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Siderophores and Outer Membrane Proteins of Antagonistic, Plant-Growth-Stimulating, Root-Colonizing *Pseudomonas* spp.

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As an approach to understanding the molecular basis of the reduction in plant yield depression by root-colonizing *Pseudomonas* spp. and especially of the role of the bacterial cell surfaces in this process, we characterized 30 plant-root-colonizing *Pseudomonas* spp. with respect to siderophore production, antagonistic activity, plasmid content, and sodium dodecyl sulphate-polyacrylamide gel electrophoresis patterns of their cell envelope proteins. The results showed that all strains produce hydroxamate-type siderophores which, because of the correlation with Fe^{3+} limitation, are thought to be the major factor responsible for antagonistic activity. Siderophore-negative mutants of two strains had a strongly decreased antagonistic activity. Five strains maintained their antagonistic activity under conditions of iron excess. Analysis of cell envelope protein patterns of cells grown in excess Fe^{3+} showed that most strains differed from each other, although two classes of similar or identical strains were found. In one case such a class was subdivided on the basis of the patterns of proteins derepressed by iron limitation. Small plasmids were not detected in any of the strains, and only one of the four tested strains contained a large plasmid. Therefore, it is unlikely that the Fe^{3+} uptake system of the antagonistic strains is usually plasmid encoded.

Yield depressions of plant growth in high-frequency cropping soil caused by an increase of deleterious microorganisms or their products can be reduced by seed inoculation with fluorescent root-colonizing *Pseudomonas* spp. (11, 34). These *Pseudomonas* spp. rapidly colonize the plant roots and cause statistically significant yield increases (11, 19). Furthermore, a significant reduction of the fungal and bacterial population in the rhizosphere was observed (17, 38). To explain these phenomena, a mechanism has been suggested (17) in which competition for limiting Fe^{3+} in soil plays a central role.

It is known that many bacteria, including *Pseudomonas* spp., react to limiting Fe^{3+} concentrations by inducing a high-affinity iron uptake system (5, 30) consisting of siderophores, Fe^{3+}-chelating molecules, and outer membrane receptor proteins with a high affinity for the matching Fe^{3+}-siderophore complex. In *Escherichia coli* K-12 (30) and several *Pseudomonas* spp. (28), such receptors have been identified as outer membrane proteins with a relatively high apparent molecular weight (approximately 80,000). For some plant-growth-promoting *Pseudomonas* spp. it has been shown that the production of siderophores during iron starvation on laboratory media was accompanied by growth inhibition of other microorganisms. Neither this antagonistic activity to other microorganisms nor siderophore production was observed when the Fe^{3+} supply was sufficient (10). The following scenario was proposed to account for the enhancement of plant growth by the *Pseudomonas* spp. (17). After the inoculation of seeds, the *Pseudomonas* bacteria rapidly colonize the roots of the developing plant. The limiting Fe^{3+} concentration in the soil induces the high-affinity iron uptake system. The siderophores bind Fe^{3+}, and as uptake of this Fe^{3+}-siderophore complex requires a very specific uptake mechanism, this binding makes this essential element unavailable for many other rhizomicroorganisms. These microorganisms, including deleterious species, then are unable to obtain sufficient iron for optimal growth since they produce either no siderophores at all or less efficient ones. Thus the population of deleterious microorganisms is reduced, creating a favorable environment for the development of the plants.

The present work is part of a study of the molecular microbiological aspects of this plant-growth-promoting process, and it is focused on the cell surface proteins of these antagonistic *Pseudomonas* spp. A study of the cell surface of these bacteria is expected to be important for the following reasons. (i) Cell surfaces of rhizobacteria are largely unstudied. (ii) Adherence of bacteria to plant cells has been reported in many cases (9, 20, 33). It therefore is conceivable that the cell surface of the growth-promoting *Pseudomonas* spp. is important for the interactions with the plant. (iii) Iron starvation induces a series of membrane proteins involved in the uptake of Fe^{3+}-siderophore complexes. Identification of these inducible proteins is an important step in the study of the uptake of the Fe^{3+}-siderophore complexes. (iv) For application of the growth-stimulating properties, it is important to know whether this ability is restricted to one or a few strains or whether it is widespread among *Pseudomonas* spp. In the last few years, outer membrane protein patterns have appeared to be useful for characterizing clones and subclones within bacterial species (e.g. *Bordetella bronchiseptica* [24], *E. coli* [1, 32], and *Haemophilus influenzae* [39]). We have now used cell envelope protein patterns obtained by sodium dodecyl sulphate (SDS)-gel electrophoresis to study the variety among the antagonistic *Pseudomonas* root isolates.

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MATERIALS AND METHODS

Strains and growth conditions. The relevant characteristics of the *Pseudomonas* root isolates used in this study are listed in Table 1. The well-studied *Pseudomonas aeruginosa* PAO1, which we used in this study as a reference strain, was obtained from H. S. Felix, Phabagen Collection, Utrecht, The Netherlands. Unless otherwise indicated, cells were grown after diluting stationary-phase cultures 100-fold into fresh King B medium, a ferric-iron-deficient medium (16), followed by growth at 28°C under vigorous aeration. Cells were harvested after 64 h at which time A_{620} values varying from 5 to 9 had been reached and the pH value had increased from 7.3 to approximately 7.7. Occasionally, minimal salt media (7) with 10 mM succinate or citrate as the carbon source were used; however, A_{620} values of only 0.2 were reached, and the pH increased from 7.3 to 9. In Tris-buffered medium (37) with 0.5% glucose as the carbon source (Tris-glucose medium), final A_{620} values varied from 0.3 to 0.5, and the pH decreased from 7.3 to 4.0. For the separation of cytoplasmic and outer membranes, cells were grown to an A_{620} value of 1.0 in the complex medium described by Hancock and Nikaido (14). When required, the media were supplemented with $FeCl_3$ to a final concentration of 100 μM from a 100 mM $FeCl_3$ stock solution in 0.1 M HCl. Nonfluorescent mutants of strains WCS358 and WCS374 were induced by incubating 1 ml of a stationary-phase culture for 2 h in the presence of 2% ethyl methane sulfonate.

Determination of the in vitro antagonistic activity. The *Pseudomonas* strains were screened on King B plates for their antagonistic properties as described by Geels and Schippers (10). The potato root isolates were screened with a series of 14 test organisms consisting of gram-positive and gram-negative bacteria found in the potato rhizosphere and fungi pathogenic to potatoes (see Table 2). The wheat isolates were screened against gram-positive and gram-negative bacteria isolated from the wheat rhizosphere and against fungi pathogenic to wheat. The *Pseudomonas* strain was spot inoculated on King B plates on three locations half the radius from the center to the edge of the petri dish. When fungi served as the test organisms, the King B plates inoculated with *Pseudomonas* spp. were simultaneously inoculated with the test fungus. This was done by placing a 2-mm agar disk, cut from the margin of a 4-day-old agar culture of the test fungus, in the center of the plate. When bacteria served as the test organisms, the King B plates inoculated with *Pseudomonas* spp. were incubated at 24°C for 24 h before the test bacteria (approximately 10^5 cells) were atomized over the plates. The inhibition zones (x) were measured in millimeters and indicated by the following values: no inhibition, 0; $x \leq 2$ mm, 1; 2 mm $< x \leq 10$ mm, 2; 10 mm $< x \leq 20$ mm, 3; $x > 20$ mm, 4. The degree of antagonism was calculated by first determining separately the average of these inhibition values for fungi, gram-positive and gram-negative bacteria. Subsequently, the average of these three values was calculated and rounded off. This figure was designated as the degree of antagonism.

Siderophores. Cell-free culture supernatants of cells grown in King B medium for 64 h were assayed for the presence of hydroxamate-type and phenolate-type siderophores as described by Czaky (8) and Arnow (2), respectively. Spectra of these supernatants were automatically recorded with a Pye Unicam SP 1700 double beam spectrophotometer.

Separation of cytoplasmic and outer membranes. The method of Hancock and Nikaido (14), in which the use of EDTA (a damaging agent for outer membranes of *P. aeruginosa*) was omitted, was used for the separation of cytoplasmic and outer membranes with two modifications. (i) Dithiothreitol (0.2 mM) was added after disruption of the cells and was present throughout the remaining part of the procedure. (ii) Sucrose gradient centrifugation was carried out in a Beckman SW27 rotor at 70,000 × *g* for 34 h. Visible bands were removed with the aid of a capillary tube connected to a peristaltic pump.

Isolation of other cell envelope fractions. Cell envelopes were isolated by differential centrifugation after disruption of the cells by ultrasonic treatment (21). Extraction of cell envelopes with 2% Trition X-100 in the presence of 10 mM $MgCl_2$ was carried out as described by Schnaitman (35) with minor modifications (22). For extraction of cell envelopes with Sarkosyl, the method described by Achtman et al. (1) was followed except that Sarkosyl was used in a final concentration of 3% instead of 1.67%. Extraction with phenol and precipitation of cell envelopes with trichloroacetic acid was carried out as described (14). Procedures used for treatment of cell envelopes with trypsin and for the isolation of proteins in association with peptidoglycan were as described previously (32) except that for the latter procedure other temperatures than 60°C were also used.

SDS-polyacrylamide gel electrophoresis. Unless otherwise indicated, samples were solubilized by incubation for 15 min at 95°C in the standard sample mixture, described previously (21) prior to separation of membrane proteins by SDS-polyacrylamide gel electrophoresis. Three different gel systems were used for the electrophoretic analysis of the samples. Unless otherwise indicated, the 11% gel system described previously (21) was used (system A). Modifications of this system included the use of highly purified SDS (Serva) (system B) and the addition of 4 M urea to the running gel (system C) (24). The molecular weights of the standard proteins are: phosphorylase *b* 97,000; bovine serum albumin, 66,000; glutamate dehydrogenase, 55,000; egg albumin, 45,000; glyceraldehyde-3-phosphate dehydrogenase, 36,000; carbonic anhydrase, 29,000; trypsinogen, 24,000; β-lactoglobulin, 18,000; lysozyme, 14,000. Gels were stained under gentle shaking for 1.5 h at 45°C in a solution of 0.1% Fast Green FCF in 50% methanol—10% acetic acid and destained in 50% methanol—10% acetic acid.

Other analytical procedures. Protein was determined by the method of Markwell et al. (25) with bovine serum albumin as a reference. The lipopolysaccharide-specific sugar 2-keto-3-deoxyoctanate (KDO) was measured by the thiobarbituric acid method (15) with commercial KDO (Sigma Chemical Co.) as a standard. To remove sucrose, which interferes with the determination of KDO, samples of the cytoplasmic and outer membrane were first precipitated with 10% trichloroacetic acid. Precipitates were successively washed with 5 and 2.5% trichloroacetic acid and finally suspended in deionized water. NADH oxidase activity was determined as described by Osborn et al. (31).

Isolation and analysis of plasmids. For the isolation and analysis of small and large plasmids, the methods described by Birnboim and Doly (4) and Wijffelman et al. (40), respectively, were used.

RESULTS

General properties of the *Pseudomonas* isolates. On the basis of nutritional and physiological characteristics, several of the fluorescent root-colonizing antagonistic *Pseudomonas* isolates used in this study were tentatively characterized as belonging to the fluorescent species *Pseudomonas fluorescens* and *Pseudomonas putida* (Table 1). The isolates

TABLE 1. Fluorescent root-colonizing *Pseudomonas* spp. isolates

Strain[a]	Host plant[b]	*Pseudomonas* species[c]	Degree of antagonism[d]
WCS007	W	*P. putida*	1.5
WCS085	W	*P. putida*	2.5
WCS134	W	ND[e]	1.5
WCS141	W	*P. fluorescens*	1.5
WCS429	W	*P. putida*	ND
WCS307	P	*P. fluorescens*	2.0[f]
WCS312	P	ND	1.0
WCS314	P	ND	1.5
WCS315	P	ND	1.0[f]
WCS317	P	ND	0.5
WCS321	P	ND	1.0
WCS324	P	ND	1.5
WCS326	P	ND	1.5[f]
WCS327	P	ND	0.5
WCS345	P	*P. putida*	2.0
WCS348	P	*P. putida*	2.0
WCS357	P	ND	2.0
WCS358	P	*P. putida*	2.0
WCS359	P	ND	2.0
WCS360	P	ND	2.0
WCS361	P	*P. putida*	3.0[f]
WCS364	P	ND	2.0
WCS365	P	*P. fluorescens*	2.5
WCS366	P	ND	2.0
WCS374	P	*P. fluorescens*	3.0
WCS375	P	ND	3.0
WCS379	P	ND	1.5[f]
A1	P	ND	ND
B10	P	ND	ND
E6	C	ND	ND

[a] Strains with the prefix WCS were isolated at Willie Commelin Scholten Phytopathological Laboratory, Baarn, the Netherlands. Isolates WCS374 and WCS375 were colony variants of one isolate. Strains A1, B10, and E6 (18) were obtained from M. N. Schroth, Berkeley, Calif.

[b] Strains were isolated from the roots of wheat (W), potato (P), and celery (C).

[c] Some strains were identified by H. J. Miller, Bacteriological Phytopathological Service, Wageningen, the Netherlands, on the basis of the following characteristics: production of fluorescent and phenazine pigments, arginine dihydrolase, catalase, growth at 4 and 41°C, levan formation from sucrose, oxidase reaction, hydrolysis of starch and gelatin, utilization of trehalose, mesoinositol, D-mannose, and D-galactose, and denitrification.

[d] The degree of antagonism of the *Pseudomonas* spp. strains towards 14 test organisms is calculated from inhibition values (Table 2) corresponding to the width of the inhibition zones on King B plates (see Materials and Methods). The degree of antagonism was rated from 0 to 4, where 0 indicates no inhibition and 4 indicates total inhibition of all test organisms. Some of these values are from previous work (11).

[e] ND, Not determined.

[f] For these strains, antagonism is not substantially influenced by the addition of Fe^{3+} to the medium.

were grown on solid King B medium at different temperatures. After incubation at 4°C for 1 week, most of the WCS root isolates had formed colonies with diameters of up to 1 mm, whereas the three North American isolates (A1, B10, and E6) grew more slowly, the largest colony diameter being approximately 0.25 mm. The optimal growth temperature for all strains was between 24 and 30°C, and growth at 37°C was significantly reduced.

Antagonistic activity and siderophores. The in vitro antagonistic activity on King B plates of all strains towards 14 test organisms was expressed by the degree of antagonism, with ratings from 0 to 4 (Table 1). For the potato root isolates, the inhibition towards each test organism indicates variability in the antagonistic spectrum (Table 2). Most strains (e.g., WCS358, WCS361, and WCS374) showed a strong antagonistic activity (degree of antagonism, 1.5 to 3.0), whereas some strains (e.g., WCS312 and WCS327) showed a poor antagonism (degree of antagonism, 0.5 to 1.0). For most strains, the antagonistic activity decreased when iron concentrations in the medium were increased. However, the antagonistic activity of five strains Table 1, (see footnote *f*) was not affected by this procedure. When cells had been grown with iron limitation, culture supernatants of the 25 tested strains were strongly positive in the assay for hydroxamate-type siderophores and negative in the assay for phenolate-type siderophores. Spectra of these supernatant fluids showed a characteristic peak at 410 nm (maxima varied between 402 to 415 nm), consistent with the presence of pyoverdine-type siderophores (27). When cells had grown in excess Fe^{3+}, the supernatant fluids were devoid of hydroxamate-type siderophores, and the A_{410} peak was absent. Nonfluorescent mutants of strains WCS358 and WCS374 grown with iron limitation had a strongly decreased antagonistic activity, whereas their supernatant fluids lacked both hydroxamate-type siderophores and the A_{410} peak, indicating a causal relationship among these three properties. The nonfluorescent mutant of strain WCS358 was tested for growth on King B agar plates containing 800 μM 2.2 Bipyridyl, a synthetic iron chelator. In contrast to the parent strain, it did not grow, but growth was restored upon addition of a few drops of sterile fluorescent culture supernatant of the wild-type strain to the plates.

Cell envelope protein patterns. No differences in resolution were observed with gel systems A and B, whereas the resolution increased for one strain with gel system C and decreased for another strain. Analysis of cell envelopes of all 30 antagonistic *Pseudomonas* strains by SDS-polyacrylamide gel electrophoresis showed many different protein patterns. Classification of the strains on the basis of these patterns was somewhat arbitrary, but two major classes were recognized. One pattern (Fig. 1A) was shared by seven different WCS potato isolates and one potato isolate from the United States. The cell envelope protein bands, characteristic for the protein patterns of these class A strains, have apparent molecular weights of 19,000, 22,000, 28,000, 39,000, 43,000, 46,000, 49,000, 55,000, and 66,000 (Fig. 1A). The difference between strains in the intensity of the 55,000-molecular weight (55K) protein band (Fig. 1A) was not reproducible in that the intensity of this band varied among independent cell envelope preparations. The differences in the protein bands with apparent molecular weights below 18,000 (Fig. 1A) were reproducible. Another pattern was found in class B strains (Fig. 1B) consisting of one wheat isolate and six potato isolates. The cell envelope protein pattern of these strains was characterized by heavy protein bands with apparent molecular weights of 19,000, 23,000, 31,000, 35,000, 45,000, 46,000, and 50,000. The disturbance observed in the cell envelope protein pattern of these strains in the lower part of the gel was never observed for strains WCS374 (lanes 6) and WCS375 (lanes 7), but always for the other five strains. Class B strains seem to differ from each other in a number of minor protein bands (Fig. 1B). The remaining fifteen isolates (Fig. 1C) showed unique cell envelope protein patterns. Of these isolates, 14 seemed to share the 19K and 42K protein bands, whereas one isolate, strain WCS429, showed no similarities with other strains at all (data not shown). Strains WCS358, WCS374, and WCS361 representing class A, class B, and the remaining isolates, respectively, were chosen for a more detailed study of the cell envelope proteins since their ability to reduce

TABLE 2. Inhibition of 14 test organisms by *Pseudomonas* spp. isolated from potato roots

Fluorescent *Pseudomonas* strain	Inbition of[a]:													
	Pathogenic fungi					Gram-positive bacteria					Gram-negative bacteria			
	Phoma exigua	*Alternaria solani*	*Verticilium albo-atrum*	*Fusarium tabacinum*	*Rhizoctonia solani*	Bacterium o	Bacterium m	Bacterium q	Bacterium t	*Streptomyces scabies*	*Erwina carotovora* var. *atroseptica*	*Erwina carotovora* var. *carotovora*	Bacterium i	Bacterium x
WCS307	2	2	1	0	1	2	3	2	3	3	1	1	3	2
WCS312	1	2	0	0	1	1	1	2	0	1	1	1	2	1
WCS314	3	3	1	0	3	2	2	2	2	2	0	1	2	1
WCS315	1	1	0	0	1	1	2	1	2	2	1	1	2	1
WCS317	1	1	0	0	1	1	1	1	0	1	1	1	1	1
WCS321	0	2	0	0	0	2	2	2	0	0	0	1	2	2
WCS324	2	2	1	1	2	2	2	2	1	2	1	1	2	2
WCS326	1	2	1	0	2	2	2	1	2	2	1	1	2	2
WCS327	1	1	0	0	1	1	1	1	0	0	1	1	1	1
WCS345	3	3	1	2	3	2	3	2	2	2	0	1	3	2
WCS348	3	3	2	2	3	2	2	2	2	2	0	1	3	1
WCS357	3	3	1	2	2	2	2	2	2	2	0	1	2	2
WCS358	3	3	1	2	2	2	2	2	2	2	0	1	2	1
WCS359	3	3	1	2	2	2	2	2	2	2	0	1	3	2
WCS360	3	3	1	2	2	2	2	2	2	2	0	1	2	2
WCS361	2	4	1	2	2	4	4	3	2	4	2	2	4	3
WCS364	3	3	2	2	2	2	2	2	2	2	0	1	2	2
WCS365	2	2	0	1	2	4	4	3	3	4	1	1	4	1
WCS366	3	3	2	2	2	2	2	2	2	2	0	1	3	2
WCS374	4	4	2	2	3	4	4	4	3	3	1	1	3	2
WCS375	4	4	2	2	3	4	4	3	1	3	1	1	4	2
WCS379	1	2	0	0	0	2	3	2	2	2	1	1	3	2

[a] The values correspond to the width (in millimeters) of the inhibition zone, ×, on King B plates: no inhibition, 0; × ≤2 mm, 1; 2 mm < × ≤ 10 mm, 2; 10 mm < × ≤ 20 mm, 3; × >20 mm, 4.

yield depressions in high-frequency cropping soil had been rigorously established (11).

Localization of class-characteristic proteins. The membrane separation procedure described for *P. aeruginosa* (14) was applied to strains WCS358, WCS374, and WCS361 as well as the well-studied laboratory strain *P. aeruginosa* PAO1. The band patterns observed after the second surcose gradient centrifugation of the membranes varied for the various strains (Fig. 2). Only one light and one heavy band were observed for strains PAO1 and WCS374, whereas both the light and the heavy bands of the other two strains were split into two bands. The lower heavy band (H2) of strain WCS361 was not sharp but continued to the bottom of the tube.

As judged from the distribution of the cytoplasmic membrane marker NADH oxidase and the outer membrane marker KDO (Table 3), the bands indicated as L and H in Fig. 2 represent enriched cytoplasmic and outer membranes, respectively. Analysis of the purified membrane fractions of the four strains by SDS-polyacrylamide gel electrophoresis (Fig. 3) showed that for all strains the protein patterns of the cytoplasmic and outer membrane fractions differed considerably from each other (Fig. 3), thereby confirming that the membrane separation had been successful. For strains WCS358 and WCS361, which yielded two heavy and two light bands, the protein patterns of the two outer membrane fractions were indistinguishable, whereas those of the two cytoplasmic membrane fractions showed minor differences. From a comparison of the observed protein patterns, it seems likely that the major outer membrane proteins of strain PAO1, for which apparent molecular weights of 19,000, 22,000, 38,000, 45,000, and 48,000 were determined (Fig. 3A), are identical to proteins designated as H, G, F, E, and D, respectively, by Mizuno and Kageyama (29). From a comparison of the protein patterns of total cell envelopes (Fig. 3, outside lanes) with those of purified outer and cytoplasmic membrane fractions (Fig. 3, inner lanes), it can be concluded that most of the prominent protein bands of the cell envelope fraction represent outer membrane proteins. Finally, it was established that the proteins, characteristic for the cell envelope protein pattern of strain WCS358 (class A) and WCS374 (class B), are outer membrane proteins. This also applies to the 19K and 42K proteins of strain WCS361, which are characteristic for most of the other isolates.

Influence of growth on the cell envelope protein pattern. The influences of the culture medium, the growth temperature, and the culture age on the cell envelope protein

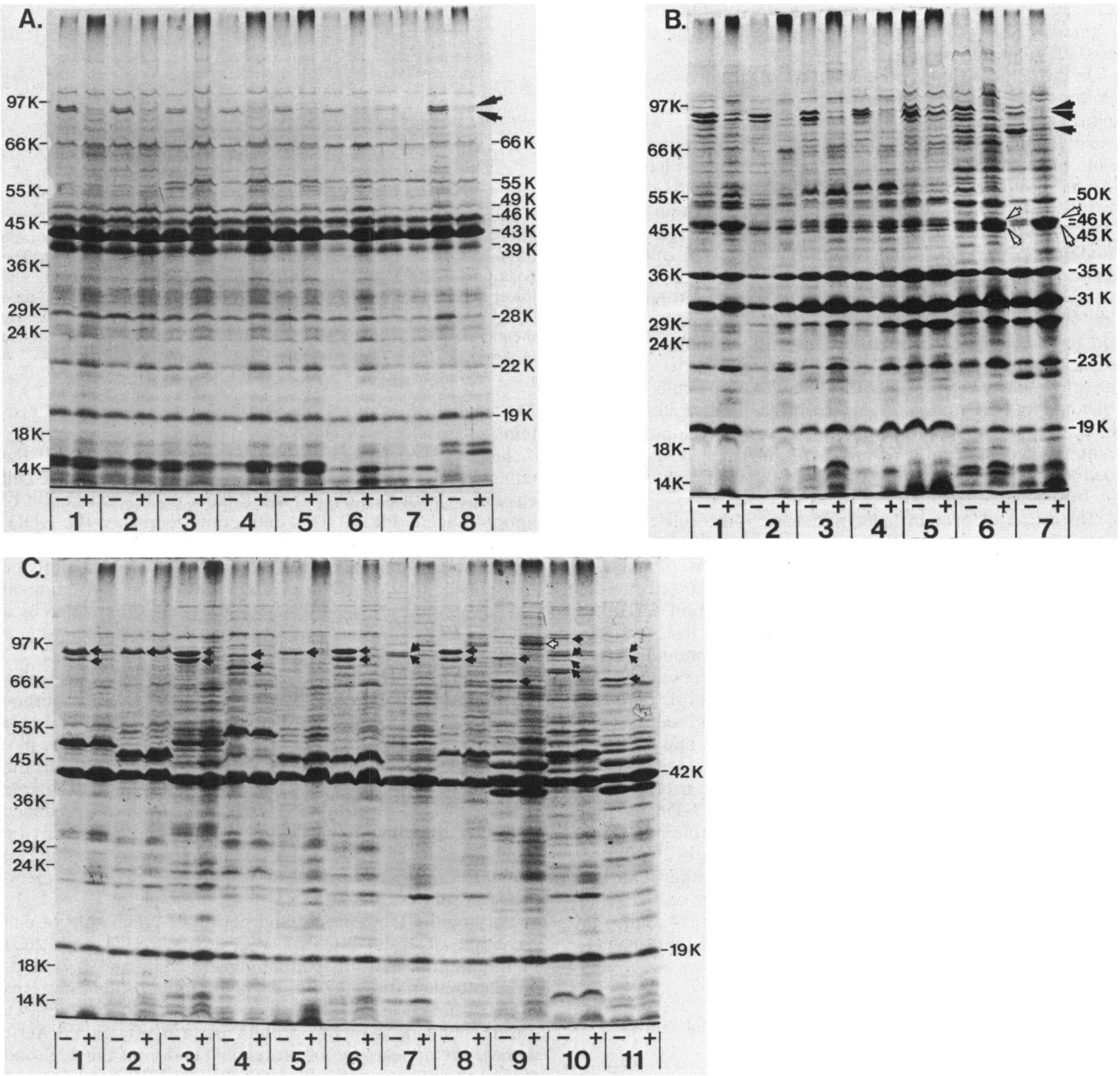

FIG. 1. Cell envelope protein patterns of root-colonizing *Pseudomonas* spp. cells grown for 64 h in King B medium (−) or in King B medium supplemented with 100 μM $FeCl_3$ (+). Positions of the molecular weight standard proteins are indicated (in thousands) at the left. Characteristic proteins in the cell envelope protein pattern are indicated by their molecular weights (in thousands) at the right. Open and solid arrows indicate proteins present in increased amounts after growth in Fe^{3+}-supplemented and Fe^{3+}-deficient medium, respectively. (A) Group A strains with similar or identical cell envelope protein patterns. Lanes: 1, WCS345; 2, WCS348; 3, WCS357; 4, WCS358; 5, WCS359; 6, WCS360; 7, WCS364; 8, A1. (B) Group B strains with similar protein pattern. Lanes: 1, WCS141; 2, WCS312; 3, WCS317; 4, WCS321; 5, WCS327; 6, WCS374; 7, WCS375. (C) Cell envelope protein pattern of most of the other antagonistic strains. Lanes: 1, WCS007; 2, WCS085; 3, WCS134; 4, WCS307; 5, WCS314; 6, WCS315; 7, WCS324; 8, WCS326; 9, WCS361; 10, WCS365; 11, WCS366.

patterns of strains WCS358, WCS361, and WCS374 were studied in more detail.

Growth in the succinate- and citrate-based minimal media and the Tris-glucose medium resulted in alterations in the relative amounts of some protein bands for each of these strains. Moreover, growth in Tris-glucose medium resulted in the appearance of a new, 43K, protein band for strain WCS374. For the cell envelope protein pattern of strain WCS358, growth in minimal media caused a reduction in the degree of distortions of the protein bands discussed below (data not shown).

Growth in King B medium at temperatures varying from 4 to 33°C usually only resulted in minor alterations in the cell envelope protein patterns of these strains. The only clear

change was an increase in the amount of 49K protein in strain WCS358 grown at 4°C compared with cells grown at 15 to 33°C (data not shown).

In the cell envelope protein pattern of strain WCS358 from cultures up to 24 h old, corresponding to early-stationary phase, the amount of 46K protein increased, while the relative amount of 45K protein decreased. Furthermore, the 66K protein appeared in these early-stationary-phase cells. Aging of cultures of strain WCS358 from 24 to 64 h did not result in any changes in the cell envelope protein pattern. The cell envelope protein pattern of strain WCS361 did not undergo remarkable changes during the 64 h cultivation period. Cells of strain WCS374 showed only minor changes: (i) the 29K protein was absent in early-logarithmic-phase cells (up to 8 h) and (ii) the 47K outer membrane protein present in young cultures, up to 24 h old, was not detected in cells cultured for 24 to 64 h. These differences in cell envelope protein patterns during growth of the cells account for the fact that some proteins in enriched outer membranes (Fig. 3) cannot be detected in the cell envelope protein pattern of cells from a 64-h-old culture (Fig. 1), as 6-h-old cells were used for the separation of outer and cytoplasmic membranes.

Distortion of protein band patterns. Some cell envelope protein patterns of *Pseudomonas* strains showed distortion in the protein band pattern. Protein patterns of class A strains, e.g., WCS358, especially contain distorted protein bands throughout the lane, while the distortion for other strains was limited to one part of the gel (e.g., five strains in the left part of Fig. 1B). Reduction of the amount of cell envelope of strain WCS358 applied to the gel resulted in an improved resolution and straighter bands (Fig. 4, compare lanes 1 and 2). Distorted protein bands in *P. aeruginosa* outer membrane fractions have been reported, and lipopolysaccharide has been identified as the causative agent (14).

Extraction with phenol or precipitation with trichloroacetic acid (14) of cell envelopes of strain WCS358 did not improve the resolution of the cell envelope protein pattern. Extraction of cell envelopes of WCS358 with 2% Triton X-100 in the presence of 10 mM $MgCl_2$ (35) neither removed all cytoplasmic membrane proteins nor improved the resolution of the protein bands on the gel (Fig. 4, lane 3). However, extraction of cell envelopes with 3% Sarkosyl (1, 23) resulted in removal of the cytoplasmic membrane proteins and in a largely improved resolution of the pattern (Fig. 4, lane 4). The reason for the improved resolution of the outer membrane protein pattern due to extraction of cell envelopes with Sarkosyl is most likely due to extraction of lipopolysaccharide. (i) This extraction decreased the KDO-to-protein ratio from 25 to 40 down to 5 to 15 μg of KDO per mg of protein. (ii) The reduction in the level of distortion due to a change in the growth medium from King B to minimal media reported previously appeared to be accompanied by a shift in the KDO-to-protein ratio from 25 to 40 down to 5 to 15 μg KDO per mg of protein.

TABLE 3. Properties of membrane fractions of various *Pseudomonas* spp. isolates

Strain	Fraction[a]	Buoyant density (g/ml)	NADH oxidase[b]	KDO (μg/mg of protein)
PAO1	L	1.16	500	1.7
	H	1.22	112	10.9
WCS358	L1	1.14	1,700	2.2
	L2	1.16	580	4.4
	H1	1.20	84	11.0
	H2	1.22	82	10.7
WCS361	L1	1.13	57	1.8
	L2	1.16	440	5.3
	H1	1.22	136	37.0
	H2	1.24	39	40.0
WCS374	L	1.16	480	1.9
	H	1.22	50	19.4

[a] The fractions represent the bands indicated in the sucrose gradients shown in Fig. 2. L, Light; H, heavy.

[b] NADH oxidase activity is expressed as micromoles of NADH oxidized per minute per milligram of protein of the added membrane fraction.

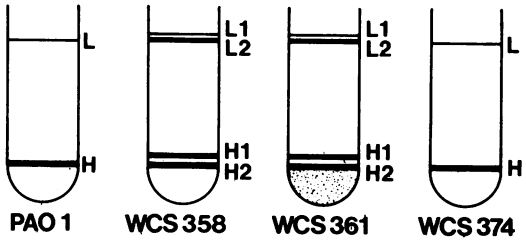

FIG. 2. Schematic representation of the band patterns observed after isopycnic sucrose gradient centrifugation of the membrane fragments of strains PAO1, WCS358, WCS361, and WCS374. The positions of the light (L) and heavy (H) bands are indicated. The lower H band of strain WCS361 continued to the bottom of the tube (□). Cells of strains PAO1, WCS361, and WCS374 were grown in the medium described by Hancock and Nikaido (14), whereas those of strain WCS358 were grown in King B medium.

Further characterization and properties of membrane proteins. We compared a number of properties of the membrane protein of the root isolates WCS358, WCS361, and WCS374 with those of the well-studied *P. aeruginosa* PAO1. We studied the influence on the cell envelope proteins of (i) the incubation temperature of the membranes in the sample mixture prior to electrophoresis (12 and 29), (ii) the presence of β-mercaptoethanol in the sample mixture (12), and (iii) the incubation of the membranes with trypsin (23), and we studied the in vitro association of peptidoglycan with membrane proteins (13).

Varying the temperature and period of incubation of cell envelopes of strains WCS358, WCS361, WCS374, and PAO1 in the sample mixture containing 2% SDS and 0.7 M β-mercaptoethanol resulted in changes in the protein patterns of the strains (Fig. 5). Consistent with the literature (12), we observed changes in the protein pattern of strain PAO1, namely the appearance of proteins D and G at the 22K and 48K positions, respectively, after 20 min at 70°C and 3 min at 95°C, respectively (Fig. 5). Furthermore, heating at 95°C for 15 min resulted in the partial transition of protein F to a slower-running form, indicated by F* (Fig. 5) (12, 29). A similar effect was observed in the cell envelope protein pattern of strain WCS374 (Fig. 5). Heating at 95°C for 15 min resulted in the appearance of a 35K protein band which most likely is a modified form of the 31K protein band as its appearance was accompanied by some loss in the amount of the 31K protein band. Transition of a protein band was also likely in cell envelopes of strains WCS358 and WCS361, in which 43K and 41K protein bands, respectively, increased at the expense of 39K and 38K protein bands, respectively (Fig. 5). Prolonged heating resulted in a deterioration of the patterns (Fig. 5, lanes 5).

Omission of β-mercaptoethanol from the sample mixture caused an increase in the electrophoretic mobility of the 43K and 41K protein bands of strains WCS358 and WCS361,

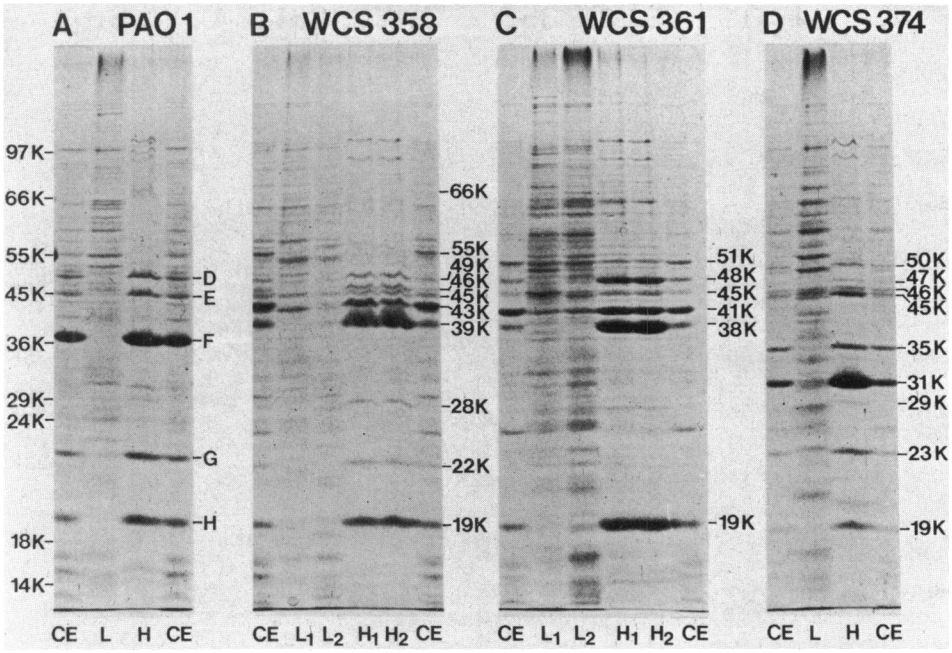

FIG. 3. Protein patterns of cell envelopes and purified outer and cytoplasmic membranes of strains PAO1 (A), WCS358 (B), WCS361 (C), and WCS374 (D). For outer membrane proteins of strain PAO1, the nomenclature of Mizuno and Kageyama (29) was used. Major outer membrane proteins of strains WCS358, WCS361, and WCS374 are indicated by their apparent molecular weights. The 55K and 66K proteins of strain WCS358 and the 29K protein of strain WCS374 cannot be observed in these specific outer membrane fractions, but they were present in purified outer membranes of 24-h-old cells. Fractions L1, L2, H1, and H2 represent bands isolated from the sucrose gradients shown in Fig. 2. CE, Cell envelopes.

respectively, similar to the situation described for pore protein F of *P. aeruginosa* PAO1 (12). None of the outer membrane proteins of strain WCS374 was influenced by the absence of β-mercaptoethanol from the sample mixture.

Treatment of cell envelopes of the four *Pseudomonas* strains with trypsin resulted in loss of the cytoplasmic membrane proteins, whereas most outer membrane proteins appeared not to be susceptible to trypsin, except for the 46K protein of strain WCS374. Furthermore, a 25K protein band appeared in the protein pattern of strain WCS374, which probably was a fragment of a trypsin-sensitive protein (data not shown).

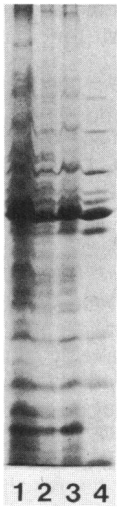

FIG. 4. Cell envelope protein pattern of strain WCS358 after various treatments of the cell envelopes. Lanes: 1, control cell envelopes (30 μg of protein); 2, same preparation (7.5 μg of protein); 3, insoluble fraction obtained after extraction of cell envelopes (50 μg of protein) with 2% Triton X-100 in the presence of 10 mM $MgCl_2$; 4, insoluble fraction obtained after extraction of cell envelopes (50 μg of protein) with 3% Sarkosyl.

We studied the root-colonizing *Pseudomonas* spp. strains WCS358, WCS361, and WCS374 for the presence of cell envelope proteins that can be isolated complexed with peptidoglycan. After incubation of the cell envelopes of these strains and *P. aeruginosa* PAO1 in the presence of 2% SDS and 0.7 M β-mercaptoethanol at 25 and 37°C, some outer membrane proteins were coisolated with peptidoglycan (Table 4), while after incubation at 50°C only trace amounts of protein were recovered in the peptidoglycan pellet (Table 4).

Cell envelope protein regulation by Fe^{3+}. As siderophores are essential for obtaining yield increases due to "bacterization" of potato tubers with *Pseudomonas* cells (P. Bakker, J. Marugg, B. Schippers, unpublished observations) and as outer membrane protein receptors for Fe^{3+}-siderophore complexes are extremely specific for the siderophores (5, 30), we studied the patterns of cell envelope proteins which are induced by Fe^{3+} limitation in some detail. In general, the amount of the cell envelope proteins of the root isolates induced by Fe^{3+} limitation increased with increasing growth temperature up to 28°C, with increasing culture age, and with increasing aeration. Therefore, cells were grown at 28°C for 64 h under vigorous aeration. Proteins present after growth in King B medium but absent after growth in King B medium supplemented with 100 μM $FeCl_3$ are visible in Fig. 1. Some of these proteins were resolved better after extraction of the cell envelopes with Sarkosyl (Fig. 4). For the 15 strains with unique cell envelope protein patterns (Fig. 1C),

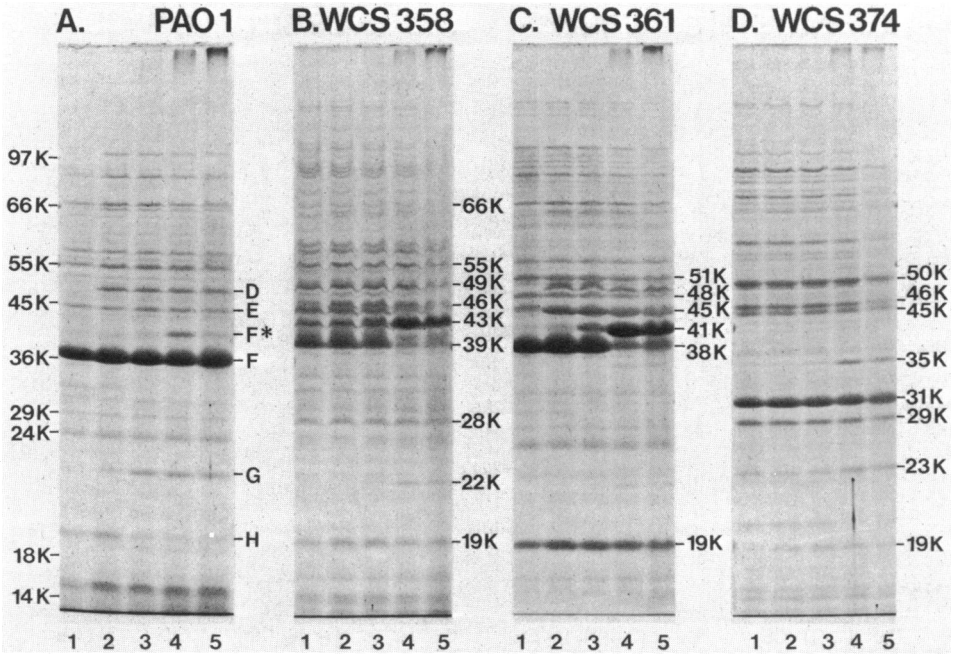

FIG. 5. Cell envelope protein patterns of strains PAO1 (A), WCS358 (B), WCS361 (C), and WCS374 (D) after incubation in the sample mixture at various temperatures for various periods of time. Lanes show incubation for: 1, 20-min at 37°C; 2, 20-min at 70°C; 3, 3-min at 95°C; 4, 15-min at 95°C (our standard condition); 5, 60-min at 95°C. The molecular weights (in thousands) of standard proteins are indicated at the left. At the right-hand side of each lane 5, the apparent molecular weights (in thousands) of the outer membrane proteins of strains WCS358, WCS361, and WCS374 are indicated. For outer membrane proteins of strain PAO1 the nomenclature of Mizuno and Kageyama (29) is used, in which F* indicates the modified form of protein F.

the number (one to four) and the apparent molecular weight(s) (68,000 to 100,000) of the protein(s) induced by iron starvation differed from strain to strain. Among the seven class B strains (Fig. 1B) three different sets of proteins induced by Fe^{3+} limitation were observed, namely a 88K and a 92K protein in strains WCS141, WCS312, WCS317, and WCS327 (Fig. 1B, lanes 1, 2, 3, and 5); a 89K and a 92K protein in strain WCS321 (Fig. 1B, lane 4); and a 76K, a 88K, and a 92K protein in strains WCS374 and WCS375 (Fig. 1B, lanes 6 and 7). All class A strains (Fig. 1A) produced 90K and 92K proteins upon iron starvation. In contrast to these iron-limitation-induced proteins, other proteins were present in increased amounts after growth in Fe^{3+}-enriched media, e.g., the 45K and 46K proteins in strains WCS374 and WCS375 and the 93K protein in strain WCS361 (Fig. 1B and C).

TABLE 4. Proteins recovered in the pellet after incubation of cell envelopes in the presence of 2% SDS plus 0.7 M β-mercaptoethanol[a]

Strain	Protein recovered after incubation at (°C)[b]:	
	25	37
PAO1	H, F, E, D	H, F,[c] E, D
WCS358	19K, 39K, 43K, 46K, 49K[c]	19K,[c] 43K,[c] 46K[c]
WCS361	19K, 38K, 41K, 45K, 48K	19K,[c] 41K,[c] 45K, 48K[c]
WCS374	19K, 31K, 35K	19K,[c] 35K[c]

[a] Pellet fractions of strain PAO1, WCS358, WCS361, and WCS374, three times more concentrated than cell envelopes, were analyzed by SDS-polyacrylamide gel electrophoresis after incubation for 15 min at 95°C in sample mixture.

[b] Only trace amounts of proteins were detected in the pellet incubated at 50°C.

[c] The amount of the indicated protein recovered in the peptidoglycan pellet is strongly reduced compared with the amount of this protein present in cell envelopes.

The Fe^{3+}-limitation-induced proteins in strains WCS358, WCS361, and WCS374 appeared to be sensitive to trypsin, giving rise to high-molecular-weight fragments, which were not observed in trypsin-treated cell envelopes of cells grown in King B medium supplemented with $FeCl_3$. Furthermore, small amounts of these proteins were coisolated with peptidoglycan after incubation of the cell envelopes in the presence of 2% SDS and 0.7 M β-mercaptoethanol at 25°C (data not shown).

Plasmid content. By the isolation procedure for small plasmids (4) on all strains no plasmids were recovered. Four isolates, WCS345, WCS358, WCS361, and WCS374, were probed for the presence of large plasmids up to 300 megadaltons. A plasmid of 71 megadaltons was recovered only from WCS374.

DISCUSSION

Siderophores and antagonistic activity. During iron-limited growth all the tested plant-root-colonizing *Pseudomonas* spp. strains produced a fluorescent pigment in the supernatant, which reacted positively in the assay for hydroxamate-type siderophores and negatively in the assay for phenolate-type siderophores. This fluorescent pigment, which was absent when cells had been grown in excess iron, was apparently able to rescue a nonfluorescent mutant on iron-limited plates. The results are consistent with the observation (27) that fluorescent pigments of *Pseudomonas* spp. strains can be siderophores. Marugg et al. (26) have studied the siderophore of one of our *Pseudomonas* spp. strains in more detail. Using Tn*5*-induced mutants of strain WCS358,

they have isolated genes involved in siderophore synthesis, and they are analyzing the genetics in more detail.

All our fluorescent *Pseudomonas* spp. strains expressed antagonistic activity towards a number of test organisms in vitro (Table 1 and 2). For five strains, this antagonistic activity was not affected by the iron concentration in the medium, suggesting that the antagonistic activity of these strains is largely due to inhibitory compounds other than siderophores. In all the remaining 25 strains, the antagonistic activity was decreased by increasing iron concentrations in the medium. This was taken as an indication that siderophores are usually responsible for the antagonistic activity. In the case of strains WCS358 and WCS374, this notion was further supported by the behavior of siderophore-negative mutants which had lost their antagonistic activity. In conclusion, these experiments indicate that hydroxamate-type siderophores are usually the major cause of in vitro antagonistic activity.

Outer membrane protein patterns as a tool for the characterization of plant-root-colonizing antagonistic *Pseudomonas* spp. Analysis of cell envelope proteins of the 30 antagonistic root-colonizing *Pseudomonas* spp. strains revealed a great diversity among these strains. Fifteen strains showed unique cell envelope protein patterns, indicating a large variety among these *Pseudomonas* spp. in the rhizosphere. Based on their cell envelope protein patterns, the remaining strains separated into two major classes. Cell envelope protein patterns of seven WCS isolates and one strain from the United States (A1) showed a very strong resemblance (class A, Fig. 1A). Furthermore, the resemblance among these seven WCS isolates was emphasized by the observation that they cannot be distinguished with respect to their iron-limitation-induced outer membrane proteins (Fig. 1A), their taxonomic reactions, or their antagonistic behavior (Table 1 and 2). When the origin of the seven similar WCS isolates was checked after completion of these experiments, we learned that they all were isolated from one potato plant, grown in a pot in the greenhouse. Therefore it is likely that they are descendants of one parent strain. Besides these seven strains, three other antagonistic strains (WCS361, WCS365, and WCS366) were isolated from this plant. These latter strains did not show any resemblance at all in cell envelope protein pattern to that of the class A bacteria (compare Fig. 1A with Fig. 1C, lanes 9, 10, and 11), indicating that more than one antagonistic strain can exist on one plant. Another type of cell envelope protein pattern, the class-B pattern (Fig. 1B), was shared by one wheat isolate and six isolates from potato plants located in different fields. The cell envelope protein patterns of these strains show some differences, especially in the Fe^{3+}-limitation-induced proteins (Fig. 1B), and also differences in the in vitro antagonistic activities (Table 1), indicating that most of these strains are similar but not identical.

The existing methods used for identification of *Pseudomonas* spp. root isolates are not satisfactory. According to Schroth (36), these strains do not fit any described taxonomical group, although they are similar to *P. fluorescens* and *P. putida*. Our results show that the cell envelope protein patterns can reveal differences between strains which, according to existing taxonomical rules, belong to one species (Table 1; Fig. 1). Although it must be stressed that the observed similarities in cell envelope protein patterns do not imply that the strains are identical, we strongly feel that characterization of these strains with the help of cell envelope protein patterns is a very useful and fast method. Therefore, we attempted to establish the basis for this characterization method in more detail. Firstly, we showed that the proteins characteristic of the strains are located in the outer membrane (Fig. 3). Secondly, we found that the levels of these characteristic proteins are hardly influenced by growth conditions like culture age and growth temperature, which ensures that the method can be reproducibly introduced in other laboratories.

Comparison of outer membrane proteins of *Pseudomonas* spp. root isolates with those of other gram-negative bacteria. To our knowledge, no information is available on comparisons of outer membrane proteins of rhizobacteria with those of other bacteria. It has recently been suggested that disulfide bonding between outer membrane proteins is a prerequisite for intracellular growth of gram-negative bacteria (3, 6). Similarly, the rhizosphere might constitute an ecological niche which requires specific properties of the outer membrane proteins of its inhabitants. Consistent with this notion is the recent finding in our laboratory that several *Rhizobium* outer membrane proteins are extremely strongly complexed with other cell envelope constituents (R. A. de Maagd and B. Lugtenberg, unpublished observations). Analysis of several properties of the outer membrane proteins of *Pseudomonas* spp. root isolates, e.g., association with the peptidoglycan, sensitivity to trypsin treatment, and the influence of detergents, heat, and β-mercaptoethanol on their solubility and electrophoretic mobility, revealed that their properties are similar to those of outer membrane proteins of many other gram-negative bacteria, especially those of the *P. aeruginosa* reference strain PAO1, and it must be concluded that there is no reason to believe that plant-associated bacteria in general have peculiar properties in common.

Fe^{3+} limitation and outer membrane proteins. Although the various cell envelope protein patterns observed (Fig. 1) clearly show the great diversity among the isolates, this diversity does not necessarily mean that the different isolates differ in the properties involved in antagonism and growth stimulation, e.g., it is conceivable that different strains carry the same plasmid or the same set of chromosomal genes involved in Fe^{3+} uptake. Although very large plasmids may have been missed, our results show that plasmids rarely occur, and therefore plasmid-located genes involved in Fe^{3+} uptake cannot be a general feature of these *Pseudomonas* spp. strains. Analysis of the proteins induced by Fe^{3+} limitation also indicates that, with the exception of the eight class A isolates (seven derived from the same plant and one American isolate), these proteins induced by ferric iron limitation vary among most of the isolates.

Based on the situation described for *E. coli* (5, 23, 30), one would expect that iron-limitation-induced outer membrane proteins play a role in recognition and transport of Fe^{3+}-siderophore complexes. Consistent with this notion is the observation that fluorescent siderophores are produced by all 30 strains upon iron limitation. Considering the variety in iron-limitation-induced proteins among these strains, it therefore seems that a variety of high-affinity Fe^{3+} uptake systems exists among *Pseudomonas* spp. isolated from the rhizosphere.

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