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Pyoverdin Is Essential for Virulence of *Pseudomonas aeruginosa*

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The role of pyoverdin, the main siderophore in iron-gathering capacity produced by *Pseudomonas aeruginosa*, in bacterial growth in vivo is controversial, although iron is important for virulence. To determine the ability of pyoverdin to compete for iron with the human iron-binding protein transferrin, wild-type *P. aeruginosa* ATCC 15692 (PAO1 strain) and PAO pyoverdin-deficient mutants were grown at 37°C in bicarbonate-containing succinate medium to which apotransferrin had been added. Growth of the pyoverdin-deficient mutants was fully inhibited compared with that of the wild type but was restored when pyoverdin was added to the medium. Moreover, when growth took place at a temperature at which no pyoverdin production occurred (43°C), the wild-type PAO1 strain behaved the same as the pyoverdin-deficient mutants, with growth inhibited by apotransferrin in the presence of bicarbonate and restored by pyoverdin supplementation. Growth inhibition was never observed in bicarbonate-free succinate medium, whatever the strain and the temperature for growth. In vivo, in contrast to results obtained with the wild-type strain, pyoverdin-deficient mutants demonstrated no virulence when injected at 10^2 CFU into burned mice. However, virulence was restored when purified pyoverdin originating from the wild-type strain was supplemented during the infection. These results strongly suggest that pyoverdin competes directly with transferrin for iron and that it is an essential element for in vivo iron gathering and virulence expression in *P. aeruginosa*. Rapid removal of iron from [^{59}Fe]ferritransferrin by pyoverdin in vitro supports this view.

Pseudomonas aeruginosa, an opportunistic human pathogen, is strongly involved in severe and often fatal infections in patients with cystic fibrosis (15), burns (28, 29), ocular diseases (2, 17), pneumonia (40), and other immunosuppressive illnesses (4). As an aerobic bacterium (39), *P. aeruginosa* has a strong iron requirement, and the mechanisms by which it fulfills its iron needs in vitro are well known. As a free-living organism, it is able to excrete large amounts of two chemically unrelated siderophores, mainly pyoverdin (PVD) (5, 10) and to a lesser extent pyochelin (11, 32), into its environment. These siderophores function as powerful iron chelators, solubilizing and transporting iron through the bacterial membranes via specific receptor proteins at the level of the outer membrane (23, 38), and have a TonB-like system for the translocation of iron through the cytoplasmic membrane (12). When inside the cell, iron is released from the ferrisiderophores by a reductive process before it reaches its targets (20, 21). Also, iron transport into *P. aeruginosa* may be facilitated by siderophores of foreign origin, i.e., PVDs from *Pseudomonas fluorescens* ATCC 13525 and *Pseudomonas chlororaphis* ATCC 9446 (26), cepabactin from *Burkholderia* (*Pseudomonas*) *cepacia* (34), desferriferrioxamine B (8), and enterobactin (41). These (ferri)siderophores penetrate the cells through specialized constitutive or siderophore-inducible receptors (8, 14) or porin F (34). Finally, small iron chelators, such as citrate (9, 22), salicylic acid (34), nitrilotriacetic acid (36), and inositol polyphosphate (47), may also actively transport iron into *P. aeruginosa* cells.

In order to grow in vivo, *P. aeruginosa* must fulfill its nutritional requirements within the human or animal host. For instance, to meet the needs for iron, bacteria must compete with the host for an element which is tightly bound to host proteins, e.g., ferritin, transferrin (Tsf), lactoferrin, and hemoglobin. How *P. aeruginosa* is able to pick up iron from these iron-binding proteins is not well understood. Many different explanations have been postulated: (i) the direct use of iron from host iron proteins via specialized, usually inducible outer membrane receptors, as is the case for many human pathogens, e.g., the uptake of Tsf and lactoferrin by *Neisseria gonorrhoeae* (46) and *Haemophilus influenzae* (45) and the uptake of heme and hemoglobin by *Vibrio cholerae* (51) and *Plesiomonas shigelloides* (13); (ii) a siderophore-mediated competition for the iron scavenged by the proteins, as has been demonstrated for various siderophores, e.g., vibriobactin (51), aerobactin (31), and amonabactin (33); and (iii) the release of iron by proteolytic cleavage of the scavenging proteins as a result of the action of bacterial proteases, with the siderophores being used by the bacteria for the translocation of the already released iron (16). Our knowledge of the exact mechanism for *P. aeruginosa* is incomplete and controversial, with some authors supporting the siderophore-mediated mechanism (1, 49) and others favoring the proteolytic one (16). Although the first mechanism—direct translocation of iron from iron-loaded proteins—has not been thoroughly investigated with *P. aeruginosa*, the mechanism involving the siderophore-mediated iron release from iron proteins has received support from in vitro dialysis experiments which suggested that PVD and pyochelin were able to release iron from ferritransferrin (ferri-Tsf) (49). Moreover, the promoting effects of these siderophores on bacterial growth in media supplemented with ferri-Tsf or human serum as the iron source also strengthened this view and further supported the primacy of PVD over pyochelin: a PVD-deficient mutant had severe growth deficiencies in human serum-supplemented medium, whereas a pyochelin-deficient

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mutant was unaffected in its growth in the same medium (1). Iron release from ferri-Tsf by proteolytic cleavage was suggested by experiments which showed that *P. aeruginosa* proteases could cleave Tsf (6, 16). While experimental results which denied that PVD had any direct Tsf-iron-releasing potential have been published (16), a recent report modulated this conclusion by recognizing that protease-deficient (LasB) mutants were still able to release iron from Fe_2-Tsf (54).

In the present study, the primary involvement of PVD in Tsf iron release was assessed by in vitro competition experiments with [^{59}Fe]ferri-Tsf, whereas growth experiments conducted with both wild-type *P. aeruginosa* ATCC 15692 (PAO1 strain) and PAO PVD-defective mutants assessed the primacy of the PVD system for iron incorporation into *P. aeruginosa*. Finally, the function of PVD as a virulence-associated factor was analyzed by comparing the virulence of PVD-producing strains with that of PVD-defective mutants in vivo.

MATERIALS AND METHODS

Bacteria and growth conditions. The *P. aeruginosa* strains used in this study were *P. aeruginosa* ATCC 15692 (PAO1 strain); *P. aeruginosa* PAO6049, a methionine auxotroph of PAO1 (43); and PVD-deficient strains PAO6606, PAO6609, PAO6616, and PAO6622 (PVD mutants) (25). These mutants were obtained by UV mutagenesis of methionine auxotroph PAO6049 and selected for the absence of a fluorescent halo around the colonies on King's B medium (24). On the basis of their growth sensitivities to ethylenediamine di(hydroxyphenyl)acetic acid, two classes of mutants were obtained. Mutations were mapped at 35 min of the PAO chromosome for PAO6616 and PAO6622 and at 60 to 65 min for PAO6606 and PAO6609 (25). These values corresponded, respectively, to 23 and 47 min of the most recent chromosomal map (30). When tested on skim milk agar for protease detection (48) or on elastin-Congo red agar for elastase detection (3), these mutants showed no significant differences compared with parental strain PAO6049 (24).

Growth experiments were performed in succinate medium (35) which, with a contaminating iron concentration of about 2.5 μM, allowed iron-starved bacterial growth with concomitant siderophore excretion. In some experiments, succinate medium was supplemented, as indicated in the text, with sodium bicarbonate and/or human apotransferrin (apo-Tsf) and/or siderophores at 20 mM, 5 μM, and 40 μM (final concentrations), respectively. For growth of PAO6049 and PAO6049 derivatives, the culture medium was supplemented with 1 mM methionine.

Supplementations were made from filter-sterilized (Millipore filter units; pore diameter, 0.45 μm) solutions in succinate medium. Iron, when needed, was added to the sterilized medium from a freshly prepared 20 mM $FeCl_3$ sterile solution. Iron-saturated human Tsf was purified by the method of Ratefiarivelo et al. (42) from normal human serum obtained from the Centre de Transfusion Sanguine, Strasbourg, France. Apo-Tsf was obtained by the deferration procedure of Warner and Weber (53), with the iron content of the preparations being monitored with the M + D Iron kit from Merz + Dade AG, Düdingen, Switzerland. The siderophores used were PVDs (succinamide form) from *P. aeruginosa* ATCC 15692 (PAO1 strain), *P. aeruginosa* ATCC 27853, and *P. aeruginosa* Pa6 (PVD_{PAO}, PVD_{27853}, and PVD_{Pa6}, respectively), purified as described previously (7), and pyochelin from PAO1 and cepabactin from *B. cepacia* ATCC 25416, both purified as described previously (37).

Bacterial cultures were grown in 180-by-18-mm capped test tubes with 7.5 ml of growth medium and were shaken at 200 rpm. The growth temperature was usually 37°C, except for some experiments as indicated in the text. Growth was measured with a Chemtrix T24 colorimeter (Chemtrix Inc., Hillsboro, Oreg.) with a 610-nm filter.

Burned mouse model. The burned mouse model of Stieritz and Holder (50) was used. Anesthetized mice received a partial thickness burn on their shaven backs (15% of the total body surface), with 0.5 ml of sterile saline given intraperitoneally for fluid resuscitation. This injury was nonlethal. Immediately postburn, 10^2 CFU of the wild type (PAO1 or PAO6049) or PVD mutants was injected subcutaneously into the burned site. The mice were monitored for death for 10 days. In some cases, purified PVD (100 μg in 100 μl of saline) was injected into the burned infected site immediately postburn and at daily intervals for 9 days, if required.

Labelling procedure for [^{59}Fe]ferri-Tsf. Apo-Tsf was saturated with iron in the presence of ^{59}Fe as follows. Apo-Tsf (0.5 μM) was dissolved in 2 ml of 10 mM bicarbonate and 150 mM NaCl buffer (pH 7.4) to which 500 μl of $^{59}FeCl_3$ (11.5 μg of ^{59}Fe per ml; specific activity, 25 mCi/mg; Amersham), 5 μl of 0.2 M $FeCl_3$ (to ensure 100% iron saturation of Tsf), and 1 ml of 5 mM nitrilotriacetic acid had been added. After pH adjustment to 8.5 with 10% Na_2CO_3 and incubation at room temperature for 30 min, [^{59}Fe]ferri-Tsf was removed from free iron by filtration through a Sephadex G-25 column equilibrated with 10 mM bicarbonate and 150 mM NaCl buffer (pH 7.4). ^{59}Fe-saturated Tsf fractions were collected and stored at −20°C until use.

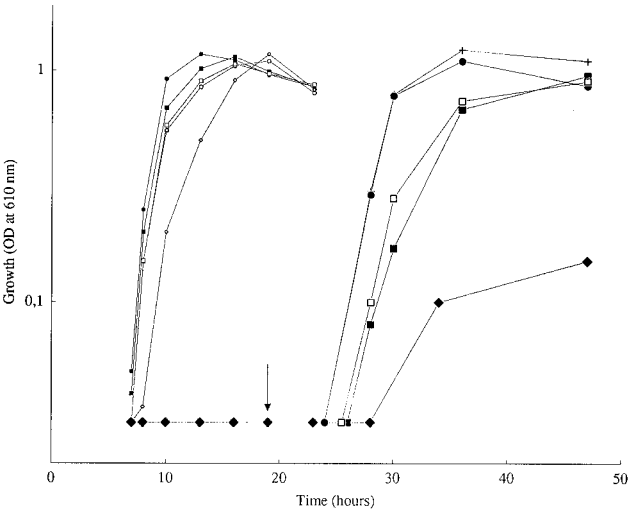

FIG. 1. Effects of bicarbonate, apo-Tsf, siderophore, and iron supplementations on growth of *P. aeruginosa* PAO6609 at 37°C. The bacteria were grown in succinate (S) medium (○); S medium supplemented with 5 μM apo-Tsf (◇); succinate and 20 mM bicarbonate (S+B) medium supplemented with 40 μM PVD_{PAO} (●), 40 μM pyochelin (□), or 40 μM cepabactin (■); S+B medium supplemented with 5 μM apo-Tsf (S+B+apo-Tsf) (◆); and S+B+apo-Tsf medium supplemented during growth (arrow) with 40 μM PVD_{PAO} (●), 40 μM pyochelin (□), 40 μM cepabactin (■), or 100 μM $FeCl_3$ (+). Growth in S+B medium was similar to growth in S medium. OD, optical density.

Assays of competition between siderophores and Tsf for ^{59}Fe. A constant concentration of [^{59}Fe]ferri-Tsf (0.5 μM in Tsf) was added to different concentrations of siderophores (0 to 12 μM), and the volume was adjusted to 200 μl with 10 mM bicarbonate and 150 mM NaCl buffer (pH 7.4). After gentle agitation, the mixture was incubated for 15 min at room temperature, and then cold acetone (400 μl) was added to precipitate the proteins (apo-Tsf and ferri-Tsf). The proteins were removed by centrifugation (20,000 × *g* for 10 min) after a 15-min incubation on ice. Controls demonstrated that 100% of the protein was removed by this procedure, with the siderophores or ferrisiderophores remaining in solution. Supernatant fluid (200 μl) was taken to measure the radioactivity with a Gamma 4000 counter (Beckman, Palo Alto, Calif.).

RESULTS

Tsf-mediated growth inhibition of PVD-deficient mutants. PVD-deficient mutants, represented in Fig. 1 by strain PAO6609, grew in succinate medium at a rate only slightly different from that at 37°C of wild-type PAO1 (25). Supplementation of the growth medium with bicarbonate (20 mM) did not affect the growth of PAO6609, whereas supplementation with apo-Tsf (5 μM) instead of bicarbonate resulted in slightly delayed growth and a small decrease in the growth rate (Fig. 1). However, supplementation of the succinate medium with both bicarbonate and apo-Tsf had a strong inhibitory effect on PAO6609 growth. Growth was not detectable before 30 to 35 h postinoculation, and even after 50 h, the optical density at 610 nm reflected about 1/10 of the cell yield reached in less than 20 h under the previous growth conditions (Fig. 1).

Supplementation of the bicarbonate–apo-Tsf–succinate medium with PVD_{PAO} (final concentration, 40 μM) at 18 h postinoculation resulted in pronounced growth following a lag phase of 5 to 6 h, with a cell yield and growth rate identical to those of the controls (PVD-supplemented and bicarbonate-supplemented succinate media) (Fig. 1). The growth-stimulating effect of PVD appeared to be highly specific to PVD_{PAO}, since the addition of PVD_{27853} or PVD_{Pa6} instead of PVD_{PAO} at 18 h postinoculation was unable to reverse the growth inhi-

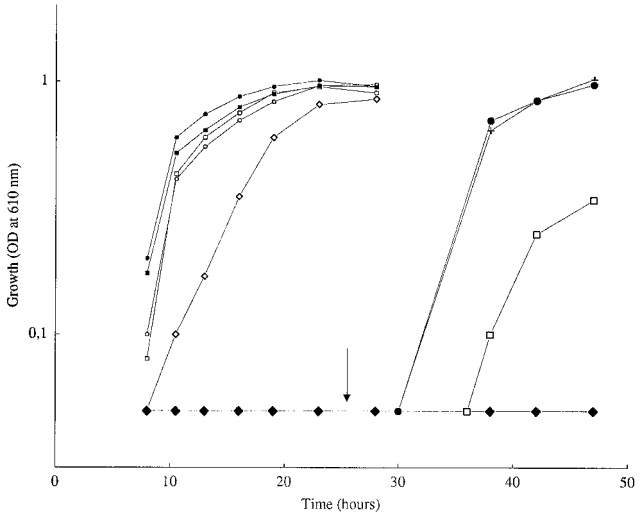

FIG. 2. Effects of bicarbonate, apo-Tsf, siderophore, and iron supplementations on growth of *P. aeruginosa* PAO1 in S medium incubated at 43°C. Conditions of growth and symbols are the same as those given in the legend to Fig. 1. No growth was observed in the S+B+apo-Tsf medium supplemented with cepabactin, PVD_{27853}, or PVD_{Pa6} at 40 μM. OD, optical density.

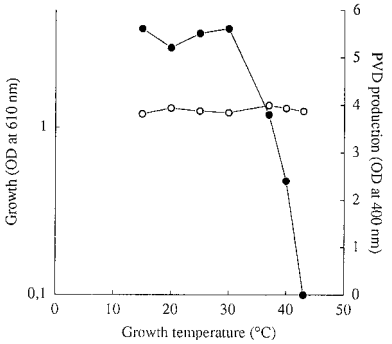

FIG. 3. Growth of (○) and PVD production by (●) *P. aeruginosa* PAO1 in S medium as a function of the temperature of incubation. Values were taken when the maximal growth was reached. OD, optical density.

bition of apo-Tsf–bicarbonate (data not shown). Figure 1 also shows growth curves for identical experiments including controls, with PVD_{PAO} being replaced by pyochelin or cepabactin at the same final concentration. Both siderophores overcame the growth inhibition properties of apo-Tsf in the presence of bicarbonate, but they resulted in lower growth rates compared with that obtained with PVD_{PAO}. The maximal growth with PVD_{PAO}, as measured by optical density at 610 nm, was 1.10, while growth with pyochelin and cepabactin at the same time was 0.75 and 0.68, respectively (35 h) (Fig. 1). Supplementation with 100 μM iron (as $FeCl_3$) instead of siderophores resulted in a growth curve identical to the one reached with PVD_{PAO} supplementation and a slight increase in cell yield (Fig. 1). Growth experiments conducted with another PVD mutant strain, PAO6606, produced results identical to those described above for PAO6609 (data not shown).

Tsf-mediated growth inhibition of wild-type PAO1 grown at 43°C. Growth experiments in succinate medium supplemented with 20 mM bicarbonate and 5 μM apo-Tsf were conducted with wild-type PAO1. Contrary to the result obtained with PAO6609, PAO1 was not affected in its growth by the bicarbonate–apo-Tsf supplementations when the experiment was conducted at 37°C; growth at this temperature was very similar to that obtained in succinate medium without supplementation, and the culture rapidly turned yellow-green because of PVD production (data not shown). At 43°C, however, the same pattern as that for PAO6609 was observed, with an even more drastic growth inhibition by apo-Tsf in the presence of bicarbonate, since no growth at all occurred, even after a prolonged incubation period (50 h) (Fig. 2). As was observed for PAO6609, supplementation of PAO1 at 43°C with PVD_{PAO} during the incubation (25 h) resulted in very good growth following a 5- to 6-h lag. Supplementation with pyochelin instead of PVD_{PAO} permitted slight growth, whereas supplementation with cepabactin or PVDs of foreign origin (PVD_{27853} and PVD_{Pa6}) had no effect (Fig. 2). The controls in succinate medium, bicarbonate-supplemented succinate medium, and apo-Tsf-supplemented succinate medium (without bicarbonate) were incubated at 43°C and showed normal growth; however, no pigmentation occurred in these media, suggesting inhibition of PVD synthesis at this growth temperature. A study of growth and PVD production by PAO1 in succinate medium as a function of the growth temperature was then undertaken, and it effectively demonstrated a drastic loss of the ability of PAO1 to produce PVD when the strain was grown at temperatures ranging from 40 to 43°C, although growth itself was unaffected at this temperature range (Fig. 3). The complete absence of PVD in the culture supernatant of cells grown at 43°C was assessed by a lack of fluorescence and by a visible spectrum missing the typical 400- to 405-nm absorption peak for PVD. Moreover, extraction of this supernatant (acidified with HCl to pH 3.0) with chloroform followed by addition of iron ($FeCl_3$) to the chloroform phase did not show the typical red wine color resulting from the formation of a ferripyochelin complex (9), as was observed with the supernatant of PAO1 cells grown at 37°C. Thus, neither PVD nor pyochelin was produced by PAO1 during growth at 43°C. It should be emphasized that a shift of the growth temperature from 43 to 37°C after 20 h of incubation in the succinate–apo-Tsf–bicarbonate medium had the same effect as the PVD_{PAO} supplementation, with good growth starting 5 to 6 h after the temperature shift. Indeed, this growth was accompanied by production of PVD and pyochelin (data not shown).

In vitro siderophore-mediated removal of iron from [^{59}Fe]Tsf. When fully iron-saturated Tsf labelled with ^{59}Fe and siderophore were mixed in an in vitro assay, it was possible to detect the presence of some radioactivity remaining in the supernatant after the protein was removed by acetone precipitation. For an assay in which PVD_{PAO} was involved, autoradiography of the electrophoresed pigmented material contained in the supernatant revealed that the solubilized ^{59}Fe was in the form of a PVD-iron complex. Controls demonstrated that all of the protein and all of the radioactivity were recovered in the precipitate when siderophore supplementation was omitted from the assay and conversely that PVD or its iron complex remained totally in the supernatant after acetone treatment. For all the siderophores tested, the maximal amount of radioactivity removed from the ferri-Tsf was reached very rapidly, within less than 1 min. As can be seen from Fig. 4, this amount increased as a function of the siderophore concentration and tended to plateau for siderophore concentrations up to 4 to 8 μM, depending on the siderophore tested. The intensity of the iron removal was also siderophore dependent, with PVDs usually demonstrating a better efficiency compared with that of pyochelin or cepabactin. The three PVDs that originated from *P. aeruginosa* strains and differed from each other in their peptide chains (5, 7, 52)

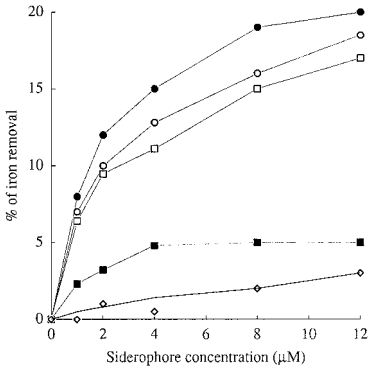

FIG. 4. Removal of iron from [^{59}Fe]ferri-Tsf by siderophores. The siderophores added at various concentrations to 0.5 μM [^{59}Fe]ferri-Tsf in 10 mM bicarbonate and 150 mM NaCl buffer (pH 7.4) were PVD from *P. aeruginosa* ATCC 15692 (PVD_{PAO}) (○), PVD from *P. aeruginosa* ATCC 27853 (PVD_{27853}) (●), PVD from *P. aeruginosa* Pa6 (PVD_{Pa6}) (□), pyochelin from *P. aeruginosa* ATCC 15692 (◇), and cepabactin from *B. cepacia* ATCC 25416 (■). The percentage of iron removed from [^{59}Fe]ferri-Tsf by the siderophore was determined by measuring the radioactivity remaining in the supernatant after an acetone treatment of the mixture (see Materials and Methods).

showed similar efficiencies in ferri-Tsf–iron removal, removing 15 to 20% of the total iron at a concentration of 12 μM PVD (Fig. 4). Other PVDs, however, showed various efficiencies; the PVD from *P. fluorescens* ATCC 13525 was the most powerful compound, removing 28% of Tsf-iron at 12 μM, whereas the PVD from *P. fluorescens* CCM 2798 showed a much lower capacity, with only 5% of the iron being removed at the same molar concentration (data not shown). Pyochelin and cepabactin reacted similarly, with iron removal capacities not exceeding 5% (Fig. 4).

In vivo virulence of PVD-producing and PVD-deficient *P. aeruginosa* strains. Fourteen mice were infected postburn with 10^2 CFU of wild-type PAO1 or its methionine auxotroph derivative PAO6049, the mother strain of PVD mutants PAO6606, PAO6609, PAO6616, and PAO6622. Groups of four other mice were infected postburn with the PVD mutants. Maximal mortality for the mice infected with the PVD-producing strains (PAO1 and PAO6049) occurred within 3 days postinfection for PAO1 and within 4 days for PAO6049 (Table 1). Ninety-three percent mortality was reached with PAO1, while mortality in the PAO6049 group was 57%. This result was not significantly different from results reported for PAO1. A significant difference, however, occurred with the PVD mutants, which appeared fully avirulent since no mice died because of infection with any of the four strains (Table 1).

TABLE 1. Comparison of mortalities of burned mice infected with *P. aeruginosa* PAO parental strains and their PVD mutants

Organism[a]	Genotype	Mortality at day[b]:			
		1	2	3	4
PAO1	Wild type	0/14	9/14	13/14	13/14
PAO6049	*met-9011 amiE200 strA*	0/14	2/14	7/14	8/14
PAO6606	*met-9011 amiE200 strA* (PVD-6)	0/4	0/4	0/4	0/4
PAO6609	*met-9011 amiE200 strA* (PVD-9)	0/4	0/4	0/4	0/4
PAO6616	*met-9011 amiE200 strA* (PVD-16)	0/4	0/4	0/4	0/4
PAO6622	*met-9011 amiE200 strA* (PVD-22)	0/4	0/4	0/4	0/4

[a] A total of 10^2 CFU of each organism was injected subcutaneously into the burned site immediately postburn.

[b] Number of dead mice/number of mice tested. The mortality numbers remained the same from day 4 to day 10. Total mortality for all PVD mutants was 0/16; this mortality was significantly different from those obtained with PAO1 and PAO6049 by chi-square analysis, with Yates correction for continuity ($P < 0.001$).

TABLE 2. Mortalities of burned mice infected with parental strain *P. aeruginosa* PAO6049 or the PVD-deficient mutants PAO6606 and PAO6609 and given supplemental injections of PVDs of different bacterial origin or saline into the burned and infected site

Organism[a]	Supplement			Mortality at day[b]:				
	Saline	PVD_{PAO}[c]	PVD_{27853}[d]	1	2	3	4	10
PAO6049	+	−	−	0/8	3/8	3/8	3/8	4/8
	−	+	−	0/8	3/8	7/8	8/8	8/8
	−	−	+	0/8	3/8	4/8	4/8	4/8
PAO6606	+	−	−	0/8	0/8	0/8	0/8	0/8
	−	+	−	0/8	3/8	4/8	4/8	4/8
	−	−	+	0/8	0/8	0/8	0/8	0/8
PAO6609	+	−	−	0/8	0/8	2/8	2/8	2/8
	−	+	−	0/8	3/8	5/8	5/8	5/8
	−	−	+	0/8	0/8	1/8	2/8	2/8

[a] A total of 10^2 CFU of each organism was injected subcutaneously into the burn site immediately postburn.

[b] Number of dead mice/number of mice tested. The mortality numbers remained the same from day 4 to day 9.

[c] PVD from *P. aeruginosa* PAO1 (100 μg) was injected subcutaneously into the burn site daily for 9 days.

[d] PVD from *P. aeruginosa* ATCC 27853 (100 μg) was injected subcutaneously into the burn site daily for 9 days.

Restoration of virulence by coinjection of PVD during infection. To assess the apparent correlation between the lack of PVD biosynthesis and the lack of virulence observed for the PVD mutants, strains PAO6606 and PAO6609 were injected into burned mice with a simultaneous coinjection of pure PVD or saline as a control. In the PVD-supplemented mice, various mortalities were observed, depending on the bacterial strain and the bacterial origin of the coinjected PVD (Table 2). With PVD_{PAO} (homologous PVD) coinjection, a significative increase in mortality was observed with PAO6606 and PAO6609, whereas the injection of PVD_{27853} (originating from *P. aeruginosa* ATCC 27853) had no effect, with mortality identical to that of the control (saline injection). Injection of PVDs into burned mice without bacterial infection had no lethal effect (data not shown).

DISCUSSION

This study demonstrated that Tsf (apo-Tsf) was able to inhibit the growth of *P. aeruginosa* fully, providing (i) that the protein was in a bicarbonate-enriched environment and (ii) that the bacteria were unable to synthesize the siderophores PVD and pyochelin. This result was particularly evident for wild-type PAO1 grown at 43°C. At that temperature, which corresponds to the temperature limit of *P. aeruginosa*, bacterial growth was unchanged compared with growth at lower temperatures but as was demonstrated in the present study, no siderophores at all were produced by the bacteria. Neither PVD nor pyochelin was detectable in the growth supernatants obtained from iron-starved cultures incubated at 43°C. In this respect, *P. aeruginosa* behaved the same as other bacteria which have been previously described as lacking siderophore production while growing at sublethal temperatures (18, 19). The absence of siderophore production by *P. aeruginosa* correlated with a full inhibition of growth by apo-Tsf in the presence of bicarbonate (Fig. 2). On the other hand, in all experimental conditions which allowed a siderophore to be

produced in the culture, the apo-Tsf inhibition effect was either reduced or totally suppressed. This outcome was particularly clear from the experiments with siderophore supplementation during growth. PVD addition fully restored the growth of PAO1 at 43°C (Fig. 2) and of PAO6609 at 37°C (Fig. 1), and pyochelin had similar but smaller effects on both strains. Furthermore, it can be assumed that the residual growth observed for PAO6609, although in the presence of apo-Tsf and bicarbonate, can be attributed to the ability of the bacteria to still produce pyochelin under these conditions of growth (37°C). Finally, when PAO1 was grown at 37°C, the growth inhibition of apo-Tsf in the presence of bicarbonate was not observed, since the bacterium was able to synthesize both PVD and pyochelin at that temperature. The observation that a heavy (100 μM) iron supplementation in the apo-Tsf–bicarbonate medium also reversed the apo-Tsf–bicarbonate growth inhibition for bacteria grown at 43°C strongly suggested, indeed, that the siderophores acted through their iron-scavenging properties. Bicarbonate ions are known to be required for apo-Tsf to bind iron (44). The growth inhibition effect of this protein, which also was strictly dependent on the presence of bicarbonate, suggested that this effect was related to the iron-binding capacity of apo-Tsf. Thus, a possible explanation of the growth experiment results would be as follows. Apo-Tsf, when added to the succinate medium, bound the traces of contaminant iron present in the medium, provided that bicarbonate ions were also added. In such conditions, a bacterium unable to synthesize its siderophores, i.e., wild-type PAO1 grown at 43°C, is unable to grow because it is unable to use directly the ferri-Tsf complex as an iron source. Moreover, this hypothesis suggests that once a siderophore is available, the bacteria will be able to grow because of a transfer of iron from ferri-Tsf to the siderophore. PVD was much more efficient in this respect than pyochelin. This observation was in agreement with and completed the conclusions reached by other investigators (1), who stated that PVD appeared to be the most important siderophore for the growth of *P. aeruginosa* in human serum. The ability of these siderophores to pick up iron in the growth medium from ferri-Tsf was strongly supported by in vitro competition experiments (Fig. 4). Although more detailed studies are needed to reach a better understanding of the iron exchange reaction, it is clear from these experiments that PVD is able to remove some iron from ferri-Tsf instantaneously and without the help of other bacterial compounds, i.e., proteases. This rapid removal could represent up to 20% of the total Tsf-bound iron under the most favorable conditions used (12 μM concentration of PVD for assays with 0.5 μM apo-Tsf). PVDs from other *P. aeruginosa* strains showed efficiencies similar to that of PVD_{PAO}, whereas pyochelin at the same concentration displaced only 5% iron. Cepabactin, a siderophore not produced by but efficiently used as an iron transporter by *P. aeruginosa* (34), also had a low efficiency, like pyochelin. These two compounds have weak affinities for iron compared with that of PVD (37), a feature which should explain their lower potentials to remove the iron bound to apo-Tsf and thus their lower potentials to reverse the apo-Tsf–bicarbonate growth inhibitory effect.

P. aeruginosa is considered an important opportunistic pathogen. The pathogenicity of *P. aeruginosa* PAO1 was assessed in this study by testing the strain in the burned mouse infection model (50). An inoculation of as few as 10^2 CFU was sufficient to reach close to 100% mortality for the mice within a few days. Strain PAO6049, a methionine auxotroph of PAO1 from which the PVD mutants were raised (25), showed a lower but still significant virulence compared with that of the wild type. This peculiarity could be correlated to the auxotrophy of this strain, which needed first to fulfill its methionine requirements before growing and developing its virulence factors. However, another trait of this strain which also might contribute to its lower virulence was that under iron starvation, it produced 40% less PVD compared with that produced by PAO1 (27). The infection data presented for mutants fully devoid of PVD production confirmed this view, since no mice infected with any of the four different PVD mutant strains died. On the other hand, supplementation with purified PVD (PVD_{PAO}) by repeated injections into the burned and infected sites increased mortality to 30 to 80% and thus restored the virulence of the PVD mutants to some extent. PVD by itself had no lethal activity when injected into burned uninfected mice. Therefore, we concluded that the PVD virulence-associated effect was related to its iron-scavenging and iron transport properties. We concluded further that the PVD effects on growth and virulence were strain specific: PVD from *P. aeruginosa* ATCC 27853 was able neither to support growth nor to restore the virulence of the PAO PVD-deficient strains. This result was as expected because of the strict uptake specificity which characterizes the PAO1-ferripyoverdin outer membrane receptor (7).

Our data from in vitro and in vivo experiments strongly support the view that PVD, because of its capacity to compete successfully for iron with Tsf, should be considered a primary essential virulence-associated factor in *P. aeruginosa.*

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