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Role of Pyocyanin in the Acquisition of Iron from Transferrin

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***Pseudomonas aeruginosa* produces a blue pigment called pyocyanin. In the presence of oxidizable substrates, bacteria reduce this pigment to a colorless product, leukopyocyanin. Pyocyanin can also be nonenzymatically reduced by NADH. Leukopyocyanin formed by cell- or NADH-mediated reduction nonenzymatically reduces oxygen or Fe(III). Pyocyanin-dependent iron reduction by whole bacterial cells was measured by the formation of the ferrous-ferrozine complex. In addition, leukopyocyanin reduced chelated Fe(III) including ferric iron in complex with transferrin, the serum iron-binding protein. High-pressure liquid chromatography was used to display the reductive removal of iron from transferrin and the accumulation of iron in the ferrous-ferrozine complex. Pyocyanin stimulated the accumulation of ^{55}Fe from [^{55}Fe]transferrin when it was added to bacteria incubated under low-oxygen conditions. Although bacteria grown in the presence of 100 μM $FeCl_3$ reduced pyocyanin just as rapidly as iron-limited bacteria, these cells did not accumulate iron in the presence or absence of pyocyanin. Therefore, *P. aeruginosa* participates indiscriminantly in the reduction of pyocyanin, but soluble or available iron generated by the pyocyanin is taken up specifically by iron-limited bacteria.**

Pyocyanin is the blue, chloroform-soluble pigment produced by *Pseudomonas aeruginosa*. This pigment has been used diagnostically to describe both the physiology (24) and the pathogenicity (blue-pus disease) of this bacterium. The synthesis of pyocyanin is affected by carbon and nitrogen sources in growth media, but most nutrients support pyocyanin production as long as the phosphate ion concentration is low and there is adequate sulfate ion present (13, 21, 22). Synthesis of this pigment also appears to be under the control of iron concentration since addition of iron to a medium containing low phosphate stimulates the synthesis of pyocyanin (13, 24) and related phenazine pigments by other species of bacteria (25, 26).

A considerable amount of effort has been invested in research on the function of pyocyanin. Pyocyanin is *N*-methyl-1-hydroxyphenazine and is capable of undergoing a two-electron reduction to a colorless product, leukopyocyanin (16). Friedheim (14, 15) theorized that the reducible nature of pyocyanin was important to the respiration of *P. aeruginosa* because addition of pyocyanin increased the oxygen uptake of *P. aeruginosa*, *Streptococcus pneumoniae*, *Staphylococcus aureus*, and erythrocytes. This theory stated that pyocyanin could function as an extracellular respiratory pigment. It was a particularly attractive theory for aerobic bacteria grown under conditions of low aeration in which the pyocyanin could be reduced, would be freely diffusable to participate in autooxidation by oxygen in more aerobic environments, and could recycle in subsequent reductive steps in bacterial respiration. The ability of pyocyanin to drain electrons from mammalian mitochondria appeared to be related to its hypothesized respiratory activity (3). However, other investigators found that pyocyanin actually inhibited the oxygen uptake of *Vibrio cholerae* and *S. aureus* (31). These conflicting data have not been resolved, but the inhibitory nature of pyocyanin suggested an antibacterial activity for the pigment. A possible explanation for the respiration-linked antibiotic activity of pyocyanin was offered by Hassan and Fridovich (20) in their finding of superoxide radical generation during the autooxidation of reduced pyocyanin. In addition to this activity, pyocyanin also inhibited keto acid oxidation in *Pseudomonas fluorescens* and *Proteus vulgaris*, but not in *Escherichia coli* (6).

The reduction of pyocyanin by *P. aeruginosa* is most apparent from the bleaching of the blue color during growth in broth medium. Leukopyocyanin can, in turn, reduce both oxygen and Fe(III). This report describes the ability of leukopyocyanin to reduce iron and to cause the release of iron from transferrin. Iron is a crucial requirement for the growth of this bacterium, and there are other well-defined mechanisms of iron acquisition in *P. aeruginosa* based upon the utilization of two siderophores (29), bacterial iron-binding compounds, pyochelin (10, 11), and pyoverdin (9, 27, 28, 34). However, siderophores may not always be produced or well suited for removing iron from transferrin (2). Pyocyanin appears to participate in a reduction mechanism which is capable of removing iron from transferrin.

MATERIALS AND METHODS

Bacteria and culture media. *P. aeruginosa* PAO1 (ATCC 15692) was maintained on brain heart infusion agar with monthly transfers. For experiments involving pyocyanin production, bacteria were inoculated into succinate minimal medium consisting of 20 mM sodium succinate, 40 mM NH_4Cl, and 2 mM K_2SO_4. This medium was autoclaved and supplemented before inoculation to make the following final concentrations: 0.4 mM $MgCl_2$, 1 μM $MnCl_2$, 1 μM $CaSO_4$, 1 μM $ZnCl_2$, 1 μM $FeCl_3$, and 10 mM morpholinepropanesulfonic acid (MOPS) buffer at pH 7.4. These supplements had been made bacterium-free by filtration through filters with 0.45-μm pore size. The complete succinate minimal medium (SMM) was constructed for pyocyanogenic cultures by adding 0.1 mM potassium phosphate buffer (pH 7.4) and for apyocyanogenic cultures by adding 4.0 mM potassium phosphate buffer. The cultures consisted of 1-liter volumes in 2.8-liter Fernbach flasks which were shaken at 160 rpm at 37°C. Bacteria were also grown in 0.5% Casamino Acids containing 0.4 mM $MgCl_2$ (CAA medium) for some of the experiments involving pyocyanin reduction and iron transport.

Purification of bacterial products. Pyocyanin was obtained by growing strain PAO1 in glycerol-alanine minimal medium (13) for 30 h. The bacteria were removed from the dark blue medium by centrifugation, and three consecutive extractions of the medium with chloroform (1 to 0.2 ratio) removed most

of the blue pigment. The purification of pyocyanin (16) involved both alternate extractions of the red, acid form of pyocyanin from the chloroform layers with acidified water and repeated chloroform extractions of the blue pyocyanin from neutralized water layers. After five base-to-acid conversions, the pyocyanin was concentrated in a 3-ml volume of slightly acidified water. This was done so that the pH of the isolated water layer could be adjusted to pH 7.5 with a minimum volume of 0.1 M NaOH. Needlelike crystals formed in the chilled solution over the following 2 h. These were trapped on a 0.45-μm (pore size) filter (Nuclepore Corp.), washed with water, dried under vacuum, and weighed.

Pyochelin was extracted and purified by methods which have been described previously (10). Pyoverdin was purified by a modification of methods which have been described previously (9). Cell-free medium from SMM cultures of strain PAO1 were concentrated by rotary evaporation and separated into fluorescent fractions by chromatography on a P-2 Bio-Gel polyacrylamide column with a water-methanol solvent flowing against gravity. The major fluorescent peak was concentrated and applied to a preparative C8 reversed-phase high-pressure liquid chromatography (HPLC) column (10 by 250 mm). A 10% acetonitrile concentration in water solvent flowing at 2 ml/min yielded a major fluorescent peak eluting at 28 min. This fraction was concentrated by lyophilization, and the dried material was weighed. This was pure pyoverdin (see Cox and Adams [9]) as determined by thin-layer chromatography and HPLC. A molecular weight of 1,300 was taken from the data of Wendenbaum et al. (34).

Assay of bacterial growth and pigment production. Two 2-liter volumes of SMM culture medium designed for pyocyanogenic and apyocyanogenic conditions, respectively, were placed in each of two 4-liter containers. These media were inoculated with 10^5 bacteria per ml, incubated at 37°C, and stirred to yield vortices which extended from the surface of the medium to stirring bars on the bottom of each vessel. Samples were withdrawn at intervals for determinations of A_{600} to quantitate growth. Other samples (50 ml) were extracted twice with 20-ml volumes of chloroform. The extracted pyocyanin was measured by A_{690} using an extinction coefficient of $E_1^{1\%} = 164$ (22). A 0.1-ml portion was taken from the aqueous phase of this extract and diluted into 0.05 M Tris hydrochloride buffer (pH 7.4) to estimate the quantity of pyoverdin by fluorescence. Fluorescence was determined by exciting the solutions at 400 nm and measuring the emission at 460 nm with an Aminco Bowman spectrofluorometer which had been calibrated with quinine sulfate. The remainder of the aqueous layer from the chloroform extraction was acidified to pH 2.5 using 1 M HCl and then extracted with 20 ml of ethyl acetate. Following the analysis of pyocyanin by spectrophotometry, the chloroform and ethyl acetate extracts were pooled and then concentrated by rotary evaporation, and the concentrates were spotted on thin layers of silica gel. Chromatography was performed in chloroform-acetic acid-ethanol (90:5:2.5), and spots were scraped from the plate where standards of pyochelin had migrated. Pyochelin was estimated in ethanol solutions of the material eluted from this silica by exciting the solutions at 350 nm and measuring the fluorescence at 440 nm. Quantitation was achieved by relating fluorescence values to a standard linear curve of weights of pure pyochelin.

The culture medium was continuously monitored for redox potential using platinum electrodes with calomel reference electrodes. A pH meter (model 26; Radiometer) was calibrated with ferrocyanide-ferricyanide mixture (23), and the millivolt values were recorded as E_{cal} (potentials with reference to a calomel electrode). E_n values may be determined by adding 242 mV to the E_{cal} values. Changes in the pH of the cultures were also measured by using a Beckman pH meter. These changes were the same in the pyocyanogenic and apyocyanogenic cultures.

Iron reduction assays. The first portion of the iron reduction reaction involved the reduction of pyocyanin. Pyocyanin reduction was assayed in suspensions of washed bacteria at 0.8 A_{600} in 2 mM sodium succinate, 5 μg pyocyanin per ml, and 1 mM MOPS buffer containing 1 mM $MgCl_2$ (MOPS-magnesium buffer). Changes in A_{690} were measured with a Gilford 240 spectrophotometer with a reference cuvette containing a cell suspension lacking pyocyanin. Pyocyanin reduction was also measured in the absence of bacteria after the addition of 1 μmol of NADH per ml.

Iron reduction was measured by using conditions described by Dailey and Lascelles (12). Cuvettes contained 2.4 ml of 0.05 M Tris hydrochloride buffer (pH 7.7) and 2 μmol of ferrozine [3-(2-pyridyl)-5,6-bis(4-phenylsulfonic acid)]. Iron-containing substrates which were consistently tested to establish the reproducibility of the reactions were 0.6 μmol of ferric citrate, 0.2 μmol of $FeCl_3$, and 0.24 μmol of ferripyochelin per ml. Pyocyanin was added from 1 to 5 μg/ml, and the reactions were initiated by the addition of 1 μmol of NADH per ml. Product formation [Fe(II) in complex with ferrozine] was measured under anaerobic conditions at 562 nm with reference to cuvettes lacking reductant. In some reactions, cell extracts of bacteria were prepared by sonication of suspensions of 5 g (wet weight) of bacteria per 10 ml of distilled water, centrifugation at 5,856 × *g* for 30 min, and pressurized filtration of the extract through a 0.45-μm (pore size) filter.

Iron reduction was also assayed by chromatography of reactants on a TSK3000 column (60 by 10 mm; Beckman) washed in a 0.005 M ammonium phosphate buffer (pH 6.5) containing 1% glycerol (or ethylene glycol) and 2 mM EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′,-tetraacetic acid] flowing at 0.8 to 1.0 ml/min. A Constametric I high-pressure pump (LDC/Milton Roy) was used to deliver solvent. The effluent of the column was monitored at 254 nm, and fractions were collected every minute in scintillation vials. These vials were then filled with 6 ml of Budgetsolve (RPI) scintillation fluid for counting. To make [^{55}Fe]transferrin for the iron reduction assays, a ferric nitrilotriacetic acid (NTA) stock was made with final concentrations of 20 μCi of ^{55}Fe per ml (10.0 nmol/ml) and 0.02 M NTA in distilled water. Radioactive iron (2 nmol) was mixed with 64.1 nmol of human transferrin (no. T2252, substantially iron-free; Sigma Chemical Co.) in a 0.25-ml total volume. Excess NTA (20 μmol/ml) and nonradioactive iron (100 nmol) were added for some experiments. This solution was allowed to incubate at 25°C for 15 min, and this was made 10 mM with MOPS and 20 mM with sodium bicarbonate. The complete assay mixture contained 5 μg of pyocyanin, 2 μmol of ferrozine, and 1 μmol of NADH per ml. This solution was placed under repeated vacuum conditions interspersed with flushes of nitrogen gas. The reaction was initiated by injecting the NADH through the rubber serum stopper which sealed the vial. At intervals, this solution was sampled by removing 40-μl portions with an HPLC syringe for injection through the septum injector of an HPLC. The chromatograph had been calibrated with proteins from molecular weight standard kits (Sigma). Individual components of the reaction mixture were also injected to determine their retention times.

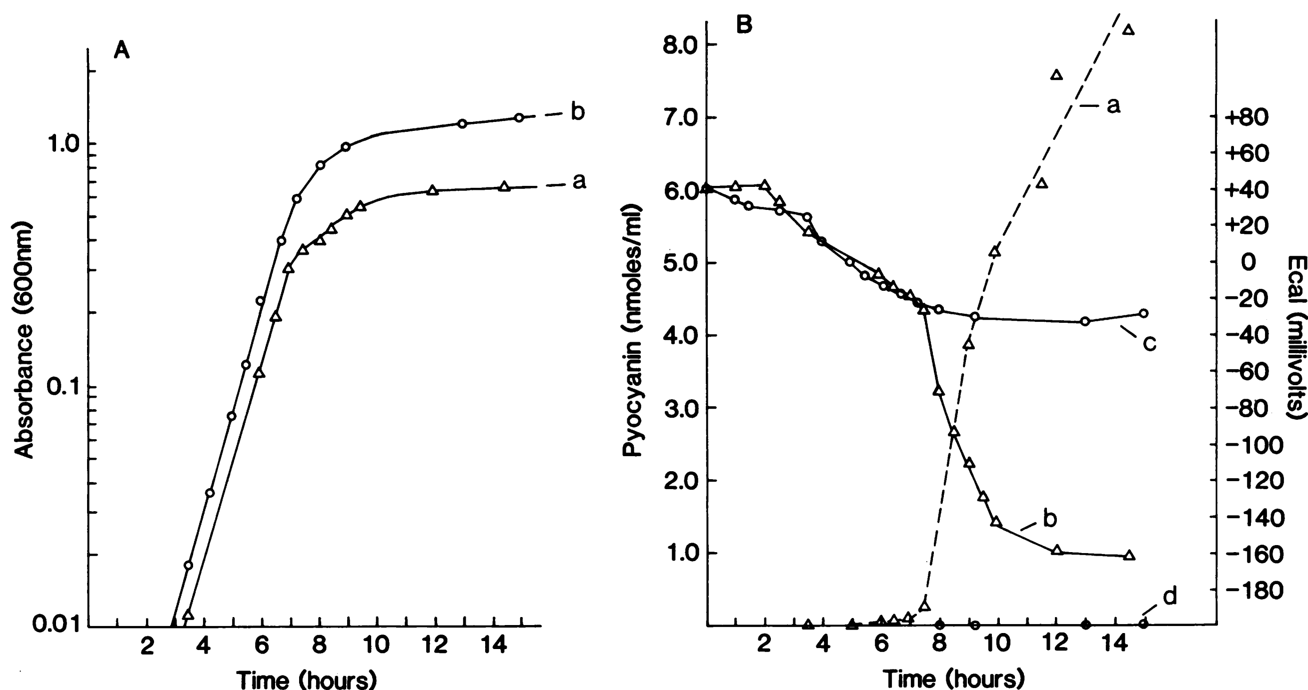

FIG. 1. Relationships between growth of strain PAO1 (A) measured by A_{600} in pyocyanogenic (curve a) and apyocyanogenic (curve b) cultures; pyocyanin production (B) measured by A_{690} in chloroform extracts of pyocyanogenic (curve a) and apyocyanogenic (curve d) growth media; and redox potential (B), measured as E_{cal} with a platinum electrode in pyocyanogenic (curve b) and apyocyanogenic (curve c) growth media. Growth media consisted of SMM and 1 μM $FeCl_3$ with 0.1 mM potassium phosphate for pyocyanogenic conditions and 4 mM potassium phosphate for apyocyanogenic conditions and was inoculated with approximately 10^6 CFU/ml.

Iron uptake assays. Suspensions of washed bacteria which had been grown in CAA medium were prepared as described before (7) and were adjusted to an A_{600} of 0.8 in MOPS-magnesium buffer. The bacterial suspension was supplied with oxidizable substrates of either 10 mM sodium succinate or 10 mM glutamine. [^{55}Fe]transferrin was prepared as described for the iron reduction assays except that no nonradioactive iron was added. The NTA which was added with the transferrin does not inhibit low-affinity uptake in *P. aeruginosa* in the same manner that has been reported for *E. coli* (17). Therefore, 10 mM EGTA was mixed with the bacteria to inhibit low-affinity iron uptake (9). The transport assays were initiated by the addition of [^{55}Fe]transferrin. Samples (1 ml) were removed at times following transferrin addition and poured through 0.45-μm (pore size) filters which were under vacuum. These filters were washed with 10 ml of distilled water, and then dried and placed in scintillation vials with Budgetsolve scintillation fluid for counting. Control reactions were conducted in the absence of bacteria or in the presence of heat-killed bacteria to determine the amount of iron precipitating on the filters or being nonspecifically absorbed by the bacteria. These values were subtracted from the quantities of iron accumulated and trapped by living bacteria on the filters. In some assays, filtrates were taken before the filters were washed and 40 μl quantities were injected onto a TSK3000 column to determine the ^{55}Fe concentration remaining in complex with transferrin.

RESULTS

Production and reduction of pyocyanin. Strain PAO1 grown in SMM containing 1 μM $FeCl_3$ and a limiting phosphate concentration, 0.1 mM potassium phosphate, (curve a, Fig. 1A) did not grow as well as a culture of PAO1 supplied with 4 mM potassium phosphate (curve b, Fig. 1A), but it did produce pyocyanin. Pyocyanin appeared in the phosphate-limited culture medium beginning at approximately 8 h (curve a, Fig. 1B), which corresponded to the entry of the culture into stationary phase. In addition, the E_{cal} of the pyocyanogenic culture medium, measured with a platinum electrode, decreased as the pyocyanin was produced (curve b, Fig. 1B). The culture containing 4 mM potassium phosphate demonstrated no significant decrease in E_{cal} (curve c, Fig. 1B) and no detectable pyocyanin synthesis (curve d, Fig. 1B). In subsequent experiments, pyocyanin was typically added to reactions at 1 μg/ml, which corresponds to approximately 5 nmol/ml (Fig. 1B). A concentration of 5 μg/ml was used in some experiments and represents the typical accumulation of pyocyanin in a late-stationary-phase culture similar to that in Fig. 1B.

Involvement of pyocyanin in Fe(III) reduction. Although the culture medium was blue, the low E_{cal} in the pyocyanogenic culture indicated the presence of reduced leukopyocyanin. The reaction between leukopyocyanin and oxygen was observed in other experiments by stopping the aeration, which was followed by pyocyanin reduction, and then restoring the aeration and observing the subsequent return of blue color due to the oxidation of pyocyanin. The reduction of Fe(III) was measured by the formation of the ferrous-ferrozine complex, which absorbs maximally at 562 nm. In the absence of NADH, pyocyanin could not stimulate the formation of Fe(II) (curve a, Fig. 2A). Likewise, there was no Fe(III) reduction when NADH was present in the absence of pyocyanin (curve equivalent to curve a, Fig. 2A). Addition of NADH to a reaction mixture containing 1 μg of pyocyanin per ml allowed the rapid reduction of iron from ferric citrate (curve b, Fig. 2A). Iron was also nonenzymatically reduced from ferripyochelin (curve c, Fig. 2A). Cell

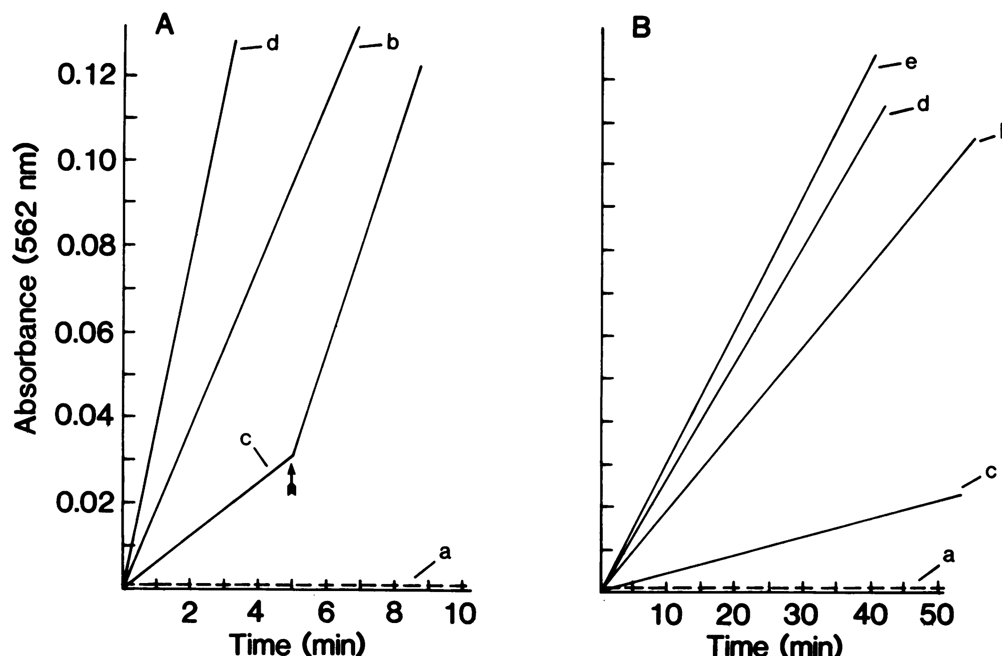

FIG. 2. (A) Pyocyanin- and enzyme-mediated reduction of iron. Ferrous-ferrozine complex formation was measured at 562 nm in reaction mixtures containing 1 μg of pyocyanin per ml to which was added 0.6 μmol of ferric citrate per ml (curve a), 0.6 μmol of ferric citrate and 1 μmol of NADH per ml (curve b), 0.31 μmol of ferripyochelin and 1 μmol of NADH per ml (curve c), and 0.2 μmol of $FeCl_3$ with 1 μmol of NADH per ml (curve d). Dialyzed cell extract from pyocyanogenic bacteria was added (50 μg/ml) at the arrow in curve c. (B) Iron reduction by whole bacteria. Ferrous-ferrozine complex was measured at 562 nm with 0.2 μmol of ferric citrate per ml (curve a), 0.2 μmol of ferric citrate with 1 μg of pyocyanin per ml (curve b), 0.2 μmol of ferric citrate with 1 μg of pyocyanin per ml and 2 mM KCN (curve c), 0.2 μmol of $FeCl_3$ with 1 μg of pyocyanin per ml (curve d), and pyocyanogenic bacteria. Apyocyanogenic bacteria were mixed with 1 μg of pyocyanin and 0.2 μmol of ferric citrate per ml (curve e). Bacteria were suspended at 0.4 A_{600} in 2 μmol of ferrozine and 2 mM sodium succinate. The reference cuvette contained bacteria without pyocyanin.

extracts are known to possess ferripyochelin iron reductase activity for these two iron chelates (8). Addition of extract to the assay containing ferripyochelin increased the rate compared to that for the added activities of the reductase- and pyocyanin-mediated reactions (curve c [arrow], Fig. 2A). Therefore, no enzymatic activity to increase pyocyanin reduction or pyocyanin-mediated iron reduction could be found for either ferripyochelin or ferric citrate. However, leukopyocyanin reduced iron in the form of $FeCl_3$ (curve d, Fig. 2A), and no other reductase activity was previously found in *P. aeruginosa* for this form of iron (8).

Spectrophotometric assays of this reaction were also conducted with whole bacteria. No reduction of iron from ferric citrate occurred in the absence of pyocyanin (curve a, Fig. 2B), but a rapid reduction reaction occurred in its presence (curve b, Fig. 2B). Bacterial respiration was apparently responsible for the reduction of extracellular iron in these assays because the process was inhibited to a degree by the addition of 2 mM KCN (curve c, Fig. 2B). Reduction of iron was most rapid when $FeCl_3$ was added to the cuvette (curve d, Fig. 2B). The process of extracellular iron reduction was not dependent upon pyocyanogenic bacteria. Addition of apyocyanogenic bacteria (derived from a culture similar to curve b [Fig. 1]) allowed iron reduction from $FeCl_3$ at equivalent or more rapid rates (curve e, Fig. 2B) than did pyocyanogenic bacteria.

Reduction of iron from ferritransferrin. The spectrophotometric assay indicated that pyocyanin reduced iron in addition to oxygen. Pyocyanin also reduced iron from certain iron chelates including ferripyoverdin, iron dextran, ferric NTA, ferric choline citrate, ferric phosphate, and transferrin. No reduction could be measured when ferric EDTA or ferric EDDA [ethylenediamine-di(*o*-hydroxyphenyl acetic acid)] were used. The reduction of iron from transferrin was of most interest. Transferrin was made by mixing ferric NTA with apotransferrin to preferentially load the A site (high-affinity site) of the protein. The transferrin was then added to the bacterial suspension in a cuvette containing buffer, oxidizable substrate, and ferrozine, but no pyocyanin. Bacteria possessed no detectable ability to reduce iron bound to transferrin (curve a, Fig. 3). However, when pyocyanin was added in a final concentration of 5 μg/ml, there was a long lag followed by a relatively rapid formation of ferrous-ferrozine complex (curve, b, Fig. 3). The lag in the formation of the ferrous-ferrozine complex was due to the presence of oxygen. If the cuvette was flushed with nitrogen before the assay, the lag was dramatically shortened to less than 5 min. Likewise, when air was introduced into the cuvette containing an ongoing reaction (curve c [arrow], Fig. 3), iron reduction ceased until the bacteria depleted the oxygen concentration.

To verify the activity of pyocyanin, the reactants in this assay were analyzed by injection onto a TSK3000 molecular sieving, HPLC column. The initial iron chelate, [^{55}Fe]NTA, eluted in a broad peak beginning at 30 min (panel A, Fig. 4). Addition of transferrin and ferrozine to the [^{55}Fe]NTA resulted in the movement of all of the ^{55}Fe into the transferrin peak eluting at 18 min (panel B, Fig. 4). A fourfold increase in the injection volume was used in this analysis to detect small amounts of [^{55}Fe]NTA eluting at 30 min, but none was detectable. The addition of pyocyanin and NADH resulted in the formation of [^{55}Fe]ferrozine, eluting at 24 min

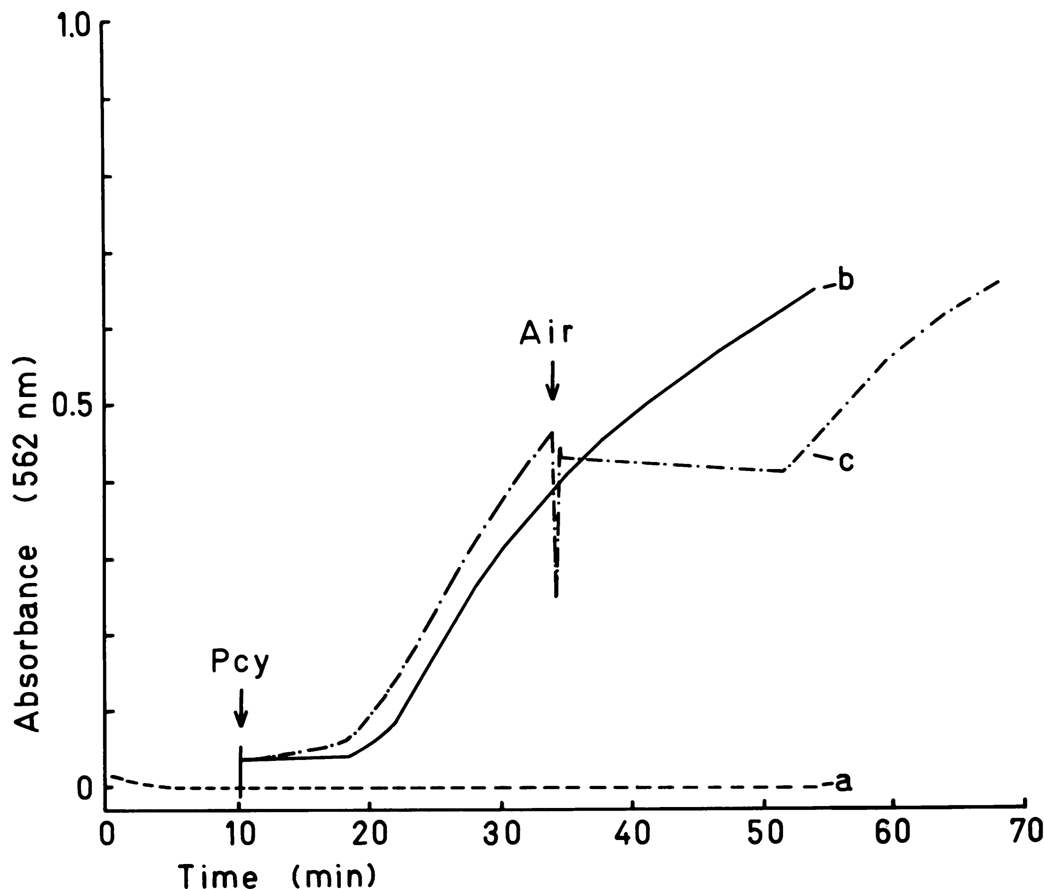

FIG. 3. Time course of iron reduction from transferrin by pyocyanin. Ferrous release was measured at 562 nm from transferrin mixed with 1 μmol of NADH per ml (curve a) and with 1 μmol of NADH and 5 μg of pyocyanin per ml (curves b and c). Pyocyanin (Pcy) was injected into the mixures at the arrow. The effect of aeration was measured by injecting air through the stopper at the arrow in curve c. Reaction mixtures contained MOPS-magnesium buffer with 2 μmol of ferrozine, 5 mg of transferrin, and 100 nmol of iron per ml (78% saturation of transferrin) in 10 mM NTA-20 mM sodium bicarbonate.

(panel C, Fig. 4) following 10 min of incubation. Pyocyanin eluted from the column at approximately 70 min and is not shown in the chromatograms. No ^{55}Fe was detected in the pyocyanin peak at any time during the incubation. Therefore, pyocyanin reduced the iron from transferrin, and no binding of iron to pyocyanin was observed.

Iron transport following leukopyocyanin-mediated iron reduction. The fate of the iron reduced from transferrin was assayed by mixing the reactants in the iron reduction assay with a bacterial suspension containing 10^9 CFU/ml. A high density of bacteria was used to assure low oxygen tensions necessary for pyocyanin reduction of iron, and the reaction vials were flushed with nitrogen. Table 1 shows that 5 μg of pyocyanin per ml dramatically stimulated the accumulation of iron from [^{55}Fe]transferrin. Siderophores, pyochelin, and pyoverdin, added in combination at 10 μg/ml, allowed 4.5 pmol of iron uptake per 10 min, but this amount was not equal to that observed in the presence of pyocyanin. When siderophores were used in combination with pyocyanin, the rates of uptake appeared to be additive. The filtrates from these assays were also injected onto the TSK3000 column, and ^{55}Fe could be detected in the ferrozine peak. The accumulation of the iron resulting from the leukopyocyanin-mediated reduction was observed with apyocyanogenic and pyocyanogenic bacteria grown in media containing iron concentrations from 0 to 15 μM added $FeCl_3$ (data not shown). However, there was a decreasing amount of transport with increasing iron concentrations, and bacteria grown in 100 μM $FeCl_3$ could accumulate only 0.4 pmol of ^{55}Fe over 10 min in the presence of pyocyanin (Table 1).

DISCUSSION

This research has described three reactions through which pyocyanin may participate in the iron metabolism of *P. aeruginosa*. The first reaction, the reduction of pyocyanin, may be linked to bacterial respiration since reduction is partially blocked by the addition of KCN (Fig. 2B). Bacterial reduction of pyocyanin proceeded more rapidly under conditions of high cell densities because bacterial respiration lowered the oxygen concentration, thus limiting the autooxidation of leukopyocyanin. The efficiency of pyocyanin for oxygen reduction as opposed to iron reduction has not been determined. However, oxygen must be at a level below the detectable level of the polarographic oxygen electrode because pyocyanin was reduced 10 min after the meter had indicated 0% oxygen saturation (<0.3 μmol/ml). In addition to bacterial respiration, NADH nonenzymatically reduced pyocyanin. This reaction was anticipated because of the similar reaction between NADH and phenazine methosulfate (30).

The second reaction, the reduction of Fe(III) by leukopyocyanin, has been measured in both cell-free reactions

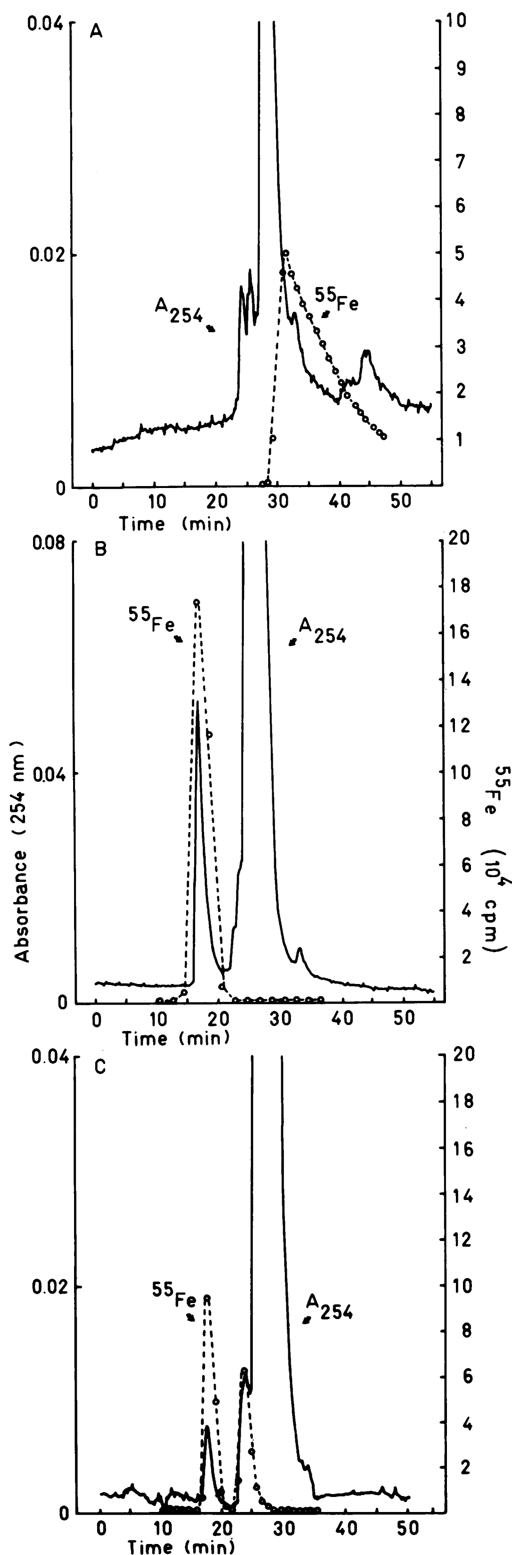

FIG. 4. HPLC analysis of iron release from transferrin on a TSK3000 protein column. (A) Elution of ^{55}Fe in complex with NTA, created by mixing 20 μmol of NTA with 10 nmol of $^{55}FeCl_3$ (10-μl injection). (B) Elution of transferrin created by mixing 64.1 nmol of transferrin with the mixture in panel A (40-μl injection). (C) Elution of ferrous-ferrozine complex generated following a 10-min reaction with 5 μg of pyocyanin, 1 μmol of NADH, and 2 μmol of ferrozine per ml (10-μl injection). Reaction vials were evacuated and flushed with nitrogen. Transferrin standards eluted at 18 min, ferrozine at 24 min, and ferric NTA beginning at 30 min.

TABLE 1. ^{55}Fe Accumulation from transferrin by strain PAO1

Assay conditions[a]	Iron uptake (pmol/10 min per 10^9 bacteria)
Bacteria alone	1.8[b]
Bacteria + Pcy	12.0
Bacteria + Pch + Pvd	4.5
Bacteria + Pch + Pvd + Pcy	18.0
Bacteria[c] (100 μM $FeCl_3$) + Pcy	0.4

[a] Abbreviations: Pcy, pyocyanin (5 μg/ml); Pch, pyochelin (10 μg/ml); Pvd, pyoverdin (10 μg/ml).

[b] Approximately 10^9 PAO1 bacteria grown in apyocyanogenic SMM lacking iron were mixed with 10 mM succinate, 64.1 nmol of transferrin, and 20 μCi of ^{55}Fe in 4 mM sodium-potassium phosphate buffer (pH 7.4). Reaction mixtures were evacuated and flushed with nitrogen repetitively. Bacteria were preincubated in this mixture for 10 min before 5 μg of pyocyanin per ml was added with a nitrogen-flushed syringe. Bacteria were trapped on filters, and ^{55}Fe was trapped with bacteria in comparison to reactions lacking bacteria, which were determined by scintillation counting.

[c] Bacteria were grown in apyocyanogenic SMM containing 100 μM added $FeCl_3$.

using NADH as a reductant and in whole-cell experiments, during which respiration supplied the reducing power. Leukopyocyanin did not have unlimited potential to reduce iron from chelates, since no reaction was detectable with iron in complex with EDDA and EDTA, chelators with binding coefficients similar to those of transferrin (1) and pyoverdin (34). Most of these reactions were measured by the formation of the ferrous-ferrozine complex, which absorbs maximally at 562 nm. However, the TSK3000 protein column was used to analyze the form of the iron. By maintaining NTA in the transferrin solution and EGTA in the mobile phase of the column, all of the iron was recovered from the column. It was clearly observed that the generation of leukopyocyanin in these reactions caused the release of ^{55}Fe and its chelation in the ferrous-ferrozine complex. In the absence of pyocyanin or reducing power, or in the presence of oxygen, no iron release or reduction could be detected. No preferential reactivity of leukopyocyanin with either of the two binding sites of transferrin could be detected. [^{55}Fe]NTA had been used to preferentially label the A binding site, but when [^{55}Fe]citrate was used, no appreciable differences were noticed in the formation of ferrous-ferrozine complex.

The third reaction, the stimulation of iron uptake from transferrin (Table 1), has reproducibly yielded six- to tenfold the levels of iron transport by bacteria over reactions run in the absence of pyocyanin. These levels of stimulation were found in reaction vessels containing high cell densities or those which had been flushed with nitrogen. The presence of oxygen decreased the level of stimulation. The conditions of the assays, low oxygen concentration and lack of amino source, limited the concern about siderophore production during the assay. Siderophores did stimulate the accumulation of iron from ferritransferrin when they were added to the assays, but not to the extent observed with pyocyanin addition (Table 1). It is uncertain whether Fe(III) or Fe(II) is the form of iron taken across the membrane. If Fe(II) is the form, then it may be taken in directly or as the ferrous-ferrozine complex. The latter possibility is not essential for

transport since the reaction proceeded at the same rate in the absence of ferrozine (data not shown). Ferrozine had been added to these assays so that HPLC analysis could detect the ferrous product being formed from ferritransferrin during the transport reaction. It is difficult at this time to comprehend how Fe(III) could accumulate in these assays and be the substrate for transport. There appears to be an insufficiency of both oxygen to explain oxidation of the Fe(II) and siderophores to explain possible Fe(III)-siderophore transport in these reactions. Although the NTA in the reaction can mediate iron transport in *P. aeruginosa* (9), no [^{55}Fe]NTA was detected during analysis of reaction contents on the TSK3000 column. Current research is focused on the functions of the siderophores and pyocyanin in iron transport during these reactions.

This laboratory is most interested in the production and potential activity of pyocyanin during infections. Its potential effects upon the iron supply for bacteria in mammalian tissues would be important to the outcome of the infection (5). However, whether it is artificially applied or produced in situ, pyocyanin is difficult to observe in living tissue. This laboratory and others (3, 14, 15) have observed the reactions of mammalian tissues with pyocyanin, so it is reasonable to theorize that pyocyanin is largely in the reduced state in mammalian tissue. Although it is not a common occurrence in health care today, pyocyanin can cause a pigmentation of pus. More commonly, the event which initially called the attention of Gessard to this bacterium is observed, i.e., the blue pigmentation of bandages (19). The two prominent possibilities for the presence of pyocyanin in infections are (i) that it is present in the oxidized state in pus and on bandages because the bacteria are growing in these areas or (ii) that pyocyanin is in the reduced state in the tissue and autooxidizes when it diffuses into the dead environments of purulent exudates and bandage materials. We are currently attempting to construct experiments which would provide some clues to answer this dilemma.

The massive burst of pyocyanin synthesis late in the logarithmic phase and in the early stationary phase (Fig. 1) has been taken as an indication that pyocyanin is a secondary metabolite (32). I have noticed that the burst of pyocyanin synthesis occurs at the same time as that of both pyoverdin and pyochelin synthesis (C. D. Cox, Curr. Microbiol., submitted for publication). I have been investigating this phenomenon and have found that there is an iron demand for cytochrome synthesis at this stage of growth which may be responsible for the burst of siderophore and pyocyanin synthesis (Cox, submitted). Another suggestion for the late synthesis of compounds involved in iron metabolism states that iron may be important for the longevity of nongrowing bacteria. Gentry et al. (18) have reported that the longevity of *P. aeruginosa* was maximal when cells were grown in low-phosphate medium and were allowed to complete secondary metabolism. Weinberg and Goodnight (33) found that five of six strains of *P. aeruginosa* grown at 37°C demonstrated increased longevity when supplied increased iron concentrations. Iron demand by bacteria in stationary phase or in resting cultures appears to be a metabolic requirement which has not been adequately studied.

Reduction of Fe(III) is a well-known mechanism for iron release from transferrin (1, 35) and siderophores (4, 8). Although the emphasis of this research has been on bacterial iron metabolism during infections, leukopyocyanin reduced iron from numerous chelates and is potentially important in free-living environments. The reductive release of iron from transferrin by leukopyocyanin is important because it requires little additional compromise to the iron-binding capacity of transferrin (enzymatic nicking or decreases in pH). However, the production and reduction of pyocyanin may require a localized, high density of bacteria (an existing bacterial infection). Therefore, this may be an example of iron metabolism which cannot allow the initiation of an infection, but would be capable of contributing to the iron supply for persistent and rapidly expanding infections.

Many experiments have been constructed to observe an effect of pyocyanin on growth. No positive effects on oxygen uptake or growth in early log phase have been observed in this laboratory. However, the findings of this research suggest that pyocyanin would only be active in metabolism once the bacteria are in dense suspension and capable of limiting the oxygen concentration. Alternatively, it is possible that pyocyanin fulfills some primary function other than iron metabolism or that it fulfills many functions so that no single selective advantage will be defined as the one contributed by pyocyanin. However, this research has shown that if pyocyanin is present, there is the possibility that it will be participating in iron-solubilizing and -releasing reactions and that the Fe(II) product is somehow accumulated by the bacteria.

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