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Antibody Inhibition of Ferripyochelin Binding to *Pseudomonas aeruginosa* Cell Envelopes†

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ABSTRACT: A 14K molecular weight protein which has been shown to bind ferripyochelin has been purified from cell envelopes of *Pseudomonas aeruginosa* low iron grown cells. The purified protein migrated as a single band on sodium dodecyl sulfate–polyacrylamide gel electrophoresis and was shown to be free of contamination by lipopolysaccharide or carbohydrate. Antiserum to this protein was made in rabbits and was shown to react with the purified protein by immunoblot assay. The immunoglobulin G fraction of this antiserum blocked binding of [^{59}Fe]pyochelin to isolated cell envelopes of *P. aeruginosa* in a dose-dependent fashion.

Iron is an essential requirement for the establishment and maintenance of bacterial infections (Bullen et al., 1978); Payne & Finkelstein, 1978; Weinberg, 1978). Since iron is either insoluble or complexed with host iron binding proteins, bacteria must possess iron sequestration and transport systems in order to compete for available iron (Neilands, 1981).

Most microorganisms excrete highly specific iron chelators, termed siderophores, which are taken up by cells in complex with Fe^{III} ion (Neilands, 1981). In many bacteria, proteins in the outer membrane have been determined to act as specific receptors for these iron–siderophore complexes. The function of these receptors is to bring the ferrisiderophore to, or through, the envelope where the iron undergoes a reductive separation from the chelator (Neilands, 1982).

A number of siderophore systems have been described for *Pseudomonas aeruginosa*; however, little is understood about the mechanism of iron transport in this organism (Neilands, 1982). Pyochelin is probably the most well-characterized siderophore of *P. aeruginosa*. Pyochelin is a salicylic acid substituted cysteinyl peptide (Cox et al., 1981). Uptake of label from [^{55}Fe]pyochelin has been shown to occur in two stages, an energy-independent step, where it presumably binds to the cell surface, followed by an energy-dependent process (Cox, 1980). We have recently identified a cell envelope of *P. aeruginosa* which binds [^{59}Fe]pyochelin (Sokol & Woods, 1983). This protein has a molecular weight of 14 000 and is produced in high concentrations in iron-starved glucose-grown cells. This protein was shown to bind [^{59}Fe]pyochelin but not $^{59}FeCl_3$. This protein is produced by all strains of *P. aeruginosa* examined, as well as several other species of *Pseudomonas* (Sokol, 1984).

In this paper, we describe the purification of this ferripyochelin binding protein and the production of antibody to the purified protein. Further, the ability of this antibody to

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block [^{59}Fe]pyochelin binding to isolated cell envelopes of *P. aeruginosa* was examined in an in vitro iron binding assay.

Materials and Methods

Materials. $^{59}FeCl_3$ (specific activity 13.3 μCi/μg) was purchased from Amersham Corp. G100 Superfine and DEAE-Sephacel were obtained from Pharmacia. Nitrocellulose membrane filters (0.2 μM) were purchased from Sartorius. Pyochelin was purified as previously described (Cox, 1980).

Bacterial Strains. Bacterial strains used in this study were *P. aeruginosa* PAO (Holloway et al., 1979), PA103 (Liu, 1974), and DG1 (Cash et al., 1979).

Preparation of Cell Envelopes. Envelopes, enriched for outer membrane, were isolated as previously described (Sokol & Woods, 1983). Briefly, cells were grown in M9 minimal salts medium (Pugsley & Reeves, 1976) supplemented with 0.5% glucose. Cells were harvested by centrifugation and washed with 30 mM tris(hydroxymethyl)aminomethane (Tris), pH 8. The cells were broken by sonication with a Braun-sonic 1510 sonicator (Braun) for 2 min in an ice bath. Cell debris was removed by low-speed centrifugation, and the membranes in the supernatant were layered onto a two-step sucrose gradient (60–70%). The gradients were centrifuged at 183000*g* for 3 h in an SW41 Ti rotor (Beckman Instruments). The lower bands consisting primarily of outer membrane were collected and stored at −70 °C.

Chromatography. Material that was extracted from the cell envelope (see Results) was dissolved in 0.01 M Tris (pH 7.5), 1.5% sodium dodecyl sulfate (SDS), 5 mM ethylenediaminetetraacetic acid (EDTA), and 0.01% sodium azide. Gel filtration on G100 Superfine (26 × 70 cm) was carried out at room temperature in the same buffer (Tris/SDS/EDTA). The flow rate was 8 mL/h (by gravity), and 5-mL fractions were collected.

Removal of LPS. The purified protein was treated with phenol to remove LPS (Hancock & Nikaido, 1978). The column fractions containing the protein were pooled, extracted with an equal volume of 90% phenol at 70 °C for 10 min, cooled to 4 °C, and centrifuged at 5000*g* for 10 min. The upper aqueous layer was discarded. The interface and phenol layer were treated once more with distilled water at 70 °C and centrifuged, and the aqueous layer was discarded. The lower phase was treated twice with 2 volumes of acetone and once with 2 volumes of ether. The protein was collected by centrifugation at 15000*g* and the supernatant discarded after each treatment.

Chemical Analysis. Protein concentration was measured by the method of Bradford (1976), modified by using a commercial reagent, Bio-Rad protein assay reagent (Bio-Rad). Lipopolysaccharide (LPS) contamination was estimated by measuring the content of 2-keto-3-deoxyoctonic acid (KDO) (Karkhanis et al., 1978). Carbohydrate concentration was determined by the method of Dubois & Gilles (1956).

SDS–Polyacrylamide Gel Electrophoresis. Sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (PAGE) was performed on 12.5% acrylamide/0.17% bis(acrylamide)/SDS slab gels (Laemmli, 1970).

Preparation of Specific Antisera. A 1-mL mixture of equal parts of Freund's complete adjuvant (FCA) and ferripyochelin binding protein (300–500 μg/mL) was injected into female New Zealand white rabbits (2–2.5 kg) The animals were injected 3 times at 2-week intervals. Ten days after the last injection, the rabbits were exsanguinated by cardiac puncture. The serum was separated by centrifugation (15000*g*, 10 min) and stored in aliquots at −70 °C. The γ-globulin fraction was obtained from the antiserum by batch binding to DEAE-Sephacel as described (Baumstark et al., 1964). The material obtained was lyophilized and resuspended in buffer to the original volume of antisera.

Electrophoretic Blotting Procedure. The specificity of the antiserum to the ferripyochelin binding protein was determined by using the electroblotting technique. Cell envelopes were electrophorsed as described above and transferred to nitrocellulose as described by Towbin et al. (1979) employing a Hoeffer transblot apparatus (Hoeffer) for 30 min at 1 A. The nitrocellulose was incubated at 37 °C for 1 h in 3% bovine serum albumin (BSA) in 10 mM Tris (pH 7.4) and 0.9% NaCl (Tris–saline) to block nonspecific binding of antiserum. The nitrocellulose paper was incubated for 2 h at 37 °C with the immunoglobulin G (IgG) fraction of the antiserum at a dilution of 1:1000. The blots were then incubated with horseradish peroxidase conjugated protein A from *Staphylococcus aureus* (Sigma) at a 1:2000 dilution in Tris–saline containing 3% BSA for 2 h at 37 °C. The blots were then immersed in a solution of 25 μg/mL *o*-dianisidine/0.01% H_2O_2/10 mM Tris, pH 7.4, as described by Ayrameas & Guilbert (1971). Positive reactions were noted by the presence of a strong brown color in the area of reactivity.

Ferripyochelin Binding to Cell Envelopes. Ferripyochelin binding to cell envelopes was measured as previously described (Sokol & Woods, 1983). To test blocking activity, the γ-globulin fraction of specific antiserum or normal rabbit serum was preincubated with envelopes for 30 min at 37 °C prior to the addition of [^{59}Fe]pyochelin. ^{59}[Fe]pyochelin was then added to the reaction mixtures; these were incubated 5 min at 22 °C and then filtered through nitrocellulose filters. The filters were washed extensively with 10 mM Tris, pH 7.5, and 0.9% saline. The ^{59}Fe retained by the filters was counted in an LKB Commpugamma counter, and the results are expressed as cpm of bound ^{59}Fe.

Results

Purification of the Ferripyochelin Binding Protein. A purification scheme has been developed for the ferripyochelin binding protein based on the procedure of Hidennach & Henning (1975) for the purification of the major outer membrane proteins in *Escherichia coli.*

P. aeruginosa strain PAO was used for purification of the ferripyochelin binding protein. Twenty-four liters of culture was grown at 37 °C in M9 minimal salts medium with 0.5% glucose for 40 h. All glassware were acid washed and rinsed with water purified by the Milli–RQ system (Millipore). The cells were harvested by centrifugation at 1000*g* for 15 min. The pellets were washed once in 10 mM Tris, pH 7.5 (Tris buffer), and resuspended in 200 mL of Tris buffer containing 2 mg of DNase. The suspension was sonicated for 4 min annd then centrifuged at 1000*g* for 10 min to remove unbroken cells. The supernatant was then centrifuged at 20000*g* for 20 min. The pellet containing the cell envelope fraction was resuspended in 100 mL of Tris buffer with 4% SDS/0.5 mM $MgCl_2$ and stirred for 30 min at 22 °C. This suspension was centrifuged at 40000*g* for 30 min and the pellet reextracted in an identical manner. The pellet was then extracted twice with 100 mL of Tris buffer, 2% SDS, and 5 mM EDTA for 30 min at room temperature, followed by centrifugation for 30 min at 48000*g* each time. The supernatant (100 mL) was precipitated with 900 mL of acetone overnight at 4 °C. The precipitate was washed twice with 90% acetone at 4 °C and then dried. The precipitate was then extracted twice with 100 mL of chloroform/methanol (2:1). The residue was extracted 3 times with Tris, 1.5% Triton X-100, and 5 mM EDTA. The

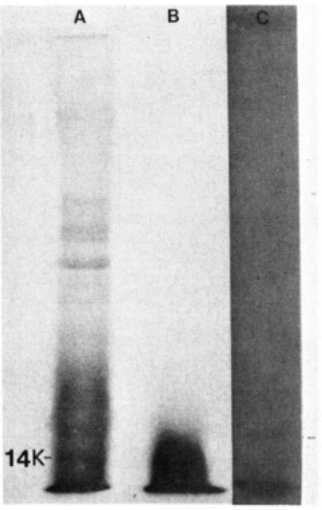

FIGURE 1: SDS–polyacrylamide gel electrophoresis of purified ferripyochelin binding protein. (A) Material applied to the G100 column containing 15 μg of protein; (B) column fraction containing 5 μg of purified protein. This lane was overloaded to demonstrate the absence of contaminating larger molecular weight proteins. (C) Column fraction containing 0.5 μg of purified protein.

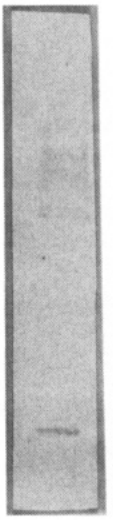

FIGURE 2: Immunoblot of antisera to the ferripyochelin binding protein reacted against cell envelopes. Nitrocellulose blots were incubated with 100 μL of antisera followed by incubation with horseradish peroxidase conjugated protein A. The blots were immersed in a solution of 25 μg/mL *o*-dianisidine/0.01% H_2O_2/10 mM Tris, pH 7.4, for color development. The reaction was stopped by washing with H_2O.

first two extractions were done in 30-mL volume; the final extraction was in 15 mL. The supernatants from all three extractions were combined and precipitated with acetone (final concentration of acetone 90%). The residue was washed with 90% acetone. The residue was resuspended in Tris/SDS/EDTA buffer and chromatographed on a G100 Superfine column. Fractions were analyzed by SDS–PAGE, and those fractions containing purified ferripyochelin binding protein were pooled.

The combined fractions were then extracted with phenol as described under Materials and Methods to remove any contaminating LPS. The residue was then resuspended in Tris/SDS/EDTA buffer. Figure 1 shows the SDS–PAGE profiles of the purified ferripyochelin binding protein as well as the material which was applied to the G100 column. The cell envelope fraction contained 1.25 g of protein prior to detergent extraction. The final yield of purified material was 1 mg of protein; therefore, the purified protein represents 0.08% of the total cell envelope protein. The purified material combined less than 0.1% LPS, as determined by KDO analysis. No carbohydrate was detectable.

Specificity of Antibody with the Ferripyochelin Binding Protein. Cell envelopes were electrophoresed on SDS–PAGE, transferred to nitrocellulose, and then reacted with antiserum. The antiserum reacts primarily with the ferripyochelin binding protein (Figure 2). It reacts strongly with a single protein of 14000 molecular weight and weakly with some higher molecular weight material. This reaction may be due to slight contamination by LPS in the material used in immunization.

Effect of Antibody on [^{59}Fe]Pyochelin Binding to Cell Envelopes. To determine the effect of antibody on ferripyochelin binding to cell envelopes of *P. aeruginosa*, envelopes were preincubated with antiserum prior to incubation with [^{59}Fe]pyochelin. Envelope preparations containing 100 μg of protein were incubated at 37 °C with 100 μL of specific antiserum or 100 μL of normal rabbit serum as a control. The serum IgG fraction was used in this assay in order to eliminate any competitive binding effects due to transferrin present in whole serum.

When envelopes of PAO were preincubated with the IgG fraction of normal rabbit serum, there was no difference in the amount of [^{59}Fe]pyochelin bound (Table I). When the envelopes were preincubated with antiserum to the ferripyochelin binding protein, there was a significant decrease in the amount of [^{59}Fe]pyochelin bound ($p < 0.01$). Envelopes preincubated with immune serum bound only 62–66% as much [^{59}Fe]pyochelin as envelopes preincubated with normal serum or no serum (Table I).

Table I: Effect of Antibody to the Ferripyochelin Binding Protein on [^{59}Fe]Pyochelin Binding to Cell Envelopes of PAO

serum	cpm ± SEM[a]	% control[b]
no serum	7287 ± 222	100.0
normal rabbit serum	7422 ± 208	101.8
antiserum 1	4463 ± 100	66.6
antiserum 2	4576 ± 179	62.8

[a] Total cpm added to reaction mixture, 18000. Envelopes were preincubated with 100 μL of serum containing 1 mg/mL IgG prior to reaction with [^{59}Fe]pyochelin. [b] Percent ^{59}Fe bound when samples with no sera added were regarded as 100%.

Table II: Effect of Antibody to the Ferripyochelin Binding Protein on [^{59}Fe]Pyochelin Binding to Cell Envelopes of PA103

serum	cpm ± SEM[a]	% control[b]
no serum	8092 ± 253	100.0
normal rabbit serum	8783 ± 58	108.5
antiserum 1	6259 ± 65	74.4
antiserum 2	6402 ± 225	79.1

[a] Total cpm added to reaction mixture, 18000. Envelopes (100 μg of protein) were preincubated with 100 μL of serum containing 1 mg/mL IgG prior to reaction with [^{59}Fe]pyochelin. [b] Percent ^{59}Fe bound when samples with no sera added were regarded as 100%.

To demonstrate that this inhibition of iron binding to the envelopes was due to antibody directed against the ferripyochelin binding protein and was serotype independent, the experriment was repeated with envelopes of a different LPS serotype of *P. aeruginosa*. When envelopes of PA103 (serotype 2) were preincubated with anti-ferripyochelin binding protein serum, the amount of ^{59}Fe bound by these envelopes was also reduced ($p < 0.01$) (Table II).

To demonstrate that this antibody inhibition of [^{59}Fe]-pyochelin binding is specific, the effects of antibody to LPS and antibody to outer membrane protein F on [^{59}Fe]pyochelin

Table III: Effect of Antibody to Serotype 1 LPS on [^{59}Fe]Pyochelin Binding to Cell Envelopes

strain	serotype[a]	cpm ± SEM NRS[b]	cpm ± SEM anti-LPS[b]
PAO	1	6643 ± 330	5667 ± 509
PA103	2	2781 ± 183	2599 ± 793
DG1	5	8023 ± 606	7933 ± 623

[a] Fisher–Devlin serotype. [b] Envelope protein (100 μg of protein) was preincubated with 100 μL of normal rabbbit serum (NRS) or antiserum to serotype 1 LPS (anti-LPS) for 30 min at 37 °C prior to addition of [^{59}Fe]pyochelin.

binding were examined. When cell envelopes from strain PAO are preincubated with antisera prepared against LPS serotype 1, the amount of [^{59}Fe]pyochelin bound is slightly less than that observed when the envelopes are preincubated with normal sera prior to iron binding. However, the decrease is not statistically significant (Table III). When envelopes prepared from strains of serotypes 2 and 5 are preincubated with antiserum to type 1 LPS prior to [^{59}Fe]pyochelin binding, there is no significant different in the amount of [^{59}Fe]pyochelin bound when compared to normal rabbit serum (Table III). When cell envelopes from strain PAO were preincubated with antiserum to outer membrane protein F, 4576 cpm of [^{59}Fe]pyochelin were bound per 100 μg of envelope protein as compared to 4451 cpm with no sera added. Therefore, it appears that antibody directed against the 14 000 molecular weight protein specifically blocks [^{59}Fe]pyochelin binding and antibody directed against LPS of protein F has no effect on iron binding.

The degree of reduction in [^{59}Fe]pyochelin binding to cell envelopes was shown to depend on the amount of antiserum added (Figure 3). As the amount of antiserum was increased, the percent ^{59}Fe bound decreased. No effect on ^{59}Fe binding was observed when envelopes were preincubated with 25 μL of antiserum (1.0 mg/mL IgG); however, the addition of 200 μL of antiserum reduced the amount of ^{59}Fe bound by 75%. Thus, the effect of antibody on Fe binding was dose dependent. The same concentrations of normal rabbit serum had no effect on ^{59}Fe binding up to and including 100 μL of serum or 0.1 mg of IgG. When 200 μL of normal rabbit was added, the percent ^{59}Fe bound was decreased 20%, indicating that high concentrations of normal serum may cause some nonspecific reduction of iron binding.

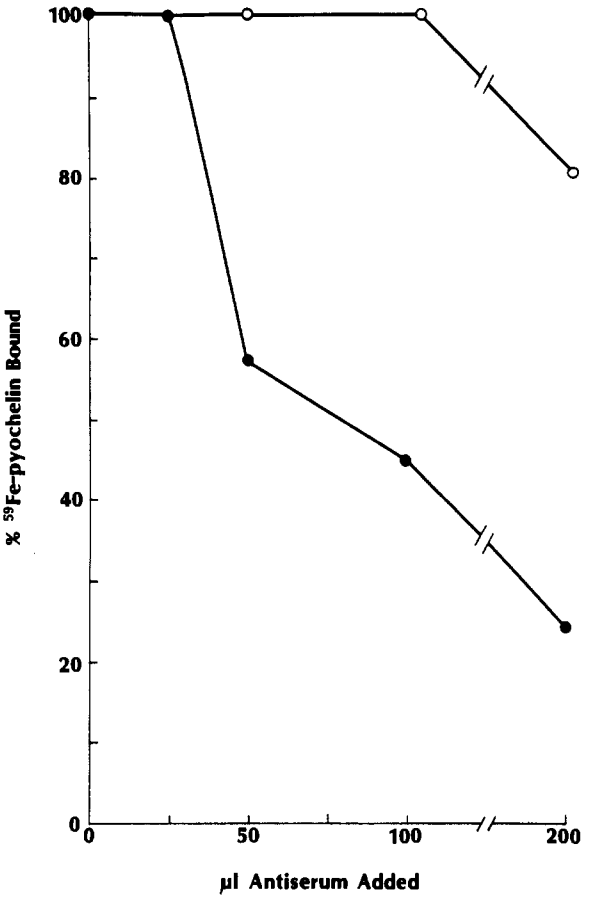

FIGURE 3: Antibody dose response. Isolated cell envelope preparations containing 100 μg of protein were preincubated with increasing concentrations of serum, prior to reacting with [^{59}Fe]pyochelin. Values represent the percentage of [^{59}Fe]pyochelin bound when the amount of [^{59}Fe]pyochelin bound by envelopes with no serum added is defined as 100%. The concentration of IgG in the serum was 1 mg/mL. Envelopes preincubated with antiserum to ferripyochelin protein (●); envelopes preincubated with normal serum (○).

Discussion

Outer membrane proteins have been shown to be involved in iron uptake by a variety of organisms (Bennett & Rothfield, 1976; Hollifield & Neilands, 1978; Ichihara & Muzushima, 1978). These proteins are induced in conditions of iron deprivation. Isolated outer membranes have been shown to be capable of siderophore-mediated iron binding in *E. coli* (Hollifield & Neilands, 1978; Ichihara & Mizushima, 1978), *Salmonella typhimurium* (Bennett & Rothfield, 1976), and *P. aeruginosa* (Sokol & Woods, 1983). The binding of ferric enterobactin to a purified 81K protein isolated from *E. coli* outer membranes has been demonstrated (Fiss et al., 1982). Mutants deficient in iron transport have been isolated that lack outer membrane proteins, indicating the involvement of these proteins in siderophore-mediated iron transport (McIntosh et al., 1979).

In the present study, we have used an immunological approach to demonstrate the involvement of a cell envelope protein in iron acquisition. The 14K protein from *P. aeruginosa* envelopes which has previously been shown to bind ferripyochelin was purified by differential detergent solubilization followed by gel filtration chromatography. Antibody to this protein was shown to inhibit ferripyochelin binding to isolated cell envelopes of *P. aeruginosa*.

Although the purified protein migrated as a single band on SDS–PAGE and was shown to be free of contamination by LPS or carbohydrate, there are disadvantages to the purification scheme used.

The procedures involve the use of a variety of reagents including acetone, methanol, phenol, and detergents which may have adverse effects on the protein. Certainly, the protein would not be expected to be functional following this treatment. However, these procedures are necessary when the present purification scheme is used. Proteins cannot be removed from gram-negative bacteria without the use of detergents (Hindennach & Henning, 1975). The presence of detergent, however, does not appear to inhibit the binding of ferrisiderophores to outer membrane proteins (Fiss et al., 1982; Sokol & Woods, 1983). Removal of contaminating LPS from outer membrane proteins is also a common problem in gram-negative organisms. The use of hot phenol to extract the LPS appears to be effective and necessary, as small amounts of residual LPS are often more immunogenic than protein antigens.

Although the protein may not be functional after purification, it maintains its antigenicity. Antibody to the purified protein reacts with isolated cell envelopes as well as the purified protein.

The blocking activity of antibody to the ferripyochelin binding protein is specific. Anti-LPS does not significantly block ^{59}Fe binding to cell envelopes. The blocking activity of antibody to the ferripyochelin binding protein is effective against different LPS types and therefore not serotype specific. It does not appear that steric hindrance is involved because antibody to the outer membrane protein F or porin protein has no effect on [^{59}Fe]pyochelin binding to isolated cell envelopes.

Further evidence that antibody to the ferripyochelin binding protein specifically blocks ferripyochelin binding was provided from experiments which measured blocking of $^{59}FeCl_3$ binding. No significant difference was noted between the ability of normal rabbit serum and antibody to ferripyochelin binding protein to inhibit $^{59}FeCl_3$ binding to cell envelopes of *P. aeruginosa*.

It may be possible to use this antibody to purify functional ferripyochelin binding protein. Studies are currently in progress to attempt affinity purification of this protein.

The data presented show that antibody to the 14K outer membrane protein blocks [^{59}Fe]pyochelin binding to isolated cell envelopes. These data provide further evidence that this protein is involved in iron transport by *P. aeruginosa*.

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