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Siderophore-Mediated Iron Acquisition from Transferrin by *Pseudomonas aeruginosa*

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Pseudomonas aeruginosa **placed across a dialysis membrane from [^{55}Fe]transferrin caused the mobilization of the iron from the transferrin side to the bacterial or dialysate side of the membrane. Although the bacteria were capable of obtaining iron from transferrin for growth, the siderophores of *P. aeruginosa* failed to convert iron bound to transferrin into dialyzable, low-molecular-weight chelates. The crucial factor produced by the bacteria which was not present when the siderophores were added alone was the acid produced from the glucose minimal medium. The siderophores mobilized considerable iron from transferrin when used in the dialysis assay at pH values between 5.0 and 6.0, values which were commonly found during incubation of bacteria in the assays. When the siderophores were tested individually, pyoverdin was more effective than pyochelin in mobilizing iron across dialysis membranes at pH values of 5.0 and 6.0, but neither had appreciable activity at pH 7.4. The amounts of iron mobilized from conalbumin were comparable to the amounts from transferrin, but there was negligible release from lactoferrin at the three pH values. When the two siderophores were combined, the level of iron mobilization was identical to that demonstrated by pyoverdin alone. When the dialysis membrane was removed and the bacteria were mixed with the siderophores and transferrin, pyoverdin was again more active than pyochelin in mediating iron transport. Although no pyochelin-mediated iron mobilization could be detected at pH 7.4, there was transport. Therefore, the bacteria appeared to be aiding the siderophores in iron mobilization from transferrin.**

A number of bacterial species have demonstrated enhanced lethality when injected into experimental animals to which iron had been administered (5, 9, 27). The administration of iron presumably results in saturation of the iron-binding sites on transferrin, resulting in uncomplexed iron which appears to be available for bacterial growth. Transferrin, a serum glycoprotein, has an extremely high binding coefficient for iron (1, 2); lactoferrin is a glycoprotein which is found in mucosal secretions and which has a similar iron-binding function. Iron complexed to these proteins is not usually available to bacteria (2, 5, 27). However, there are two mechanisms which bacteria are known to use to acquire iron from transferrin and lactoferrin without the need for iron saturation. *Neisseria gonorrhoeae* and *Neisseria meningitidis* bind transferrin and appear to acquire iron directly through a process which has not yet been resolved (18, 19, 23). There has been a suggestion that an iron-binding compound may play a role in this process (31).

The topic of the present report involves the bacterial production of extracellular iron-binding compounds called siderophores (20). Siderophores convert polymeric ferric oxyhydroxides, present in aerobic environments, into soluble chelates which are substrates for high-affinity bacterial transport mechanisms. Some of these compounds are also able to deliver iron from transferrin to bacteria. For example, enterochelin (21) from *Escherichia coli*, which is identical to enterobactin (22) from *Salmonella typhimurium*, has a very high binding coefficient for iron (20) and should be capable of competing with transferrin for iron. However, invasive strains of *E. coli* have rapid growth rates in serum because of their ability to produce aerobactin (24, 25, 26), another siderophore with a high binding coefficient (10^{30} [20]). The ability of aerobactin to selectively contribute to the pathogenicity of *E. coli* may be explained by the instability and antigenicity of enterochelin and its binding to serum albumin, which effectively lowers the useful concentration for bacterial needs (14). This example suggests that when two siderophores are present, the iron-mobilizing activity of each siderophore with transferrin should be determined experimentally and not merely assumed from the binding coefficients.

Pseudomonas aeruginosa produces two siderophores, pyochelin and pyoverdin (10, 16). Pyochelin (12) is active in iron transport (8) and is capable of stimulating bacterial growth during infections in mice (9). Pyochelin contains a salicylate ring which is amide bonded to a thiazoline ring which is, in turn, amide bonded to a terminal *N*-methylthiazolidine ring (13). The stoichiometry of iron binding appears to be two pyochelins to one iron, and the binding coefficient of pyochelin for iron appears to be very low (5×10^5 [12]). However, pyoverdin has a binding coefficient of approximately 10^{32} (28). Pyoverdin produced by *P. aeruginosa* contains a dihydroxyquinoline moiety bonded to an octapeptide containing two *N*-hydroxyornithine residues (28). This siderophore appears to have an iron-binding stoichiometry of 1:1 (11, 28) and appears to mediate iron uptake and stimulate bacterial growth in human plasma (11).

The present report describes the activities of these two siderophores in iron acquisition assays with iron-binding proteins, particularly transferrin. Although we previously observed that pyoverdin production is the crucial event allowing bacterial growth in glucose minimal medium containing transferrin (3), recent experiments, described here, reveal that both pyoverdin and pyochelin can participate in iron mobilization from transferrin at pH values lower than neutrality. The term iron mobilization will be used to indicate the conversion of unavailable iron bound to transferrin into siderophore-bound iron or iron which is utilizable by the

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bacteria. This term is used to avoid any implication of an interaction between the siderophores and transferrin.

MATERIALS AND METHODS

Culture conditions. Assays to measure iron mobilization and acquisition by bacteria were conducted in glucose minimal medium (GMM). This medium is identical to that used previously for growth experiments with human plasma and transferrin (3, 11) and was designed to provide a minimal medium based upon a common catabolite in serum, glucose. GMM consisted of 20 mM dextrose, 15 mM $(NH_4)_2SO_4$, 20 mM sodium bicarbonate, 0.4 mM $MgCl_2$, and 5 mM potassium phosphate buffer (pH 7.4). Alternatively, arginine minimal medium (AMM) was used in certain experiments and contained 20 mM arginine in place of glucose and $(NH_4)_2SO_4$. Casamino Acids (CAA) medium was used to grow cells for inocula and for siderophore production and consisted of 0.5% CAA and 0.4 mM $MgCl_2$. Bacteria were maintained on meat infusion agar slants and were inoculated either into 50 ml of medium in 250-ml Erlenmeyer flasks or into 1 liter of medium in 2.8-liter Fernbach flasks. Cultures were incubated by shaking at 37°C for 20 h. Viable bacteria were numerically determined by spreading dilutions of culture media on 1% tryptic soy agar plates and counting the colonies after incubation of the plates at 37°C for 24 h. Solid media, used in the selection of mutants (3), were CAA agar, GMM agar, and AMM agar and contained 1.5% agar. Media which were more selective against siderophore-defective mutants were based on AMM, GMM, or CAA agar but contained either 200 μM ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA) or ethylenediamine-di-(*o*-hydroxyphenylacetic acid) (EDDA) and 0.9% Gelrite (Kelco, San Diego, Calif.) as a replacement for the agar (3).

Bacteria. *P. aeruginosa* PAO1 (ATCC 15692) was obtained from the American Type Culture Collection, Rockville, Md. Mutant strains defective in siderophore synthesis originated from strain PAO1 and have been described previously (3). These mutants are strain 211-5 (pyoverdin deficient and pyochelin proficient [Pvd^- Pch^+]), strain IA5 (pyoverdin proficient and pyochelin deficient [Pvd^+ Pch^-]), and strain IA1 (pyoverdin deficient and pyochelin deficient [Pvd^- Pch^-]).

Preparation of iron-binding proteins and siderophores. Transferrin, conalbumin, and lactoferrin were obtained in the purest forms available from Sigma Chemical Co., St. Louis, Mo., and were converted to their iron-free forms by dialyzing preparations containing 20 mg of protein per ml against 0.1 M sodium citrate-sodium acetate buffer (23). The dialysis process was conducted at pH 4.5 for transferrin and conalbumin and at pH 2.0 for lactoferrin. Portions of these iron-free forms were brought to 100% saturation levels with ^{55}Fe by the procedure of Simonson et al. (23). Sodium citrate (1 mM) was mixed with $^{55}FeCl_3$ and then incubated with the iron-free proteins in 40 mM Tris hydrochloride buffer (pH 7.4) containing 20 mM sodium bicarbonate for 30 min. The mixture was dialyzed against the same buffer until a minimal, constant level of ^{55}Fe appeared in the dialysate over a 24-h period.

Pyochelin was purified by thin-layer chromatography (12). Pyoverdin was purified by passing cell-free spent CAA medium from PAO1 cultures through Sep-Pak (Waters Associates, Inc., Milford, Mass.) C18 cartridges. The cartridges were subsequently washed with 40 ml of distilled water per cartridge and then with 5 ml of a water-methanol (1:1) mixture. The pyoverdin eluate was chilled in an ice bath and mixed with 250 ml of ethyl acetate. The resulting precipitate was collected on a Nylon 66 filter and washed on the filter with 20 ml of ethyl acetate and then with 20 ml of ethanol. The filter was dried under vacuum, and the pyoverdin was measured by weight (C. D. Cox, submitted for publication). This preparation has been shown to be pure pyoverdin by high-pressure liquid chromatography and to be active in iron transport (Cox, submitted). Pyoverdin was maintained in water solutions, and pyochelin was maintained in ethanol solutions. Before use in experiments, the ethanol in the pyochelin solutions was removed under vacuum and the pyochelin was dissolved in buffer.

Dialysis membrane experiments. The formation of ferrisiderophores in siderophore-transferrin mixtures was determined in dialysis experiments. These were run either in a Plexiglas dialysis chamber with Teflon plugs for sampling ports or in chambers constructed from scintillation vials. These vials, used in experiments with multiple samples, were plastic vials (1.7 by 4.9 cm) with the bottoms cut off. The smooth top of the vial was used to seal a dialysis membrane between the vial cap, containing 0.5 ml of a [^{55}Fe]transferrin solution (the total volume of this space was 1 ml), and the vial itself, containing 0.5 ml of buffer to which were added siderophores or bacteria or both. The smooth edge of the vial and the cap trapped the membrane in a liquid-tight seal, and the open end of the vial was closed with a rubber serum stopper. The vial was shaken at an angle which permitted both fluid phases to mix on the surface of the dialysis membrane. All experiments were conducted at room temperature, and 20-μl samples were removed from the upper (dialysate) fluid at various times to measure the amounts of ^{55}Fe in the low-molecular-weight chelates which diffused through the membrane.

The initial concentration of iron-binding protein was 12 μg/ml, and that of ^{55}Fe was 0.38 μCi/ml. At the counting efficiencies found in conventional scintillation counters, approximately 30% (with Budgetsolve scintillation fluid; Research Products International, Mount Prospect, Ill., the 20-μl samples from the [^{55}Fe]transferrin side of the chamber contained at least 10,000 cpm. An initial 20-μl sample was removed from the [^{55}Fe]transferrin side of the membrane, and the amounts of low-molecular-weight iron in the dialysate were calculated as percentages of the iron concentration in this initial sample. Apotransferrin (100 μg/ml) was also added to both sides of the dialysis membrane. This transferrin substrate was 10.5% saturated with ^{55}Fe. Iron assays were conducted with ferrozine (6), and the iron saturation levels of transferrin were determined by a single-tube method (29). Controls were used to assay the amount of iron appearing in the dialysate in the presence of buffer without bacteria or siderophores. The buffer used in the dialysis experiments contained 20 mM MOPS (morpholinepropanesulfonic acid) (pH 7.4), 20 mM sodium bicarbonate, 6.4 mM sodium phosphate buffer (pH 7.4), 3.2 mM potassium phosphate buffer (pH 7.4), and 0.4 mM $MgCl_2$. The sodium bicarbonate stock solution was depleted of iron by adding 5% magnesium carbonate with stirring for 5 min and then removing the precipitate by centrifugation and filtration. This treatment lowered the iron concentration in the final medium from 1.7 μM to an undetectable level. When pH modification was called for, the buffer was adjusted to the desired level with 1 N HCl and the concentrate was brought to the correct volume. The pH of the dialysate was checked during experiments at each sampling by spotting samples on pH paper. Bacteria were added to some experiments on the dialysate side of the membrane at a density of 0.8 A_{600} units.

This corresponded to approximately 5×10^9 CFU/ml. When bacteria were used, GMM was used instead of buffer. Other media (CAA medium and AMM) were used in place of GMM in specified experiments. Bacterial accumulation of ^{55}Fe on the dialysate side was measured by removing 100- to 200-μl samples of dialysate and treating them in a similar fashion to the samples obtained in the bacterial iron uptake assays described in the next section.

Bacterial iron uptake from [^{55}Fe]transferrin. Bacteria were grown in CAA medium, washed, and suspended in the buffer used in dialysis assays at a density of 0.8 A_{600} units. The bacteria were incubated at 37°C in this buffer containing 10 mM glutamate (added as oxidizable substrate) for 10 min before the addition of the iron-containing substrate, [^{55}Fe]transferrin. The transferrin (or other iron-containing proteins) was added to yield 12 μg of protein per ml and 0.38 μCi of ^{55}Fe per ml. In addition, 100 μg of the iron-free protein per ml was also included in the assay buffer. Samples (1 ml) were removed at various times and poured through filters (pore size, 0.45 μm) which were held in a vacuum manifold. The bacteria trapped on the filters were washed with 10 ml of distilled water, and the filters were dried and placed in scintillation fluid for counting. Control reactions were conducted without bacteria to determine the amount of iron nonspecifically trapped by the membranes. Control reactions which checked the active-transport nature of the iron accumulation were conducted by incubating bacteria at 4°C and by adding 5 mM KCN during the preincubation period.

RESULTS

Effective, dialyzable bacterial factors in iron mobilization from [^{55}Fe]transferrin. A dialysis assay patterned after that of Simonson et al. (23) was used to determine siderophore activity while minimizing direct contact between bacteria, their enzymes, and the ferritransferrin. This assay allowed measurement of the conversion of nondialyzable ^{55}Fe bound to transferrin into dialyzable forms of iron (ferrisiderophores). Apotransferrin (100 μg/ml) was included on both sides of the membrane to bind iron which was spontaneously released and not bound or iron which was not tightly bound by siderophores (i.e., ferripyochelin in a 1:1 ratio of pyochelin to iron). These assay conditions appeared to be effective; very little iron appeared in the dialysate when 12 μg of [^{55}Fe]transferrin per ml containing 0.38 μCi of ^{55}Fe per ml was dialyzed against GMM containing MOPS buffer (medium control, Fig. 1). The inclusion of a mixture of 10 μg of pyochelin and 40 μg of pyoverdin per ml (siderophores, Fig. 1) did not result in a mobilization of iron across the dialysis membrane. However, the inclusion of approximately 10^9 bacteria (strain PAO1) on the dialysate side of the membrane allowed ^{55}Fe to appear in the dialysate after 40 h (bacteria, Fig. 1). The addition of 10 μg of pyochelin and 40 μg of pyoverdin per ml to the bacteria on the dialysate side of the chamber allowed a more rapid onset of equilibration of ^{55}Fe with the dialysate (bacteria + siderophores, Fig. 1). These siderophore concentrations were produced in most culture media and represented an equimolar ratio at 0.03 μmol of each per ml for the sake of comparing activities. There was no indication that the added siderophores were acting alone or in concert with the bacteria because a time span of at least 20 h was required before detectable iron appeared in the dialysate. This time span appeared to be a result of the bacterial production of acid by-products from GMM, resulting in pH levels between 5.0 and 6.0 at 20 to 24 h of incubation. The times of the appearances of acid and iron in the dialysate coincided. Iron-mobilizing activity depended upon the addition of GMM. When CAA medium or AMM was used, the pH increased during the experiment and no iron appeared in the dialysates. During experiments in which the buffer (MOPS) concentration was increased, the onset of mobilization was delayed, but 200 mM MOPS was required before significant decreases in overall activity were noticed.

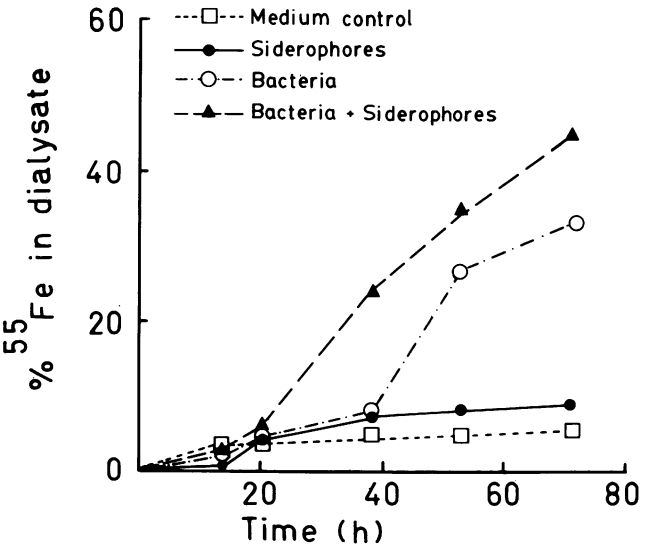

FIG. 1. Iron mobilization from [^{55}Fe]transferrin, which was placed on one side of a dialysis membrane containing 12 μg of transferrin and 0.38 μCi of ^{55}Fe per ml, to the opposite or dialysate side of the membrane, which contained GMM (medium control), a combination of 10 μg of pyochelin and 40 μg of pyoverdin per ml (siderophores), 5×10^9 washed PAO1 bacteria per ml (bacteria), or a combination of bacteria and siderophores in the same concentrations as those listed above (bacteria + siderophores). Both sides contained 100 μg of apotransferrin per ml. Samples (20 μl) of the dialysate were removed at various times of incubation to determine by scintillation counting the mobilization of ^{55}Fe to the dialysate side of the membrane. The overall saturation of transferrin was 88%, 10.5% of which was ^{55}Fe.

Activities of siderophore-defective mutants in iron mobilization. Siderophore-deficient strains of *P. aeruginosa* (3) were washed and placed on the dialysate side of the membrane to compare the relative effects of individual siderophore production with those demonstrated by the parent strain (PAO1, Fig. 2). Pvd^- Pch^+ (211-5, Fig. 2) and Pvd^+ Pch^- (IA5, Fig. 2) strains yielded positive iron mobilization in contrast to a strain which was deficient for the production of both siderophores (IA1, Fig. 2). Although these patterns were reproducible during four separate repetitions of this experiment, there were variations in the relative percentages. Strains IA5 and 211-5 always generated dialyzable iron before the PAO1 strain did. However, in some experiments the IA5 strain was equal to or exceeded the 211-5 strain in the percentage of iron generated. The finding that the IA1 strain was reproducibly defective in iron mobilization suggested that either pyochelin or pyoverdin was necessary for iron mobilization in this assay. When either siderophore was added to the IA1 culture, the resultant curve was similar to that for strain PAO1 in the presence of siderophores (Fig. 1).

Effects of pH on the iron from [^{55}Fe]transferrin. The bacteria were omitted from the dialysis assay, and the buffer was adjusted to three different pH values with HCl to study

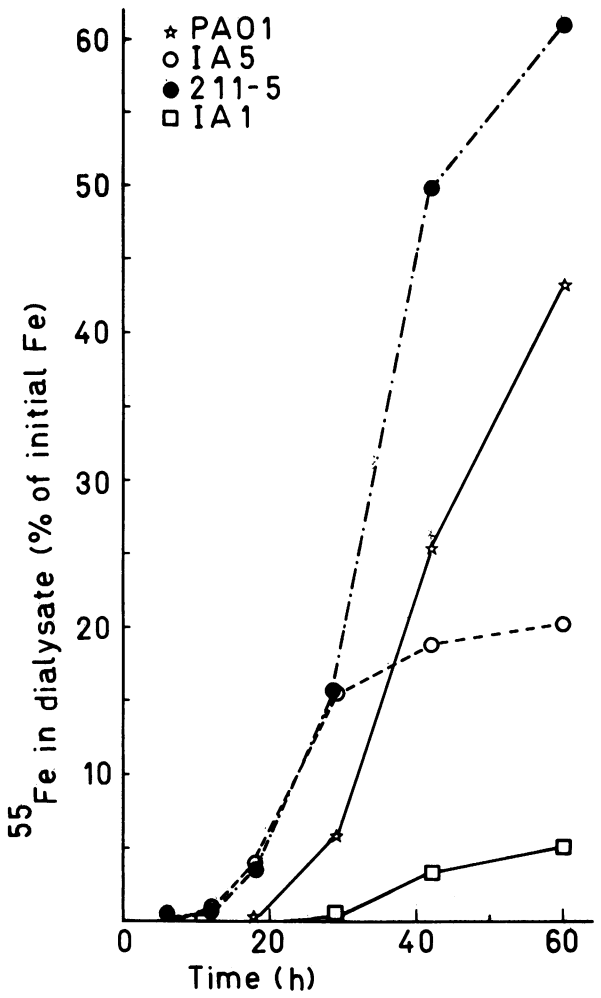

FIG. 2. Iron mobilization from [^{55}Fe]transferrin by the siderophore-defective mutant strains 211-5 Pvd$^-$ Pch$^+$, IA5 Pvd$^+$ Pch$^-$, and IA1 Pvd$^-$ Pch$^-$ in comparison with mobilization by strain PAO1 Pvd$^+$ Pch$^+$. The bacteria were added at 5×10^9 CFU/ml to GMM on the dialysate side of the membrane. Conditions and analyses were identical to those described in the legend to Fig. 1.

the effects of siderophores on iron mobilization from transferrin. In the absence of siderophores, there was a slight but reproducible amount of iron in the dialysate at pH 5.0 but none at pH 6.0 or 7.4 (Fig. 3A). The addition of pyochelin and pyoverdin to the dialysate side of the membrane resulted in a minute but reproducible increase in ^{55}Fe mobilization at pH 7.4 and dramatic increases in the amounts of ^{55}Fe appearing in the dialysates at pH values of 6.0 and 5.0 (Fig. 3B).

Effects of pyoverdin and pyochelin on iron release from transferrin, conalbumin, and lactoferrin. The buffer was adjusted to pH 6.0 to determine which of the siderophores was most important in the iron equilibration across the dialysis membrane. A pH of 6.0 was chosen because the siderophores were active at this pH and this pH may exist in tissue under certain conditions (17). Pyoverdin had more activity in iron release from transferrin and conalbumin, with very little iron appearing in the controls lacking siderophores (Table 1). Pyochelin was also effective, but there were no additive effects when pyochelin was combined with pyoverdin; the levels of release were similar to those with pyoverdin alone. It is very important that minimal siderophore activity was observed when lactoferrin was used (Table 1). Although the data were not included in Table 1, the same trends in siderophore activities were observed at pH 5.0. At pH 7.4, only pyoverdin demonstrated appreciable iron mobilization from conalbumin and transferrin; there was no detectable mobilization from lactoferrin.

Effects of siderophores on iron uptake from [^{55}Fe]transferrin. An analysis of the effects of pH on iron uptake was conducted by mixing bacteria and [^{55}Fe]transferrin with no intervening dialysis membrane and measuring the amount of ^{55}Fe accumulated by the bacteria at pH 6.0. There was a detectable amount of uptake by the bacteria in the absence of siderophores (control, Fig. 4). As was the case in the dialysis assays, pyoverdin (Pvd, Fig. 4) demonstrated greater activity in transport than did pyochelin (Pch, Fig. 4). There was also no additive effect when pyochelin was combined with pyoverdin (Pvd + Pch, Fig. 4) in equimolar amounts (0.03 mM). When these reactions were conducted at pH 7.4, the same general trends were observed, but with lower amounts of transport. However, there was more transport observed with pyochelin alone and pyoverdin alone (2.9 ± 0.13 and 0.88 ± 0.07 pmol of ^{55}Fe for pyoverdin and pyochelin, respectively, at 30 min) than had been anticipated from the negligible iron mobilization at this pH during the dialysis assays.

DISCUSSION

The dialysis assay described by Simonson et al. (23) was used to estimate the activities of *P. aeruginosa* siderophores with minimum contact between bacteria and transferrin. Theoretically, if a siderophore is capable of mobilizing the iron from transferrin, the resultant ferrisiderophore will equilibrate to both sides of the membrane and ^{55}Fe will be detectable in the dialysate. When *P. aeruginosa* siderophores were tested in this assay, they lacked activity to mobilize iron across the dialysis membrane during a 70-h incubation at pH 7.4 (Fig. 1). The inclusion of bacteria in this assay, with or without siderophores, initiated the dialysis of ^{55}Fe. The bacteria in the dialysis assay accumulated the iron which appeared in the dialysate; therefore, values approaching and in excess of 50% were to be expected (Fig. 1 and 2). A great deal of effort was committed to the isolation and analysis of bacterial factors which could have resulted in this activity. Certain inorganic ions (PP_i) and organic derivatives of glucose (autoclaved glucose) were found to be capable of yielding dialyzable iron from transferrin, but no bacterial siderophore could be detected other than pyochelin and pyoverdin.

The explanation for the bacterial activity in the dialysis assay became apparent when it was discovered that 50 to 100 mM MOPS buffer was incapable of maintaining the pH of the bacterial suspensions. The inclusion of bacteria in the assay was followed by a decreasing pH to 5.1 to 5.4 because of the catabolism of glucose. Any medium which resulted in an increasing pH (CAA medium or AMM) did not allow iron equilibration by the bacteria. The lack of bacterial activity in these media suggested that either there is no additional siderophore capable of competing with transferrin or the production of such a siderophore is dependent upon the catabolism of glucose. The suggestion that there is no additional siderophore for *P. aeruginosa* was also supported by the lack of iron equilibration when strain IA1 was placed in the dialysis assay (Fig. 2). Only the siderophore-producing strains 211-5 (Pch$^+$ Pvd$^-$) and IA5 (Pch$^-$ Pvd$^+$) were effec-

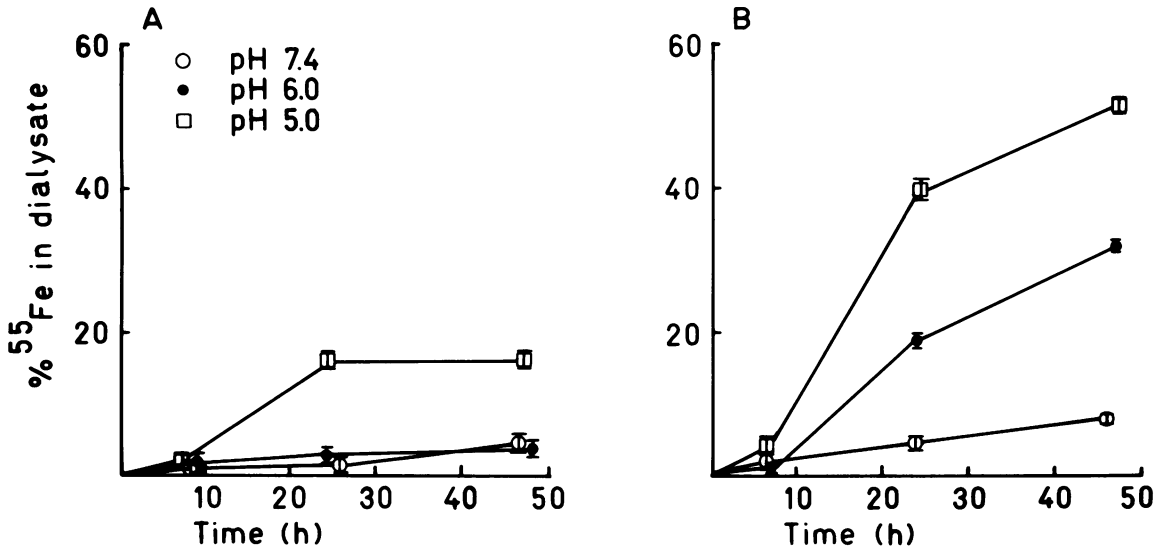

FIG. 3. Effects of pH on iron mobilization from [^{55}Fe]transferrin in buffer (A) and in buffer containing 10 μg of pyochelin and 40 μg of pyoverdin per ml (B) at pHs 7.4 (○), 6.0 (●), and 5.0 (□). Conditions and analyses were identical to those described in the legend to Fig. 1 except that no GMM was added. The overall saturation of transferrin was 11.5%, 10.5% of which was ^{55}Fe. Values are the means of three determinations with standard deviations from the means indicated by bars.

tive. Strain PAO1 lagged behind these strains because siderophore-deficient mutants were routinely found to hyperexcrete the siderophore which they are capable of producing. We had found previously that strain 211-5 did not grow well in a medium containing transferrin and did not produce pyochelin in substantial concentrations in the presence of glucose (3). The appreciable activity demonstrated by strain 211-5 in the dialysis assay appears to have been a result of the use of a suspension of cells at high density (0.8 A_{600} units). An analysis of these suspension conditions did confirm the production of pyochelin during the incubations (Fig. 1 and 2).

An investigation of the low pH conditions necessary for the demonstration of siderophore activity in the absence of bacteria indicated that iron freely dissociates from transferrin at about pH 4.0 (data not shown; 1, 2). A low level of iron dialysis from transferrin at pH 5.0 is shown in Fig. 3A, and the effects of siderophores on the mobilization of iron at pHs 5.0 and 6.0 is shown in Fig. 3B. A pH of 6.0 was chosen for further analyses of siderophore activity because at this pH there was measurable activity and active transport from the siderophores (Fig. 4A) and because this pH has been found to exist in inflammatory exudates (17).

TABLE 1. Percentage of initial ^{55}Fe bound to protein[a] appearing in the dialysate at pH 6.0

Siderophore added to dialysate	% with indicated iron-binding protein opposing the dialysate[b]		
	Transferrin	Conalbumin	Lactoferrin
None	4.09 ± 0.21	2.03 ± 0.25	0.17 ± 0.02
Pyochelin[c]	21.63 ± 0.52	36.10 ± 0.40	2.64 ± 0.08
Pyoverdin[d]	32.12 ± 0.78	41.61 ± 0.25	4.25 ± 0.04
Pyochelin and pyoverdin[e]	32.31 ± 0.16	42.55 ± 4.00	3.75 ± 0.09

[a] ^{55}Fe-labeled proteins were prepared by methods described by Simonson et al. (23); final concentrations were 12 μg of protein, 0.38 μCi of ^{55}Fe, and 100 μg of apotransferrin per ml.

[b] The overall saturation of iron-binding proteins was 11.5%, 10.5% of which was ^{55}Fe. Values are the means of three determinations and standard deviations from the means taken at 47 h of incubation.

[c] 10 μg of purified pyochelin per ml (0.03 mM).

[d] 40 μg of purified pyoverdin per ml (0.03 mM).

[e] 10 μg of pyochelin and 40 μg of pyoverdin per ml (0.03 mM each).

Pyoverdin demonstrated greater activity than did pyochelin in the dialysis assay when the two siderophores were tested separately (Table 1). In addition, pyoverdin mobilized iron bound to conalbumin better than pyochelin did at pH 6.0 (Table 1). However, the finding that neither siderophore had significant mobilizing activity when used with lactoferrin evokes interesting theories concerning the colonization of mucosal surfaces by *P. aeruginosa*. Theoretically, this organism should have more difficulty obtaining iron for growth during the initial stages of colonization of host surfaces, which are bathed in lactoferrin, than during colonization of tissue (burn infections) or growth in tissue once infections have been established. The fact that lactoferrin forms more stable complexes with iron at acid pH values and maintains its activity in the gut after ingestion (2) explains the lack of siderophore activity in these assays at acid pH values. There may be correlative information in the finding by Brown et al. (4) that mucoid *P. aeruginosa* harvested from the lungs of cystic fibrosis patients, a site which would have lactoferrin present, demonstrated the outer membrane protein profiles of iron-deficient bacteria.

Bacteria accumulated the iron which came through the dialysis membrane. Therefore, it was expected that iron transport would occur from transferrin at pH 6.0 in the presence of siderophores (Fig. 4). The amount of transport mediated by pyochelin at pH 6.0 and the stimulation of transport by both siderophores at pH 7.4 were not expected. There are many possible explanations for this activity. One of the most important considerations is the action of bacterial enzymes and surface binding proteins on transferrin when there was no dialysis membrane separating them. The finding of transport over a 30-min interval when there was negligible iron mobilization over 47 h at pH 7.4 indicated the possibility of interactions between bacteria and siderophores when mixed with transferrin. Although the transport assays were limited to 30 min to minimize the production of enzymes and siderophores, the effects of these factors

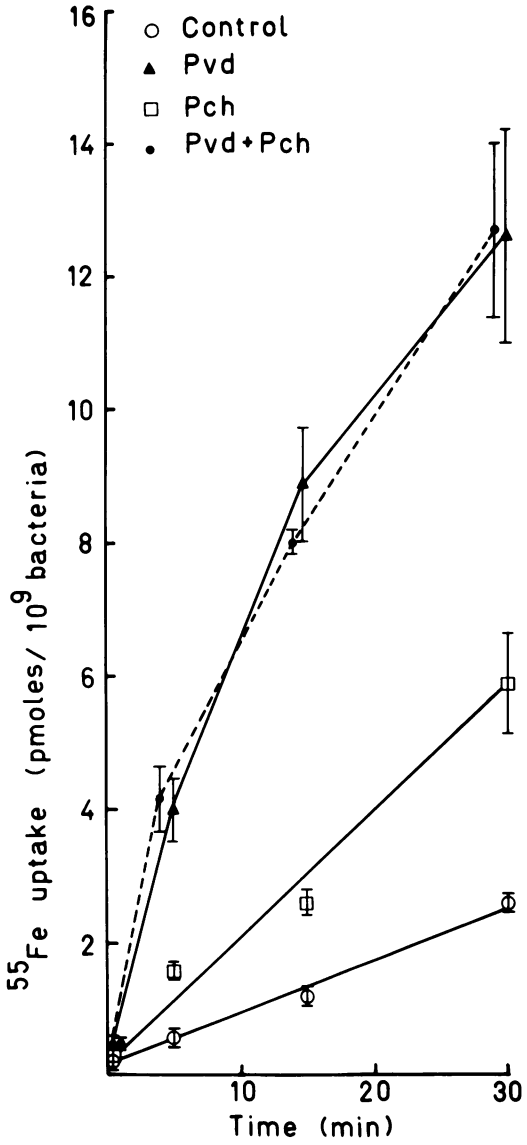

FIG. 4. Effects of siderophores on ^{55}Fe transport by strain PAO1 from [^{55}Fe]transferrin at pH 6.0. ^{55}Fe trapped on membrane filters with bacteria was determined in buffer containing glutamate only (control), glutamate with 40 μg of pyoverdin per ml (Pvd), glutamate with 10 μg of pyochelin per ml (Pch), and glutamate with 40 μg of pyoverdin and 10 μg of pyochelin per ml (Pvd + Pch). The reaction mixtures contained 5×10^9 bacteria per ml, and ^{55}Fe accumulation was measured in a scintillation counter. The values reported have had nonbacterial iron trapping subtracted and represent the means of three determinations, with bars indicating standard deviations from the means. The overall saturation of transferrin was 11.5%, 10.5% of which was ^{55}Fe.

cannot be ruled out. Siderophores and acids accumulating during incubation should have generated increasing rates of transport. However, the rates were nearly linear over the 30-min assay (Fig. 4). These findings have stimulated our current investigations into the effects of bacteria on transferrin and siderophore-mediated iron mobilization from transferrin.

Although both siderophores were active in mobilizing iron from transferrin, pyochelin did not appear to add to the activity of pyoverdin when the siderophores were combined during dialysis or transport assays. First, additive activity could not be observed between the siderophores in the absence of transferrin, and we are currently using iron-transport-deficient mutants to determine the mechanisms of ferrisiderophore transport. Second, the data suggest that the iron bound in transferrin exchanged with the solvent at a constant rate independently of siderophore presence or concentration. We used the siderophores at concentrations which are present in most culture media and at equal molarities to compare activities. However, increasing concentrations of siderophores did not yield corresponding increases in activities. Our present theory is that when siderophores are present during the iron exchange with the solvent, there is a chance that the iron will collide with the siderophores rather than transferrin. Because pyoverdin has the higher binding coefficient for iron (28) and the iron exchange with the solvent is the rate-limiting reaction, the rate of pyoverdin mobilization is the process which was apparent during dialysis and transport assays with combined siderophores. In other experiments which supported this theory, decreasing the apotransferrin concentration in the dialysis or transport assays yielded higher siderophore activities because of the decreased chances for the Fe(III) to collide with an apotransferrin molecule. Finally, the siderophores had no mobilizing activity with transferrin at pH 7.4. The necessity of lowering the pH to observe activity indicates the necessity for increasing the rate of exchange of iron with the solvent (1, 15) and thus increasing the probability of siderophore collision with Fe(III).

This research concerned the initial steps of siderophore-mediated iron mobilization by *P. aeruginosa*. The conclusion is that the siderophores of *P. aeruginosa* cannot appreciably affect iron release from transferrin. However, pyochelin and pyoverdin can be effective at the acid pH values which may exist during inflammation reactions (17). The utilization of glucose by bacteria and the effects of lipopolysaccharide on the inflammatory response may accentuate the acid accumulation in tissue. During the period of initial colonization of the host and periods of low inflammatory response, the effects of acid pH and siderophores on transferrin may most easily be imagined when the bacteria are present in tissue in the form of microcolonies. Microcolonies have been studied in many different environments and are thought to be significant during the colonization of mammalian tissue (7, 30). Iron accumulation by localization of acid metabolites and siderophores in the microcolony may allow these important factors to work for the bacteria without being lost by diffusion. In this model, the interaction of transferrin with the microcolony might allow sufficient iron accumulation by the bacteria for colonization and persistence. Any further decrease in the nutritional immunity (5, 27) of the host (a decrease in the serum transferrin concentration or an increase in the iron saturation of transferrin) could allow increased iron supply to the bacteria, which could lead to local tissue invasion and spread of microcolonies via the bloodstream. Although work is in progress on bacterial accumulation of iron from transferrin, the hypothesis resulting from the present results is compatible with the type of infection that might be expected for an opportunistic pathogen.

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