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NOTES

Isolation and Characterization of *Pseudomonas aeruginosa* Mutants Blocked in the Synthesis of Pyoverdin

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We have isolated and characterized by chemical and enzymatic analyses three distinct types of pyoverdin-defective (*pvd*) mutants of *Pseudomonas aeruginosa* PAO1. The *pvd-1* mutant is an L-N^5-hydroxyornithine (L-N^5-OH-Orn) auxotroph unable to hydroxylate L-ornithine (L-Orn) in a cell-free system and requiring L-N^5-OH-Orn for pyoverdin production. The other two types of mutants appear to be blocked in further steps of the biosynthetic pathway leading to pyoverdin, namely, the acylation of L-N^5-OH-Orn (*pvd-2*) and chromophore synthesis (*pvd-3*). The different *pvd* mutations were all found to be located in the *catA1* region at 47 min of the genetic map of *P. aeruginosa* PAO1.

Under iron limitation, *Pseudomonas aeruginosa* synthesizes two siderophores, pyochelin and pyoverdin. Pyochelin is a unique phenolate siderophore which has been assigned the chemical structure 2-[2-(*o*-hydroxyphenyl)-2-thiazoline-4-yl]-3-methyl-4-thiazolidine carboxylic acid (12). Ferripyochelin uptake occurs via two outer membrane proteins of 14 kDa (38) and 75 kDa (16). Pyoverdin is a hydroxamate siderophore composed of a 6,7-dihydroxyquinoline-containing fluorescent chromophore, bound to the N terminus of an octapeptide (D-Ser-L-Arg-D-Ser-L-N^5-OH-Orn-L-Thr-L-Thr-L-Lys-L-N^5-OH-Orn, in *P. aeruginosa* PAO1) (7). Recent studies of different isolates of *P. aeruginosa* have demonstrated a strict strain specificity of the pyoverdin-mediated Fe(III) uptake system associated with a remarkable heterogeneity in the amino acid composition of the peptide moiety and in the outer membrane proteins involved in ferripyoverdin uptake (10). In *P. aeruginosa* PAO1, two high-molecular-mass outer membrane proteins of 80 kDa (28) and 90 kDa (34) have been shown to function in ferripyoverdin transport. The different pyoverdins from fluorescent pseudomonads (or pseudobactins from plant-related isolates) and the yellow-green siderophore azotobactin from *Azotobacter vinelandii* have similar spectral characteristics and extensive structural homologies (reference 1 and references therein). As a general rule, the same dihydroxyquinoline-derived chromophore is present in all these compounds, with minor structural differences. Moreover, they all contain hydroxamate groups provided by one or two residues of L-N^5-OH-Orn which participate in Fe(III) coordination with the quinoline hydroxyls of the chromophore (1). These similarities also extend to the DNA level, since an extensive homology has recently been shown for pyoverdin genes from different fluorescent *Pseudomonas* spp. (35).

Pyoverdins are powerful iron chelators, with Fe(III)-binding constants about 25 orders of magnitude higher than that of pyochelin (11). The different affinities of the two *P. aeruginosa* siderophores for Fe(III) may also account for the higher growth-promoting activity of pyoverdin in an iron-restricted environment. In fact, the importance of pyoverdin compared with pyochelin in the removal of transferrin-bound Fe(III) has been deduced from the fact that the ability of pyoverdin-deficient mutants to multiply in the presence of this serum protein is greatly reduced while the pyochelin-deficient mutant grows as well as the wild type (5). Therefore, it has been proposed that pyoverdin plays a relevant role in the infectious process of *P. aeruginosa* (5).

The biosynthetic pathway leading to pyoverdin has not yet been elucidated. Taking into account that hydroxylation of ω-amino acids is an enzymatic reaction involved in the formation of a number of hydroxamate siderophores (2, 3, 14, 17, 20, 39), we assumed that conversion of L-Orn to L-N^5-OH-Orn might represent an early biosynthetic step in the generation of the peptidic moiety of pyoverdin. In this report we substantiate this hypothesis by showing that a *P. aeruginosa* PAO1 mutant defective in pyoverdin production lacked the L-Orn-N^5-hydroxylating activity and required L-N^5-OH-Orn for pyoverdin synthesis.

The strains of *P. aeruginosa* used in this study are listed in Table 1. The strains were grown in nutrient-yeast extract medium (32) or in the minimal medium M9 (21) supplemented with 20 mM sodium succinate (SM9), sodium benzoate, or mannitol as the carbon source. Amino acids required for auxotrophs were added at a concentration of 1 mM. Streptomycin sulfate was used at 500 μg/ml. To reduce iron availability, the chelator 2,2′-dipyridyl (9) was added to SM9 at 500 μM, corresponding to the maximal nontoxic concentration of the compound. L-N^5-OH-Orn was obtained from acid hydrolysis of rhodotorulic acid (6). The molecule was checked by paper electrophoresis on Whatman no. 1 filter paper in a water-cooled apparatus at 30 V/cm with pyridine-acetic acid-water (7:5:465) (pH 5.5) as a solvent. The cathodic migration rate of L-N^5-OH-Orn was 0.6 to 0.7 with respect to L-Orn, as determined after ninhydrin and alkaline tetrazolium spraying (6, 39). L-N^5-OH-Orn was stored at −20°C in acid solution (39) and employed in biochemical feeding assays at 400 μM. *P. aeruginosa* PAO1 was mutagenized with ethyl methanesulfonate as previously

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TABLE 1. *P. aeruginosa* strains

Strain	Genotype[a] and/or phenotype	Reference or source
PAO1 (ATCC 15692)	Prototroph	American Type Culture Collection
PAO3-sr	*trp-54* Str^r	15
PAO25[b]	*argF10 leu-10*	15
PAO222-sr	*trpC6 proA82 met-28 lysA12 his-4 ilv-226* Str^r	40
PAO307-sr	*argC54* Str^r	22
PAO503-sr	*met-9011* Str^r	30
PAO1035-sr	*pur-67 thr-9001 cys-54 pro-63* Str^r	36
PAO4032-sr	*met-9020 catA1 nar-9011 mtu-9002 tyu-9030 dcu-9013* Str^r	27
PALS124	*pvd-1*	This study
PALS128	*pvd-2*	This study
PALS106	*pvd-3A*	This study
PALS115	*pvd-3B*	This study
PALS125	*pvd-3C*	This study
PALS132	*pvd-3D*	This study
PALS147	*pvd-3E*	This study
PALS149	*pvd-3F*	This study

[a] Genotype symbols follow the nomenclature of Royle et al. (36); *pvd*, defect in pyoverdin biosynthesis.

[b] Host of plasmid R68.45 (Cb, Km, Tc, Tra, and Cma) (15).

described (24). For enzymatic analysis, cell-free lysates were prepared essentially as reported elsewhere (33). *P. aeruginosa* was grown to mid-logarithmic phase (A_{620}, 0.30 to 0.40) in SM9 supplemented with either 150 μM nitrilotriacetic acid (to minimize protease activity and to reduce iron availability [8]) or 100 μM $FeCl_3$. Cells (approximately 40 mg [dry weight]) were collected by centrifugation (2,000 × *g*, 15 min, 4°C), washed once with 0.85% NaCl, and resuspended in 1 ml of 100 mM sodium phosphate buffer (pH 7.0) containing 2 mg of lysozyme per ml and 20 mM EDTA. After 15 min of incubation at 20°C, cells were put in ice and subjected to ultrasonic disruption for a total of 3 min. The cell-free lysate was supplemented with 1 ml of 100 mM sodium phosphate buffer containing 40 μg of DNase per ml, 100 μg of RNase per ml, 4 mM dithiothreitol, and 4 mM glutamine. After 30 min of incubation at 4°C, cell debris was removed by centrifugation (2,000 × *g*, 15 min, 4°C) and the resulting supernatant (containing less than 10^2 viable cells per ml) was immediately assayed for hydroxylase activity. The enzyme assay was performed by mixing 2 ml of crude lysate and 2 ml of 100 mM sodium phosphate buffer containing 2 mM sodium pyruvate and 4 mM L-Orn. Incubation was carried out at 30°C for up to 6 h in 10-ml tubes flushed with air (4 h was used as the standard incubation time for most of the assays performed). In some experiments, the tubes were stoppered and the air was replaced with nitrogen. The reaction was terminated by the addition of 10% trichloroacetic acid. The supernatant obtained after centrifugation (6,000 × *g*, 10 min, 20°C) was assayed for the presence of hydroxamate and hydroxylamine nitrogen by iodine oxidation (13) with and without acid hydrolysis (for 30 min at 121°C in 6 N H_2SO_4 under N_2), respectively. L-N^5-OH-Orn was identified after ion-exchange chromatography and gel filtration. The hydroxylamine-positive material was loaded on a column (2 by 7 cm) of Dowex 50W-X8 (H^+ form) resin and washed first with 100 ml of 0.1 N HCl and then with 50 ml of 6 N HCl. The latter fraction, containing all the hydroxylamine-positive material, was freeze-dried, resuspended in 2 ml of water, and loaded onto a Sephadex G-10 column (2.5 by 52 cm, containing about 250 ml of resin) equilibrated and eluted with water. The hydroxylamine-positive material which was eluted at approximately 150 ml was freeze-dried and characterized as detailed above. Further analysis of L-N^5-OH-Orn was carried out by performic acid oxidation (29). Glutamic acid formed upon performic acid oxidation of L-N^5-OH-Orn was identified in an LKB Pharmacia 4400 amino acid analyzer. Pyoverdin from early-stationary-phase cultures in SM9 was purified by gel filtration and high-pressure liquid chromatography as described elsewhere (1). Amino acid analysis was carried out after acid hydrolysis (6 N HCl, 12 h, 100°C, in sealed, evacuated Carius tubes) with an LKB Pharmacia 4400 analyzer. Pyochelin was extracted with ethyl acetate from Fe(III)-poor culture supernatants at pH 1.5 to 2.0 and analyzed by thin-layer chromatography on Silica Gel G (11).

A total of eight independent, nonfluorescent, pyoverdin-defective (*pvd*) mutants were selected on SM9 agar plates after ethyl methanesulfonate mutagenesis. The *pvd* mutants were of three distinct classes, designated *pvd-1*, *pvd-2*, and *pvd-3*. The *pvd-1* mutant strain PALS124 produced neither hydroxylamine nor hydroxamate compounds when grown in SM9. Under these conditions, the *pvd-2* mutant strain PALS128 synthesized low levels of hydroxylamine nitrogen (approximately 30 μM), which also reacted quantitatively in the hydroxamate assay, while the *pvd-3* mutants produced hydroxamate nitrogen at wild-type levels (from 95 to 272 μM) but no hydroxylamine compounds. The synthesis of hydroxylamine and hydroxamate compounds by the *pvd-2* and *pvd-3* mutants, respectively, was totally repressed by the addition of 100 μM $FeCl_3$ to SM9, as was true also for pyoverdin synthesized by *P. aeruginosa* PAO1. In comparison with the wild type, the *pvd-1* and *pvd-2* mutants grew poorly in SM9 supplemented with 500 μM 2,2′-dipyridyl, while the *pvd-3* mutants grew as well as the parental strain, PAO1, did. However, all the *pvd* mutants produced pyochelin at wild-type levels (data not shown). Therefore, we hypothesized that the *pvd-1* mutant might be blocked in the reactions generating the hydroxylamine groups of pyoverdin (i.e., the L-N^5-OH-Orn residues) whereas the *pvd-2* mutant might be impaired in the formation of the hydroxamate functions (by acylation of L-N^5-OH-Orn). Indeed, the presence of L-N^5-OH-Orn in culture supernatants of strain PALS128 was confirmed by paper electrophoresis and amino acid analysis of the hydroxylamine-positive material purified by Dowex 50W-X8 ion-exchange chromatography and Sephadex G-10 gel filtration. Additional evidence for this hydroxylamine-positive material being the N^5-hydroxy derivative of L-Orn was provided by the formation of glutamic acid upon performic acid oxidation. The *pvd-3* mutants are probably blocked in later steps of the biosynthetic pathway leading to pyoverdin, most likely in chromophore synthesis. Preliminary results allowed us to identify the hydroxamate-positive compound produced by the *pvd-3A* mutant PALS106 as an acidic, hydrophilic oligopeptide containing Asx-Ser-Glx-Gly-N^5-OH-Orn, the apparent ratio being 1:2:3:1:2 (38a). This molecule is endowed with a siderophore-like activity in the Schwyn and Neilands assay (37) and might be responsible for the growth of the *pvd-3* mutants in SM9 supplemented with 500 μM 2,2′-dipyridyl.

To further characterize the nature of the *pvd-1* mutation, biochemical complementation experiments were conducted. Strains PALS124 (*pvd-1*) and PALS128 (*pvd-2*) and the prototypic *pvd-3* mutant PALS106 were grown to stationary

TABLE 2. Conversion of L-Orn to hydroxylamine and hydroxamate derivatives by cell extracts of *P. aeruginosa* strains[a]

Strain	Genotype	nmol of hydroxylamine nitrogen/min/g (dry wt) of cells[b]	nmol of hydroxamate nitrogen/min/g (dry wt) of cells[c]
PAO1	*pvd*$^+$	12.7	44.5
PALS124	*pvd-1*	0.0	0.0
PALS128	*pvd-2*	19.4	0.0
PALS106	*pvd-3A*	18.2	123.4

[a] Assays were carried out with cell extracts of bacteria grown for 10 h at 37°C in SM9. The assay system consisted of 4 ml of 100 mM sodium phosphate buffer, pH 7.0, containing 0.5 mg of lysozyme per ml, 10 μg of DNase per ml, 25 μg of RNase per ml, 5 mM EDTA, 1 mM dithiothreitol, 1 mM sodium pyruvate, 1 mM L-glutamine, 2 mM L-Orn, and the equivalent of approximately 40 mg (dry weight) of bacterial lysate. The assay system without any added L-Orn served as a control. The values were corrected by subtracting the corresponding values of the control. An endogenous activity was noted in the controls of strains PAO1, PALS128, and PALS106 (<8% of that in the L-Orn-supplemented sample), which could arise from a small amount of L-Orn in the bacterial extract.

[b] Hydroxylamine nitrogen was determined by iodine oxidation (13) with hydroxylamine hydrochloride as the standard. The lower sensitivity limit of the method was 6.25 μM hydroxylamine nitrogen.

[c] Hydroxamate nitrogen was determined by the Csàky assay (13) after acid hydrolysis. The hydroxamate nitrogen concentration was corrected by subtracting the hydroxylamine nitrogen concentration for each sample.

phase in SM9 supplemented with 400 μM L-N^5-OH-Orn. Under this condition, pyoverdin production was fully restored in the *pvd-1* mutant but not in the other pyoverdin-deficient strains. Conversion of L-N^5-OH-Orn to pyoverdin by PALS124 never exceeded 60%, assuming a theoretical L-N^5-OH-Orn/pyoverdin ratio of 2:1. This is probably because at pH values near or above the pK of the hydroxylamine group, which is about 5, L-N^5-OH-Orn is oxidatively decomposed (14) and might be partially degraded in the culture medium during bacterial growth. L-N^5-OH-Orn restored not only pyoverdin synthesis by the *pvd-1* mutant but also its growth in SM9 supplemented with 2,2′-dipyridyl. This effect was not observed for the *pvd-2* mutant. Furthermore, pyoverdin synthesis by the *pvd-1* mutant fed with L-N^5-OH-Orn was totally repressed upon addition of 100 μM $FeCl_3$ to SM9 (data not shown).

Pyoverdin produced by biochemical complementation of the putative L-N^5-OH-Orn auxotroph PALS124 was purified and analyzed in detail. The molecule showed fluorescence spectra identical to those of authentic pyoverdin from *P. aeruginosa* PAO1, with excitation and emission maxima at 405 and 455 nm, respectively. Spectrophotometric titrations of the siderophore with Fe(III) in 200 mM Tris-HCl at pH 8.8 showed a shift in the absorption maxima from 425 nm for the desferrisiderophore form to 410 nm for the ferrisiderophore, as also shown for pyoverdin from wild-type PAO1 (18). Amino acid analysis of the peptide moiety of the molecule following acid hydrolysis with HCl and reductive hydrolysis with HI gave the same composition as pyoverdin from *P. aeruginosa* PAO1, with Thr-Ser-N^5-OH-Orn-Lys-Arg in the ratio 2:2:2:1:1.

The results of enzymatic analysis confirmed expectations based on phenotypic characterization and biochemical complementation tests. Cell-free lysates of wild-type PAO1 and *pvd-2* and *pvd-3* mutants formed hydroxylamine and/or hydroxamate nitrogen when incubated at 30°C under aeration with L-Orn but not with other putative substrates or precursors, e.g., L-Lys or L-Glu (23), at the same concentration. In contrast, the L-N^5-OH-Orn auxotroph PALS124 was unable to convert L-Orn to the hydroxylamine and/or the hydroxamate derivative (Table 2). It appears also that the wild-type PAO1 and, to a greater extent, the *pvd-3* mutant formed much higher amounts of hydroxamate than hydroxylamine nitrogen. This is probably because hydroxylation of L-Orn might be readily followed by formylation or cyclization in cell-free lysates of these strains. The requirement of molecular oxygen was demonstrated by the complete inhibition of the hydroxylation process when air was replaced with N_2 in the reaction mixture. Hydroxylation was also affected by the growth stage of the cells and by the Fe(III) concentration of the culture medium; it was maximal in early-logarithmic-phase cells from Fe(III)-depleted medium and was absent when bacteria were grown to late stationary phase or in the presence of 100 μM $FeCl_3$ (data not shown).

The three different types of mutations affecting pyoverdin production were mapped by chromosome mobilization with plasmid R68.45 (15) and by phage E79-*tv2*-mediated transduction (32). Initial attempts to score the pyoverdin-proficient phenotype by selection on 2,2′-dipyridyl plates failed because at high cell densities the *pvd* mutants were not completely inhibited by the maximal nontoxic concentration of the chelator. Therefore, the eight *pvd* mutants were converted into donors by conjugative transfer of plasmid R68.45 from strain PAO25(R68.45) and mated with suitable streptomycin-resistant recipients. Exconjugants were selected for auxotrophic or catabolic markers and tested for coinheritance with *pvd* by being screened for fluorescence

TABLE 3. Linkage of *pvd* mutations with selected chromosomal markers in R68.45 matings

Selected marker(s)	Map position (min)	% Linkage[a] with:							
		pvd-1	*pvd-2*	*pvd-3A*	*pvd-3B*	*pvd-3C*	*pvd-3D*	*pvd-3E*	*pvd-3F*
trp-54	23	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
argC54	23	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
met-9011	40	5.2	8.7	5.3	9.2	2.8	4.5	4.7	2.9
met-9020	40	2.5	14.0	1.9	11.0	1.7	2.8	5.2	3.3
catA1	47	100.0	98.3	100.0	100.0	98.1	96.3	100.0	97.4
mtu-9002	48	96.7	95.2	99.6	91.3	99.2	90.9	98.4	89.3
met-9020[b] and *catA1*	40–47	93.1	100.0	95.8	94.7	91.2	95.5	96.2	93.5
met-9020[b] and *mtu-9002*	40–48	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
catA1[b] and *mtu-9002*	47–48	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
cys-54	56	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

[a] Linkage was estimated by the analysis of a minimum of 156 exconjugants.

[b] Marker used for primary selection of exconjugants.

TABLE 4. Phage E79-*tv2*-mediated cotransduction of *pvd* mutations with selected chromosomal markers

Propagating strain (mutation)	Selected marker[a]	Transduction frequency[b]	Coinherited marker	Coinheritance (%)[c]
PALS124 (*pvd-1*)	*met-9020*	1.23×10^{-6}	*pvd-1*	0/123 (0.0)
			catA1	0/123 (0.0)
	catA1	1.58×10^{-7}	*pvd-1*	106/158 (67.0)
			mtu-9002	0/158 (0.0)
PALS128 (*pvd-2*)	*met-9020*	1.34×10^{-6}	*pvd-2*	0/134 (0.0)
			catA1	0/134 (0.0)
	catA1	7.72×10^{-8}	*pvd-2*	44/77 (57.1)
			mtu-9002	0/77 (0.0)
PALS106 (*pvd-3A*)	*met-9020*	1.65×10^{-6}	*pvd-3A*	0/165 (0.0)
			catA1	0/165 (0.0)
	catA1	1.15×10^{-7}	*pvd-3A*	88/115 (76.5)
			mtu-9002	0/115 (0.0)

[a] The recipient strain was PAO4032-sr (*met-9020 catA1 nar-9011 mtu-9002 tyu-9030 dcu-9013* Str^r) (27).
[b] Expressed as transductants per PFU.
[c] Number of transductants coinheriting/number tested.

emission on SM9-streptomycin plates under UV light exposure. The results of chromosomal mapping are shown in Table 3. The three different types of *pvd* mutations were closely linked to *catA1* and *mtu-9002*, located at 47 and 48 min on the PAO1 map (19), respectively. A lower degree of linkage with *met-9020* and *met-9011*, both located at 40 min, was shown. On the basis of these results, three-factor crosses were performed with strain PAO4032. Recombinants at both *met-9020* and *mtu-9002* or at both *catA1* and *mtu-9002* gave 100% coinheritance with *pvd-1*, *pvd-2*, and *pvd-3*. The positions of the *pvd* mutations were also determined by phage E79-*tv2*-mediated transduction. The *pvd-1*, *pvd-2*, and *pvd-3* mutations were all cotransducible with *catA1* (Table 4). No transductional linkage was found with *mtu-9002*. These results are in agreement with previous reports (4, 18, 35) and indicate that the different mutations affecting pyoverdin biosynthesis might be clustered within the chromosome region near the genes responsible for benzoate catabolism, at 47 min of the revised PAO1 map (19).

The results presented in this article define at least three genetic defects affecting pyoverdin biosynthesis. This is a minimum estimation, since recent studies of different *Pseudomonas* spp. indicate that several genes are required for the biosynthesis of pyoverdins, in agreement with the structural complexity of these fluorescent siderophores (25, 26, 31, 35). It has also been reported that the synthesis of the fluorescing group is preceded by the synthesis of the peptide part of the siderophore (26). Our results strengthen this hypothesis and indicate that the formation of L-N^5-OH-Orn is an earlier step in the peptide synthesis by *P. aeruginosa* PAO1. Physiological and enzymatic criteria indicate that the *pvd-1* mutant is an L-N^5-OH-Orn auxotroph, lacking the L-Orn-N^5-hydroxylating enzyme. The *pvd-2* mutant, producing low levels of hydroxylamine nitrogen identified as N^5-OH-Orn, might be blocked in the following step of pyoverdin synthesis, i.e., the acylation of the hydroxylamine group. Accordingly, cell-free lysates of the *pvd-2* mutant converted L-Orn to the corresponding hydroxylamine but not to the hydroxamate derivative. All the other pyoverdin-deficient mutants (*pvd-3*) produced hydroxamate compounds in iron-poor medium and converted L-Orn to the hydroxamate derivative in a cell-free enzyme assay. Of note, the formation of hydroxamate and/or hydroxylamine compounds by the wild type and by *pvd-2* and *pvd-3* mutants both in liquid cultures and in a cell-free system was strictly iron regulated and oxygen dependent, as expected for a hydroxylating activity involved in siderophore synthesis.

In conclusion, although hydroxamate siderophores include a variety of structurally distinct chelators, the enzymes for the hydroxylation of the ω-amino groups seem to have been conserved over a wide evolutionary range, from bacteria to fungi (2, 3, 14, 17, 20, 39). These enzymes catalyze a unique microbial reaction, which does not occur in animal and plant cells, and hence they might constitute potential targets for new antimicrobial drugs capable of interfering with in vivo iron uptake by pathogenic microorganisms.

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