and cluster 23 (biovar VI). In contrast, the *Pythium ultimum* mycelium was very thin in SEA medium, and antagonism was observed for approximately one-third of the *Pseudomonas* clusters. There was again an elevated level of antagonism demonstrated by clusters 1 (biovar I) and 4 (biovar II/IV).

(ii) *R. solani*. Generally, *R. solani* grew well on all three test media. Table 2 shows that inhibition zones were observed on PDA for *P. fluorescens* bv. I, bv. II/IV, and bv. III, including elevated levels for clusters 3, 4, and 5 of *P. fluorescens* bv. II/IV. In contrast, no antagonism toward *R. solani* was observed for *P. fluorescens* bv. V and VI (except for cluster 23 of biovar VI). On PA medium, cluster 1 (biovar I) demonstrated a slightly higher inhibition level than it did on PDA. A large majority of the clusters of biovars II/IV and III, showing inhibition of *R. solani* growth on PDA, had lost this activity on PA. Remarkable exceptions, however, were clusters 16 and 17 of biovar III, which showed stronger antagonism toward *R. solani* on PA. Even more pronounced, however, was the stimulated antagonism on PA for cluster 23, which belongs to *P. fluorescens* bv. VI. On SEA, antagonism was observed in about one-third of the clusters representing all biovars except biovar V. Inhibition zones were generally small, but cluster 23 (biovar VI) expressed a higher activity.

Production of antifungal compounds. A number of potential mechanisms of antagonism, e.g., production of pigments, antibiotics, siderophores, cyanide, and endochitinase, in the *P. fluorescens* isolates were tested.

(i) Antibiotics. Culture extracts were analyzed by HPLC to identify compounds of possible significance in fungal antagonism. UV-absorbing (210-nm-wavelength) metabolites were identified from chromatographic peaks which were unique to a specific isolate or cluster and related to in vitro antagonism. According to the HPLC analysis, only clusters 1, 4, and 5 grown on PDA produced UV-absorbing metabolites related to the observed in vitro antagonism (data not shown). These clusters were all active against both fungi on PDA (Table 2). The corresponding culture extracts were also active against both *Pythium ultimum* and *R. solani* as tested by the filter test method, confirming the production of at least one active compound by these isolates.

In cluster 1, *P. fluorescens* isolate DR54 produced two unique compounds (retention times, 4.68 and 4.84 min, respectively). The parallel observation of the two compounds in culture extracts from both PA and SEA media indicated a medium-independent metabolite production in this isolate, which corresponded well with its antagonism against the two fungi on PDA, PA, and SEA media (Table 2). Both compounds had only one absorption maximum at 200 nm or below, marking the detection limit of the UV detector. Fractionation of the culture extract by semipreparative HPLC analysis resulted in one compound (retention time, 4.84), which was active against both *Pythium ultimum* and *R. solani* in the filter test. Further analysis of the compound by liquid chromatography-mass spectrometry and nuclear magnetic resonance analysis resulted in

TABLE 2. In vitro antagonism against *Pythium ultimum* and *R. solani* on different media and production of antagonistic agents by *P. fluorescens* isolates from sugar beet rhizosphere

Cluster no.[a]	Biovar	Antagonism[b] on indicated medium toward:						Production of[c]:			
		P. ultimum			*R. solani*						
		PDA	PA	SEA	PDA	PA	SEA	Viscosinamide	DAPG	Blue-green pigment[d]	Endochitinase[e]
1	I	++	+	++	+	++	+	+			+
2	II/IV										
3	II/IV				++		+				
4	II/IV	++		++	++		+		+		
5	II/IV	++			++				+		
6	III	+		+	+					+	
7	III	++		+	+					+	
8	III	+			+					+	
9	III	+			+					+	
10	III	+			+					+	
11	III	+			+		+			+	
12	III				+					+	
13	III	+			+	+				+	
14	III	+			+					+	+
15	III	+		+	+					+	+
16	III	+			+	++	+			+	+
17	III				+	++	+			+	+
18	V										
19	V										
20	V										
21	VI			+						+	
22	VI	+									
23	VI	+	+		+	+++	++				+

[a] Clusters are distinguished by LPS profile.
[b] Symbols refer to the sizes of the inhibition zones (distance between fungal mycelium and bacterial colony); +, <1-mm inhibition zone; ++, 1- to 5-mm inhibition zone; +++, >5-mm inhibition zone. Blanks indicate no inhibition.
[c] Blanks indicate no production.
[d] Growth on PDA.
[e] Growth on either PDA, PA, SEA, or 1/10 TSA with CM-chitin-RBV as the chromogenic substrate for endochitinase activity.
[f] Variable response between strains in cluster.
[g] Variable response in test media.

identification of a compound proposed to be named viscosinamide (32), which is very similar to the lipopeptide antibiotic viscosin produced by *P. fluorescens* (4).

In clusters 4 and 5, *P. fluorescens* isolates PS8 and PS16 produced only one potentially active compound (retention time, 3.00 min) on PDA. No unique metabolites were observed on PA or SEA, which corresponded well with the PDA-mediated in vitro antagonism exhibited by these isolates. In both isolates, the active metabolite had distinct spectral properties corresponding to those of the antibiotic DAPG, including an absorption maximum at 270 nm (28) and a molecular mass of 210 g/mol as determined by liquid chromatography-mass spectrometry. Identification was further confirmed by direct comparison with a pure DAPG standard and by the formation of orange pigmentation on PDA plates, which is characteristic of DAPG production (2). Finally, HPLC analysis of the culture extract of PDA-grown *P. fluorescens* Pf-5, known to produce DAPG (33), showed a peak with the same retention time and absorption spectrum as those of isolates PS8 and PS16.

All clusters of biovar III produced a blue-green pigment on PDA, which probably contributed to the antagonism against the two fungi, but only at a low level (Table 2). As mentioned above, we were unable to detect any activity in the corresponding cell extract, and no further studies were done with this pigment.

(ii) Siderophores. Siderophore production on CAS medium was detected in all clusters. The test reaction with CAS reagent was quite different between clusters. Most isolates produced an orange halo surrounding the colonies, but for some isolates the halo became whitish surrounded by a pink (clusters 1, 2, 3, and 22) or green (cluster 23) color. In addition, the halo could be diffuse (most clusters) or sharp (cluster 22). Variability in pigment formation on CAS medium indicated involvement of several siderophores, but the results did not indicate a direct correlation to in vitro antagonism in this study.

(iii) HCN. Cyanide production occurred in all *P. fluorescens* biovars except biovars I and III but with different intensities. Clusters 3, 5 (biovar II/IV), and 18 (biovar V) were low-intensity cyanide producers, resulting in weak orange pigmentation of the indicator paper, while clusters 2 and 4 (biovar II/IV) gave moderate orange pigmentation and clusters 22 and 23 (biovar VI) were intense cyanide producers, giving strong orange-red pigmentation. The observed pattern could not be related to patterns of in vitro antagonism, and cyanide production was therefore not considered to be a significant factor in the inhibition of fungal growth.

(iv) Endochitinase activity. None of the isolates tested produced cellulase or hemicellulase (mannanase and xylanase), which indicated that antagonism against the oomycete *Pythium ultimum* (with a cellulose-containing wall structure) by these enzymes could be excluded. In contrast, several clusters produced endochitinase activity, which could be involved in antagonism against *R. solani* (with a chitin-containing wall structure). Table 2 shows the occurrence of endochitinase activity among *Pseudomonas* clusters as tested on PDA, PA, SEA, or TSA containing chitinaceous substrate. All media consistently supported endochitinase activity in clusters 1 (biovar I) and 23 (biovar VI); as determined by the clearing zone on the test

TABLE 3. Effects of seed inoculation with *P. fluorescens* DR54 isolate or Thiram[a] fungicide on appearance of healthy seedlings in pot experiments with *Pythium ultimum*-infested soil

Treatment	% Healthy seedlings[b]	Odds ratio[c]
DR54	36 ± 29	7.6*
Thiram	26 ± 13	4.8*
None (untreated)	7 ± 8	1.0

[a] Thiram is a trade-marked fungicide.
[b] Values are the means of results ± standard deviations of two pot experiments with three replicates each (12 plants per replicate) after 2 weeks of growth.
[c] Estimate of odds ratio (Proc Genmod; SAS Institute) of treated compared to nontreated seeds. *, significantly different from results for untreated control ($P \leq 0.01$).

plates, these clusters expressed a high level of chitinolytic activity. All other endochitinase-producing isolates belonged to clusters 14, 15, 16, and 17 (biovar III). Here the activity was generally only about half of that expressed in clusters 1 and 23, and the activity was further quite variable when tested on different media and by repeated tests on the same medium. Generally, chitinolytic activity was up to five times higher on PA than on PDA medium.

Disease suppression in sugar beet seedlings. Treatment of sugar beet seeds with the DR54 isolate resulted in a significant ($P = 0.008$) increase in the number of healthy plants compared to the number resulting with the untreated control after 2 weeks of growth in soil infested with *Pythium ultimum* (Table 3). There was no significant difference between experiments as determined by the statistical analysis. The Thiram-treated control also resulted in a significant increase ($P = 0.003$) in the number of healthy seedlings, although the number was lower than that of the DR54-treated seeds. The pathogenic pressure was quite high in these experiments, as indicated by the data for fungicide-treated pots.

DISCUSSION

Diversity of antagonistic *P. fluorescens* isolates from sugar beet rhizosphere. Lambert et al. (22) used whole-cell protein patterns obtained by sodium dodecyl sulfate-polyacrylamide gel electrophoresis to cluster rhizosphere *Pseudomonas* sp. isolates from sugar beets and showed that fungal antagonism could vary within such clusters. This was also confirmed by Vlassak et al. (52), who tested root-associated fluorescent pseudomonads against several plant-pathogenic fungi. It was suggested that whole-cell protein patterns differentiated the pseudomonads at the strain level, although this may be challenged by other biochemical characteristics (23). It is likely, however, that whole-cell protein profiles provide a clustering of closely related strains. Alternatively, several studies have used LPS O-antigen patterns obtained by sodium dodecyl sulfate-polyacrylamide gel electrophoresis to resolve *Pseudomonas* isolates at the strain level (6), even if LPS clusters may also display internal variation in biochemical characteristics (51). In the present study, we first separated the isolates into subgroups based on classical *Pseudomonas* biovars (44) by using a number of selected biochemical traits (43). The LPS typing pattern was subsequently used to differentiate clusters of highly similar isolates, such that each cluster was characterized by a unique combination of phenotypic traits (biovar classification) and LPS profile. It was clear from the results that most of the classical *P. fluorescens* biovars contained isolates showing antagonism toward one or both of the test fungi, *Pythium ultimum* and *R. solani*. However, within the *P. fluorescens* biovars, both isolates showing active in vitro antagonism toward *Pythium ultimum* and *R. solani* and others showing no significant effect were found, as has also been reported earlier (22).

Effect of medium composition on fungal antagonism. The present study demonstrated that both medium-independent and medium-dependent types of antagonism against the two plant-pathogenic fungi were found among the *P. fluorescens* isolates from sugar beet rhizosphere. The antibiotic viscosinamide, produced by *P. fluorescens* DR54 (cluster 1, biovar I) on all three test media, has not previously been reported, in contrast to the very similar compound viscosin (4). Viscosin has been shown to mediate increased membrane permeability in plant cells, thus facilitating pectolytic attack (24), and has been further reported to act as a biosurfactant (29, 36). To our knowledge, this compound has not been reported to act in fungal antagonism of root pathogens.

(i) *Pythium* and *Rhizoctonia* antagonism by secondary metabolites in high-glucose PDA medium. Much attention has recently been paid to the regulation of antibiotic production in *Pseudomonas* spp. as affected by growth phase and medium composition. Stimulation of oomycin A (16) and DAPG (33) antibiotic production by glucose has thus been shown. DAPG production may also be stimulated by other carbohydrates (42, 45), and production of the antibiotics pyoluteorin and pyrrolnitrin were shown to be stimulated by glycerol (21, 33). In the present work, the PDA medium was anticipated to support antagonism controlled by a high glucose content. As demonstrated by the in vitro antagonism toward both *Pythium ultimum* and *R. solani*, certain *Pseudomonas* clusters (4 and 5, biovar II/IV) demonstrated a strong, PDA-stimulated inhibition and confirmed the important role of carbohydrates in antibiotic production. Active components in the culture extracts confirmed production of DAPG (clusters 4 and 5). DAPG production is common in *P. fluorescens* (19), and the compound is a well-studied antibiotic with regard to biological control of root pathogens in the sugar beet (9).

(ii) Antagonism toward *Rhizoctonia* by chitinolytic activity in low-glucose PA medium. Endochitinase activity has occasionally been proposed as a mechanism of antagonism of *Pseudomonas* spp. and other bacteria toward fungi, such as *R. solani* (5, 20) and *Fusarium solani* (25), with cell walls containing chitinaceous material. It should be noted that direct evidence for endochitinase activity (hydrolytic splitting within chitin polymer) may be necessary for documentation of fungal antagonism by cell wall degradation, since a highly active exochitinase (hydrolytic splitting from polymer end) in *Pseudomonas stutzeri* has been shown not to inhibit growth of *Trichoderma reesei* (38).

In the present work, we used the substrate CM-chitin-RBV to document endochitinase activity (56). The low-glucose PA medium supported a high and stable endochitinase activity in clusters 1 (biovar I) and 23 (biovar VI), which also demonstrated relatively strong in vitro antagonism toward *R. solani* on this medium. For other clusters expressing a variable endochitinase activity on different media, antagonism was also most often observed on PA medium. These results suggest a correlation between endochitinase activity and antagonism toward *R. solani* among the *P. fluorescens* clusters. Instability of enzyme production in most clusters may result from different regulatory mechanisms, e.g., induction and repression control as affected by carbon source. This was confirmed by the lower level of expression of endochitinase activity on PDA than on PA in the present study. Alternatively, multiple types of chitinolytic enzymes expressed on different media may occur in *P. fluorescens*, as has been demonstrated in *Bacillus* (49, 54), *Enterobacter* (5), and *Serratia* (11) species.

TABLE 4. Proposed mechanisms of in vitro antagonism against *Pythium ultimum* and *Rhizoctonia solani* in *P. fluorescens* biovars in different media

Cluster no.[a]	Biovar	Target fungus on[b]:			Antagonistic mechanism[b]	
		PDA	PA	SEA	Antibiotic	Enzyme
1	I	*P. ultimum*, *R. solani*	*P. ultimum*, *R. solani*	*P. ultimum*, *R. solani*	Viscosinamide	Chitinase[c]
4 and 5	II/IV	*P. ultimum*, *R. solani*			DAPG	
16 and 17	III		*R. solani*	*R. solani*		Chitinase[c]
23	VI		*R. solani*	*R. solani*		Chitinase[c]

[a] Clusters are distinguished by LPS profile.
[b] Blanks indicate no inhibition or production of antifungal compound.
[c] Active only against *R. solani*.

Different types of fungal antagonism in *P. fluorescens* biovars. The results indicated that several *P. fluorescens* biovars demonstrated their own characteristic mechanisms of fungal antagonism. A summary of these results is presented (Table 4), and a specific mechanism of fungal inhibition for most of the biovar groupings, based on the production of active metabolites and enzymes, is proposed. Despite the limited number of active clusters in some biovars, several distinct patterns of antagonism seem to appear. (i) A unique group is cluster 1 belonging to biovar I, in which the inhibition of both *Pythium ultimum* and *R. solani* was largely independent of medium composition. Viscosinamide, a metabolite showing antifungal activity, was produced on all three test media and was associated with the observed antagonism. In subsequent studies, two very similar strains have been isolated from the rhizosphere of sugar beet and barley plants; both strains also produce viscosinamide (32) and also belong to biovar I (30). (ii) Clusters 4 and 5 belonging to biovar II/IV were also active against both *Pythium ultimum* and *R. solani* but primarily on high-glucose PDA, in which DAPG production was associated with the observed antagonism. Similar observations were subsequently made with other DAPG-producing *P. fluorescens* strains which were isolated from the rhizosphere of other plant species and also assigned to biovar II/IV (30). (iii) Clusters 16 and 17 of biovar III were particularly active against *R. solani* on PA and SEA. Here, the effect was associated with endochitinase activity. (iv) No antagonistic activity was observed in clusters of biovar V. (v) Finally, cluster 23 belonging to biovar VI was also highly active against *R. solani* but only on PA and SEA. Endochitinase activity also was associated in this case with the observed antagonism.

For growth inhibition of plant-pathogenic microfungi under in situ conditions, the variability of growth conditions in the rhizosphere would suggest that antagonists, expressing multiple mechanisms of medium-independent antagonism, would be suitable for application in biological control of preemergence damping-off. As shown in this study, the *P. fluorescens* DR54 isolate (cluster 1, biovar I) produces both an antibiotic compound (viscosinamide) and a cell wall-degrading enzyme (endochitinase), which may act alone or in synergy against widely different target fungi. By comparison, the recent focus on the microfungus *Trichoderma harzianum* (26, 40) for application in biological control also has emphasized the importance of simultaneous production of antibiotics and cell wall-degrading enzymes (e.g., endochitinases) in biological control. The DR54 isolate was further shown to produce the new viscosinamide antibiotic in all test media, which indicates that in situ variation of growth conditions may not affect its antifungal effect. Pot experiments with sugar beet seeds inoculated with *P. fluorescens* DR54 in *Pythium ultimum*-infested soil indeed showed a significant decrease in damping-off disease incidence compared to that of an untreated control.

We conclude that selection for *P. fluorescens* strains showing multitarget and medium-independent fungal antagonism, as represented by DR54 and two other isolates belonging to biovar I, is promising for future applications of these bacteria in biological control of preemergent damping-off disease. In situ testing is obviously necessary before the final selection of candidate strains. However, the apparent correlation between phenotypic grouping (biovar typing) and fungal antagonism within *P. fluorescens* isolates suggests that functional characteristics may be useful for improved selection of candidate isolates for biological control.

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