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Mapping of Mutations in *Pseudomonas aeruginosa* Defective in Pyoverdin Production

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Twelve mutants of *Pseudomonas aeruginosa* PAO defective in pyoverdin production were isolated (after chemical and transposon mutagenesis) that were nonfluorescent and unable to grow on medium containing 400 μM ethylenediaminedi(*o*-hydroxyphenylacetic acid). Four mutants were unable to produce hydroxamate, six were hydroxamate positive, one was temperature sensitive for pyoverdin production, and another was unable to synthesize pyoverdin on succinate minimal medium but was capable of synthesizing pyoverdin when grown on Casamino Acids medium (Difco Laboratories, Detroit, Mich.). The mutations were mapped on the PAO chromosome. All the mutations affecting pyoverdin production were located at 65 to 70 min, between *catA1* and *mtu-9002*.

Pathogenic bacteria require iron for growth in a mammalian host (6). To obtain iron from the host, bacteria must effectively compete with the iron-sequestering proteins transferrin and lactoferrin (1). Many microbes possess iron acquisition systems mediated by siderophores, low-molecular-weight products capable of binding and delivering iron to the cell via high-affinity transport systems (31).

Pseudomonas aeruginosa is a major cause of nosocomial infections which result in high mortality. *P. aeruginosa* is known to produce two siderophores, pyochelin (9, 10, 25) and pyoverdin (8, 42). Pyochelin is a phenolic siderophore that has two sulfur-containing heterocyclic rings (9, 10). Pyoverdin, previously termed bacterial fluorescein (A. Turfreijer, Ph.D. thesis, University of Amsterdam, Amsterdam, The Netherlands, 1941), has long been thought to be involved in iron metabolism because of its hydroxamate character (16) and the inhibition of its production by iron (14, 17, 38). Only recently has the siderophore activity of pyoverdin been demonstrated (8) and its molecular structure elucidated (42). This fluorescent siderophore produced by *P. aeruginosa* is a complex peptide containing two hydroxamate groups and a dihydroxyquinoline derivative (42) as its theorized chelating moieties. Yellow-green fluorescent peptides produced by other fluorescent *Pseudomonas* species and *Azotobacter vinelandii* (5, 15, 28, 32, 33, 37, 44) have similar spectral characteristics and extensive structural homology with pyoverdin.

Recent investigations have suggested that iron acquisition by *P. aeruginosa* may play a role in its pathogenesis. The concentration of iron in culture medium has a significant effect on the production of the extracellular proteins, toxin A, alkaline protease, and elastase (3, 4). Pyochelin has been shown to increase the lethality of *P. aeruginosa* during infections in mice (7). A mutant unable to synthesize pyoverdin had an extremely depressed growth rate compared with that of wild-type strains when grown in human serum and transferrin (2).

Advances in *P. aeruginosa* genetics have allowed the map positions of several reported virulence factors to be determined. Use of the chromosome-mobilizing plasmid R68.45 (19), R-prime plasmids (21), and transposon-facilitated recombination (24) has made it possible to map loci coding for toxin A (20), elastase (22), flagella (39), extracellular proteins (43), and alginate production (12, 18).

We previously described the isolation of mutants of *P. aeruginosa* PAO which were unable to produce pyochelin and pyoverdin (2). Additional mutants that were defective in pyoverdin production have been isolated, and their map positions were located by using the chromosome-mobilizing plasmid R68.45 and linkage analysis.

MATERIALS AND METHODS

Strains. All bacterial strains, plasmids, and bacteriophages used in this study are listed in Table 1. The *P. aeruginosa* strains used were derived from the prototrophic parent strain PAO. Strains were maintained in frozen solutions of 20% glycerol–5% tryptic soy broth at −70°C between experiments.

Media. Vogel-Bonner minimal medium (41) without citrate was used for all bacterial matings. Glucose and sodium succinate were incorporated into minimal medium at concentrations of 20 mM. Benzoate and mannitol were used at 15 mM concentrations. Amino acids and other nutritional supplements were added at 1 mM. CAA medium contained 0.5% Casamino Acids (Difco Laboratories, Detroit, Mich.) and 1 mM $MgSO_4$ in distilled H_2O. LB medium contained 1% tryptone, 0.5% yeast extract, and 0.5% NaCl. Antibiotics were used at the following concentrations: ampicillin, 1 mg/ml; kanamycin sulfate, 500 μg/ml; streptomycin sulfate, 500 μg/ml; tetracycline hydrochloride, 100 μg/ml; and rifampin (50 μg/ml) for *P. aeruginosa* or kanamycin sulfate and tetracycline hydrochloride (50 and 20 μg/ml, respectively) for *Escherichia coli*. Ethylenediaminedi(*o*-hydroxyphenylacetic acid) (EDDA) was incorporated into medium at 400 μM concentrations to select against Pvd^- strains.

Isolation and analysis of mutants. PAO1 and its derivatives were mutagenized with ethyl methanesulfonate (2) and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (18) and by Tn*10* mutagenesis with the suicide vector pSX2 (S. N. Steenbergen, A. Bergstrom, and J. L. Ingraham, manuscript submitted). Mutants unable to produce pyoverdin were identified by one of the following criteria: (i) colonies unable to grow on

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TABLE 1. Bacterial strains, plasmids, and bacteriophages

Strain, plasmid, or bacteriophage	Genotype,[a] phenotype, or phage characteristics	Reference or source
P. aeruginosa strains		
PAO1	Prototroph	34
PAO12-sr	*leu-8 pur-136 chl-3* Str^r Rif^r	34
PAO25	*argF leu-10*	34
PAO25-sr	*argF leu-10* Str^r Rif^r	34
PAO236-sr	*his-4 ilvB lys-12 trp-6 met-28 proA28* Str^r Rif^r	19
PAO440	*pvd-12 ser-3 bla-440*	24
PAO944-sr	*thr-9001 cys-54 pur-67* Str^r Rif^r	34
PAO949-sr	*thr-9001 cys-59 pur-67* Str^r Rif^r	34
PAO2368-sr	*catA1 cnu-9001 met-9020 nar-9011 puuE8* Str^r Rif^r	20
PAO2376	*catA1 met-9020 nar-9011 tyu-9030 mtu-9002*	34
PAO2376-s	*catA1 met-9020 nar-9011 tyu-9030 mtu-9002* Str^r	34
PAO2376-sr	*catA1 met-9020 nar-9011 tyu-9030 mtu-9002* Str^r Rif^r	34
IA1	*pvd-1* Bnu$^-$	2
IA5	Pvd$^+$ transductant of IA1	2
IA31	Bnu$^+$ transductant of IA1	This study
CDC5	*pvd-2*	This study
IA121	*pvd-3 argF leu-10*	This study
IA126	*pvd-4 argF leu-10*	This study
IA130	*pvd-5 argF leu-10*	This study
ICA2	*pvd-6*	This study
ICA10	*pvd-7*	This study
ICA14	*pvd-8*	This study
ICA28	Pvd$^-$ Mtu$^-$	This study
ICC4	*pvd-9*	This study
IAJ40M1	*pvd-10*::Tn*10*	This study
IA500	*pvd-11*(Ts)	This study
E. coli strain		
JL4051	*hsdR hsdM recA13 supE44 lacZ4 leuB6 proA2 thi-1 rpsL*	J. Ingraham
Plasmids		
R68.45	Cb Km Tc Tra Cma	19
pSX2	pRK2013::Tn*10*	J. Ingraham
Bacteriophages		
E79*tv-2*	General transducing phage	30
F116L	General transducing phage	23

[a] Genotype symbols for *P. aeruginosa* are according to Royle et al. (34) except for *pvd* (inability to synthesize pyoverdin) and *bla* (resistance to carbenicillin) (24). Bnu represents benzoate utilization.

EDDA-CAA agar after replica plating from LB agar; (ii) colonies that were nonfluorescent under UV excitation after growth on CAA or succinate minimal medium agar at 37°C; and (iii) colonies that were nonfluorescent under UV excitation after growth on CAA agar at 42°C. To correlate nonfluorescence with impaired growth on iron-limiting media, pyoverdin-negative mutants were patched onto EDDA-CAA or EDDA-succinate minimal medium agar, and the plates were observed for growth after 24 h of incubation at 37°C. The biosynthesis of hydroxamate compounds was assayed by methods reported by Csaky (11). Our use of the term hydroxamate is intended to include N^6-hydroxyornithine in addition to complex hydroxamates.

Genetic techniques. R68.45 plasmid-mediated conjugation was performed by direct plate matings (19). Transductions were carried out as described by Krishnapillai (23) with minor modifications (2). Transposon mutagenesis was performed essentially as described by Selvaraj and Iyer (35) by using the suicide vector pSX2.

RESULTS

Mutants in pyoverdin production. The Pvd$^-$ mutants used in this study were nonfluorescent (apparently lacking the dihydroxyquinoline moiety) and unable to grow on EDDA-containing medium. No nonfluorescent mutants were able to grow on EDDA-containing medium, nor were any fluorescent strains unable to grow on EDDA-containing medium. In this study there was an absolute correlation between nonfluorescence and the inability to grow on EDDA-containing medium. Strain IA500 (*pvd-11*) was isolated as a nonfluorescent colony on CAA agar incubated at 42°C. After replating and incubation at 30°C, the mutant demonstrated a Pvd$^+$ phenotype, indicating a temperature-sensitive lesion in pyoverdin production. Mutant ICA2 (*pvd-6*) was isolated as a colony with negligible fluorescence on succinate minimal medium agar and was later found to be Pvd$^+$ when grown on CAA agar. ICA2 was unable to grow on EDDA-succinate minimal medium agar within 24 h, but prolonged incubation (>48 h) allowed limited growth, and some fluorescence was detected. Although we suspected that there were nutritional requirements for pyoverdin production, we were unable to determine the cause of this phenomenon in ICA2.

Since pyoverdin possesses two N^6-hydroxyornithine residues (42) (one of them cyclized as an *N*-hydroxypiperidone ring), we further analyzed our collection of Pvd$^-$ mutants for the ability to synthesize hydroxamate-containing compounds. It should be noted that the Csaky assay (11) detects

both complex hydroxamates and N^{δ}-hydroxyornithine, a putative biosynthetic intermediate of pyoverdin. The results (Table 2) are expressed as hydroxylamine equivalents produced per milliliter of medium. Mutants IA1 (*pvd-1*), IA121 (*pvd-3*), IA126 (*pvd-4*), and IA130 (*pvd-5*) accumulated negligible hydroxamate in overnight cultures. The transposon insertion mutants IAJ40M1 (*pvd-10*) and PAO440 (*pvd-12*) had markedly decreased hydroxamate character in comparison with PAO1. The remaining Pvd$^-$ mutants produced hydroxamate-containing compounds at levels comparable to PAO1. A Pvd$^+$ transductant of IA1, IA5 (2), produced hydroxamate at wild-type levels, unlike its parent strain IA1. All mutants demonstrated growth rates comparable to the parent PAO1 when grown in CAA or succinate minimal media.

Genetic mapping of lesions in pyoverdin production. An early clue to the map position of the genes necessary for pyoverdin production was deduced during an analysis of the catabolic potential of mutants IA1 and ICA28. Strain IA1 was unable to utilize benzoate, and strain ICA28 was unable to utilize mannitol. The genes responsible for benzoate utilization are located at 64 to 65 min on the PAO chromosome (34), and those for mannitol catabolism have been previously mapped at approximately 69 min (R. A. Roehl, P. V. Phibbs, Jr., and T. W. Feary, Abstr. Annu. Meet. Am. Soc. Microbiol. 1981, K70, p. 149). Another indication of the map position of pyoverdin genes was realized by analysis of a Tn*1* insertion mutant, PAO440, previously mapped by Krishnapillai et al. (24) to the region between the *catA1* and *mtu-9002* markers at approximately 67 min on the PAO chromosome. PAO440 was also found to be Pvd$^-$, whereas the rest of the Tn*1* insertion mutants isolated by Krishnapillai et al. (24) were Pvd$^+$.

To locate the region(s) of the PAO chromosome responsible for pyoverdin production, we used strain IA1 (*pvd-1*) (R68.45) as the donor. When it was found that strain IA1 was Bnu$^-$ (benzoate utilization negative), a Bnu$^+$ IA1 derivative, IA31, was prepared by transduction with bacteriophage E79*tv-2* and was subsequently used as the donor. This manipulation was necessary so that we could use this strain to transfer the *catA1*$^+$ marker to PAO2368 and PAO2376. Exconjugants which had been selected for single markers were screened for coinheritance of *pvd-1* by plating on CAA agar and observing fluorescence under UV light. The results of the chromosomal mapping of *pvd-1* are given in Table 3. We observed 77% linkage of *pvd-1* to *catA1* and only 12 and 5% linkage to *met-9020* and *nar-9011*, respectively. With the previous information provided by strains IA1, ICA28, and PAO440, we used PAO2376, which contains lesions *catA1* and *mtu-9002*, as a recipient during matings. Prototrophic recombinants at both *catA1* and *mtu-9002* gave 100% coinheritance of *pvd-1* as an unselected marker, clearly indicating that *pvd-1* was located between *catA1* and *mtu-9002*.

All other Pvd$^-$ mutants were converted into donors by conjugative transfer of R68.45 to the mutants from strain PAO25(R68.45). These were then mated with PAO2376. The mutations carried by these strains were also mapped by selecting both the *catA1*$^+$ and *mtu-9002*$^+$ markers in PAO2376 and then screening for the unselected *pvd* mutation (Table 4). Although the linkage of the various *pvd* mutations to *catA1* ranged from 41 to 92%, the coinheritance of the *pvd* lesions in recombinants repaired at both *catA1* and *mtu-9002* ranged from 94 to 100%. Thus far, all *pvd* mutations inves-

TABLE 2. Production of hydroxamates by *P. aeruginosa* strains[a]

Strain (condition)	Hydroxamates (μmol equivalents of NH_2OH/ml)[b]
PAO1	0.31
PAO1 (SMM)[c]	0.33
PAO1 (42°C)	0.11
PAO25	0.30
IA1 (*pvd-1*)	0.006
IA5	0.42
IA31 (*pvd-1*)	0.007
CDC5 (*pvd-2*)	0.32
IA121 (*pvd-3*)	0.005
IA126 (*pvd-4*)	0.008
IA130 (*pvd-5*)	0.006
ICA2 (*pvd-6*)	0.18
ICA2 (*pvd-6*) (SMM)	0.25
ICA10 (*pvd-7*)	0.27
ICA14 (*pvd-8*)	0.30
ICC4 (*pvd-9*)	0.29
IAJ40M1 (*pvd-10*)	0.095
IA500 (*pvd-11*)	0.24
IA500 (*pvd-11*) (42°C)	0.13
PAO440 (*pvd-12*)	0.069

[a] Assays were carried out on CAA cultures grown at 37°C for 24 h, with the exception of CAA cultures grown at 42°C for 120 h and succinate minimal medium cultures grown at 37°C for 48 h.
[b] Hydroxamates released by hydrolysis were determined in comparison to an NH_2OH standard curve and therefore expressed as equivalents of NH_2OH.
[c] SMM, Succinate minimal medium.

TABLE 3. Linkage of *pvd-1* to selected markers[a]

Selected marker(s)	Map location (min)	*pvd-1* linkage (%)
ilvB$^+$	8	0/20 (0)
pur-136$^+$	25	0/20 (0)
trp-6$^+$	35	0/20 (0)
leu-8$^+$	42	0/20 (0)
argF$^+$	47	0/20 (0)
met-9020$^+$	55	22/180 (12)
catA1$^+$	65	138/180 (77)
catA1$^+$ and *mtu-9002*$^+$[b]	65 and 70	99/99 (100)
nar-9011$^+$	77	1/20 (5)
cys-54$^+$	80	0/20 (0)
cys-59$^+$	95	0/20 (0)

[a] The donor strains were IA1 (*pvd-1*) (R68.45) and IA31 (*pvd-1*) (R68.45).
[b] Exconjugants which were selected for prototrophy at *catA1*$^+$ and found to be prototrophic at *mtu-9002*$^+$ were then tested for *pvd-1* coinheritance.

TABLE 4. Linkage of *pvd* mutations to *catA1*$^+$ and *catA1*$^+$ and *mtu-9002*$^+$[a]

Mutation	Linkage to *catA1*$^+$ (%)	Linkage to *catA1*$^+$ and *mtu-9002*$^+$ (%)
pvd-2	169/232 (73)	92/93 (99)
pvd-3	66/107 (62)	36/37 (97)
pvd-4	53/93 (57)	32/32 (100)
pvd-5	52/74 (70)	42/42 (100)
pvd-6	56/89 (63)	34/35 (97)
pvd-7	57/90 (63)	40/40 (100)
pvd-8	45/89 (51)	33/33 (100)
pvd-9	37/90 (41)	29/31 (94)
pvd-10	147/180 (82)	117/117 (100)
pvd-11	124/135 (92)	60/60 (100)
pvd-12	103/180 (57)	61/64 (95)

[a] Exconjugants which were selected for prototrophy at *catA1*$^+$ and found to be prototrophic at *mtu-9002*$^+$ were then screened for *pvd* coinheritance.

tigated in this laboratory have been mapped between the markers *catA1* and *mtu-9002*.

DISCUSSION

Pyoverdin has been shown to be the mediator of a very important iron acquisition system for *P. aeruginosa*. Pyoverdin possesses a very high iron-binding coefficient, 10^{32} (42), and its efficiency at reversing iron deprivation effected by high concentrations of EDDA was reported by Vandenbergh et al. (40). The possible role of pyoverdin in virulence has been inferred from the growth stimulation phenomenon following its addition to iron-deficient culture medium and defined medium containing transferrin, human serum, or plasma (8). A Pvd^- mutant possessed slower growth and lower iron uptake rates than a Pvd^+ strain when grown in the presence of human serum or transferrin (2). The present investigation underscores the importance of pyoverdin by reporting the inability of Pvd^- mutants to grow in EDDA-containing medium. Increased yields of the extracellular proteins, toxin A, alkaline protease, and elastase are observed when *P. aeruginosa* is grown in iron-limiting medium (3, 4), indicating the important regulatory role of iron in the production of these reported virulence factors (36).

Pyoverdin from *P. aeruginosa* shares structural aspects with other bacterial yellow-green fluorescent peptides: pseudobactin from *Pseudomonas* strain B10 (37), pyoverdin from *Pseudomonas fluorescens* (28, 32, 33), pseudobactin 7SR1 from a plant-deleterious *Pseudomonas* strain (44), and azotobactin from *A. vinelandii* (5, 15). The same dihydroxyquinoline derivative, responsible for the fluorescence of these compounds, is present in all these compounds with only minor structural differences. Unfortunately, little is known about the biosynthetic origin of this unique structure. These molecules, with the exception of azotobactin, all contain N^{δ}-hydroxyornithine as a probable chelating group. A preponderance of serine residues and D-amino acids occurs in these compounds, and all have approximately the same number of amino acids in their peptide chains.

Genetic studies have been carried out on siderophores with much simpler structures than that of pyoverdin, mainly enterochelin (27) and aerobactin (13). There is increasing interest in the genetics of the siderophores of the fluorescent pseudomonads. Loper et al. (26) reported the isolation of several recombinant plasmids capable of complementing lesions in the biosynthesis of a fluorescent compound produced by a *Pseudomonas syringae* isolate. Moores et al. (29), studying *Pseudomonas* strain B10 and its fluorescent siderophore pseudobactin, isolated recombinant plasmids which complemented nearly all available nonfluorescent mutants. These laboratories concluded that many genes are required for the biosynthesis of these fluorescent molecules (a minimum of 12 for pseudobactin [29]) and suggested that the biosynthetic genes of these fluorescent molecules may be dispersed around the genome, as is often the case of biosynthetic pathways in *Pseudomonas* species (34). However, data presented in this paper would suggest that in *P. aeruginosa*, at least several genes for pyoverdin production are localized in a limited region of the PAO genome.

The hydroxamate-negative mutants (IA1, IA121, IA126, and IA130) appear to lack genetic information necessary for pyoverdin, ferribactin, and all hydroxamate production, since any of these compounds should have yielded positive Csaky tests. Ferribactin (32), another iron-binding hydroxamate peptide, has previously been found in *P. aeruginosa* spent culture medium (8). The inability of these mutants to produce other hydroxamates as well as pyoverdin suggests that the lesions in pyoverdin production possessed by these mutants may be in genes necessary for overall hydroxamate production. This is also indicated by the analysis of IA5, a Pvd^+ transductant of IA1, which was found to produce hydroxamates at wild-type levels. Pvd^- mutants still capable of producing hydroxamates are most likely producing ferribactin or fragments of the pyoverdin molecule. We are presently analyzing the hydroxamate compounds produced by these mutants in hopes of further understanding pyoverdin biosynthesis and the relationship between pyoverdin and ferribactin.

This investigation reported the mapping of several mutations affecting production of pyoverdin by *P. aeruginosa*. All mutations were mapped to the same region of the PAO chromosome, the 4 to 5 min located between *catA1* and *mtu-9002*. We also reported the isolation of Pvd^- mutants unable to produce hydroxamate-containing compounds. Although multiple defects in pyoverdin biosynthesis may be present in strains IA1, IA121, IA126, and IA130, their inability to produce hydroxamate-containing compounds, presumably N^{δ}-hydroxyornithine, is a first step in understanding the functional aspects of the genes responsible for pyoverdin biosynthesis. The nature of the mutations in pyoverdin production remains obscure at this point, and the genetic complexity associated with the biosynthesis of fluorescent siderophores (26, 29) suggests that a great deal of work will be required to elucidate the organization and regulation of the genes responsible for pyoverdin biosynthesis.

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