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Iron Release from Transferrin by Pyoverdin and Elastase from *Pseudomonas aeruginosa*

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Pseudomonas aeruginosa **produces the siderophores pyoverdin and pyochelin as well as receptors for siderophores in response to iron deprivation. Previously, it has been shown in vitro that at neutral pH purified pyoverdin acquires iron from transferrin only in the presence of *P. aeruginosa* elastase (LasB), which proteolytically degrades transferrin. We constructed a LasB-negative mutant, PAO1E, by insertional mutagenesis to investigate whether this mutant differs in growth from the parental strain PAO1 in an iron-depleted medium supplemented with transferrin or human serum. PAO1 and PAO1E did not differ in growth with 1.25 μM Fe_2-transferrin as the only iron source. Urea gel electrophoresis indicated iron release from intact transferrin during the logarithmic growth phase of PAO1 and PAO1E. A total of 333 μM LasB was synthesized from PAO1 after onset of stationary-phase growth. Quantification of pyoverdin by spectroscopy revealed that up to 900 μM pyoverdin was produced during growth of the strains in medium supplemented with Fe_2-transferrin or 10% human serum. Incubation of Fe_2-transferrin and purified pyoverdin in concentrations similar to those found in the culture supernatant resulted in release of iron from transferrin after 10 h at 37°C. However, LasB significantly enhanced the rate constant for iron acquisition of pyoverdin from transferrin. We conclude that *P. aeruginosa* can use transferrin as an iron source without further need of LasB or pH changes. This is further supported by experiments with *P. aeruginosa* K437, which has a defective iron uptake system, and its LasB-negative mutant, K437E. Though K437 and K437E did not differ in growth with Fe_2-transferrin as the only iron source, their growth was significantly reduced relative to that of PAO1 and PAO1E.**

In spite of the virtual absence of freely available iron in the human body, pathogenic bacteria can successfully multiply in vivo to establish infection. The iron transport proteins transferrin and lactoferrin are thought to serve as the iron source for bacteria in the human body fluids (16). *Pseudomonas aeruginosa*, like other pathogens, produces siderophores during iron deprivation which bind Fe^{3+} and deliver the iron to the bacterial cell by high-affinity uptake systems (6). However, there are conflicting results concerning the role of the *P. aeruginosa* siderophores pyoverdin and pyochelin in iron release from the transferrin molecule. Both siderophores promoted growth of *P. aeruginosa* when added to a medium with Fe_2-transferrin or human sera as the iron source (1). But, because of the higher iron binding constant of pyoverdin (10^{32}) (8) compared with that of pyochelin (10^5) (7), pyoverdin is suggested to be more effective. Accordingly, a pyochelin mutant was still able to grow in human sera whereas a pyoverdin mutant was deficient in growth (1).

However, incubation of the purified compounds pyoverdin and Fe_2-transferrin showed that the transfer of iron from Fe_2-transferrin to pyoverdin is detectable only under certain experimental conditions. The reduction of pH after growth of bacteria in medium containing glucose is one possible mechanism to release iron from transferrin (26). Other results implied that proteolytic cleavage of transferrin may be necessary for iron release (12). *P. aeruginosa* produces several extracellular proteases, one of which, elastase (LasB), has been shown to cleave transferrin into small peptides (12). Interestingly, it was reported that the synthesis of LasB was enhanced in iron-deficient media (2, 5, 27). Thus, the synthesis of transferrin-hydrolyzing enzymes in concert with siderophores may allow the pathogens to overcome iron limitations during infection. To test this hypothesis, we examined the ability of LasB-negative mutants of *P. aeruginosa* to release iron from Fe_2-transferrin during growth. The LasB structural genes from strains PAO1 and K437, a derivative of PAO1 defective in pyoverdin, pyochelin, and pyoverdin uptake, were interrupted by insertional mutagenesis, resulting in strains PAO1E and K437E, respectively. We then reinvestigated the reaction between purified pyoverdin and transferrin with and without LasB in vitro. The LasB mutants were still able to release iron from the Fe_2-transferrin at a rate comparable with that of the proteolytically competent parental strain in transferrin media. Purified pyoverdin scavenges iron from transferrin without need for LasB.

MATERIALS AND METHODS

Bacterial strains and mutagenesis. *P. aeruginosa* PAO1 and the LasB-negative mutant PAO1E have been described elsewhere (28). Strain K437 is deficient in pyoverdin, pyochelin, and pyoverdin uptake (25). Strain K437E, the LasB-negative mutant derived from K437, was constructed by insertional

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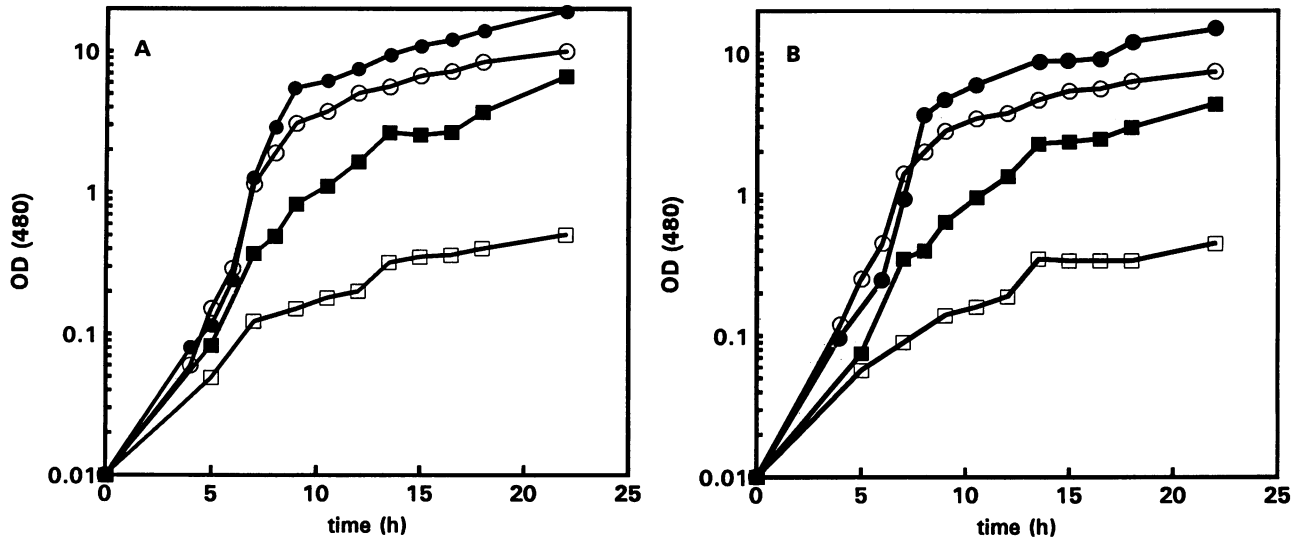

FIG. 1. Growth of *P. aeruginosa* PAO1 (A) and PAO1E (B) in CDM with 25 μM $FeSO_4$ (●), 1.25 μM apotransferrin (□), 1.25 μM Fe_2-transferrin (○), or 10% heat-inactivated human serum (■).

mutagenesis, and the insertion of the mobilization vector pBRMOB-LasB was confirmed by Southern hybridization as described previously (28).

Chemicals and glassware. Fe_2-transferrin and apotransferrin were purchased from Sigma; LasB was purchased from Nagase Biochemicals. For all iron-restricted-growth experiments, the glassware was rinsed in 1 mM EDTA overnight and then once with 0.1 N HCl and six times with deionized water (dH_2O).

Growth conditions and media. A chemically defined medium (CDM) which was optimized for LasB production (19) (14.4 mM K_2PO_4, 92 mM Na-glutamate, 24 mM valine, 8 mM phenylalanine, 70 mM glucose, 1.33 mM $MgSO_4$, 0.14 mM $CaCl_2$, 0.0085 mM $ZnSO_4$) was used. Free iron was removed with Chelex-100 (Bio-Rad Laboratories); 3× concentrated stock solutions of NaCl, K_2PO_4, Na-glutamate, valine, and phenylalanine and a 10× concentrated stock solution of glucose were stirred with Chelex-100 (5% [wt/vol]) for 2 h and sterile filtered into iron-free, sterile glassware. $MgSO_4$, $CaCl_2$, and $ZnSO_4$ were prepared as 100× stock solutions, autoclaved, and added to the media. The iron concentration of the medium was 44 μg/ml as determined by atomic absorption spectroscopy. In some experiments, iron was added from a 45 mM $FeSO_4$ solution which had been freshly prepared and sterile filtered. Human serum was freshly prepared and heat inactivated (30 min, 56°C). Apotransferrin or Fe_2-transferrin was added to a final concentration of 1.25 μM to the medium. Bacteria were inoculated in 25 ml of medium in 100-ml flasks with an initial optical density value at 480 nm (OD_{480}) of 0.01 from an overnight culture in the same medium.

Radioimmunoassay, gel electrophoresis, and immunoblotting. Radioimmunoassay for LasB was carried out as described previously (24). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method of Laemmli (20) with 12% T-3% C. The gels were stained with colloidal Coomassie G (23) or with silver (4). Urea gel electrophoresis (21) was performed with some modifications in the Mini-Protean II gel apparatus (Bio-Rad Laboratories). The gel was prepared as follows: 4.5 g of urea–625 μl of 20× concentration of TBE (2 M Tris, 0.2 M boric acid, 0.032 M EDTA; pH 8.4), was added to 2.7 ml of acrylamide stock solution (2.6% C–30% T), the volume was adjusted to 12.5 ml with dH_2O, and the gel was polymerized with 10 mg of ammonium persulfate and 20 μl of TEMED (*N,N,N',N'*-tetramethylethylenediamine). The gel was overlaid with a comb gel (1.3 ml of 20× TBE, 1.3 ml of acrylamide stock solution [2.6% C, 30% T], 6.1 ml of dH_2O, 10 μl of ammonium persulfate, 50 μl of TEMED). TBE (1×, pH 8.4) was used as electrophoresis buffer. Proteins were freshly prepared in sample buffer (10% glycerol, 0.2% bromphenol blue, 1× TBE). For samples containing serum, 100 μl of culture supernatant was mixed with 800 μl of Rivanol solution (25% glycerol, 0.38% Rivanol in 1× TBE) and left for 5 min, and the precipitated serum proteins were removed by centrifugation. Electrophoresis was carried out for 5 h at 100 V. For immunoblotting, proteins were transferred to nitrocellulose (Schleicher and Schüll) by semidry blotting in electrophoresis buffer. Blots were developed with polyclonal primary antibodies and peroxidase-conjugated secondary antibodies, and bands were visualized with chemiluminescence (ECL Kit from Amersham Buchler) according to the instructions of the manufacturer.

Pyoverdin purification and quantification. Pyoverdin was isolated from the culture supernatant of *P. aeruginosa* ATCC 15692 (9) and purified according to the hydrophobic chromatography method (8). The spectrophotometric measurements were performed with a spectrophotometer (Pharmacia, Freiburg, Germany). Iron-free pyoverdin has an absorption maximum at 400 nm, whereas binding of iron leads to a characteristic shoulder at 465 nm. Since the spectrum of pyoverdin as a free ligand is pH dependent, the quantification of pyoverdin was performed at pH 5.0. The supernatants were diluted (1:10) with 0.5 M acetic acid-sodium acetate buffer, pH 5.0. At this pH, the spectrophotometric characteristics of pyoverdin are λ_{max} = 380 nm (ε_{max} = 16,500 M × L^{-1}) (8). Upon acidification of the supernatants, the peak shifted from 400 to 380 nm as described for purified pyoverdin (22).

The fluorescence spectrum measurements were carried out with a Deltascan 4000 spectrofluorimeter (Photon Technology International, Inc.). The concentration of pyoverdin in the culture supernatant was determined with a calibration curve obtained from the fluorescence emission spectra of pure pyoverdin at pH 5.0 (in 0.5 M acetic acid-sodium acetate

TABLE 1. Cell density (OD_{480}), pyoverdin, and LasB synthesis of *P. aeruginosa* PAO1 and PAO1E in stationary phase

Growth conditions[a]	OD_{480}, pyoverdin, and LasB synthesis of *Pseudomonas* strains[b]:					
	PAO1			PAO1E		
	OD_{480} (24 h)	Pyo[c] (μM)	LasB[d] (μM)	OD_{480} (24 h)	Pyo (μM)	LasB (μM)
Apotransferrin	1.1 ± 0.3	126 ± 20	13.6 ± 6	1.4 ± 0.6	120 ± 14	ND[e]
Fe_2-transferrin	10.9 ± 1.8	582 ± 140	342 ± 30	9.1 ± 0.9	174 ± 35	ND
Serum	8.6 ± 1.2	855 ± 430	396 ± 121	5.4 ± 1.4	990 ± 126	ND
$FeSO_4$	15.2 ± 2.2	ND	324 ± 90	13.2 ± 1.2	ND	ND

[a] Iron-depleted CDM with apotransferrin (1.25 μM), Fe_2-transferrin (1.25 μM), 10% human serum, or 25 μM $FeSO_4$.
[b] Mean ± standard deviation of at least three determinations.
[c] Pyo, pyoverdin. Molarity was calculated by measuring A_{380} at pH 5.0; $\varepsilon = 16{,}500\ M^{-1}\ cm^{-1}$.
[d] Determined by radioimmunoassay.
[e] ND, not detectable (<1 ng of LasB per ml).

buffer; excitation wavelength, 400 nm; emission, 455 nm). Since both methods for pyoverdin quantification led to comparable results, pyoverdin concentrations in the culture supernatant were normally measured by spectroscopy.

Kinetics. Pyoverdin (1.8 mM) was incubated with 8 μM Fe_2-transferrin in the absence or presence of LasB (3 mM) in 0.1 M Tris-HCl, pH 7.4. The ferric solution was prepared with $Fe(ClO_4)_3 \times 9H_2O$. The kinetic measurements were done by classical absorption spectrophotometry at 465 nm (Kontron; Uvikon 860). The fastest step observed in the presence of LasB was checked by a rapid mixing technique at 465 nm (Stopped-Flow Spectrophotometer; Applied Photophysics). The temperature was kept at 25.0 ± 0.1°C for all the experiments. The kinetic data have been fitted by statistical method (Biokine V 3.0 software; Bio-Logic Co., Echirolles, France).

RESULTS

Characterization of *P. aeruginosa* strains grown in a chemically defined medium. To investigate the role of LasB in iron acquisition from transferrin, we compared the LasB-negative mutants (PAO1E and K437E) with the proteolytic wild-type strains. The PAO1 derivative K437, a strain defective in pyoverdin and pyochelin synthesis as well as in pyoverdin uptake (25), was mutagenized by insertional mutagenesis, resulting in the LasB-negative strain K437E. Mutagenesis was confirmed by Southern hybridization of *Bgl*II-digested chromosomal DNA with *lasB*. The size of the restriction fragment carrying *lasB* increased in the mutants K437E and PAO1E (28) by the size of the inserted plasmid (data not shown). LasB was produced only in PAO1 and K437, not in the mutants PAO1E and K437E, as shown by a radioimmunoassay which has a sensitivity limit of 1 ng/ml.

For growth experiments, an iron-depleted medium (CDM) was used, which was optimized for LasB production. All strains grew equally well in CDM supplemented with 20 μM $FeSO_4$. Without added iron, PAO1 and PAO1E showed growth inhibition and produced pyoverdin in equal concentrations (100 μM) (data not shown). The strains K437 and K437E did not grow at all without additional iron. The pH in the growth media was maintained at pH 8 throughout the experiments.

Growth of *P. aeruginosa* PAO1 and PAO1E with transferrin. Strains were grown in CDM with Fe_2-transferrin, apotransferrin, or human sera. Whereas apotransferrin has no growth-stimulating effect on the strains, addition of 1.25 μM Fe_2-transferrin or 10% human serum resulted in growth promotion similar to that of addition of free iron. PAO1 and PAO1E did not differ in growth in CDM with Fe_2-transferrin (Fig. 1; Table 1). However, addition of 10% human serum resulted in higher cell density in the stationary phase with PAO1 compared with PAO1E (Table 1). Under these conditions, the pH decreased to pH 5.0 in PAO1 but remained stable in PAO1E.

Cleavage of transferrin by LasB during growth of *P. aeruginosa* PAO1 and PAO1E. The proteins of the culture supernatants were analyzed by SDS-PAGE (Fig. 2), and transferrin and LasB were identified by immunoblotting (data not shown). As expected in PAO1, LasB was produced after 24 h of growth and transferrin was cleaved in the stationary phase. In PAO1E, LasB was undetectable and transferrin was still intact even after 36 h of growth (Fig. 2, lane 5). In media supplemented with human sera, besides transferrin other proteins were also cleaved in PAO1 (Fig. 2, lane 2).

Iron release from transferrin by *P. aeruginosa* PAO1 and PAO1E. Since the transferrin molecule was still intact after 36 h of growth of the LasB-negative mutant, we examined the iron load of the transferrin by urea gel electrophoresis. Because of the increase in stability as iron is bound to transferrin, four molecular forms of transferrin are separated with gels with 6 M urea: iron-free apotransferrin, iron bound only to the carboxyl

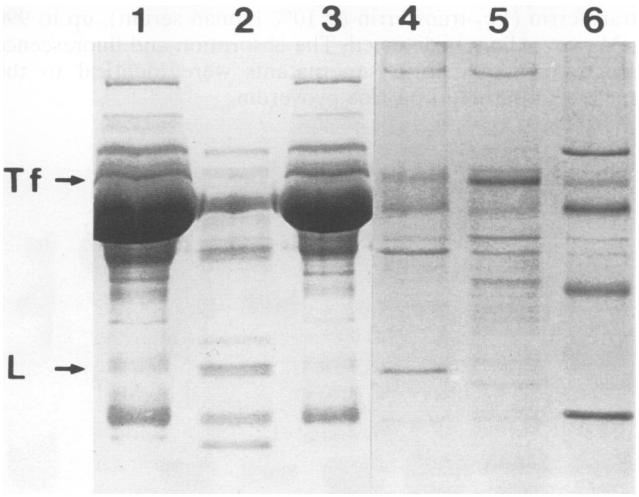

FIG. 2. SDS-PAGE of proteins from the culture supernatant (10 μl) collected after 24 h of growth of *P. aeruginosa* PAO1 and PAO1E in CDM with 10% human serum or Fe_2-transferrin (1.25 μM). Tf, transferrin; L, elastase. Lane 1, 10% human serum in CDM after 24 h at 37°C without bacteria; lane 2, PAO1 grown with human serum; lane 3, PAO1E grown with human serum; lane 4, PAO1 grown with Fe_2-transferrin; lane 5, PAO1E grown with Fe_2-transferrin; lane 6, molecular mass marker (94, 67, 43, and 30 kDa).

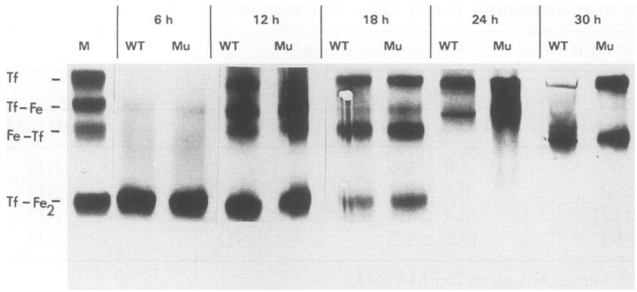

FIG. 3. Urea-polyacrylamide gel from culture supernatant collected during growth of *P. aeruginosa* PAO1 (WT) and PAO1E (Mu). M, mixture of Fe_2-transferrin, N-terminal monoferric transferrin (Fe-Tf), C-terminal monoferric transferrin (Tf-Fe), and apotransferrin.

(C)-terminal site of transferrin, iron bound only to the amino (N)-terminal site of transferrin, and iron bound to both iron binding sites (14). Whereas incubation of Fe_2-transferrin in CDM without bacteria at 37°C for up to 8 days in the medium resulted in no iron release from Fe_2-transferrin, transferrin lost the iron when bacteria were grown in the media as seen by the appearance of apotransferrin after onset of bacterial growth. This was independent of LasB, since growth of strain PAO1E also led to iron release from Fe_2-transferrin (Fig. 3).

When the bacterial strains were inoculated in media with 10% human serum, similar results were found. The bands representing the four molecular forms of transferrin in the sera were identified by immunoblotting with antitransferrin antibodies (data not shown). Again, *P. aeruginosa* PAO1 and PAO1E used the iron bound to the transferrin, since after growth only apotransferrin (PAO1E) or no detectable transferrin (PAO1) was present in the media.

Pyoverdin production of *P. aeruginosa* PAO1 and PAO1E under different growth conditions (Fig. 1; Table 1). Addition of free iron resulted in no detectable pyoverdin. With apotransferrin in the media, growth was minimal but pyoverdin was produced. When the iron in the medium was bound to transferrin (Fe_2-transferrin or 10% human serum), up to 990 μM pyoverdin was detected. The absorption and fluorescence spectra of the culture supernatants were identical to the spectra of purified iron-free pyoverdin.

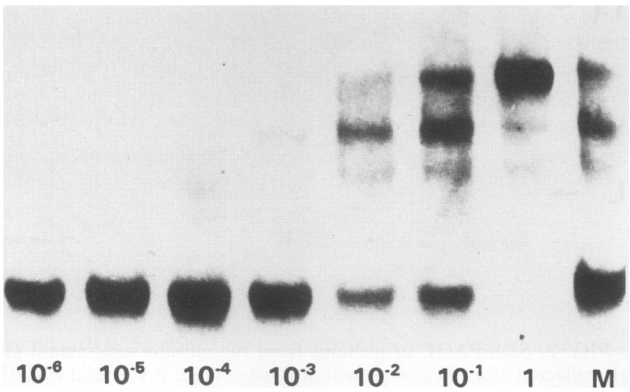

FIG. 4. Urea-polyacrylamide gel after incubation (10 h, 37°C) of Fe_2-transferrin (12.5 μM) with increasing concentrations of pyoverdin (0.749 nM to 0.749 mM). M, mixture of Fe_2-transferrin, N-terminal monoferric transferrin (Fe-Tf), C-terminal monoferric transferrin (Tf-Fe), and apotransferrin.

TABLE 2. Incubation of Fe_2-transferrin and pyoverdin with and without LasB in phosphate-buffered saline[a]

Incubation	Filtrate[b]	
	A_{400}	A_{465}
Transferrin-Fe_2	0.001	0.000
Transferrin-Fe_2 + LasB	0.008	0.003
Pyoverdin	0.603	0.008
Transferrin-Fe_2 + pyoverdin + LasB	0.578	0.207
Transferrin-Fe_2 + pyoverdin	0.601	0.137

[a] Incubation was carried out for 10 h at 37°C; amounts were as follows: Fe_2-transferrin (40 μM); pyoverdin (40 μM); LasB (9 mM).
[b] After incubation, pyoverdin was separated from transferrin and LasB by ultrafiltration, and A_{400} and A_{465} were determined in the filtrate.

Incubation of pyoverdin and Fe_2-transferrin with and without LasB. From the observation that the LasB mutant was able to release iron from Fe_2-transferrin, we assumed that the siderophores produced by this strain are able to get the iron directly from transferrin. Therefore, purified pyoverdin and Fe_2-transferrin were incubated at 37°C, and the molecules were separated by ultrafiltration. The iron saturation of transferrin was monitored by urea gel electrophoresis (Fig. 4), and the iron acquisition of pyoverdin was monitored by spectroscopy (Table 2). With increasing pyoverdin concentrations, iron was lost from transferrin, and the spectrum of the pyoverdin showed the typical shoulder at 465 nm, indicating iron acquisition. This iron transfer was more efficient when, at lower pyoverdin concentrations, LasB was added to the incubation mixture. The transferrin was cleaved within 10 h of incubation at 37°C (Fig. 5, lane 5), and the pyoverdin bound more iron in the presence of LasB (Table 2).

In order to investigate the reaction between Fe_2-transferrin and pyoverdin with and without LasB in more detail, a kinetic analysis was performed. The kinetic measurements of the iron(III) exchange reaction between Fe_2-transferrin and pyoverdin in the absence of LasB (Fig. 6A) showed an exponential increase of the absorption versus time with a pseudo-first-order rate constant = $(3.6 \pm 0.4) \times 10^{-5}$ s^{-1}.

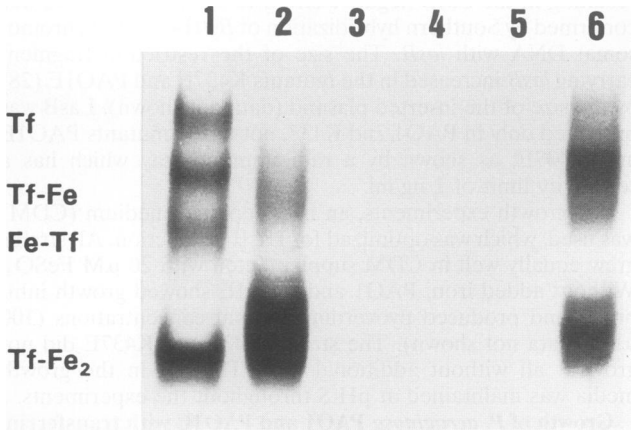

FIG. 5. Urea gel electrophoresis after incubation (10 h, 37°C) of Fe_2-transferrin (40 μM) and pyoverdin (40 μM) with (9 mM) and without LasB. Lane 1, mixture of Fe_2-transferrin, N-terminal monoferric transferrin (Fe-Tf), C-terminal monoferric transferrin (Tf-Fe), and apotransferrin; lane 2, Fe_2-transferrin; lane 3, Fe_2-transferrin with LasB; lane 4, Fe_2-transferrin with LasB and pyoverdin; lane 5, pyoverdin; lane 6, Fe_2-transferrin with pyoverdin.

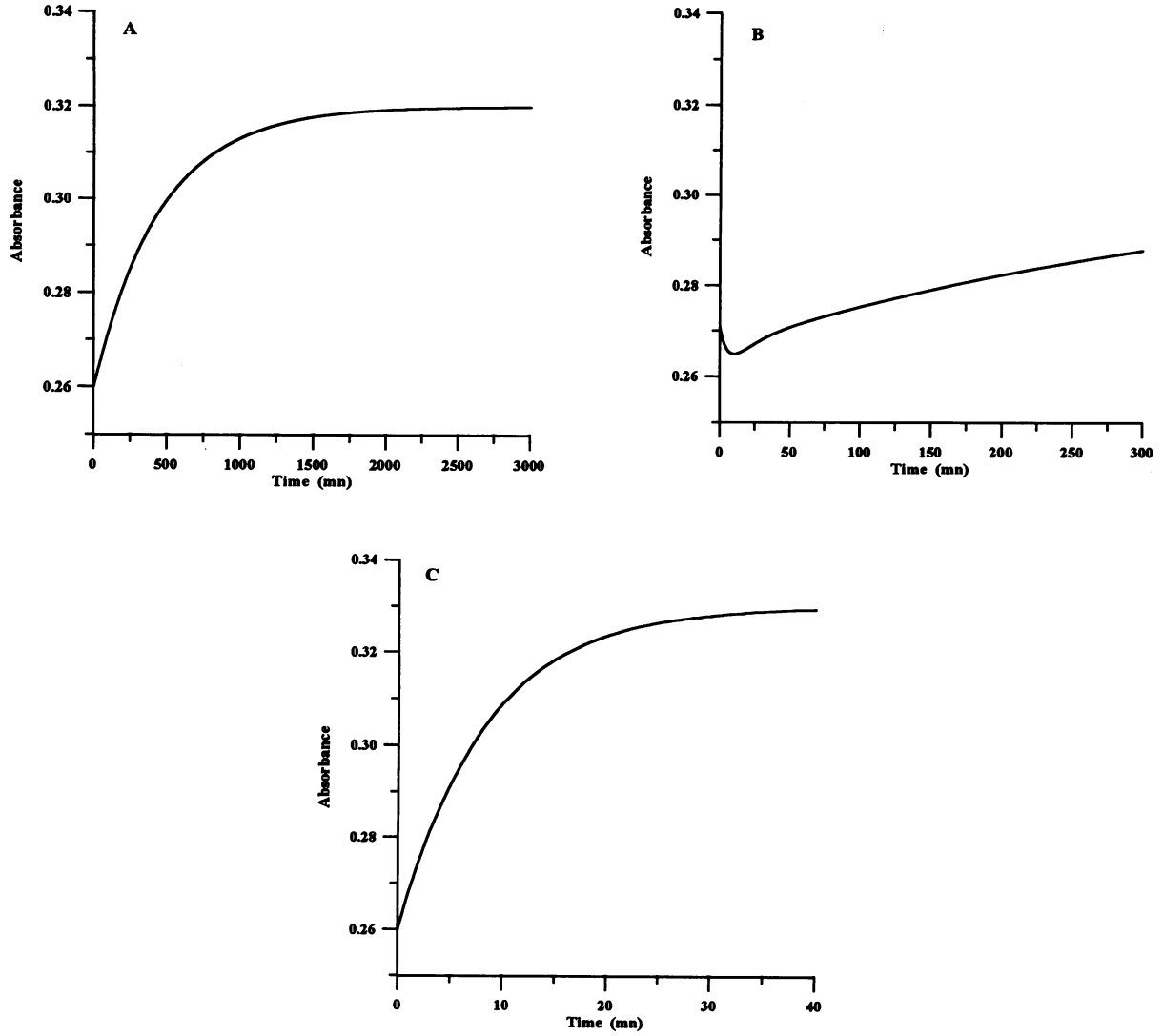

FIG. 6. Kinetics of iron(III) exchange performed in 0.1 M Tris-HCl (pH 7.4, 25.0 ± 0.1°C, λ = 465 nm). (A) Fe_2-transferrin (8 μM) with pyoverdin (1.8 mM); (B) Fe_2-transferrin (8 μM) with pyoverdin (1.8 mM) and LasB (3 mM); (C) Fe $(ClO_4)_3$ (16 μM) with pyoverdin (1.8 mM).

Assuming a maximum iron saturation (1.6×10^{-5} M iron) of pyoverdin during the reaction, a maximum increase of A_{465} of 0.06 can be calculated with the known extinction coefficients of the reactants: pyoverdin (free ligand), ε_{465} M^{-1} cm^{-1} = 140 (3); pyoverdin (ferric complex), ε_{465} M^{-1} cm^{-1} = 6,500 (3); apotransferrin, ε_{465} M^{-1} cm^{-1} = 0 (15); Fe_2-transferrin, ε_{465} M^{-1} cm^{-1} = 4,800 (15). This is in good agreement with the increase in absorption measured.

In the presence of LasB (Fig. 6B), three steps were observed at 465 nm: a fast decay of the absorption and a fast increase and a second slow increase of absorption. The pseudo-first-order rate constants for the second and third step are (1.2 ± 0.1) × 10^{-3} s^{-1} and (5.0 ± 0.5) × 10^{-5} s^{-1}, respectively.

The rate constant of the reaction between free iron and pyoverdin was (2 ± 0.2) 10^{-3} s^{-1} (Fig. 6C). Therefore, we attributed the fast increase of absorption in Fig. 6B to the acquisition of free iron by pyoverdin. Control experiments with Fe_2-transferrin and LasB without pyoverdin (data not shown) showed that the initial decay in Fig. 6B is due to cleavage of transferrin, which leads to the release of free iron. Apparently, cleavage of transferrin is not completed, since the rate constant decreased during the reaction to (5.0 ± 0.5) × 10^{-5} s^{-1}, comparable to the rate constant of the reaction of pyoverdin with Fe_2-transferrin (Fig. 6A). This was confirmed by SDS and urea gel electrophoresis showing uncleaved apotransferrin after overnight incubation at room temperature.

Growth and iron release from transferrin by *P. aeruginosa* K437 and K437E. Since from the above experiments we assumed that pyoverdin is probably effective in iron release without LasB, we further investigated this notion with the siderophore-deficient derivative K437 of *P. aeruginosa* PAO1. K437 is defective in synthesis of pyoverdin, pyochelin, and pyoverdin uptake. After a variable long lag phase (1 to 7 days), this strain was able to grow in CDM with Fe_2-transferrin as the iron source. Analysis of the culture supernatant revealed that after onset of growth K437 started to produce a fluorescent green pigment. The fluorescence spectrum was identical to that of purified pyoverdin. The concentration, however, was reduced to 10% of the PAO1 pyoverdin concentration. K437 produced LasB, and therefore, the cleavage of the transferrin

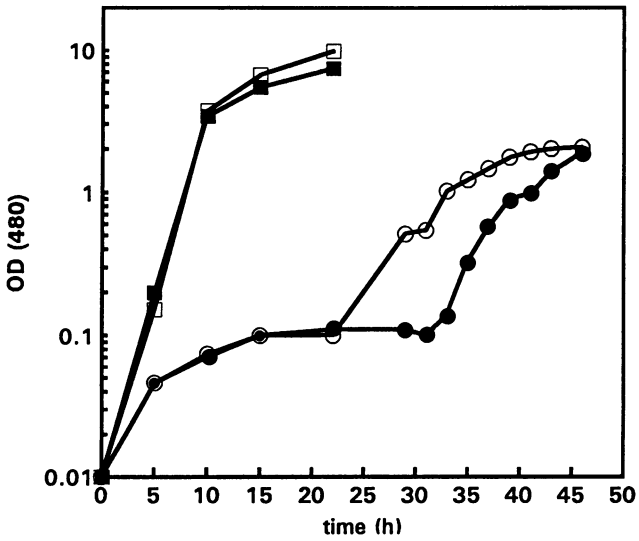

FIG. 7. Typical growth curve of *P. aeruginosa* PAO1 (□), PAO1E (■), K437 (○), and K437E (●) in CDM with Fe_2-transferrin (1.25 μM).

may account for the growth promotion by transferrin in this mutant. In this case, a LasB mutant of K437 should not grow at all in Fe_2-transferrin. Therefore, the LasB gene of K437 was interrupted by insertional mutagenesis, resulting in strain K437E.

Figure 7 shows a typical growth curve of K437 and K437E. The lag phase in both strains was variable; however, the doubling time and the final cell density did not differ between both strains. Analysis of the transferrin after growth of strain K437 and K437E by urea gel electrophoresis (Fig. 8) revealed again that growth of the proteolytic strain K437 led to cleavage of the transferrin in the stationary phase (Fig. 8, lane 5), in contrast to strain K437E, in which cleavage was not observed (lane 6). The absence of Fe_2-transferrin both in K437 and in K437E in the stationary phase (lanes 5 and 6) shows that iron was released from the transferrin after onset of growth (Fig. 8). Therefore, even under conditions in which the high-affinity iron uptake system is highly defective, the remaining iron uptake system seems to be sufficient to supply the bacteria with iron from transferrin without need of proteolytic cleavage.

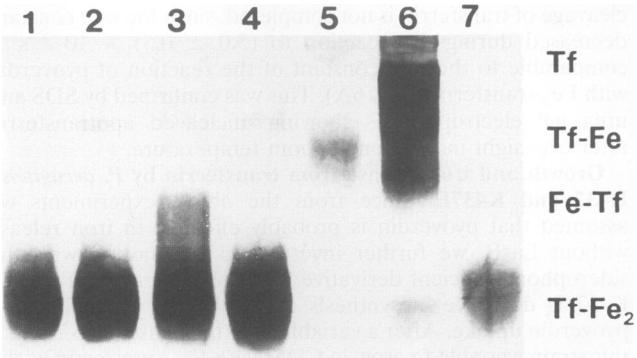

FIG. 8. Urea-polyacrylamide gel of culture supernatants (10 μl) collected during growth of *P. aeruginosa* K437 and K437E. Lane 1, K437 after 12 h; lane 2, K437E after 20 h; lane 3, K437 after 20 h; lane 4, K437E after 35 h; lane 5, K437 in stationary phase; lane 6, K437E in stationary phase; lane 7, Fe_2-transferrin incubated (37°C, 7 days) in CDM without bacteria.

DISCUSSION

The ability of siderophores to scavenge host iron has been related to their capacity to remove iron from transferrin. It has been shown previously that the iron transfer from transferrin to pyoverdin was detected only by lowering the pH (26) or by proteolytic cleavage of the transferrin molecule (12). From these in vitro experiments, it was suggested that for in vivo growth *P. aeruginosa* needs either a low pH (as present in inflammatory tissues) or secretion of extracellular LasB. In the present study, a medium in which the pH does not decrease during growth with Fe_2-transferrin was selected. Since the bacteria were still able to utilize the transferrin-bound iron, a pH decrease was not necessary for iron mobilization from transferrin.

LasB is the only extracellular protease produced by *P. aeruginosa* with transferrin-hydrolyzing activity, since incubation of concentrated culture supernatant of the LasB-negative mutant PAO1E resulted in no cleavage of transferrin (28). A role of LasB in iron acquisition from transferrin was suggested by several reports describing the enhancement of LasB production under iron restriction (2, 5, 27). Nevertheless, we found no inhibition of LasB synthesis in PAO1 by addition of free iron into the medium. Furthermore, as shown here, LasB is not necessary for utilization of transferrin-bound iron. Analysis of iron saturation of transferrin by urea gel electrophoresis during growth revealed that iron is removed from transferrin independently from LasB. In the proteolytically competent wild-type strain, cleavage of transferrin started after onset of the stationary phase and did not yield a significantly higher cell density than strain PAO1E when purified transferrin was used as the only iron source. Cleavage is coincident with LasB synthesis at the end of exponential phase (this work and reference 18), when most iron is already removed from the transferrin. In addition, Fe_2-transferrin in general is more stable towards proteolytic cleavage than apotransferrin (13, 14).

When bacteria were inoculated in 10% human sera, PAO1 reached a slightly higher cell density than PAO1E. However, the doubling times during exponential growth were identical. One explanation may be that the proteolytic cleavage of serum proteins (e.g., immunoglobulins) by LasB is not only responsible for decreasing pH but may also generate better nutritional conditions for PAO1. From the gel analysis, it is evident that the LasB mutant PAO1E released iron from transferrin in human sera without decreasing pH. However, we cannot rule out that under these conditions the wild type indeed has better access to iron by proteolytic cleavage of transferrin or by iron mobilization due to a decrease in pH.

Thus, from the experiments with whole bacteria, it is clear that iron is utilized for growth in the absence of LasB at neutral pH. The discrepancy with previous results from our group (12) may be explained by the high pyoverdin concentration (up to 900 μM) produced during bacterial growth with Fe_2-transferrin as the single iron source. Such a high pyoverdin/transferrin ratio also resulted in iron mobilization from transferrin in in vitro incubation experiments. Döring et al. (12) used an equal molar ratio of transferrin and pyoverdin, and under this condition, iron exchange between transferrin and pyoverdin indeed is very slow and can be enhanced by the rapid proteolytic cleavage of transferrin by LasB.

To draw conclusions about iron-exchange reactions between transferrin and pyoverdin during infection, one has to consider

concentrations of the reactants. However, little is known about the concentrations of pyoverdin and LasB produced during infections. Although pyoverdin (17) and LasB transcription (27) were detected in sputa from patients with cystic fibrosis infected with *P. aeruginosa*, quantitative measurement was performed only for LasB. Depending on the strain used, between 2.4 and 24 μM LasB was detected in an animal model (10). Detection of LasB in sputa of *P. aeruginosa*-infected cystic fibrosis patients was successful only in the absence of specific antibodies (11). However, other transferrin-hydrolyzing enzymes derived from the host or other microorganisms may be present during infection. Under conditions of high proteolytic activity combined with low siderophore activity, the cleavage of the transferrin molecule may have a more profound effect on iron acquisition for bacteria. Therefore, we used a *P. aeruginosa* strain (K437) with a highly defective iron uptake system (25) to investigate whether under these conditions the cleavage of LasB plays a role in iron acquisition. This strain showed diminished growth compared with PAO1. Regardless, the strain was still able to utilize iron bound to transferrin even when *lasB* was interrupted by insertional mutagenesis. Urea gel analysis again showed that in the proteolytically competent strain the transferrin is cleaved and in the LasB mutant the iron is released from the transferrin. Onset of growth in both strains was accompanied by pyoverdin synthesis. This was probably due to a regulatory mechanism and not to spontaneous mutation since subculturing of colonies grown with Fe_2-transferrin as the only iron source showed the same long lag phases for pyoverdin synthesis and growth. Although the genetic background of this regulatory process remains unclear, we conclude that a strain defective in iron uptake as well as in LasB synthesis was able to chelate iron from transferrin. Therefore, the iron uptake system of *P. aeruginosa* may also be sufficient during infection to supply bacteria with this essential ion.

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REFERENCES

1. **Ankenbauer, R., S. Sriyosachati, and C. D. Cox.** 1985. Effects of siderophores on growth of *Pseudomonas aeruginosa* in human serum and transferrin. Infect. Immun. **49**:132–140.
2. **Bjorn, M. J., P. A. Sokol, and B. H. Iglewski.** 1979. Influence of iron on extracellular products in *Pseudomonas aeruginosa* cultures. J. Bacteriol. **138**:193–200.
3. **Blanc-Parasote, S.** 1989. Etude physico-chimique d'ionophores naturels: antibiotiques carboxyliques et sidérophores bactériens. Doctoral thesis. l'Université Louis Pasteur, Strasbourg, France.
4. **Blum, H., H. Beier, and H. J. Gross.** 1987. Improved silver staining of plant proteins, RNA and DNA in polyacrylamide gels. Electrophoresis **8**:93–99.
5. **Brumlik, M. J., and D. G. Storey.** 1992. Zinc and iron regulate translation of the gene encoding *Pseudomonas aeruginosa* elastase. Mol. Microbiol. **6**:337–344.
6. **Cox, D. C., and P. Adams.** 1985. Siderophore activity of pyoverdin for *Pseudomonas aeruginosa*. Infect. Immun. **48**:130–138.
7. **Cox, D. C., and R. Graham.** 1979. Isolation of an iron-binding compound from *Pseudomonas aeruginosa*. J. Bacteriol. **137**:357–364.
8. **Demange, P., S. Wendenbaum, A. Bateman, A. Dell, and M. A. Abdallah.** 1987. Bacterial siderophores: structure and physiochemical properties of pyoverdins and related compounds, p. 167–187. *In* G. Winkelmann, D. Van der Helm, and J. B. Neilands (ed.), Iron transport in microbes, plants and animals. VCH Verlagsgesellschaft, Weilheim, Germany.
9. **Demange, P., S. Wendenbaum, C. Linget, C. Mertz, M. T. Cung, A. Dell, and M. A. Abdallah.** 1990. Bacterial siderophores: structure and NMR assignment of pyoverdins Pa, siderophores of *Pseudomonas aeruginosa* ATCC 15692. Biol. Metals **3**:155–170.
10. **Döring, G., A. Dalhoff, O. Vogel, H. Brunner, U. Droge, and K. Botzenhart.** 1984. In vivo activity of proteases of *Pseudomonas aeruginosa* in a rat model. J. Infect. Dis. **149**:532–537.
11. **Döring, G., H.-J. Obernesser, K. Botzenhart, B. Flehmig, N. Høiby, and A. J. Hofmann.** 1983. Proteases of *Pseudomonas aeruginosa* in cystic fibrosis. J. Infect. Dis. **147**:744–750.
12. **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.
13. **Esparaza, I., and J. H. Brock.** 1980. The effect of trypsin digestion on the structure and iron-donation properties of transferrins from several species. Biochim. Biophys. Acta **622**:297–307.
14. **Evans, R., and J. Williams.** 1978. Studies of the binding of different iron donors to human serum transferrin and isolation of iron-binding fragments from the N- and C-terminal regions of the protein. Biochem. J. **173**:543–552.
15. **Frieden, E., and P. Aisen.** 1980. Forms of iron transferrin. Trends Biochem. Sci. **5**:xi.
16. **Griffiths, E.** 1987. The iron-uptake systems of pathogenic bacteria, p. 69–137. *In* J. J. Bullen and E. Griffiths (ed.), Iron and infection. John Wiley and Sons Ltd., Chichester, United Kingdom.
17. **Haas, B., J. Kraut, J. Marks, S. C. Zanker, and D. Castignetti.** 1991. Siderophore presence in sputa of cystic fibrosis patients. Infect. Immun. **59**:3997–4000.
18. **Iglewski, B. H., L. Rust, and R. A. Bever.** 1990. Molecular analysis of *Pseudomonas aeruginosa* elastase, p. 36–43. *In* S. Silver, A. M. Chakrabarty, B. Iglewski, and S. Kaplan (ed.), *Pseudomonas*: biotransformations, pathogenesis, and evolving biotechnology. American Society for Microbiology, Washington, D.C.
19. **Jensen, S. E., I. T. Fecycz, and J. N. Campbell.** 1980. Nutritional factors controlling exocellular protease production by *Pseudomonas aeruginosa*. J. Bacteriol. **144**:844–847.
20. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
21. **Makey, D. G., and U. S. Seal.** 1976. The detection of four molecular forms of human transferrin during the iron binding process. Biochim. Biophys. Acta **453**:250–256.
22. **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.
23. **Neuhoff, V., R. Stamm, and J. Eibl.** 1985. Clear background and highly sensitive protein staining with Coomassie blue dyes in polyacrylamide gels: a systematic analysis. Electrophoresis **6**:427–448.
24. **Obernesser, H. J., and G. Döring.** 1982. Extracellular toxins of *Pseudomonas aeruginosa*. IV. Radioimmunoassay for detection of elastase. Zentralbl. Bakteriol. Mikrobiol. Hyg. (A) **252**:248–256.
25. **Poole, K., S. Neshat, and D. Heinrichs.** 1990. Pyoverdine-mediated iron transport in *Pseudomonas aeruginosa*: involvement of a high-molecular-mass outer membrane protein. FEMS Microbiol. Lett. **78**:1–6.
26. **Sriyosachati, S., and C. D. Cox.** 1986. Siderophore-mediated iron acquisition from transferrin by *Pseudomonas aeruginosa*. Infect. Immun. **52**:885–891.
27. **Storey, D. G., E. E. Ujack, and H. R. Rabin.** 1992. Population transcript accumulation of *Pseudomonas aeruginosa* exotoxin A and elastase in sputa from patients with cystic fibrosis. Infect. Immun. **60**:4687–4694.
28. **Wolz, C., E. Hellstern, M. Haug, D. R. Galloway, M. L. Vasil, and G. Döring.** 1991. *Pseudomonas aeruginosa* LasB mutant constructed by insertional mutagenesis reveals elastolytic activity due to alkaline proteinase and the LasA fragment. Mol. Microbiol. **5**:2125–2131.