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Demonstration of Ferric L-Parabactin-Binding Activity in the Outer Membrane of *Paracoccus denitrificans*

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Under low-iron conditions, *Paracoccus denitrificans* excretes a catecholamide siderophore, L-parabactin, to sequester and utilize iron. In this report, we demonstrate the presence of stereospecific high-affinity ferric L-parabactin-binding activity associated with *P. denitrificans* membranes grown in low-iron medium. Isolated outer membrane components were shown to be three to four times higher in specific activity for ferric L-parabactin. The same amount of binding activity existed whether or not the radiolabel was present in the metal (^{55}Fe) or the ligand (^{3}H) portion of ferric parabactin chelate, suggesting that binding was to the intact complex. Ion-exchange chromatography of a Triton X-100-solubilized outer membrane mixture on DEAE-cellulose resulted in a 10-fold increase in binding activity relative to that present in whole membranes. Polypeptide profiles by sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the products of each stage of the purification showed that binding activity copurified with one or more of the low-iron-induced outer membrane proteins in the 80-kilodalton (kDa) region. Membrane proteins and [^{55}Fe]ferric L-parabactin electrophoresed in nondenaturing gels demonstrated the presence of membrane component(s) which stereospecifically bound ferric L-parabactin, thus providing independent confirmation of the binding assay results. Moreover, when the band labeled by [^{55}Fe]ferric L-parabactin was excised and profiled by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, 80-kDa polypeptides were the major components present. These results demonstrate the presence of a high-affinity ferric L-parabactin receptor in *P. denitrificans* membranes and suggest that one or more of the 80-kDa low-iron-induced polypeptides are components of the ferric L-parabactin receptor.

Although iron composes 5% of the earth's crust, it is difficult for living systems to access this essential metal. This inaccessibility is due in part to the extreme insolubility of the ferric iron at physiological pH [K_{sp} of $Fe(OH)_3 = 10^{-39}$ M] and in part to the tendency of ferric hydroxide to precipitate as polymerized oxyhydroxides (22).

The critical roles which iron plays in many biological redox systems are certainly well documented (29). In microorganisms, these roles include the fixation of molecular nitrogen, photosynthesis, DNA synthesis, and electron transport involved in oxidative phosphorylation. To overcome the problems associated with accessing the metal, bacteria have evolved a group of low-molecular-weight, virtually ferric ion-specific ligands, the siderophores. These chelators are excreted into the extracellular medium to sequester exogenous ferric iron as soluble chelation complexes, which the organism can then recognize and transport into the cell (16, 17).

Since ferric siderophore complexes are typically heavier than 700 daltons, they do not permeate porin structures in bacterial outer membranes. Because of this, microorganisms have developed specific high-affinity transport systems to assimilate iron from ferric siderophore chelates (8). The gram-negative soil bacterium *Paracoccus denitrificans* excretes a hexacoordinate catecholamide iron chelator, a siderophore, parabactin (Fig. 1a) (28). The five aromatic hydroxyls and the oxazoline ring nitrogen of this ligand have been shown to be the donor centers coordinated to iron in the ferric L-parabactin chelate complex (19). We previously demonstrated that when grown in iron-deficient media, *P. denitrificans* is able to assimilate iron from the ferric complex of parabactin (2) in an energy-dependent process. Moreover, we showed that *P. denitrificans* exhibits stereospecificity in its siderophore requirement. The ferric chelate of the organism's native siderophore, L-parabactin, was the most effective in supplying iron to the cells. The ferric chelate of the synthetic enantiomer, D-parabactin, was unable to supply iron to the microorganism in various experimental protocols, including growth stimulation, iron transport (accumulation), and reversal of ethylenediamine di-*o*-hydroxyphenylacetic acid (EDDA)-induced iron starvation (2). In the present study, we isolated and partially purified a membrane-associated, stereospecific ferric L-parabactin-binding protein, parabactin receptor, with a K_d estimated to be 0.7 μM.

MATERIALS AND METHODS

Materials. EDDA was deferrated and purified by the method described by Rogers (25). D-Parabactin was synthesized and purified in a manner identical to that for L-parabactin but with D-BOC-Thr instead of L-BOC-Thr (3). (L)-[^{3}H]parabactin was prepared analogously by starting with (L)-[^{3}H]threonine. The final specific activity of (L)-[^{3}H]parabactin was 10.5×10^{3} dpm/μg. Salicylic acid was recrystallized twice from hot 0.01 N HCl and sublimed at 70°C in high vacuum.

Bacterial strain and culture conditions. *P. denitrificans* (ATCC 19367) was maintained on trypticase agar plates. Individual colonies were inoculated into 20 ml of trypticase soy broth and incubated with rotary shaking for 24 h at 30°C. Inoculations were then made from this culture into a low-iron minimal salts liquid medium at 200 μl of culture inoculum per 100 ml of new medium and were incubated with shaking at 30°C. The low-iron minimal salts medium was prepared as previously described (2) but with the addition of 0.2 mg of EDDA per ml and 0.5% Tween 80.

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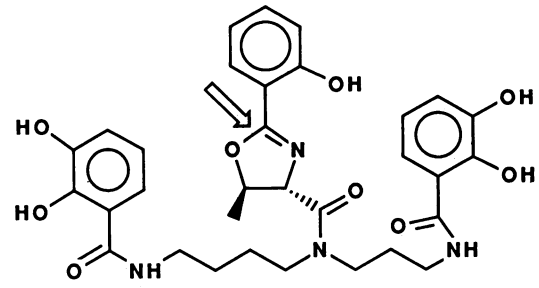
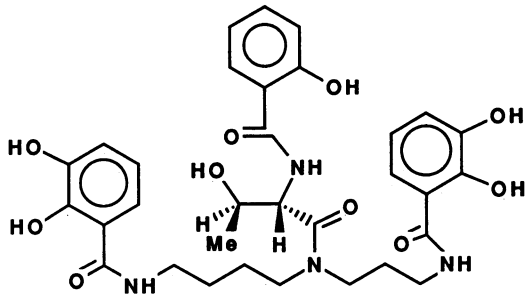


(a) L-Parabactin



(b) L-Parabactin A

FIG. 1. Hydrolysis of the oxazoline ring in L-parabactin (a) (arrow), gives the open chain L-threonyl form, L-parabactin A (b).

Protein determination. The protein concentration was estimated by using bovine serum albumin as the standard, following the method of Lowry et al. (14). Modifications were made for membrane protein samples as described by Markwel et al. (15).

Isolation of membranes. Cells in middle- to late-log phase were harvested by centrifugation at 4°C and washed twice in 50 mM Tris hydrochloride (pH 7.5) containing 10 mM $MgCl_2$. The washed pellet was mixed with a small volume of this buffer containing DNase (1 μg/ml) and RNase (1 μg/ml) to give a thick suspension. The cells were homogenized by two passages of this suspension through a prechilled French pressure cell (18,000 lb/in^2). The crude homogenate was centrifuged twice (5,000 × *g*, 30 min, 4°C) to remove unbroken cells and large debris. The resulting clear supernatant was then ultracentrifuged (105,000 × *g*, 60 min, 4°C) to isolate the cell envelopes from the supernatant containing soluble cytosol proteins. The pellet was suspended in 50 mM Tris hydrochloride (pH 7.5) to a protein concentration between 10 and 20 mg/ml to give a clear liquid which could be stored at 4°C for at least 2 weeks without the formation of sediment or apparent loss of ferric parabactin-binding activity.

Extraction of membrane proteins. Solubilized protein extracts selectively enriched in inner (cytoplasmic) or outer (cell wall) membrane components were prepared on the basis of the method described by Schnaitman for *Escherichia coli* (26). Briefly, whole-cell envelopes were extracted first with 100 mM Tris hydrochloride (pH 7.5) containing 2% Triton X-100 and 10 mM $MgCl_2$, which selectively solubilizes cytoplasmic membrane proteins. Then, the high-speed pellet of this extraction was reextracted with 2% Triton X-100–Tris containing 5 mM EDTA instead of Mg^{2+}. This EDTA–Triton X-100 extract was enriched with outer membrane proteins, including porin, and had none of the red-brown tinge (cytochromes) present at earlier stages of purification.

Preparation of iron(III) parabactin chelate. An equivalent of parabactin was dissolved in 0.01 N NaOH (under argon), sufficient to neutralize the five equivalents of H^+ produced upon chelation as well as any excess acid present in the iron(III) stock solution. After the addition of one equivalent of iron(III), there should be a slight excess of base present. This chelate solution is then buffered at 7.5 with 100 mM Tris.

Sephadex G-25 column-centrifugation binding assay. A method modified from that described by Penefsky (21) was used to evaluate ferric parabactin-binding activity. Control experiments to measure the retention of free ligand consisted of placing on the column and centrifuging 100 μl of the incubation mixture without protein and containing either 8.8 × 10^5 cpm of [^{55}Fe]ferric parabactin or 2 × 10^4 cpm of ferric (L)-[^{3}H]parabactin. Eluates contained 70 to 180 cpm for ^{55}Fe or background for ^{3}H, indicating 99.99+% retention of free ligand on the column. Separate control experiments demonstrated complete passage of macromolecules into the eluate. Recoveries in the eluate ranged from 95 to 97% for membrane vesicles to 99 to 100% for soluble proteins.

The typical binding assay consisted of incubating a mixture of the protein (50 to 200 μg), chelate (50 nM to 90 μM), 100 mM Tris hydrochloride (pH 7.5), and 0.25 M sodium salicylate (pH adjusted to 7.5) in a total volume of 200 μl at room temperature for 1 h. In experiments to determine the binding constant (K_d), all mixtures contained 50 nM [^{55}Fe]ferric L-parabactin of very high specific activity (2.4 mCi/μmol). Datum points for higher ligand concentrations were derived from mixtures to which an appropriate amount of unlabeled ferric L-parabactin had been added. Thus, for example, the 1 μM mixture contained 50 nM [^{55}Fe]ferric L-parabactin and 950 nM cold ferric L-parabactin. The data were then analyzed as the displacement of radioactive ligand by nonradioactive ligand by the method of Akera and Cheng (1).

DEAE-cellulose ion-exchange column. DEAE-cellulose was equilibrated in 50 mM Tris hydrochloride (pH 7.5) containing 5 mM EDTA and 0.4% Triton X-100. EDTA–Triton X-100-solubilized receptor was dialyzed against this buffer for 24 h at 4°C. The column was then eluted with the same buffer and a 0 to 0.20 M NaCl gradient, representing a total of 10 to 12 bed volumes of eluate. Protein contents of the fractions were salted out by bringing them to 90% saturation with ammonium sulfate. The protein contents were analyzed for binding activity and by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE).

SDS-PAGE. SDS-PAGE was performed as described by Laemmli (12).

Nondenaturing gel electrophoresis and autoradiography. Slab gels were prepared with a 3% stacking gel and either a 7.5 or 10% separating gel as for SDS-PAGE, with the exception that gels contained 0.1% Triton X-100 and SDS was omitted from sample and running buffers. Membrane preparations were incubated with [^{55}Fe]ferric L- or D-parabactin and electrophoresed at a constant current of 15 mA per slab for 5 h at 4°C. This gel was not stained for protein but instead was dried for autoradiography. For each experiment, a twin, nonradiolabeled gel was run in parallel. Instead of [^{55}Fe]ferric parabactin, the samples for this gel were incubated with the nonradiolabeled ligand and electrophoresed. The gel was stained for protein to serve as the reference for the autoradiography. The autoradiograms were

TABLE 1. Effect of various ionic conditions on [^{55}Fe]ferric L-parabactin and [^{55}Fe]ferric D-parabactin binding in membrane microvesicles

Additions[a]	Binding (pmol/mg of protein) of:		L/D[b]
	[^{55}Fe]ferric L-parabactin	[^{55}Fe]ferric D-parabactin	
None	88	33	2.7
0.9% NaCl	96	24	4.0
0.5 mM Cyanocobalmin	84	50	1.7
1 M Guanidine-hydrochloride	86	8.6	10.0
0.25 M Salicylate	90	9	10.0

[a] Ligand concentration, 1.0 μM; 100 μg of protein per 200 μl of incubation mixture, all in 50 mM Tris hydrochloride (pH 7.5), with additions as indicated.
[b] Ratio of bound (L)-[^{55}Fe]parabactin to bound (D)-[^{55}Fe]parabactin.

prepared by exposing the dried untreated gels to Kodak X-Omatic AR film in a Kodak X-Omatic X-Ray cassette for 5 to 7 days at −70°C. In one experiment, the Coomassie blue-stained band, corresponding to the band which was radiolabeled by [^{55}Fe]ferric L-parabactin in a twin 10% nondenaturing gel, was excised, placed in the sample well of a standard SDS gel together with SDS sample buffer, and electrophoresed in a second dimension.

RESULTS

Demonstration of high-affinity binding activity for ferric L-parabactin in *P. denitrificans* membranes. The binding assay conditions were developed by using whole-cell membranes as the source of receptor-binding activity and showed a linear dependence on receptor concentration. Incubation periods ranging from 45 min to 3 h at room temperature yielded similar results. Assays done in triplicate typically exhibit standard deviations on the order of ±10% when substantial specific binding activity is present in the sample. When low binding activity is present, standard deviations may be ±25% or larger (corresponding to ±50 to 200 cpm). In initial experiments with 1 μM ^{55}Fe-labeled chelate, 100 μg of membrane protein bound about 8.6% of the ^{55}Fe in the incubation mixture. In the presence of excess (100 μM) unlabeled ferric L-parabactin, binding was reduced to 1.5%. In binding experiments with 1 μM [^{55}Fe]ferric D-parabactin, 100 μg of membrane protein bound 3.3% of the ligand. In the presence of excess (100 μM) unlabeled ferric D-parabactin, there was only a modest reduction in this binding, to 1.5%. Clearly, binding sites for ferric D-parabactin are far fewer in number than for ferric L-parabactin. The difference between the means for L-parabactin (8.6%) and D-parabactin (3.3%) binding was significant ($P < 0.005$), and the magnitude and significance of this difference were reproducible from one culture batch to another, thus demonstrating the stereospecific, high-affinity ferric L-parabactin binding in *P. denitrificans* membranes.

Taken together, these data suggested that *P. denitrificans* membranes exhibit at least two types of binding activity: (i) a specific, saturable, high-affinity binding which is stereospecific for ferric L-parabactin; and (ii) nonspecific binding which is nonsaturable, and linear up to at least 100 μM ferric parabactin, and nonstereospecific, so that the amount of nonspecific background binding is identical for ferric D- and ferric L-parabactin. A number of ionic conditions in the assay mixture were explored with the goal of optimizing specific binding while minimizing nonspecific binding and maximizing stereospecificity (Table 1). In these examples, the binding of ferric L-parabactin was similar under very different ionic conditions. In contrast to the minimal influence on high-affinity ferric L-parabactin binding, certain conditions, such as the presence of 0.25 M sodium salicylate, markedly reduced the amount of nonspecific (i.e., ferric D-parabactin) binding. For this reason, we chose subsequently to routinely include 0.25 M sodium salicylate in our incubation mixtures.

TABLE 2. Stereospecificity of ferric parabactin binding in *P. denitrificans* membrane microvesicles[a]

Ligand concn (μM)	L/D[b]
91	1.0
51	1.0
21	1.0
10	1.0
5	1.2
2	4.0
0.040	14.0

[a] Conditions: 100 μg of membrane protein per 200 μl of incubation mixture.
[b] Ratio of total bound counts of [^{55}Fe]ferric L-parabactin at a particular concentration to total bound counts of [^{55}Fe]ferric D-parabactin at the same concentration.

With optimized assay conditions, the binding of ferric L-parabactin and ferric D-parabactin to *P. denitrificans* membranes was examined in triplicate over a broad range of ligand concentrations. The stereospecific, high-affinity and the low-affinity, nonsaturable, nonstereospecific binding activities are each clearly demonstrated in these data. At low ligand concentrations, the high-affinity, stereospecific system recognizing ferric L-parabactin dominated, whereas at high ligand concentrations, this specific system became saturated and only nonsaturable, nonstereospecific binding was seen (Table 2). An apparent upper limit of 1 μM for K_d in microvesicular membranes is indicated.

Solubilization of ferric L-parabactin-binding activity in apparent association with outer membrane proteins. In the Schnaitman stepwise detergent extraction of the cell envelope (26), ferric L-parabactin activity was mainly found in the Mg^{2+}–Triton X-100-insoluble fraction (Fig. 2) and then in the EDTA–Triton X-100-soluble fraction, which was enriched in 80-kilodalton (kDa) low-iron induced proteins (Fig. 3). This fractionation behavior was as expected for outer membrane proteins, at least in gram-negative enteric bacteria. Although the Schnaitman scheme has not been critically evaluated for *P. denitrificans*, there are several reasons to suggest that the EDTA–Triton X-100-soluble fraction was indeed enriched in outer membrane proteins. Firstly, the EDTA–Triton X-100-soluble fraction was colorless, indicating the absence of cytochromes, which are major components of the cytoplasmic membrane in *P. denitrificans*. The Mg^{2+}–Triton X-100-soluble fraction, in contrast, was a deep red-brown color, indicating that this fraction was selectively enriched in cytoplasmic membrane components. Secondly, there appeared to be selective enrichment of the 33-kDa outer membrane porin (31) in the EDTA–Triton X-100-soluble fraction (Fig. 3). Finally, the SDS-PAGE profile of outer membranes, isolated free from cytoplasmic membrane by sucrose density gradient centrifugation (9) of French pressure cell-sheared *P. denitrificans* cells which had been iron starved, was similar in all major details to the SDS-PAGE profile of the EDTA–Triton X-100-soluble fraction, including the presence of the low-iron-induced, 80-kDa proteins (Fig. 3).

Binding properties of the Triton X-100-solubilized receptor. The binding assay normally used ^{55}Fe as the radiolabel. The

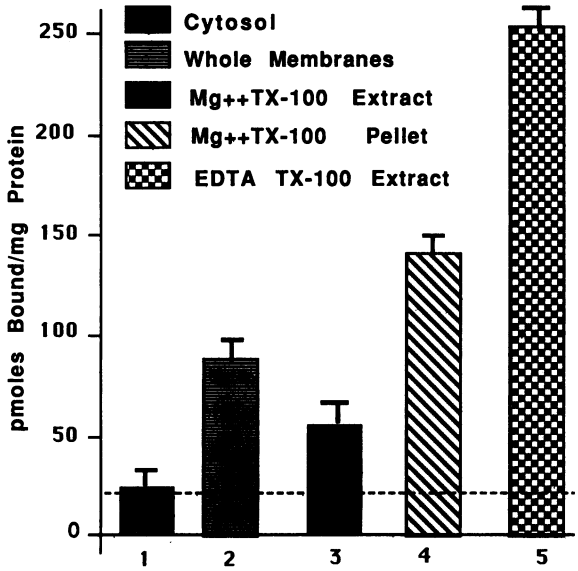


FIG. 2. Purification of ferric L-parabactin-binding activity from *P. denitrificans*. Briefly, *P. denitrificans* cells were disrupted by hydraulic shear in a French pressure cell. The high-speed supernatant contained soluble proteins, most of which are cytoplasmic. Hence this fraction was termed the Cytosol. The high-speed pellet consists of Whole Membranes. Extraction of whole membranes with 50 mM Tris hydrochloride (pH 7.5) containing 2% Triton X-100 and 10 mM $MgCl_2$ (Mg^{++}TX-100 Extract) selectively solubilizes inner membrane proteins as judged by the presence of cytochromes. The high-speed pellet of this extraction (Mg^{++}TX-100 Pellet), depleted of inner membrane components, is extracted with 50 mM Tris hydrochloride (pH 7.5) containing 2% Triton X-100 and 5 mM EDTA to solubilize outer membrane proteins (EDTA TX-100 Extract) such as porin (see the 33-kDa band in Fig. 3 and text). All data represent preparations from the same culture batch and are expressed as picomoles of [^{55}Fe]ferric L-parabactin bound per milligram of protein and are uncorrected for nonspecific low-affinity binding, which under the assay conditions ([ligand] = 1 μM) is about 15 ± 7 pmol/mg of protein. The dashed line represents the base line after correction for this nonspecific background.

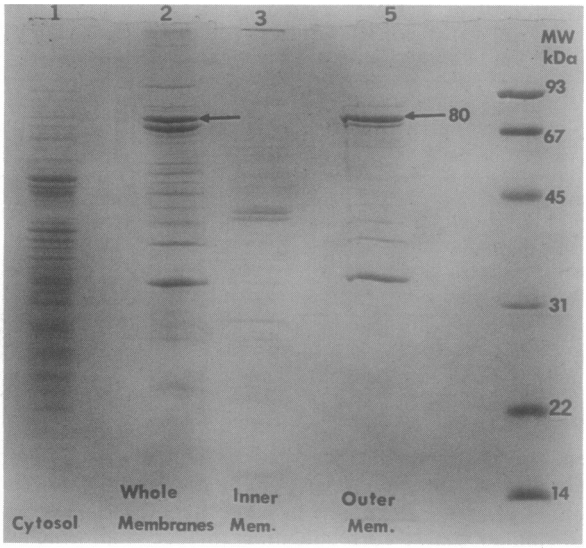


FIG. 3. Polypeptide compositions of purification fractions profiled by SDS-PAGE. Samples of the same fractions for which biochemical binding data were presented in Fig. 2 were subjected to SDS-PAGE and stained with Coomassie blue. Thus, bar 1 in Fig. 2 corresponds to lane 1 in Fig. 3, bar 2 corresponds to lane 2, bar 3 corresponds to lane 3, and bar 5 corresponds to lane 5. Arrows point to a group of several ca. 80-kDa polypeptides which are induced in *P. denitrificans* grown under low-iron conditions.

assumption was that ^{55}Fe remained in the form of [^{55}Fe] parabactin chelate and that any counts bound to the protein thus represented bound chelate. It was important to demonstrate that this was indeed the case, and that, for example, ^{55}Fe was not stripped from the parabactin ligand so that iron became protein bound in a form not associated with parabactin. We synthesized (L)-[^{3}H]parabactin for use as a ligand. In side-by-side experiments, an equal amount of solubilized protein was assayed for the binding of [^{55}Fe] ferric L-parabactin or ferric (L)-[^{3}H]parabactin in each case at a chelate concentration of 1 μM. This protein preparation had a specific activity of 226 ± 14 pmol/mg of protein for the binding of [^{55}Fe]ferric L-parabactin and 243 ± 13 pmol/mg of protein for ferric (L)-[^{3}H]parabactin. Thus for each mole of iron bound, there was 1 mol of L-parabactin bound. The gallium(III) (L)-[^{3}H]parabactin chelate, also at a concentration of 1 μM, was also bound well by this preparation (149 ± 20 pmol/mg of protein). Ga(III) cannot be reduced under physiological conditions. Thus, this result argues against an obligate redox stripping of metal from its L-parabactin chelate under binding assay conditions. These results are consistent with a receptor protein recognizing and binding the ferric L-parabactin chelate and then carrying it intact in the column assay and into the eluate.

An apparent dissociation constant (K_d) for ferric L-parabactin binding to the partially purified Triton X-100-solubilized receptor was estimated by incubation in the presence of 50 nM [^{55}Fe]ferric L-parabactin of very high specific activity and then adding various amounts of unlabeled ferric L-parabactin (0 to 89.95 μM) to achieve a total ligand concentration range of 50 nM to 90 μM. Data were then analyzed as the displacement of [^{55}Fe]ferric L-parabactin by the unlabeled ferric L-parabactin (1). The total ligand concentration at which 50% maximal specific binding activity is observed is estimated to be K_d = 0.7 μM (Fig. 4). This corresponds to an apparent K_m of 0.5 to 1 μM, as measured for the overall rate of iron transport in whole cells (2; unpublished observations). For comparison, the ferric enterobactin receptor in *E. coli* has a K_d of 0.3 μM (10).

Ferric L-parabactin-binding assays performed at pH values of 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, and 9.0 varied only slightly (±10%), demonstrating that there was remarkably little effect of pH on binding over the pH range of 6.0 to 9.0.

Demonstration of stereospecific ferric parabactin-binding activity in electrophoresis gels. When membrane proteins were incubated with [^{55}Fe]ferric L- or D-parabactin and then subjected to SDS-PAGE, autoradiograms revealed that all of the radiolabel moved with the dye front (i.e., none remained protein bound under these conditions). We therefore performed the same experiment by using the nondenaturing, nonionic detergent Triton X-100 in place of SDS. Figure 5a shows the results of such an experiment in which the gel matrix was polymerized from 10% monomers. Distinct bands of radiolabel were seen near the top of this gel with [^{55}Fe]ferric D- or L-parabactin as the radioligand. By far the most intensely labeled band was seen with [^{55}Fe]ferric L-parabactin (arrow). [^{55}Fe]ferric D-parabactin did label some bands, the most intense of which (arrow) was clearly different from the most intensely labeled band seen with [^{55}Fe]ferric L-parabactin as the ligand. Because of the poor mobility experienced in this 10% gel, we prepared a gel from

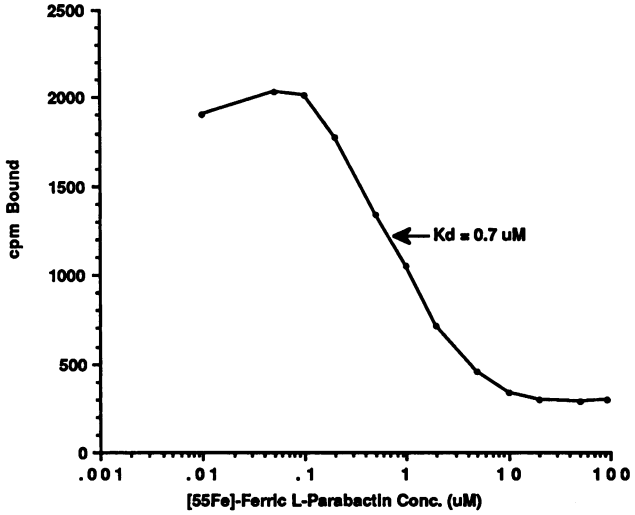


FIG. 4. [^{55}Fe]ferric L-parabactin-binding curve. With the exception of the 10 nM concentration, all assay mixtures contained 50 nM [^{55}Fe]ferric L-parabactin of very high specific activity. For each concentration above 50 nM, sufficient nonradioactive ferric L-parabactin was added to give the nominal total ligand concentration, e.g., 100 nM = 50 nM [^{55}Fe]ferric L-parabactin plus 50 nM unlabeled ferric L-parabactin; 1 μM = 50 nM [^{55}Fe]ferric L-parabactin plus 950 nM unlabeled ferric L-parabactin, etc. The data were then analyzed as radioactive ligand being displaced competitively by nonradioactive ligand. The affinity of receptor protein for ferric L-parabactin is expressed as the concentration at which 50% of binding sites are occupied, i.e., K_d = ca. 0.7 μM.

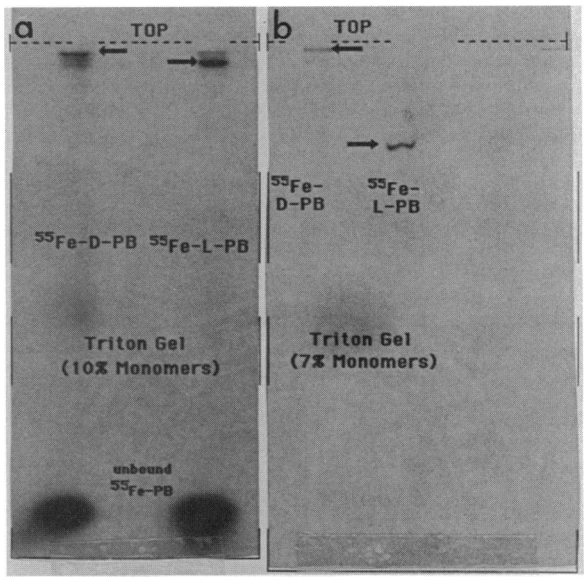


FIG. 5. Autoradiograms following Triton X-100–PAGE of *P. denitrificans* membrane proteins in the presence of [^{55}Fe]ferric D- or L-parabactin. When membrane proteins were incubated with (D)- or (L)-[^{55}Fe]parabactin (PB) and subjected to SDS-PAGE, all radiolabel moved with the dye front (i.e., none was protein bound). We therefore performed the same experiment with the nondenaturing, nonionic detergent Triton X-100 in place of SDS. (a) Results of such an experiment in which the gel matrix was polymerized from 10% monomers. The large diffuse spot at the bottom represents unbound radiolabel. Both (D)- and (L)-[^{55}Fe]parabactin were associated with distinct bands near the top of the gel. By far the most intensely labeled band is seen with L-[^{55}Fe]parabactin (arrow). (D)-[^{55}Fe]parabactin (arrow) did label at least one band, but it was clearly different in mobility from that labeled by (L)-[^{55}Fe]parabactin. We then repeated the experiment with a gel prepared from 7% monomers to form a more porous matrix. (b) Results demonstrating more clearly the difference between the (D)- and (L)-[^{55}Fe]parabactin-labeled bands (arrows). (D)-[^{55}Fe]parabactin labeled only one band of very low mobility near the top of the gel; this clearly differed from the strongly labeled single band of comparatively high mobility seen with (L)-[^{55}Fe]parabactin.

7% monomers to form a more porous matrix in hopes of better separation of these bands. Figure 5b demonstrates more clearly the difference between the D- and L-chelate-labeled bands (arrows). [^{55}Fe]ferric D-parabactin labeled only one band of very low mobility near the top of the gel. This clearly differed from the strongly labeled single band of comparatively high mobility seen with [^{55}Fe]ferric L-parabactin as the ligand.

On a 10% gel, a twin of that shown in Fig. 5a, Coomassie blue staining revealed that of the five or six distinct bands, only one was labeled by [^{55}Fe]ferric L-parabactin. These stained bands were excised with a razor blade and electrophoresed in a second dimension by SDS-PAGE. The radiolabeled band contained 80-kDa polypeptides as the major components.

DEAE-cellulose ion-exchange chromatography. The SDS-PAGE protein profiles of fractions from DEAE-cellulose column chromatography with ferric L-parabactin-binding activity are shown in Fig. 6b. Many lower-molecular-weight proteins eluted from the column in the earlier fractions and prior to NaCl reaching its maximum value. The 80-kDa proteins then eluted from the column at the end of the gradient (lanes 2 and 3), and they were substantially free of major contaminants. The samples corresponding to lanes 1 to 3 all contained ferric L-parabactin-binding activity and were combined to give a preparation with higher specific activity for [^{55}Fe]ferric L-parabactin binding (824 pmol/mg of protein) than the protein mixture applied to the column (306 pmol/mg of protein). Clearly, the 80-kDa proteins predominate. Nonetheless, several minor contaminants remained as revealed by SDS-PAGE.

DISCUSSION

In gram-negative bacteria, the expression of outer membrane proteins in response to iron starvation is common. Examples include *Vibrio cholerae* (27), *Klebsiella aerogenes* (30), *Campylobacter jejuni* (6), and *Agrobacterium tumefaciens* (13). Typically, these proteins are in the molecular mass range of 70 to 90 kDa and are widely assumed to function as receptors for ferric siderophores. However, only for *E. coli* has there been any biochemical evidence presented in support of this view. An 80-kDa outer membrane ferric enterobactin receptor has been purified and characterized with a K_d of ca. 0.3 μM (7, 10). Strong genetic evidence supports the proposed role in iron transport of the ferric enterobactin receptor encoded by the *fepA* gene, which has been mapped and sequenced (11).

In this report, we confirm the observation of Hoe et al. (9) that several 80-kDa region outer membrane proteins were derepressed by *P. denitrificans* in response to iron starvation (Fig. 3; unpublished observations). In addition, we present biochemical data which strongly implicate at least one of these 80-kDa region polypeptides to be responsible for stereospecific high-affinity binding of ferric L-parabactin by *P. denitrificans* membranes. Although we have yet to

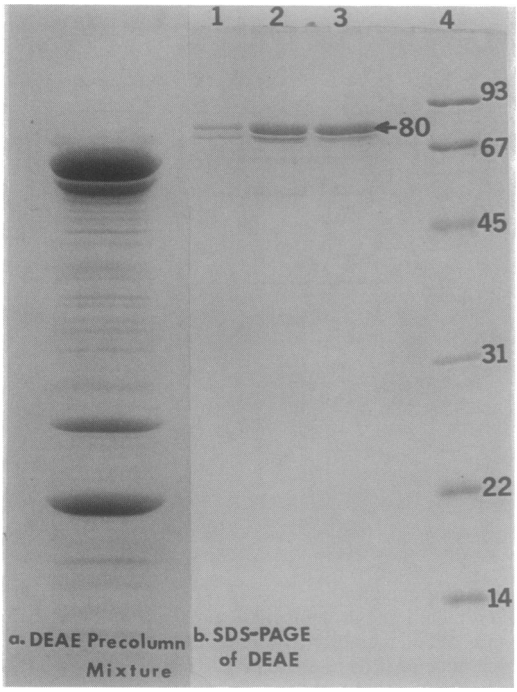


FIG. 6. SDS-PAGE polypeptide profile of DEAE-cellulose chromatography fractions containing [^{55}Fe]ferric L-parabactin-binding activity. (a) Enlarged view of the SDS-PAGE profile of the Triton X-100–EDTA-solubilized mixture of *P. denitrificans* outer membrane proteins applied to a DEAE-cellulose column which had been equilibrated with 50 mM Tris hydrochloride (pH 7.5) containing 0.4% Triton X-100 and 5 mM EDTA. After application, the column was eluated with several column bed volumes of this buffer, followed by buffer containing a 0 to 100 mM NaCl gradient. The protein in fractions of eluate was concentrated by salting out with 90% saturated ammonium sulfate at 4°C. Samples were then assayed for binding activity by the column centrifugation method, and the polypeptide composition was profiled by SDS-PAGE. No ferric L-parabactin-binding activity eluted from the column until the end of the gradient ($[NaCl]_{eluate}$ = 100 mM), when all the binding activity came off the column in a bolus. Lane 1 in panel B corresponds to combined fractions 36 and 37, in which the binding activity was first detected in the eluate; the remainder of the binding activity then eluted in fractions 38 to 43 (lane 2) and 44 (lane 3). SDS-PAGE showed that the low-iron-induced 80-kDa proteins copurified with the binding activity and that these were substantially free of major contaminants of lower molecular weight (see panel a), most of which eluted from the column in earlier fractions. The specific activity of the protein mixture put on the column was 306 pmol/mg of protein, compared to that of combined fractions 36 through 44, which had a specific activity of 824 pmol of [^{55}Fe]ferric parabactin bound per mg of protein.

achieve a homogeneous preparation, we found that the binding activity and the 80-kDa components on SDS-polyacrylamide gels copurified. The preparation which had the highest specific activity (824 pmol/mg of protein) for ferric L-parabactin binding was found to contain predominately 80-kDa components, with species of other molecular weights present only in minor amounts as determined by SDS-PAGE (Fig. 6). The view that one or more of these 80-kDa components are responsible for the binding activity was further supported by electrophoresis in nondenaturing gels, which allowed the band binding [^{55}Fe]ferric L-parabactin to be identified by autoradiography. When this band was excised and examined by SDS-PAGE, 80-kDa polypeptides were the major components present.

An unexpected finding was the presence of bands of protein in the nondenaturing gels which apparently stereospecifically bound ferric D-parabactin, the synthetic enantiomer of the natural siderophore of *P. denitrificans*. While the amount of this apparent high-affinity binding activity for ferric D-parabactin was much less than the amount of ferric L-parabactin-binding activity present in these same preparations (Fig. 5), the observation was reproducible. Moreover, [^{55}Fe]ferric D-parabactin-binding activity clearly differed in electrophoretic mobility from the band heavily labeled by [^{55}Fe]ferric L-parabactin. That *P. denitrificans* should contain even minor amounts of proteins stereospecifically recognizing the unnatural mirror image of its natural siderophore, L-parabactin is a puzzling finding. We are exploring this further.

Note that the oxazoline ring of L- or D-parabactin contains two asymmetric carbons derived from L-(2R,3S)- or D-(2S,3R)-threonine. In addition, ferric parabactin chelate exhibits chirality around the metal coordination center, as is characteristic of coordination compounds with three sets of bidentate ligands (5, 18). We have 300-MHz nuclear magnetic resonance evidence that ferric D-parabactin is mostly, if not exclusively, a right-hand (Δ) coordination propeller while ferric L-parabactin is a left-hand (Λ) coordination propeller (4). Our nuclear magnetic resonance data support the original interpretation by Neilands of circular dichroism spectra of ferric L-parabactin (19). Thus ferric L- and D-parabactin are of opposite configuration at all chiral centers. It is of interest, however, that the closely related compound ferric L-parabactin A, in which the oxazoline ring is hydrolyzed to give the open chain L-threonyl form (Fig. 1b), is of opposite coordination chirality (i.e., Δ) to that of ferric L-parabactin (19). An intriguing possibility is that *P. denitrificans* produces proteins which stereospecifically recognize the chiral metal coordination center of ferric L-parabactin A and that such a protein might well also recognize and bind ferric D-parabactin, since both have the same chiral coordination configuration (Δ).

Ferric siderophore outer membrane receptors, as a general rule, may recognize the chirality at the metal center as a primary prerequisite to binding (18). The Δ-chelates ferric L-parabactin A and ferric D-parabactin are unable to effectively reverse EDDA-induced iron starvation in *P. denitrificans* (2), possibly because the ferric L-parabactin outer membrane receptor is stereospecific for Λ-chelates. While they remain to be experimentally defined, proteins that recognize and bind ferric L-parabactin A may nonetheless be of importance in the *P. denitrificans* iron uptake apparatus. Of particular interest is the possibility that processing of ferric L-parabactin, after it is bound by the outer membrane receptor, could include hydrolysis of the oxazoline ring to give ferric L-parabactin A, which is much more readily reduced than ferric L-parabactin (24), to provide the cell iron in readily usable form, since parabactin does not form tight complexes with Fe(II). While current data are consistent with the ferric L-parabactin receptor primarily recognizing Λ-chirality at the metal center as the basis for its stereospecificity, further binding studies of parabactin analogs and related compounds of known coordination chirality are required. Such binding studies, as well as experiments to identify apparent specific ferric L-parabactin A-binding proteins, are in progress in our laboratory.

In summary, our current view is that iron transport in *P. denitrificans* involves the recognition and binding of ferric L-parabactin present in the extracellular milieu by a high-affinity receptor in the bacterial outer membrane. The poly-

peptide component(s) of this outer membrane receptor has been tentatively identified as one or more of the several low-iron-induced polypeptides in the 80-kDa region on SDS-polyacrylamide gels. Our laboratory is currently involved in the further characterization of the receptor, as well as in studies aimed at understanding and defining the steps involved in processing the complex between the initial binding to the receptor and the final delivery of iron to the cytoplasm.

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