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Antibiotic and Biosurfactant Properties of Cyclic Lipopeptides Produced by Fluorescent *Pseudomonas* spp. from the Sugar Beet Rhizosphere

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Cyclic lipopeptides (CLPs) with antibiotic and biosurfactant properties are produced by a number of soil bacteria, including fluorescent *Pseudomonas* spp. To provide new and efficient strains for the biological control of root-pathogenic fungi in agricultural crops, we isolated approximately 600 fluorescent *Pseudomonas* spp. from two different agricultural soils by using three different growth media. CLP production was observed in a large proportion of the strains (approximately 60%) inhabiting the sandy soil, compared to a low proportion (approximately 6%) in the loamy soil. Chemical structure analysis revealed that all CLPs could be clustered into two major groups, each consisting of four subgroups. The two major groups varied primarily in the number of amino acids in the cyclic peptide moiety, while each of the subgroups could be differentiated by substitutions of specific amino acids in the peptide moiety. Production of specific CLPs could be affiliated with *Pseudomonas fluorescens* strain groups belonging to biotype I, V, or VI. In vitro analysis using both purified CLPs and whole-cell *P. fluorescens* preparations demonstrated that all CLPs exhibited strong biosurfactant properties and that some also had antibiotic properties towards root-pathogenic microfungi. The CLP-producing *P. fluorescens* strains provide a useful resource for selection of biological control agents, whether a single strain or a consortium of strains was used to maximize the synergistic effect of multiple antagonistic traits in the inoculum.

Cyclic lipopeptides (CLPs) are produced by distinctively different groups of bacteria, both gram-positive (20) and gram-negative (28). The high diversity of CLP-producing microorganisms (28) and differences in chemical structure suggest that the CLP compounds may serve different, and possibly multiple, purposes. This may explain why the specific role of CLP production is often unclear (28, 40). For a limited number of CLPs (28), the reported functions include promotion of bacterial swarming (12, 26) and biosurfactant properties (19, 24, 41). In many cases, CLP compounds are also known to exert a role in antagonistic interactions with other organisms (28), e.g., plant pathogenicity (5) and antifungal (19, 30, 31, 38, 44), antibacterial (11), antiviral (49), or cytotoxic (16) activity.

Synthesis of CLPs is nonribosomal and catalyzed by large peptide synthetase complexes (27). Various environmental stimuli may affect CLP production, i.e., carbon substrate (36), limitation by C, N, or P (15, 37), Fe limitation (15), growth phase conditions (15), and interaction with interfaces (32). Little information is available on production rates and regulating factors for the compounds in natural environments. Asaka and Shoda (2) detected surfactin and iturine production by *Bacillus subtilis* RB14 in a sterilized vermiculite-soil system, and Nakayama et al. (31) detected xanthobaccin A production by a *Stenotrophomonas* sp. strain, SB-K88, in a hydroponic sugar beet rhizosphere system, but documentation for in situ production of CLPs in natural soils has been lacking.

Despite the fact that several new CLP-producing *Pseudomonas* spp. strains have recently been isolated from soil (37, 38), an overview of the different types (chemical structure) and properties (surface tension, antibiosis) of CLP compounds, together with frequencies of the producing *Pseudomonas* spp. strains, has never been made. In this study, we present an extensive survey on lipopeptide production among approximately 600 fluorescent *Pseudomonas* spp. isolated from the sugar beet rhizosphere. Several new CLP compounds are reported. A total of eight subgroups of strains producing CLPs of different molecular weights (MWs) (amino acid number) and compositions (amino acid substitution) were identified among the *Pseudomonas* spp. isolates. The strain groups and hence the production of specific CLPs could be affiliated to a limited number of *Pseudomonas fluorescens* biovars (I, V, and VI). Important differences in biosurfactant and antagonistic properties of the CLPs were documented.

MATERIALS AND METHODS

Isolation of surfactant-producing *Pseudomonas* spp. strains. Soil was collected from a fallow field (loamy sand; Højbakkegård field station, Tåstrup [near Copenhagen], Denmark) and a sugar beet field (sandy loam; Danisco Seed, Holeby, Lolland, Denmark) and kept at 5°C until use. The loamy sand and sandy loam contained 46.3 and 21.1% coarse sand, 39.1 and 39.0% fine sand, 5.9 and 17.8% silt, and 7.2 and 19.5% clay, respectively. Total organic C in the Højbakkegård and Danisco soils was 9 and 17 g kg of dry soil^{-1} and total N was 0.6 and

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0.9 g kg of dry soil^{-1}, respectively. The pH values of the two soils in $CaCl_2$ were 6.3 and 7.3, respectively. In all experiments, the soils were air dried to 10% (wt/wt) before sieving through a 2-mm-diameter mesh screen. The water content was then adjusted to 13% (wt/dry weight) by spraying with tap water and gently mixing in a polyethylene bag.

Fifty grams of soil (wet weight) was weighed into 50-ml polyethylene vials and compressed to a bulk density of 1.1 g cm^{-3}. Three sugar beet seeds (Madison variety) were sown in each vial before samples were incubated at 15°C under a 16-h light and 8-h dark cycle. After 7 days, the plants were excavated and five roots plus adhering soil were transferred to 10 ml of sterile 0.9% NaCl to comprise one combined sample. The sample was vortexed for 1 min and sonicated for 0.5 min before the liquid phase was diluted and plated onto solid media for isolation of fluorescent *Pseudomonas* spp. colonies.

To obtain a high diversity among the isolated *Pseudomonas* spp., three different isolation media were used. (i) Gould's S1 medium, containing (per liter) 10 g of sucrose, 10 ml of glycerol, 5 g of Casamino Acids, 1 g of $NaHCO_3$, 1 g of $MgSO_4 \cdot 7H_2O$, 2.3 g of K_2HPO_4, 1.2 g of Na-lauryl sarcosin, and 15 g of agar, was autoclaved, and then 5 ml of solution containing 100 mg of trimethoprim (Sigma T-7883), 8.5 ml of methanol, and 16.5 ml of Milli-Q water was added to 1 liter of the medium. Due to the selectivity of Gould's S1 medium for *Pseudomonas* spp. (13), all colonies appearing on this medium were eligible for random picking. (ii) On King's B medium (*Pseudomonas* F; Difco catalog no. 0448-17-1), fluorescent *Pseudomonas* spp. could be detected by illuminating the agar plates with UV light (254 nm) and randomly picking the fluorescent colonies. (iii) To isolate *Pseudomonas* spp. from plates covered with R2A medium (Difco catalog no. 1826-15-3), a polyclonal antibody targeting outer membrane protein in genuine *Pseudomonas* spp. (rRNA homology group I) was used in a colony blot procedure (22). For all media, the R2A plates were incubated at 15°C and colonies were picked randomly from plates with the highest dilution (containing more than 10 *Pseudomonas* spp. colonies). Eventually, all isolates from the three media were streaked again onto Gould's S1 agar and checked for fluorescence before culturing in 3 ml of Luria-Bertani medium containing (per liter) 10 g of tryptone, 5 g of yeast extract, 10 g of NaCl, and 1 g of glucose (pH 7.2) for subsequent preservation at −80°C.

The first screening step for surfactant production among a total of 581 fluorescent *Pseudomonas* spp. isolates was a test for each isolate to lower the interfacial tension between an oil-covered surface and a drop of spent growth medium, as described by Bodour and Miller-Maier (6). All isolates were cultured in 3 ml of Luria-Bertani medium (room temperature, overnight), and 5 μl of culture was transferred into the 10W-40 Pennzoil-coated wells of a microtiter lid. Surfactant production was determined up to 3 min after incubation, and a comparison to a surfactant-producing *P. fluorescens* strain, DR54 (37), and a nonproducing *P. fluorescens* strain, DF57 (43), was made.

Structural diversity of *Pseudomonas* spp. surfactants. The first characterization of the *Pseudomonas* spp. surfactants by high-pressure liquid chromatography (HPLC) analysis was performed after culturing of all isolates at 20°C for 2 days in 25-ml glass tubes with 3 ml of Davis minimal broth (DMB) medium. DMB contained 30 mM K_2HPO_4, 14 mM KH_2PO_4, 0.4 mM $MgSO_4$, 7.6 mM $(NH_4)_2SO_4$, 120 mM carbon-C (glucose), and 1 ml of trace element solution per liter (pH 7.3); the trace element solution contained (per liter) 20 mg of $CoCl_2 \cdot 6H_2O$, 30 mg of H_3BO_3, 10 mg of $ZnSO_4 \cdot 7H_2O$, 1 mg of $CuCl_2 \cdot 2H_2O$, 2 mg of $NiCl_2 \cdot 6H_2O$, 3 mg of $NaMoO_4 \cdot 2H_2O$, 10 mg of $FeSO_4 \cdot 7H_2O$, and 2.6 mg of $MnSO_4 \cdot H_2O$. Samples were obtained by extraction for 1 h with 5 ml of ethyl acetate containing 1% formic acid. HPLC analysis of surfactant compounds was performed by using a Hypersil BDS C_{18} column (100 by 4.6 mm; 3-μm particle diameter) held at 40°C, and UV detection (200 to 400 nM) was performed on a Hewlett-Packard model 1100 HPLC diode array detector. The samples were analyzed in a gradient of 85% eluent A (0.1% *o*-phosphoric acid) and 15% eluent B (acetonitrile) at 0 min, increasing eluent B to 100% after 40 min. Eluent flow rate was 1 ml per min. Chromatograms were analyzed using the Hewlett-Packard ChemStation software package. Surfactants were considered identical when retention times in HPLC chromatograms varied by less than 0.1 min. The retention times of one (occasionally two) major surfactant peak were used to cluster the isolates, hereafter referred to as *Pseudomonas* spp. strain groups.

Final purification of surfactants from representative *Pseudomonas* strains was conducted by preparative HPLC (Waters Delta-Pak C_{18} column; 15-μm inner diameter, 100 Å, 300 by 19 mm) using gradients of acetonitrile in water with 0.1% trifluoroacetic acid at a flow rate of 20 ml min^{-1}. Structural analyses of the surfactants were based on one-dimensional and two-dimensional nuclear magnetic resonance (NMR) spectra recorded on a Varian 400 MHz FT-NMR spectrometer with deuterated dimethyl sulfoxide (DMSO-d_6) as solvent. Fast atom bombardment-mass spectrometry (MS) data were obtained on a JMS-HX/HX110A tandem mass spectrometer (JEOL, USA Inc.) (positive ion mode), and stereochemical data were obtained by Chiral gas chromatography. In the case of tensin and amphisin, the complete structure was established by X-ray crystallographic studies (17, 42).

Diversity of surfactant-producing *Pseudomonas* spp. strains. *Pseudomonas* strains representing the production of all surfactant groups were tentatively identified to the species and biovar levels described by Palleroni (39). The simplified scheme suggested by Sørensen et al. (43) and Nielsen et al. (34) based on the occurrence of denitrification, levan production, and growth in mannitol, *meso*-inositol, or sorbitol was used. Strains representing all the surfactant groups were also compared by their C utilization patterns by using Biolog GN plates as described by the manufacturer (Biolog Inc., Hayward, Calif.).

Additional phenotypic characteristics tested included extracellular enzymes and secondary metabolites often associated with antagonism by the fluorescent *Pseudomonas* spp. Endochitinase activity (hydrolytic splitting of chitin polymer) was tested on potato dextrose broth-Bacto agar (PDA; Difco catalog no. 0013-17) medium containing 1.5 mg of chromogenic substrate (carboxymethylcellulose-chitin-RBV; Loewe, Sauerlach, Germany) per ml. Protease activity (casein degradation) was tested as described by Nielsen and Sørensen (35), except using PDA instead of one-fifth-strength tryptic soy agar. Both endochitinase and protease were determined semiquantitatively by the diameters of clearing zones around colonies on the agar plates. Hydrogen cyanide (HCN) production was determined as described by Bakker and Schippers (4) by using the protocol of Nielsen et al. (34). All antagonistic traits were classified visually into categories of no, weak, moderate, or strong activity. Other metabolites tested included diacetyl phloroglucinol, phenazine-1-carboxylic acid, pyrrolnitrin, and pyoluteorin by following the HPLC protocol of Nielsen et al. (37). Absorption spectra (200 to 400 nm) were compared to library spectra by the Hewlett-Packard three-dimensional ChemStation software.

Surface-active properties of *Pseudomonas* spp. surfactants. Biosurfactant properties (surface tension) were tested using the spent growth medium of *Pseudomonas* spp. cultures. One representative strain from each strain group producing a specific CLP profile was cultured in DMB with 20 mM glucose in triplicate for 2 days at 15°C. Cells were removed by centrifugation (10,000 × *g*) for 10 min at 4°C, and the supernatant was frozen for later analysis. Reduction of surface tension was measured on a Wilhelmy plate mounted in a Sigma model 7^{03} instrument (KSV Instruments Ltd., Helsinki, Finland) (1).

To test the effect of surfactant production on the swarming abilities of the bacteria, randomly selected isolates from each strain group were dotted on soft-agar medium composed of potato dextrose broth (Difco) with 0.6% Bacto agar (Difco) or ABTG (AB medium of Clark and Maaløe [7] containing [per liter] 2.5 mg of thiamine, 4 g of glucose, and 4 g of Casamino Acids [Difco]) with 0.6% Bacto agar (Difco). After inoculation for 1 or 2 days at 20°C, swarming was recorded by the radial growth of the colonies, measured in millimeters per hour.

Antagonistic activity of *Pseudomonas* spp. surfactants. Antagonistic properties of purified surfactants were tested by growing the pathogenic fungi *Pythium ultimum* and *Rhizoctonia solani* in the vicinity of the compounds on PDA medium. Purified surfactant (0.1 mg) in a 5-mm Whatman GF/C filter was placed 10 mm from the edge of the fungal inoculum. Triplicate plates were then incubated at 25°C, and the antagonistic activity was registered after 1 and 2 days by measuring the inhibition zone between the filter and the growing mycelium (37). In parallel, whole-cell antagonistic activity by representative cultures from each of the strain groups was tested by dotting the cultures 20 mm from the edge of an agar plug with mycelium on PDA or ABTG medium. Triplicate plates were incubated at 25°C, and inhibition zones were recorded after 1 and 2 days.

RESULTS

Abundance of surfactant-producing *Pseudomonas* spp. isolates in the sugar beet rhizosphere. The abundance of fluorescent *Pseudomonas* spp. was approximately 5×10^6 colonies per g of rhizosphere soil sample (Danisco sandy loam) and approximately 1.5×10^6 colonies per g of rhizosphere soil sample (Højbakkegård loamy sand) when tested on the three different media (data not shown). When a total of 353 and 228 fluorescent *Pseudomonas* spp. isolates from Danisco and Højbakkegård soils, respectively, were tested, their frequencies of surfactant production by the drop assay (6) were highly variable in the two soils. As shown in Fig. 1, approximately 6% of the fluorescent *Pseudomonas* spp. isolated on the three media

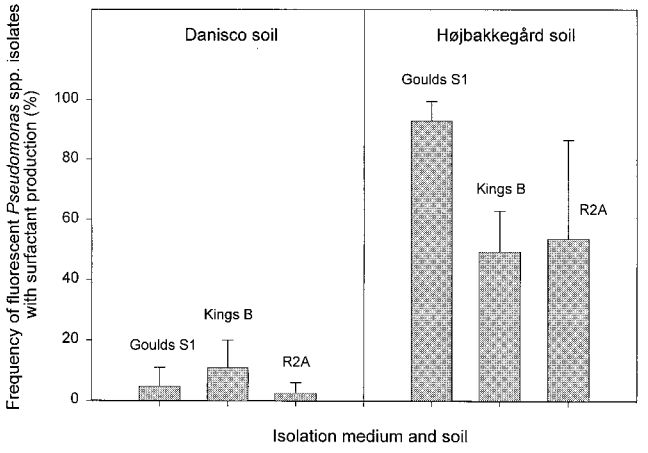

FIG. 1. Frequency of surfactant-producing fluorescent *Pseudomonas* spp. isolates from the sugar beet rhizosphere in Danisco and Højbakkegård soils. Error bars indicate standard deviations.

from the Danisco soil were able to produce surfactants while the average value was approximately 60% for isolates from the Højbakkegård soil. For both soils, the frequencies of surfactant producers among the *Pseudomonas* spp. isolates retrieved on the three different media were quite similar. Finally, when calculating the total number of surfactant-producing fluorescent *Pseudomonas* spp. per gram of rhizosphere soil sample, their abundance was approximately three times higher in the Højbakkegård soil than in the Danisco soil.

CLP surfactants produced in *Pseudomonas* spp. isolates. The initial screening step for surfactant-producing *Pseudomonas* spp. strains was based on the drop collapse assay, but CLP production was subsequently verified by HPLC analysis. By determining the retention time of all major peaks (retention times between 27 and 36 min), we found that 155 out of 169 surfactant-producing isolates from the Danisco and Højbakkegård soils were clustered into eight distinct strain groups. Figure 2 shows representative chromatograms from each of these groups. One major surfactant peak was present in most strain groups, while two peaks representing different surfactants appeared in groups V3 and V4. The smaller peaks surrounding the major surfactant peak in all chromatograms are derivates of the major compounds (D. Sørensen, unpublished data). No major peaks with retention times between 27 and 36 min were observed for strains testing negative in the drop collapse assay. The absorption spectra recorded by the HPLC diode array detector showed that all surfactants had a maximum absorption at approximately 200 nm (endpoint absorption).

Further analysis of purified surfactants by MS and NMR demonstrated two major groupings of the eight strain groups producing CLPs. In Fig. 2 are included MW data for all the major CLPs and their chemical names, where available (37, 38, 42). Four of the strain groups (A1 through A4) produced CLPs with MW values of approximately 1,350 to 1,430. This A group (so called because it is associated with the production of amphisin-like compounds) produced amphisin (A1), lokisin (A2), hodersin (A3), and tensin (A4), all of which contained 11 amino acids in the cyclic peptide structure and a 3-hydroxydecanoyl moiety. The group A1 surfactant amphisin, produced by a single isolate, *P. fluorescens* DSS73 (42), contains leucine

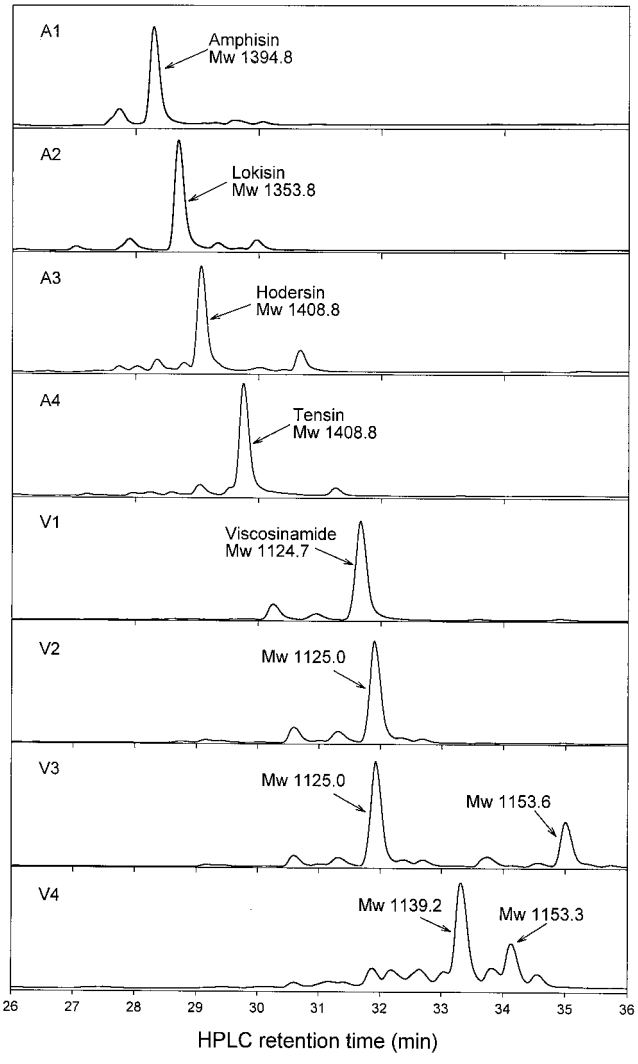

FIG. 2. HPLC chromatograms showing major surfactants (CLPs) produced in batch culture (spent growth medium) by fluorescent *Pseudomonas* spp. strain groups isolated from the sugar beet rhizosphere.

(L-Leu, positions 7 and 9, and D-Leu, positions 1, 4, and 5), isoleucine (L-Ile, position 10), aspartic acid (D-Asp, position 2, and L-Asp, position 11), glutamine (D-Gln, position 8), *allo*-threonine (D-*allo*-Thr, position 3), and serine (D-Ser, position 6) (42). By comparison, the group A2 surfactant lokisin, produced by 6% of the CLP-positive isolates, differs from the amphisin compound by replacement of the glutamine (D-Gln, position 8) with a serine (D-Ser, position 8) (Sørensen, unpublished). The group A3 surfactant hodersin, produced by 12% of the CLP-positive isolates, is currently being investigated to determine the chemical structure. Finally, the group A4 compound tensin, produced by 29% of the surfactant-producing isolates, differs from amphisin by replacement of the aspartic acid (L-Asp, position 11) with glutamic acid (L-Glu, position 11) (17).

By comparison, the V group (so called because it is associated with the production of viscosinamide-like compounds) produced CLPs with MW values of approximately 1,120 to

TABLE 1. Phenotypic variation among CLP-producing *P. fluorescens* strain groups isolated from the sugar beet rhizosphere

P. fluorescens		Biolog GN carbon utilization activity of[b]:								
Strain group	Biovar[a]	Ino	Sor	Ado	Gla	Glu	Ita	Hyd	Ery	Put
A1	VI	−	−	−	−	−	−	−	−	+
A2	VI	−	−	−	−	−	−	−	−	+
A3	VI	−	−	−	−	−	−	−	−	+
A4	VI	−	−	−	−	−	−	−	−	+
V1	I	+	+	+	+	+	+	+	+	−
V2	I	+	+	+	+	+	+	−	−	−
V3	V	+	+	+	+	+	+	−	−	−
V4	I	+	+	+	+	+	+	+	+	−

[a] Biovar affiliation was based on levan production, denitrification activity, and growth on mannitol, *meso*-inositol, and sorbitol.
[b] Utilization of *meso*-inositol (Ino), D-sorbitol (Sor), adonitol (Ado), D-galacturonic acid (Gla), D-glucuronic acid (Glu), itaconic acid (Ita), *p*-hydroxy phenyl acetic acid (Hyd), *i*-erythritol (Ery), and putrescine (Put).

TABLE 2. Surface tension (in spent DMB medium) and swarming ability (on PDA and ABTG soft-agar media) recorded in CLP-producing *P. fluorescens* strain groups isolated from the sugar beet rhizosphere

P. fluorescens strain group	Surface tension (mN m^{-1}) in spent DMB medium[a]	Swarming motility (mm h^{-1}) on[b]:	
		PDA	ABTG
A1	27.4 ± 0.7	1	1
A2	27.4 ± 0.4	1.0 ± 0.3	1.6 ± 0.5
A3	26.9 ± 1.2	1.2 ± 0.6	1.7 ± 0.5
A4	26.7 ± 0.0	1.3 ± 0.5	1.2 ± 0.4
V1	26.8 ± 0.2	0	0.3 ± 0.1
V2	26.7 ± 0.2	0	0.4 ± 0.3
V3	24.7 ± 0.3	0	0.7 ± 0.3
V4	24.5 ± 0.1	0.5 ± 0.5	0.7 ± 0.3
Control 1[c]	63.3 ± 6.6	0	0
Control 2[d]	48.3 ± 5.7	0	0

[a] Surface tension in spent DMB medium after 2 days of incubation at 20°C is given as the mean of three replicates ± the standard deviation, except for control 1, which is given as the mean of six strains ± the standard deviation. Surface tension in uninoculated DMB medium was 71 mN m^{-1}.
[b] Swarming velocity on PDA and ABTG media after 24 and 48 h of incubation at 20°C is given as the mean of 3 to 22 strains ± the standard deviation, except for those for groups A1 and control 2, which represent one strain.
[c] Strain group without CLP production.
[d] A1 mutant impaired in the peptide synthetase responsible for amphisin production (Koch et al., submitted).

1,150. The four strain groups produced viscosinamide (V1), an unnamed 1,125.0-MW compound (V2), two unnamed 1,125.0-MW and 1,153.6-MW compounds (V3), and two unnamed 1,139.2-MW and 1,153.3-MW compounds (V4). Although the chemical structures of all the viscosinamide-like compounds were not fully investigated, HPLC and MS analyses so far indicate a close similarity between them (Sørensen, unpublished). The V1 group surfactant viscosinamide, produced by less than 1% of the surfactant-producing isolates, thus contains only 9 amino acid residues in the peptide moiety but has the same 3-hydroxydecanoyl residue as the amphisin-like compounds described above. The surfactants from groups V2 and V3, representing 12 and 28% of the surfactant-producing isolates, respectively, have not been chemically characterized, but the two groups share one of the surfactants (MW, 1125.0) produced. Finally, the group V4 surfactants, produced by 8% of the surfactant-producing isolates, are also under further study but have shown high similarity to viscosinamide based on preliminary NMR data (Sørensen, unpublished), indicating amino acid replacement(s) in the peptide moiety.

Affiliation of CLP surfactant production to *Pseudomonas* species and biovars. Table 1 shows a comparison of selected phenotypic characters among the biosurfactant-producing *Pseudomonas* spp. strains. By using the limited number of growth characteristics (43), it was possible to tentatively assign each surfactant-producing strain group to a specific *P. fluorescens* biovar. The groups producing the relatively large amphisin-like lipopeptides (A1 through A4) could all be assigned to biovar VI, while most of the groups producing the relatively small viscosinamide-like lipopeptides (V1, V2, and V4) could be assigned to biovar I. One exception was group V3, which differed from the V1, V2, and V4 groups only by the absence of levan production on sucrose medium; this group was tentatively assigned to biovar V (Table 1).

Also included in Table 1 is a number of selected items from the Biolog GN carbon utilization pattern, including the presence of glucuronidase and galacturonidase activity and the utilization of adonitol, *i*-erythritol, *p*-hydroxy phenylacetic acid, and putrescine. These selected substances primarily affiliated the two major A and V groups with biovars VI and I, respectively. Even if levan production affiliated group V3 with biovar V, the additional test substances in Table 1 suggested that group V3 was closely related to group V2, representing biovar I.

Role of CLP surfactants in surface motility and fungal antagonism. The reduction in surface tension in spent growth medium was indicative of surfactant activity. In Table 2, representative strains from the A and V groups are shown to lower the surface tension of DMB from 71 to 27 mN m^{-1} in the A groups and to 25 through 27 mN m^{-1} in the V groups. For control strains, those with no CLP production, and for a mutant impaired in the peptide synthetase responsible for amphisin production (B. Koch et al., submitted for publication), surface tension reductions were 63 and 48 mN m^{-1}, respectively.

Surface motility may be greatly stimulated by surfactants, as is known for the CLP serrawettin produced by *Serratia* spp. (26). Inoculation of the *P. fluorescens* strains on soft-agar medium (Table 2) revealed that strains producing the relatively large, amphisin-like CLPs (group A) had a higher swarming motility on both PDA and ABTG media than the strains in group V. In contrast, all strains producing the relatively small, viscosinamide-like CLPs (group V) all exhibited surface motility when propagated on soft-agar ABTG medium whereas only strains belonging to group V4 demonstrated swarming motility on the soft-agar PDA medium. Finally, control strains, including the A1 mutant that did not produce CLPs, had no swarming motility on either of the two media.

CLP compounds may also play a role in antagonism, which was the basis for the selection of fluorescent *Pseudomonas* spp. strains in the biological control of root-pathogenic microfungi (37, 38, 45). In the present study, whole-cell incubation of representative strains from all eight surfactant-producing

TABLE 3. Whole-cell antagonistic activity towards *P. ultimum* and *R. solani* (on PDA and ABTG media) and selected antagonistic traits recorded in CLP-producing *P. fluorescens* strain groups isolated from the sugar beet rhizosphere

P. fluorescens strain group	Antagonism to *P. ultimum* and *R. solani*[a]		Production of[b]:		
	PDA	ABTG	Protease	Chitinase	HCN
A1	4.0, 6.3	1.6, 2.3	++	++	+++
A2	1.9 ± 1.4, 0.9 ± 0.7	0.9 ± 0.7, 1.0 ± 0.6	++	++	+++
A3	2.3 ± 0.9, 1.4 ± 0.6	0.6 ± 0.6, 0.7 ± 0.5	++	++	+++
A4	0.6 ± 0.3, 0.7 ± 0.4	0.6 ± 0.4, 1.2 ± 0.5	++	++	+++
V1	1.6 ± 0.5, 2.6 ± 0.2	0.2 ± 0.4, 0.3 ± 0.4	+++	+++	−
V2	0, 0.2 ± 0.3	0, 0	−	−	−
V3	0, 0	0, 0	−	−	−
V4	0, 0.4 ± 0.2	0.1 ± 0.3, 0.4 ± 0.8	+++	+++	−
Control[c]	0, 0	0.1 ± 0.1, 0.2 ± 0.3	+/−	−	+/−

[a] Inhibition zone (in millimeters) between fungal mycelium (*P. ultimum* or *R. solani*) and a bacterial colony after 1 or 2 days of incubation at 25°C on PDA or ABTG medium. Values are means of 3 to 22 strains ± standard deviations, except for those for group A1, which are given as data from one strain.
[b] These antagonistic traits were visually classified into categories of no (−), weak (+), moderate (++), or strong (+++) activity. Values are means of 3 to 22 strains ± standard deviations, except for those for group A1, which are data from one strain. +/−, variable activity.
[c] Strain group without CLP production. Values are means of 14 strains ± standard deviations.

strain groups was performed to test their effects against *P. ultimum* and *R. solani* on PDA and ABTG media. As shown in Table 3, the strain groups A1 through A4, producing the relatively large amphisin-like CLPs, generally demonstrated the highest level of antagonism against the microfungi. One exception was the V1 group, which was already known to be very active against the two fungi on PDA medium (37). Interestingly, the highly antagonistic strain groups A1 through A4 also had the most complete set of antagonistic traits tested (protease, chitinase, and HCN production) while one or two of these substances were always absent in the strain groups V1 through V4.

As shown in Table 4, the purified CLPs from the most antagonistic *P. fluorescens* groups, A1 through A4 and V1, were also tested against *P. ultimum* and *R. solani* on PDA medium. Except for the completely inactive lipopeptide (hodersin) from strain group A3, the relatively large lipopeptides (amphisin, lokisin, and tensin) generally appeared to be more antagonistic towards the fungi than the smaller viscosinamide from *P. fluorescens* DR54 (37).

DISCUSSION

Surfactant-producing *Pseudomonas* spp. in two Danish agricultural soils. It was surprising to find that approximately 60% or more of the fluorescent *Pseudomonas* spp. isolates inhabiting the sugar beet rhizosphere from loamy sand soil (Højbakkegård field station) were able to produce CLP. The high frequency of CLP-producing isolates in this soil was confirmed by other isolations from the sugar beet rhizosphere, showing approximately 40% CLP producers within the fluorescent *Pseudomonas* spp. population (data not shown). This suggests that the trait may be of ecological importance in the soil environment. The data further indicate that the soil type may be important for the frequency of CLP-producing strains, since only approximately 6% of the fluorescent *Pseudomonas* spp. isolated from the sandy loam (Danisco soil) were found to be CLP producers.

The total number of surfactant-producing *Pseudomonas* spp. isolates retrieved from the sandy loam (Danisco soil) was only 17 compared to 152 from the loamy sand (Højbakkegård soil), and the surfactant groups identified in the Danisco soil were already represented in the Højbakkegård soil samples (data not shown). Most noteworthy in the whole strain collection was that some surfactants appeared to be rare whereas others appeared to be very common. For instance, amphisin (group A1) was found in only one strain (*P. fluorescens* DSS73) while tensin (group A4) was observed in as much as 29% of the surfactant-producing *Pseudomonas* spp. population in the Højbakkegård soil. The high abundance of isolates producing tensin was confirmed in a different isolation, showing that 56% of all surfactant-producing *Pseudomonas* spp. strains retrieved from this soil actually produced this surfactant (data not shown). Even if tensin production among the fluorescent *Pseudomonas* spp. population appears to be common in the Højbakkegård soil, the reason will remain obscure until a functional role, including a possible selective value of the CLP production, has been identified.

The possible advantage of surfactant production among *Pseudomonas* spp. in the rhizosphere could involve such traits as facilitated surface motility, adhesion, nutrient availability, competition, or antagonism. Still, the task of identifying the actual role of CLP production may be difficult, and predicting the selective value (if there is one) may be just too difficult,

TABLE 4. In vitro antagonistic activity to *P. ultimum* and *R. solani* (on PDA medium) by purified CLPs produced in *P. fluorescens* strain groups isolated from the sugar beet rhizosphere

P. fluorescens strain group	CLP compound	Antagonism to *P. ultimum* and *R. solani*[a]
A1	Amphisin	2.3 ± 0.6, 1.7 ± 0.6
A2	Lokisin	2.7 ± 0.6, 3.3 ± 0.6
A3	Hodersin	0.0 ± 0.0, 0.0 ± 0.0
A4	Tensin	1.7 ± 0.6, 2.3 ± 0.8
V1	Viscosinamide	1.0 ± 0.9, 0.7 ± 0.3

[a] Inhibition zone (in millimeters) between fungal mycelium (*P. ultimum* or *R. solani*) and a GF/C filter with purified CLP compound (0.1 mg) after 1 or 2 days of incubation at 25°C on PDA medium. Values are means of three replicates ± standard deviations.

since many factors influence the diversity and activity of soil *Pseudomonas* spp. populations. Hence, when looking at *Pseudomonas* spp. populations in tomato and flax rhizospheres in different soil types, Latour et al. (23) observed that diversity was most influenced by soil type and less by crop type. Bachmann and Kinzel (3) also suggested that soil type was the most important factor determining the overall diversity of bacterial populations in the rhizosphere. Höper et al. (18) suggested that basic soil characteristics such as pH and texture may influence the density of fluorescent pseudomonads in soil. Indeed, the two soils at Danisco and Højbakkegård differed significantly in both pH value and texture, but at the moment, we are unable to identify a particular role or selective value of CLP productions that would explain the very different frequencies of surfactant-producing *Pseudomonas* spp. strains in the two soils.

An attempt to link CLP production to the abundance of specific *Pseudomonas* spp. was made by affiliating the CLPs produced with specific *P. fluorescens* biovars. This resulted in the almost exclusive assignment of CLP production to *P. fluorescens* biovars I, V, and VI. In a study on CLP production in *Pseudomonas* spp. isolates from the sugar beet rhizosphere (34), the exclusive assignment of viscosinamide production (group V1 [this study]) to *P. fluorescens* biovar I was reported. For the viscosinamide-like compounds, Laycock et al. (24) affiliated the viscosin-producing strain SH10-3B with *P. fluorescens* biovar II. The extensive survey of the present study strongly supports the predominance of a few selected *P. fluorescens* biotypes (I, V, and VI) as CLP producers in the sugar beet rhizosphere. This observation does not contradict the larger, overall diversity of fluorescent *Pseudomonas* spp. populations in the rhizosphere, which may commonly include the other *P. fluorescens* biovars II, III, and IV as well (23, 25, 34). Hence, CLP production could well be associated with only a smaller, exclusive subpopulation of the total fluorescent *Pseudomonas* spp. population in soil.

Structural differences among CLP surfactants. Although an increasing number of CLPs with surfactant properties have been described in *Pseudomonas* spp., the present survey is the first to demonstrate the predominance in a soil environment of two major groups of compounds, sharing the same 3-hydroxydecanoic acid fatty acid moiety but with either 9 or 11 amino acids in the peptide ring structure. We suggest that the group of larger compounds is termed "amphisin-like," based on a recent discovery of the highly active amphisin (42). Similarly, we suggest that the group of smaller compounds is termed "viscosinamide-like," referring to the highly active viscosinamide (37).

The structural differences in both amphisin-like and viscosinamide-like CLPs are all caused by single amino acid replacements in the peptide moiety. Compared to the amino acid sequence of the amphisin molecule (D-Leu-D-Asp-D-*allo*-Thr-D-Leu-D-Leu-D-Ser-L-Leu-D-Gln-L-Leu-L-Ile-L-Asp), tensin has L-Glu (position 11) (38) and lokisin has D-Ser (position 8) according to our investigations (Sørensen, unpublished). Lokisin has the same sequence as pholipeptin isolated from *P. fluorescens* strain BMJ279-76F1 (48), but pholipeptin features the L-isomer of threonine in position 3, all leucines are D-isomers, and the lactone ring is slightly different, since it connects the side chain of C-terminal D-Asp to L-Thr. Similarly, the amino acid sequence of viscosinamide (L-Leu-D-Glu-D-*allo*-Thr-D-Val-L-Leu-D-Ser-L-Leu-D-Ser-L-Ile) is different from viscosin isolated from *Pseudomonas viscosa* (21) by only the D-Glu substitution (position 2). In turn, the WLIP compound from *Pseudomonas reactans* (30) varies only stereochemically from viscosin by the D-Leu in position 5. Finally, the massetolides A through H from several marine *Pseudomonas* spp. strains (11) vary by amino acid substitutions (Val or Ile in position 4; Val, Leu, or Ile in position 9) as well as by the length of the fatty acid chain (10 to 12 C atoms). The MWs of massetolide F (1,125.6 kDa), massetolides A, D, and G (1,139.7 kDa), and massetolides B and H (1,153.7 kDa) are all very similar to those found for V2, V3, and V4, suggesting that these unresolved structures may be very similar to the massetolide compounds.

A possible structure-function relationship may be depicted from the amino acid sequences of CLPs. Hydrophobic amino acids (leucine, isoleucine, and valine) thus occur abundantly (positions 1, 4, 5, 7, and 9) between hydrophilic ones (aspartic acid, glutamic acid, glutamine, serine, and *allo*-threonine in positions 2, 3, 6, and 8, respectively). This provides an amphiphilic ring structure, which may facilitate molecular binding such as the formation of ligands of metal ions (siderophore function) and other cations (14) of importance for cellular (membrane) transport, e.g., Ca^{2+}. Both the topological configuration of the amino acid residues and the existence of carboxylic groups in the peptide moiety are important for water solubility and surfactant properties of CLPs (29). Three-dimensional structures depicted from crystalline amphisin (42) and tensin (17) clearly show how the localization and stereochemistry of amino acids make the ring structure hydrophobic or hydrophilic, respectively, at the two sides of the molecule. The hydrophobic fatty acid tail, together with the amphiphilic property of the peptide, structure may finally play an important role in penetration and binding of CLPs within biological membranes. This in turn may support their role as surfactants and as antibiotics, e.g., disrupting membrane functions leading to excess Ca^{2+} influx into target cells (46).

Functional roles of CLP surfactants in *P. fluorescens*. A unifying trait of the CLP compounds is the high capability to lower the surface tension in a medium. Surface tension measured in spent growth medium (DMB) was thus very low (approximately 25 to 27 mN m^{-1}), indicating that the compounds were very powerful surfactants. The recorded values are as low as the ones (approximately 27 mN m^{-1}) reported for other CLPs, surfactin from *B. subtilis* (8) and viscosin from *P. viscosa* (33). One likely role of the CLP compounds, therefore, is an enhancement of surface motility, as has previously been observed with induction of the swarming phenotype associated with serrawettin production in *Serratia* spp. (26). The strong correlation observed in this study between CLP production and swarming motility in fluorescent *Pseudomonas* spp. was further supported by results obtained by using the *P. fluorescens* DSS73-15C2 mutant impaired in the peptide synthetase responsible for amphisin production (Koch et al., submitted). This mutant lowered the surface tension to 48 mN m^{-1} in spent DMB medium, which was only slightly lower than the values obtained for strains without CLP production. The mutant, further, had no swarming motility (J. B. Andersen et al., submitted for publication). Whether CLP-enhanced surface

motility actually plays a role in the rhizosphere, e.g., for seed or root colonization, needs further investigation.

The antifungal activity of purified CLPs seemed somewhat higher for at least three of the larger amphisin-like compounds (amphisin, lokisin, and tensin) than that for the smaller viscosinamide and viscosinamide-like compounds (data not shown), but the difference could not be directly linked to the amino acid number or sequence of the peptide moiety. Most interestingly, the hodersin compound of the amphisin-like type (group A3) was completely inactive in antagonizing the two plant-pathogenic fungi, *P. ultimum* and *R. solani.* This was quite surprising, as the whole-cell assay with producing strains indeed showed inhibition of both fungi. One explanation may be that this compound is inactive because of a structural difference. Preliminary NMR results suggest that hodersin and tensin are structural isomers (Sørensen, unpublished) and that stereochemical changes may be responsible for the differences in antagonistic activity. Changes of single amino acids may indeed cause drastic changes in antagonistic activity (9, 47) and surfactant properties (14). Alternatively, compounds other than CLPs may have been produced and exerted the antagonism in the whole-cell assay.

While most of the purified CLPs showed antifungal properties, the compounds were likely to act in synergism with other antifungal elements, such as antibiotic compounds or cell wall-degrading enzymes. Hence, the differences between antagonistic activities in the strain groups tested in the whole-cell assays may be attributed to differences in lipopeptide type (amphisin-like or viscosinamide-like), though the synergistic enzyme or antibiotic production may also be important. In the present study, we observed that the *P. fluorescens* strains (groups A1 through A4) demonstrating the largest inhibition towards the fungi also had the most complete array of cell wall-degrading enzymes (protease and chitinase) and HCN production. In contrast, none of the surfactant-producing *Pseudomonas* spp. strains tested here had the often-reported antifungal traits of diacetyl phloroglucinol, phenazine-1-carboxylic acid, pyrrolnitrin, or pyoluteorin production (10). In future studies, the well-characterized collection of CLP-producing *P. fluorescens* strains and their purified compound will be used to study the functional role of CLP production on surfaces (seed, root, fungal mycelium) in soil microcosms and to identify further structure-function relationships for the different CLP compounds.

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