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Evidence for Different Pyoverdine-Mediated Iron Uptake Systems among *Pseudomonas aeruginosa* Strains

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Fourteen strains of *Pseudomonas aeruginosa* (*P. aeruginosa* ATCC 15692, *P. aeruginosa* ATCC 27853, and 12 clinical isolates) were checked for the production of pyoverdine and for pyoverdine-mediated iron uptake. Under iron restriction, two isolates produced undetectable amounts of pyoverdine, but all the other strains produced a compound with physicochemical properties identical or close to those of the pyoverdine of *P. aeruginosa* ATCC 15692 (strain PAO1). The pyoverdines were purified and tested for their growth-promoting activity and for their ability to facilitate ^{59}Fe uptake in homologous experiments involving each pyoverdine and its producing strain, as well as in heterologous systems involving all the other strains. The results of both types of experiments suggested the existence of three specificity groups. This was confirmed by analysis of the amino acid composition of the pyoverdines, which differed for each group. A partially purified polyclonal antiserum raised against a major 80-kilodalton (kDa) iron-regulated outer membrane protein (IROMP) of *P. aeruginosa* PAO1 recognized the 80-kDa IROMPs from *P. aeruginosa* PAO1 and the clinical isolates belonging to the same group, whereas the IROMPs from the strains belonging to the two other groups were not detected. A second antiserum raised against the *P. aeruginosa* ATCC 27853 80-kDa IROMP gave similar results by reacting specifically with the 80-kDa IROMP from the strains belonging to this group. Thus, together with the already known pyoverdine from *P. aeruginosa* PAO1, two new types of pyoverdines produced by strains belonging to this species were characterized.

In response to iron depletion, many bacteria and fungi excrete high-affinity chelators, specific for the ferric ion, termed siderophores (23). This potentiality is particularly important for *Pseudomonas aeruginosa*, since this bacterium, like other pathogens, is faced with a problem of iron availability in serum and secretions in which all iron is tightly bound to transferrin and lactoferrin (1, 26). The type strain *P. aeruginosa* ATCC 15692 (commonly designated as strain PAO1) produces two siderophores. One is a thiazoline derivative, pyochelin (7), which is structurally unrelated to the second siderophore, pyoverdine, a more complex molecule composed of an 8-hydroxyquinoline derivative forming a fluorescent chromophore and an octapeptide containing two residues of the rare amino acid δ-*N*-hydroxyornithine (4, 8, 27). The hydroxamate groups formed from these two particular residues participate, together with the catecholate group of the chromophore, in scavenging of the otherwise unsoluble ferric ion. The importance of pyoverdine, compared with pyochelin, in the removal of the transferrin-bound iron has been demonstrated by the greatly reduced ability of pyoverdine-deficient mutants to grow in the presence of this serum protein (2, 20). A combined effect between pyoverdine and the *P. aeruginosa* elastase has also been observed in an in vitro assay of iron exchange with transferrin (10).

Recent studies of different species of fluorescent pseudomonads have usually demonstrated strict strain specificity for the pyoverdine-mediated iron uptake. As a general rule, uptake of iron(III) by a strain is facilitated by its own pyoverdine, but not by pyoverdines produced by other strains (5, 11, 12, 20). This specificity correlated well with the structural differences of the different pyoverdines, essentially at the level of their amino acid composition. It is reasonable to assume that the specificity of the pyoverdine-mediated iron uptake is determined both by the siderophore amino acid residues not directly involved in the iron complexation and by the iron-regulated outer membrane protein (IROMP) (28), which acts as the receptor for the ferrisiderophore (12). Although the *P. aeruginosa* ferripyoverdine outer membrane receptor has not yet been identified, it is probably one of the high-molecular-mass OMPs (70 to 85 kilodaltons [kDa]) induced when *Pseudomonas* strains are grown in low-iron media (6, 9, 21), since the receptors for two ferric pseudobactins (siderophores structurally related to pyoverdines) from plant-growth-promoting fluorescent pseudomonads have been cloned and shown to correspond to an 85-kDa outer membrane protein (16, 17).

Previous studies that focused on clinical *P. aeruginosa* isolates have revealed some heterogeneity at the level of siderophore utilization (14). Differences in the IROMP content of other clinical isolates have also been described (6). These observations, when analyzed in view of our recent finding about the specificity of the pyoverdine-mediated iron uptake system among fluorescent pseudomonads (12), suggest the existence of several pyoverdine receptor systems in strains of the same *P. aeruginosa* species. This heterogeneity and hence the inability of the pyoverdine system to be used as a taxonomic marker are demonstrated in this paper.

MATERIALS AND METHODS

Strains and media. The different *P. aeruginosa* strains used in this study were *P. aeruginosa* ATCC 15692 (strain PAO1) and *P. aeruginosa* ATCC 27853 (type strains); clinical isolates Pa1, Pa2, and Pa5 (originally from the Intensive Care Unit, St Luc Hospital, Pr G. Wauters, Brussels,

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Belgium); Pa3 (isolated from a patient with cystic fibrosis at the same source); and Pa4, Pa6, Pa7, Pa8, Pa9, Pa10, Pa11, and Pa12 (isolated from patients with urinary tract infections at the same source). Pa1 and Pa2 were chosen because they did not produce pyoverdine. The above strains were bacteriophage typed at the Institut d'Hygiène et d'Epidémiologie, Brussels, Belgium, and confirmed to be *P. aeruginosa* strains. All the strains were found to be different on the basis of phage typing; only three strains were found to be resistant to all the phages used: Pa2, Pa8, and Pa12. A minimal succinate medium (19) was used throughout this study, either unsupplemented (low-iron medium) or supplemented with 4 mg of $FeCl_3$ per liter added as a sterile solution before inoculation (iron-rich medium). The cultures were incubated at 37°C with vigorous shaking (200 rpm) in 1-liter Erlenmeyer flasks containing 500 ml of medium for large-scale purification of pyoverdines (usually 5 liters of medium for one preparation) or in capped tubes (180 by 18 mm) for precultures.

Purification of pyoverdines. The different pyoverdines were purified from the low-iron medium by a method previously described (19) and summarized here briefly. The pyoverdine present in the culture supernatant was first chelated to iron by addition of 1 ml of 2 M $FeCl_3$. After centrifugation, the medium was concentrated 10-fold by in vacuo rotary evaporation and then saturated with NaCl and the chelate was extracted once with 0.5 volume of a phenol-chloroform mixture (1:1, wt/vol). The iron complex was then reextracted from the organic phase with 2 volumes of ether and 0.2 volume of distilled water. After concentration, the aqueous phase was layered on top of a carboxymethyl (CM)-Sephadex column (50 by 2.5 cm) equilibrated with 0.1 M pyridine–acetic acid (pH 5.0) buffer. After elution with the same buffer, the main absorbance peak at 380 nm was collected and treated by vigorous shaking three times with 3 volumes of 5% 8-hydroxyquinoline in chloroform to remove iron. The free ligand was then rechromatographed on CM-Sephadex with a gradient of 0.1 to 2.0 M pyridine–acetic acid (pH 5.0). The main peak, corresponding to the uncomplexed pyoverdine, was collected, lyophilized, and stored at −20°C. Its purity was checked by Cellogel electrophoresis as mentioned previously (19).

Growth stimulations with the purified pyoverdines. Bacteria were grown at 37°C overnight in tubes containing 7.5 ml of succinate medium, with agitation. Samples of 1 ml were taken, the cells were centrifuged (5 min at 10,000 × *g*) in sterile Eppendorf tubes, washed, and suspended in 1 ml of sterile saline. Then 0.1-ml volumes of the cell suspensions were spread on petri plates containing 20 ml of succinate medium (agar solidified, 1.5% agar) supplemented with 100 μg of purified ethylenediaminedihydroxyphenyl acetic acid (EDDHA), a potent synthetic iron chelator, per ml (24). Filter paper disks, each impregnated with 7 μl of the different pure pyoverdine solutions (5 mg/ml in distilled sterile water), were then deposited on the plates. The plates were incubated at 37°C for 24 h and then scored according to the effects of pyoverdines on bacterial growth around the paper disks: no stimulation (0), slight stimulation (±), or good stimulation (+). Growth inhibition was never observed.

Uptake of [^{59}Fe]pyoverdines. The labeling mixture was composed of the following: 5 μl of the $^{59}FeCl_3$ commercial solution (20 mCi/mg; Commissariat à l'Energie Atomique, Gif sur Yvette, France), 50 μl of a 1 mM solution of purified pyoverdine, and 945 μl of nitrogen-free succinate medium (the first two ingredients were added first and allowed to react for 10 min to ensure complete complexation of the iron). The cells were grown in low-iron succinate medium to the mid-exponential phase, centrifuged, washed twice with distilled water, and suspended in nitrogen-free succinate medium to a final A_{600} of 0.3. Cell suspensions (9 ml) were preincubated for 10 min at 30°C in 250-ml Erlenmeyer flasks with agitation (100 rpm) before the addition of 1 ml of the labeling mixture. Samples of 1 ml were taken at 2, 5, 10, 20, and 30 min, rapidly filtered onto membranes (HAWP; pore size, 0.45 μm; Millipore Corp., Bedford, Mass.), and washed twice with 2 ml of nitrogen-free succinate medium. Cell-associated radioactivity was counted in a gamma counter (Intertechnique). Separate control experiments (data not shown) indicated that the iron concentration used was saturating for the pyoverdine-mediated iron uptake system. These assays were previously demonstrated to reflect specific, energy-dependent iron transport (12).

Determination of the amino acid composition of pyoverdines. Approximately 10 nmol of pyoverdine (estimated from the molar absorption coefficient found for the *P. aeruginosa* PAO1 pyoverdine [8]) was hydrolyzed in 6 N HCl or 6 N HI at 105°C under vacuum for 24 h. The hydrolysates were analyzed on a Durrum amino acid analyzer. Norleucine and *allo*-threonine (Sigma Chemical Co., St. Louis, Mo.) were used as internal reference standards. δ-*N*-Hydroxyornithine was estimated by comparing the ornithine content of HI versus HCl hydrolysates (18).

Preparation of outer membranes. Cells were grown in either iron-poor or iron-rich medium, and their outer membranes were prepared by the method of Mizuno and Kageyama (22). Briefly, the cells from 500-ml cultures were resuspended after being washed in 4 ml of 10 mM sodium phosphate buffer (pH 7.2) and disrupted by sonication. The supernatant of a first centrifugation (10 min at 10,000 × *g*) was centrifuged for 30 min at 100,000 × *g*, and the resulting pellet was suspended in 4 ml of 10 mM Tris hydrochloride–2 mM $MgCl_2$ buffer (pH 7.2). After a second centrifugation for 30 min at 100,000 × *g*, the pellet was suspended in 5 ml of 10 mM Tris hydrochloride–2% Triton X-100 (pH 7.2) buffer, sonicated, and incubated for 30 min at 40°C before being subjected to a final centrifugation at 100,000 × *g* for 1 h. The final pellet was washed once with a 10 mM Tris hydrochloride (pH 7.2) solution and homogenized by sonication in 2 ml of 10 mM Tris hydrochloride (pH 6.8). The protein content of the pure outer membrane preparations was determined by the method of Lowry et al. (15) and analyzed by electrophoresis on sodium dodecyl sulfate (SDS)–10% polyacrylamide by the Laemmli procedure (13).

Immunological methods. OMPs (250 μg) from iron-starved PAO1 cells were separated by electrophoresis on an SDS–10% polyacrylamide gel with a preparative slot. The Coomassie blue-stained band corresponding to the major iron deficiency-induced 80-kDa protein was sliced, crushed, and lyophilized. Aliquots corresponding to 1/10 of the starting material were powdered, suspended in 1 ml of phosphate-buffered saline (PBS), and injected subcutaneously into 6-week-old female rabbits. The injections were repeated four times at 2-week intervals. The antiserum was checked for the presence of the iron-regulated polypeptide by Western immunoblotting by the method of Towbin et al. (25). After transfer of the OMPs from the SDS-gel to nitrocellulose sheets by an 18-h electrophoresis at 20 V (4°C), the sheets were incubated for 1 h in PBS containing 3% bovine serum albumin, washed with PBS–0.1% Tween 80, incubated for 2 h with different dilutions of the antiserum, washed three times with PBS-Tween, and further incubated

TABLE 1. Growth stimulation of the different *P. aeruginosa* strains mediated by the homologous and heterologous pyoverdines

Pyoverdine source	Growth stimulation[a] of following strain:													
	PAO1	ATCC 27853	Pa1	Pa2	Pa3	Pa4	Pa5	Pa6	Pa7	Pa8	Pa9	Pa10	Pa11	Pa12
PAO1	+	0	0	0	0	0	+	0	0	+	+	+	+	0
Pa5	+	0	0	0	0	±	+	0	0	+	+	+	+	0
Pa8	+	0	0	0	0	±	+	0	0	+	+	+	+	0
Pa9	+	0	±	0	0	0	+	0	0	+	+	+	+	+
Pa10	+	0	0	0	0	0	+	0	0	+	+	+	+	0
Pa11	+	0	0	0	0	±	+	0	0	+	+	+	+	0
ATCC 27853	0	+	+	+	+	+	0	±	+	0	0	0	0	+
Pa4	0	+	+	+	+	+	0	0	+	0	0	0	0	+
Pa7	0	+	+	+	+	+	0	+	+	0	0	0	0	+
Pa12	0	+	±	+	+	+	0	0	+	0	0	0	0	+
Pa3	0	+	0	0	+	0	0	0	0	0	0	0	0	+
Pa6	0	0	+	0	0	0	0	+	0	0	0	±	±	±

[a] Growth effects of pyoverdines were observed after 24 h of growth on EDDHA-supplemented succinate medium and are described as follows: +, strong stimulation; ±, slight stimulation; 0, no stimulation.

for 1 h with a 1/1,000 dilution of peroxidase-labeled protein A (Amersham International pLc). The nitrocellulose sheets were then washed five times in PBS-Tween and twice in PBS, and the antigenic protein bands were visualized by incubation with the substrate 4-chloro-1-naphthol (Bio-Rad Laboratories, Richmond, Calif.) in the presence of H_2O_2 as recommended by the manufacturer. The proteins of the molecular mass standard (Bio-Rad) were visualized by a separate incubation with an antiserum directed against phosphorylase *b* (92 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (21 kDa), and egg white lysozyme (14.4 kDa).

Partial purification of the crude antiserum (10 ml) was obtained by absorption for 18 h against a sonic extract from PAO1 cells grown in 300 ml of succinate medium supplemented with 4 mg of $FeCl_3$ per liter. The supernatant of a 10-min, 10,000 × *g* centrifugation was considered the purified antiserum. Crude and purified antisera raised against the 80-kDa IROMP from *P. aeruginosa* ATCC 27853 were obtained by the same procedure.

RESULTS

Pyoverdine production. All strains, except Pa1 and Pa2, produced significant amounts of pyoverdine. Pa1 did not produce any pyoverdine at 37°C, and only small quantities of a fluorescent compound were detected when the cells were grown at 25°C. The pyoverdines from the 12 other strains showed chromatographic behavior very similar to that of the PAO1 pyoverdine. The absorption spectra of the free ligand and its iron(III) complex were in good agreement with what is seen for the purified PAO1 pyoverdine, with a maximal absorption at 380 nm at pH 5 for the free ligand and a maximal absorption at 403 nm for the iron complex (8).

Growth-promoting activity of the pyoverdines. For any given strain, a strong growth enhancement after 24 h was observed around the paper disk impregnated with its homologous pyoverdine. The heterologous pyoverdines generally gave two types of response: no stimulation or a strong stimulation usually identical to that obtained in the homologous system. In a few instances, weak stimulation was also seen; however, inhibition of growth by supplemented pyoverdines was never detected. In Table 1, the pyoverdines giving similar patterns of growth stimulation are grouped. Pyoverdines from *P. aeruginosa* PAO1, Pa5, Pa8, Pa9, Pa10, and Pa11 were interchangeable and stimulated the growth of all these strains. Another specificity group was formed by the pyoverdines from *P. aeruginosa* ATCC 27853, Pa4, Pa7, and Pa12. Strain Pa3 produced a pyoverdine which stimulated only *P. aeruginosa* ATCC 27853, Pa12, and itself, but had no effect on any other strain. These results suggested that Pa3 may belong to the same specificity group as *P. aeruginosa* ATCC 27853. The isolate Pa6 was clearly different from the others and cannot be assigned to the two specificity groups described. The growth of Pa6 was well stimulated only by its own pyoverdine and by the pyoverdine from Pa7, whereas a slight stimulation was observed with the pyoverdine from *P. aeruginosa* ATCC 27853. The only heterologous strain to be really stimulated by the pyoverdine from Pa6 was Pa1. The two pyoverdine nonproducers, Pa1 and Pa2, were both stimulated by pyoverdines from the second group (*P. aeruginosa* ATCC 27853, Pa4, Pa7, and Pa12).

Uptake experiments. Table 2 shows the results obtained for uptake experiments with the [^{59}Fe]pyoverdines purified from the different strains. The levels of ^{59}Fe incorporation at 10 min were compared. The percent incorporation with heterologous combinations was calculated versus the 100% (control) level obtained in the homologous system and was found to vary over the range of 0 to 239%. The results confirmed the clustering into three specificity groups: the *P. aeruginosa* PAO1 group (Pa5, Pa8, Pa9, Pa10, and Pa11), the *P. aeruginosa* ATCC 27853 group (Pa3, Pa4, Pa7, and Pa12), and Pa6. The uptake experiments also allowed unequivocal confirmation of Pa3 as a member of the second specificity group. The pyoverdine from Pa9 was a striking exception, since it was taken up by all the bacteria to levels equal to or higher than in the homologous system. The pyoverdine from Pa6 appeared to be different from the others, as suggested by the results of the growth stimulation studies. Strains Pa1 and Pa2 were not included in Table 2, since they did not produce pyoverdines. However, the heterologous uptake studies with Pa1 revealed that this strain accepted pyoverdines from Pa6 and Pa9 only, whereas strain Pa2 incorporated iron when it was chelated by any of the pyoverdines from the *P. aeruginosa* ATCC 27853 group.

TABLE 2. Uptake of the different [^{59}Fe]pyoverdines by homologous or heterologous *P. aeruginosa* strains

[^{59}Fe]pyoverdine source	% Pyoverdine uptake[a] by following strain:											
	PAO1	ATCC 27853	Pa3	Pa4	Pa5	Pa6	Pa7	Pa8	Pa9	Pa10	Pa11	Pa12
PAO1	100	0	27	15	101	25	26	86	63	143	102	30
Pa5	75	1	0	0	100	0	0	79	48	130	81	1
Pa8	73	3	17	0	109	0	4	100	50	156	96	1
Pa9	114	90	101	113	144	122	187	112	100	239	168	155
Pa10	81	9	41	0	109	0	25	103	59	100	113	34
Pa11	90	0	0	0	102	0	2	98	59	206	100	2
ATCC 27853	0	100	153	114	0	0	57	0	0	1	0	87
Pa3	18	58	100	64	0	0	58	0	0	4	0	63
Pa4	1	105	217	100	0	0	99	0	0	2	0	122
Pa7	5	103	206	70	0	4	100	0	0	3	0	95
Pa12	11	94	136	72	1	0	85	4	3	15	4	100
Pa6	0	9	0	0	1	100	0	2	3	4	6	0

[a] Expressed as the percentage of the uptake observed for the homologous system.

Determination of the amino acid compositions of pyoverdines. The amino acid compositions of the different pyoverdines confirmed the clustering in three specificity groups (Table 3). The pyoverdines from the first group (*P. aeruginosa* PAO1, Pa5, Pa8, Pa9, Pa10, and Pa11) demonstrated an identical, previously determined (27) composition: two serines, two threonines, one arginine, one lysine, and two δ-*N*-hydroxyornithines. The pyoverdines from the second group, including *P. aeruginosa* ATCC 27853, Pa3, Pa4, Pa7, and Pa12, were all identical in their amino acid compositions: one glycine, two serines, one *allo*-threonine, one ornithine, and two δ-*N*-hydroxyornithines. The pyoverdine from Pa6 presented an original amino acid composition completely different from those of the two other types of pyoverdines. Analysis of the Pa6 pyoverdine HCl hydrolysate revealed the presence of only two residues, glutamic acid and glycine, roughly in a ratio of 2:1. The HI hydrolysis results showed the presence of these two residues in the same ratio, together with ornithine in roughly the same amount as glycine. The complexation of iron(III) requires two residues of hydroxyamino acid. By comparison with the structures of other pyoverdines (12), these results suggested that the peptide moiety of the Pa6 pyoverdine is composed of two glycines, four glutamic acids, and two δ-*N*-hydroxyornithines.

TABLE 3. Amino acid composition of pyoverdines isolated from the different *P. aeruginosa* strains

Strain	Amino acid ratio[a]							
	Gly	Glu	Ser	Thr	Arg	Lys	Orn	OHO[b]
PAO1	–	–	1.08	1	0.63	0.53	–	1.13
Pa5	–	–	1.15	1	0.65	0.54	–	0.88
Pa8	–	–	1.04	1	0.61	0.53	–	1.25
Pa9	–	–	1.03	1	0.68	0.55	–	1.03
Pa10	–	–	1.04	1	0.67	0.56	–	1.03
Pa11	–	–	1.13	1	0.60	0.51	–	1.26
ATCC 27853	1.15	–	1.90	1[c]	–	–	1.05	1.90
Pa3	1.18	–	1.89	1[c]	–	–	1.09	2.06
Pa4	1.13	–	1.81	1[c]	–	–	1.27	2.25
Pa7	1.07	–	1.87	1[c]	–	–	0.89	1.68
Pa12	1.07	–	1.80	1[c]	–	–	1.13	1.83
Pa6	0.89	1.90	–	–	–	–	–	1

[a] The values refer to the analysis of the HCl hydrolysates of the pyoverdines, except for their δ-*N*-hydroxyornithine content and the amino acids of pyoverdine from strain Pa6, which were calculated according to the HI hydrolysate analysis. Values are normalized to the threonine content, except for Pa6 pyoverdine, which was normalized to the δ-*N*-hydroxyornithine content.

[b] The values refer to the ornithine content of the HI hydrolysates minus the ornithine content of the HCl hydrolysates. OHO, δ-*N*-Hydroxyornithine.

[c] *allo*-Threonine.

IROMPs. Outer membranes from the different strains grown in iron-poor succinate medium presented very similar protein profiles to those of *P. aeruginosa* PAO1. Two major IROMP bands with apparent molecular masses of approximately 80 and 70 kDa were visualized for all the strains, together with a less pronounced IROMP band (ca. 74 kDa), which appeared to be absent for strains Pa1, Pa2, Pa3, and Pa4. The other major OMPs not regulated by iron were the same as those found for *P. aeruginosa* PAO1, with apparent molecular masses of 33, 41, and 45 kDa. The 45-kDa protein was absent or appeared as a minor band in a few clinical strains, i.e., Pa6, Pa8, and Pa11.

This apparent similarity of IROMP patterns between all these strains, based on SDS-polyacrylamide gel electrophoresis migration, was contradicted by an analysis of their immunological reactivities with an anti-80-kDa PAO1-IROMP antiserum. After absorption of this antiserum against outer membranes from iron-replete PAO1 cells, a specific reaction was obtained with only the 80-kDa IROMP in Western blots (manuscript in preparation). When the outer membranes from the different strains were similarly tested with the absorbed antiserum, a similar band of 80 kDa was strongly revealed for all the strains belonging to the *P. aeruginosa* PAO1 group (Pa5, Pa8, Pa9, Pa10, and Pa11), whereas a slight band was revealed for *P. aeruginosa* ATCC 27853 and Pa12, two strains belonging to the second specificity group (Fig. 1). No band was detected with the other isolates. Conversely, when all the strains were tested with a similarly absorbed antiserum raised against the 80-kDa IROMP from *P. aeruginosa* ATCC 27853, all the isolates belonging to this group (*P. aeruginosa* ATCC 27853, Pa3, Pa4, Pa7, and Pa12) reacted positively, whereas the others did not react, except the nonproducer strain Pa2 (Fig. 2). It should be emphasized that strain Pa6 (third specificity group), together with strain Pa1, the only heterologous strain

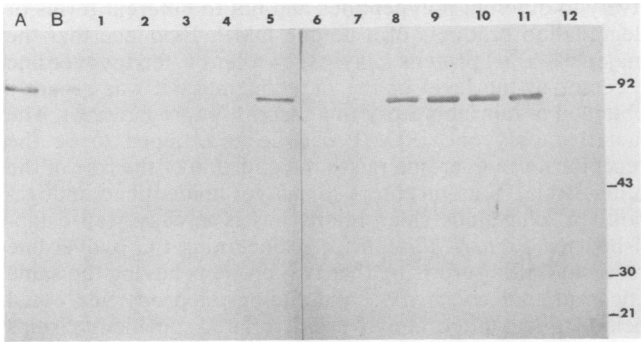

FIG. 1. Western blot of OMP preparations from the different *P. aeruginosa* strains grown under iron-deficient conditions, electrophoresed on SDS–10% polyacrylamide gels, and incubated with the purified antiserum raised against the 80-kDa IROMP of *P. aeruginosa* PAO1. Lane A contains strain PAO1, lane B contains strain ATCC 27853, and lanes 1 to 12 correspond to the different clinical isolates (Pa1 to Pa12, respectively).

to accept pyoverdine from Pa6, did not react with either of these two antisera (Fig. 1 and Fig. 2).

DISCUSSION

As revealed from pyoverdine-mediated ^{59}Fe uptake studies and cross-feeding experiments, it was possible to cluster the 12 different pyoverdine-producing *P. aeruginosa* strains into three groups. The clinical isolates Pa5, Pa8, Pa9, Pa10, and Pa11 cross-reacted in both types of experiments only with the type strain *P. aeruginosa* ATCC 15692 (PAO1 strain), whereas clinical isolates Pa3, Pa4, Pa7, and Pa12 shared features specifically with the other type strain, *P. aeruginosa* ATCC 27853. The clinical isolate Pa6 was distinct from both type strain groups and was recognized as the only representative of the third group. Two other clinical isolates, strains Pa1 and Pa2, which did not produce pyoverdine (a feature frequently observed by medical microbiologists [G. Wauters, personal communication]) or, at least, produced it at a level too low to be isolated and purified, were included in some of these studies. The heterologous pyoverdine-mediated uptake or cross-feeding experiments performed with these strains suggested an identity of strain

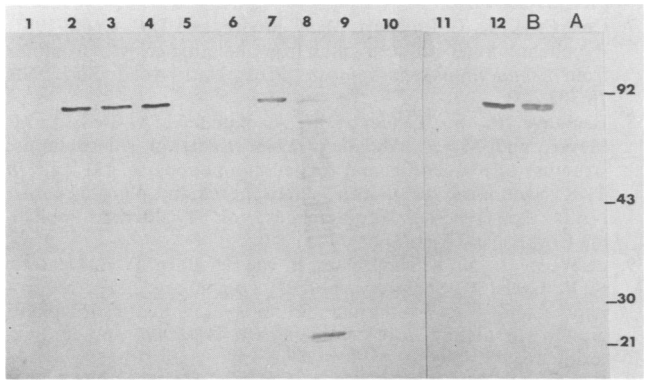

FIG. 2. Western blot of OMP preparations from the different *P. aeruginosa* strains grown under iron-deficient conditions, electrophoresed on SDS–10% polyacrylamide gels, and incubated with the purified antiserum raised against the 80-kDa IROMP of *P. aeruginosa* ATCC 27853. Lanes are as in Fig. 1.

Pa1 with strain Pa6, whereas strain Pa2 cross-reacted preferentially with the *P. aeruginosa* ATCC 27853 group. Such results indicated that these supposedly pyoverdine-negative strains still possess a recognition capacity for some pyoverdines and are able to incorporate iron through a pyoverdine-mediated iron uptake system. Perhaps these strains are stable spontaneous mutants of fluorescent *P. aeruginosa* affected in the biosynthetic pathway of pyoverdine rather than strains lacking all the genetic system involved in the pyoverdine-mediated iron uptake. Another possibility is that these strains, originally devoid of pyoverdine biosynthesis and uptake capacity, might have acquired outer membrane receptors for pyoverdine through genetic exchange. We recently analyzed the relationships existing at the level of the pyoverdine-mediated iron uptake among several pyoverdine-producing strains belonging to different *Pseudomonas* species (12). It was established that the pyoverdines produced by these strains differed from each other in their amino acid compositions. This difference in siderophore structure usually resulted in a strict strain specificity of the system, one strain being able to incorporate iron only when it was chelated by its own pyoverdine. However, exceptions to this rule were found; for example, heterologous uptake was effective between *P. aeruginosa* PAO1 and *P. chlororaphis* ATCC 9446 (or *P. fluorescens* ATCC 13525) and their respective pyoverdines. These pyoverdines shared some homology at the level of the primary structure of their peptide moiety; such results suggest that the recognition mechanism between pyoverdines and their respective outer membrane receptors involves only part of this peptide (12).

In the present study, the strong relationship between structure and specificity is again verified, since we have determined that the three specificity groups consist of bacteria synthesizing different pyoverdines. Pyoverdines from the clinical isolates Pa5, Pa8, Pa9, Pa10, and Pa11 had the same amino acid composition as the previously established *P. aeruginosa* PAO1 pyoverdine (27) (Table 3), whereas the pyoverdines from the other strains had a different amino acid composition. All the pyoverdines from strains belonging to the *P. aeruginosa* ATCC 27853 group shared with the *P. aeruginosa* PAO1 group the two serine residues together with the two δ-*N*-hydroxyornithine residues involved in the iron complexation. However, they were characterized by one glycine and one ornithine residue replacing the two basic amino acids (Arg and Lys) present in the PAO1 pyoverdine. The peptide moiety of this newly described pyoverdine was composed of seven amino acids, one *allo*-threonine being involved instead of two threonines for the PAO1 pyoverdine, which is an octapeptide. The third specificity group, recognized by the cross-feeding and uptake experiments and containing only one pyoverdine-producing strain, Pa6, differed from the two others in amino acid content; only two different amino acids were recognized, together with the two δ-*N*-hydroxyornithine residues: glutamic acid (four residues) and glycine (two residues). Among all the pyoverdines analyzed so far (about twenty), the Pa6 pyoverdine appears to be the most homogeneous in its peptide moiety.

Despite these differences in amino acid composition, the three *P. aeruginosa* pyoverdines presented identical physicochemical characteristics; this observation favors an identical chromophore for all of them, a feature verified for all pyoverdines analyzed so far. No differences were found between strains in their ability to grow in the presence of the strong iron chelator EDDHA, nor was there inhibition of growth during the cross-feeding experiments. Such results suggest similar stability constants of the iron(III) complexes

of these three pyoverdines. In general, the data from uptake experiments reaffirmed the grouping in three categories, as revealed by the amino acid analysis. The only exception was with the Pa9 strain, whose pyoverdine was taken up by all of the strains. Although its amino acid composition and growth stimulation properties clearly suggested its association with the PAO1 group, this pyoverdine apparently promoted the entry of iron in all strains tested. More investigations on the structure and properties of this pyoverdine are needed to determine whether this surprising result could be due to nonspecific interactions between its iron complex and bacterial membranes. Additional discrepancies appeared at the level of the cross-feeding experiments. For instance, the pyoverdine of strain Pa3, which, by its amino acid content, belongs to the *P. aeruginosa* ATCC 27853 group, stimulated the growth of some of the strains belonging to this group (Pa3, ATCC 27853, and Pa12) but, surprisingly, did not stimulate the growth of Pa4 and Pa7. The uptake experiments (Table 2) show clearly that the Pa3 [^{59}Fe]pyoverdine is taken up somewhat less efficiently in heterologous systems (only 60% of the homologous value) than the other [^{59}Fe]pyoverdines of the same specificity group. These results (growth promotion and uptake) suggest that the Pa3 pyoverdine could structurally differ from the other pyoverdines with the same amino acid composition (ATCC 27853, Pa4, Pa7, and Pa12), maybe in the stereochemistry of the peptide chain. Other examples of discrepancies concern strain Pa1, whose growth was stimulated by pyoverdines belonging to the three groups, or strain Pa4, which cross-reacted slightly in some assays involving pyoverdines of the *P. aeruginosa* PAO1 group (Pa5, Pa8, and Pa11). These discrepancies illustrate well the precariousness of making conclusions on the basis of cross-feeding data only. We discussed this point in a previous paper (12) by pointing out that differences in the affinity for iron or in the rate of biosynthesis and excretion between the different siderophores (endogenous and exogenous) could interfere with the recognition specificity. Another important factor is the iron requirement of the cells, which can vary from one strain to another (20). In our previous paper, we confirmed the cross-feeding results with uptake experiments and binding capacity studies of the labeled iron-pyoverdine complexes to purified outer membranes of the different strains. In the present study, uptake and immunological experiments permitted us to define more precisely the preliminary results of cross-feeding. The recognition patterns of the partially purified antisera raised against the major IROMP of each of the type strains reflect perfectly the specificity displayed by the different pyoverdines, since similar 80-kDa antigenic polypeptides were revealed for *P. aeruginosa* PAO1, Pa5, Pa8, Pa9, Pa10, and Pa11 by the *P. aeruginosa* PAO1 antiserum, whereas the *P. aeruginosa* ATCC 27853 antiserum revealed a similar 80-kDa protein exclusively in the strains belonging to the second group (*P. aeruginosa* ATCC 27853, Pa2, Pa3, Pa4, Pa7, and Pa12). Thus, this immunological procedure provides a powerful, easy method for determining pyoverdine-related specificity of a *P. aeruginosa* strain. This is exemplified by the result obtained with strain Pa2, which can be classified in the *P. aeruginosa* ATCC 27853 group, although it did not itself produce detectable amounts of pyoverdine. For the same reason, strain Pa1 should belong to the Pa6 group or to a yet unidentified group.

When grown under iron-deficient conditions, all the strains tested exhibited induction of more than one OMP (6, 21). The presence of only one major band on Western blots (Fig. 1 and 2) suggests that this plurality corresponds effectively to different polypeptides and not to different forms or degradation products of a unique OMP. Evidence that the major 80-kDa protein plays the role of ferripyoverdine receptor at the level of the outer membrane was recently obtained in our laboratory (manuscript in preparation). The fact that only one IROMP can be ascertained to be the receptor for pyoverdine raises the question of the role of the other IROMPs as receptors for as yet unidentified endogenous or exogenous siderophores, as is already well established for *Escherichia coli* (3). Concerning the pyoverdine receptors, it is surprising that two proteins having the same apparent molecular mass and biological properties could belong to two different pyoverdine-related specificity groups but present so few immunological cross-reactions. The cloning of the genes for different pyoverdine receptors and a comparison of their sequences should resolve this problem at a molecular level. It would be interesting to extend this study to a larger number of clinical isolates of *P. aeruginosa* by including the antiserum against the pyoverdine receptor of Pa6 to screen for the existence of more specificity groups in this species.

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LITERATURE CITED

1. **Aisen, P., and I. Litowski.** 1980. Iron transport and storage proteins. Annu. Rev. Biochem. **49**:357–393.
2. **Ankenbauer, R., S. Sriyosachati, and C. D. Cox.** 1985. Effect of siderophores on the growth of *Pseudomonas aeruginosa* in human serum and transferrin. Infect. Immun. **49**:132–140.
3. **Bagg, A., and J. B. Neilands.** 1987. Molecular mechanism of regulation of siderophore-mediated iron assimilation. Microbiol. Rev. **51**:509–518.
4. **Briskot, G., K. Taraz, and H. Budzikiewicz.** 1986. Siderophore vom Pyoverdin-Typ aus *Pseudomonas aeruginosa*. Z. Naturforsch. Sect. C **41**:497–506.
5. **Buyer, J. S., and J. Leong.** 1986. Iron transport-mediated antagonism between plant growth-promoting and plant deleterious *Pseudomonas* strains. J. Biol. Chem. **261**:791–794.
6. **Cornelis, P., N. Moguilevsky, J. F. Jacques, and P. L. Masson.** 1987. Study of the siderophores and receptors in different clinical isolates of *Pseudomonas aeruginosa*. Antibiot. Chemother. **39**:290–306.
7. **Cox, C. D., K. L. Rinehart, M. L. Moore, and J. C. Cook.** 1981. Pyochelin: novel structure of an iron-chelating growth promoter from *Pseudomonas aeruginosa*. Proc. Natl. Acad. Sci. USA **78**:302–308.
8. **Demange, P., S. Wendenbaum, A. Bateman, A. Dell, J. M. Meyer, and M. A. Abdallah.** 1986. Bacterial siderophores: structure of pyoverdins and related compounds, p. 131–147. *In* T. R. Swinburne (ed.), Iron, siderophores and plant diseases. NATO ASI Series A. Life Sciences, vol. 117. Plenum Publishing Corp., New York.
9. **de Weger, L. A., R. van Boxtel, B. van der Burg, R. A. Gruters, F. P. Geels, B. Schippers, and B. Lugtenberg.** 1986. Siderophores and outer membrane proteins of antagonistic, plant-growth-stimulating, root-colonizing *Pseudomonas* spp. J. Bacteriol. **165**:585–594.
10. **Döring, G., M. Pfestorf, K. Botzenhart, and M. A. Abdallah.** 1988. Impact of proteases on iron uptake of *Pseudomonas aeruginosa* pyoverdin from transferrin and lactoferrin. Infect. Immun. **56**:291–293.
11. **Hohnadel, D., and J. M. Meyer.** 1986. Pyoverdine-facilitated iron uptake among fluorescent pseudomonads, p. 119–129. *In*

T. R. Swinburne (ed.), Iron, siderophores, and plant diseases. NATO ASI Series A. Life Sciences, vol. 117. Plenum Publishing Corp., New York.

12. **Hohnadel, D., and J. M. Meyer.** 1988. Specificity of pyoverdine-mediated iron uptake among fluorescent *Pseudomonas* strains. J. Bacteriol. **170**:4865–4873.
13. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
14. **Liu, P. V., and F. Shokrani.** 1978. Biological activities of pyochelins: iron-chelating agents of *Pseudomonas aeruginosa*. Infect. Immun. **22**:878–890.
15. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurements with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
16. **Magazin, M. D., J. C. Moores, and J. Leong.** 1986. Cloning of the gene coding for the outer membrane receptor for ferric pseudobactin, a siderophore from a plant growth-promoting *Pseudomonas* strain. J. Biol. Chem. **261**:795–799.
17. **Marugg, J. D., L. A. de Weger, H. B. Nielander, M. Oorthuizen, K. Recourt, B. Lugtenberg, G. A. J. M. van der Hofstad, and P. J. Weisbeek.** 1989. Cloning and characterization of a gene encoding an outer membrane protein required for siderophore-mediated uptake of Fe^{3+} in *Pseudomonas putida* WCS358. J. Bacteriol. **171**:2819–2826.
18. **Maurer, B., A. Müller, W. Keller-Schierlein, and H. Zähner.** 1968. Ferribactin, ein Siderochrom aus *Pseudomonas fluorescens* Migula. Arch. Mikrobiol. **60**:326–339.
19. **Meyer, J. M., and M. A. Abdallah.** 1978. The fluorescent pigment of *Pseudomonas fluorescens*: biosynthesis, purification and physicochemical properties. J. Gen. Microbiol. **107**:319–328.
20. **Meyer, J. M., F. Hallé, D. Hohnadel, P. Lemanceau, and H. Ratefiarivelo.** 1987. Siderophores of *Pseudomonas*—biological properties, p. 188–205. *In* G. Winkelmann, D. van der Helm, and J. B. Neilands (ed.), Iron transport in microbes, plants and animals. VCH Verlagsgesellschaft, Weinheim, Federal Republic of Germany.
21. **Meyer, J. M., M. Mock, and M. A. Abdallah.** 1979. Effect of iron on the protein composition of the outer membrane of fluorescent pseudomonads. FEMS Microbiol. Lett. **5**:395–398.
22. **Mizuno, T., and M. Kageyama.** 1978. Separation and characterization of outer membrane of *Pseudomonas aeruginosa*. J. Biochem. **84**:179–191.
23. **Neilands, J. B.** 1981. Microbial iron compounds. Annu. Rev. Biochem. **50**:715–731.
24. **Rogers, H. J.** 1973. Iron-binding catechols and virulence in *Escherichia coli*. Infect. Immun. **7**:445–456.
25. **Towbin, H., T. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA **76**:4350–4354.
26. **Weinberg, E. D.** 1984. Iron withholding: a defense against infection and neoplasia. Physiol. Rev. **64**:65–102.
27. **Wendenbaum, S., P. Demange, A. Dell, J. M. Meyer, and M. A. Abdallah.** 1983. The structure of pyoverdine *Pa*, the siderophore of *Pseudomonas aeruginosa*. Tetrahedron Lett. **24**:4877–4880.
28. **Williams, P., H. Chart, E. Griffiths, and P. Stevenson.** 1987. Expression of high affinity iron uptake systems by clinical isolates of *Klebsiella*. FEMS Microbiol. Lett. **44**:407–412.