

Outer-membrane siderophore receptors of heterotrophic oceanic bacteria

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Abstract

Pathogenic gram-negative bacteria use specific receptors to transport ferric siderophore complexes across their outer membrane during iron (Fe)-limited growth. Receptors such as these have not yet been characterized in oceanic heterotrophic bacteria. We examined four species of γ -proteobacteria for the presence of Fe-siderophore receptors with the use of a nondenaturing polyacrylamide gel electrophoresis binding assay and the siderophore ferrioxamine B (FB) labeled with ⁵⁵Fe. Small-subunit rRNA sequence analysis assigned these bacteria to the genera *Pseudoalteromonas* and *Alteromonas*. Two oceanic species, *Pseudoalteromonas haloplanktis* (Neptune) and *Alteromonas macleodii* (Jul88), which were shown previously to transport and assimilate Fe bound to FB during growth, synthesized an outer-membrane FB receptor under Fe-limiting conditions. Only low concentrations of the receptors were detected in these bacteria when they were grown with high concentrations of Fe. The FB receptor of *P. haloplanktis* (Neptune) had an apparent molecular mass of 79 kDa and an externally oriented binding site. The molecular mass of the receptor of *A. macleodii* (Jul88) was 100 kDa. No FB receptors were detected by our methods in two coastal species, *Pseudoalteromonas rubra* (LMG1) and *Pseudoalteromonas piscicida* (PWF3). *P. haloplanktis* (Neptune) and *A. macleodii* (Jul88) also bound ⁵⁵Fe-ferrichrome, a trihydroxamate siderophore like FB. Binding assays conducted with 115 nmol L⁻¹ ⁵⁵Fe-FB in the presence of increasing concentrations of desferrioxamine B showed a progressive decrease in the amount of ⁵⁵Fe-FB bound by the receptor protein, suggesting strong affinity of the receptor for the Fe-free siderophore. Our results provide the first demonstration of Fe-siderophore receptors in oceanic heterotrophic bacteria.

Heterotrophic bacteria have evolved Fe(III) transport systems that enable them to grow in environments containing extraordinarily low concentrations of Fe. When Fe is scarce, many of these organisms excrete low-molecular mass Fe-binding compounds, called siderophores, that bind free Fe(III) in solution. The Fe-siderophore complex attaches to its cognate receptor on the outer membrane of the cell and is subsequently internalized (Braun and Killman 1999; Ratledge and Dover 2000). Some bacterial receptors recognize more than one siderophore, not necessarily of the same structural type (e.g., FhuE of *E. coli*) (van der Helm 1998). Many bacteria express receptors for siderophores released by other species or for Fe sources contained in their hosts (Braun et

al. 1998; Ratledge and Dover 2000). Such uptake systems provide a high degree of selectivity and regulation and enable microbes to scavenge and solubilize Fe from otherwise inaccessible sources. The precise molecular mechanisms of Fe-siderophore transport are now being elucidated in *E. coli* and other laboratory strains (Ferguson et al. 1998; Buchanan et al. 1999; Ratledge and Dover 2000). Much less is known about Fe transport by aquatic bacteria, particularly among the heterotrophic marine species.

Concentrations of dissolved Fe in the open sea are among the lowest of any environment on Earth (Johnson et al. 1997). Some evidence suggests heterotrophic bacteria might be limited by these concentrations (Pakulski et al. 1996; Tortell et al. 1996), although other limitations may at times be important (Cotner et al. 1997; Cochlan 2001; Hall and Safi 2001). Because of their abundance and need for Fe, bacteria must play an important role in the Fe cycle in the sea and compete with the phytoplankton for this limiting resource (Tortell et al. 1996; Price and Morel 1998). Short-term uptake experiments show that bacteria take up most of the dissolved Fe (Tortell et al. 1996; Maldonado and Price 1999) and contain relatively large amounts of Fe in their biomass compared to other living organisms (Tortell et al. 1996). The reasons for the success of bacteria in acquiring Fe under these conditions are not well understood, but they may have to do with their ability to produce siderophores and to transport ferric siderophore complexes.

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Table 1. Place of origin and identification of marine, heterotrophic γ -proteobacteria. Oceanic habitats are >50 km from the nearest continental boundary and coastal habitats are <50 km from the nearest continental boundary. ATCC, American Type Culture Collection.

Strain name	Provenance (latitude, longitude)	Habitat	ATCC No.	Related γ -proteobacteria group/subgroup
Neptune	Subarctic Pacific Ocean 49°30.0'N, 137°40.0'W	Oceanic	Not deposited	<i>Pseudoalteromonas/P. haloplanktis</i>
Jul88	Sargasso Sea 32°10.0'N, 64°30.0'W	Oceanic	ATCC BAA-444	<i>Alteromonas/A. macleodii</i>
PWF3	Port Aransas, Texas 27°50.3'N, 97°03.1'W	Coastal	ATCC BAA-445	<i>Pseudoalteromonas/P. piscicida</i>
LMG1	Laguna Madre, Texas 26°48.9'N, 97°28.2'W	Coastal	ATCC BAA-446	<i>Pseudoalteromonas/P. rubra</i>

Free-living, heterotrophic marine bacteria under Fe-limiting conditions in the laboratory produce a variety of siderophores that comprise one or more of the reactive Fe-binding functional groups: the hydroxamates and the catecholates. The molecular structures of some of these siderophores have been determined (Haygood et al. 1993; Reid et al. 1993; Martinez et al. 2001). Their role in Fe complexation and the microbial ecology of the sea are of considerable interest, but not well understood. Microbial siderophores have not yet been identified in seawater, but electrochemical analyses distinguished two classes of Fe(III)-binding ligands in seawater that possess binding constants for Fe like those of the siderophores (Rue and Bruland 1995). Chemical analyses of some of this Fe-binding dissolved organic matter confirm that it contains the typical siderophore functional groups (Macrellis et al. 2001). Thus, microbial siderophores are likely to represent some of these Fe-binding ligands and to affect the chemistry of Fe and the ecology of the sea.

Recently, we examined the role of siderophores in Fe uptake and nutrition of a number of heterotrophic marine bacteria (Granger and Price 1999). Not all of these gram-negative strains produced siderophores under the assay conditions, but they all took up Fe bound to siderophores. Five of the strains transported Fe bound to desferrioxamine B (DFB), a terrestrial bacterial siderophore. The results suggested that these marine bacteria express receptor protein(s), under conditions of Fe limitation, which recognize ferrioxamine B (FB) and can use the bound Fe for growth.

Little has been done to identify siderophore receptors of free-living marine bacteria. The outer-membrane receptor for aerobactin was recently identified by cloning and sequence analysis of the coastal species *Vibrio* SD004 (Murakami et al. 2000). The predicted molecular mass of the encoded peptide was 77.9 kDa and the gene showed 41% homology with the *iutA* gene of *E. coli*. Reid and Butler (1991) reported differential expression of outer-membrane proteins of *Alteromonas luteoviolaceus* grown in high-Fe and low-Fe media. On the basis of size and relative abundance, they identified one protein as a putative alterobactin receptor. In a similar way, Guan et al. (2001) classified an outer-membrane protein as a siderophore receptor of the marine *Vibrio* V0210. However, in none of these cases were the proteins shown to bind ferric siderophores.

Here, we examine the Fe(III)-siderophore receptors of

four species of gram-negative heterotrophic bacteria isolated from oceanic and coastal regions of the sea. Two of the strains expressed an outer-membrane receptor, when Fe-limited, that bound Fe-siderophore complexes. The receptors differed greatly in size, and both showed strong affinity for the Fe-free siderophore DFB.

Materials and methods

All chemicals were purchased from Sigma unless otherwise stated.

Bacterial strains and identification—Four strains of gram-negative heterotrophic marine bacteria were used in this study (Table 1). DNA was extracted and purified from the bacterial isolates using a QIAGEN DNeasy Tissue kit. Small-subunit (SSU) rRNA genes were amplified from the DNA using 1.25 U Taq (Roche) in a 50- μ l reaction mix containing 10 mmol L⁻¹ Tris-HCl (pH 8.3), 1.5 mmol L⁻¹ MgCl₂, 50 mmol L⁻¹ KCl, 0.1 μ mol L⁻¹ of each oligonucleotide primer (27F and 1492r; Lane 1991), and 0.2 mmol L⁻¹ deoxynucleotide triphosphate with 30 cycles of 95°C, 55°C, and 72°C each for 1 min in a BioRad iCycler.

Amplified DNA was purified with the QIAGEN QIAquick PCR Purification kit, and the entire amplified product was sequenced with the ABI Prism BigDye (version 2.0) dye-terminator chemistry following the manufacturer's directions. Close relatives of these SSU rRNA gene sequences were identified by the Sequence Match program on the ribosomal database project server (<http://rdp.cme.msu.edu/html/>) [Maidek et al. 2001]) and the standard nucleotide-nucleotide BLAST program (<http://www.ncbi.nlm.nih.gov/blast/Blast.cgi>) [Atschul et al. 1997]).

Growth media and conditions—Bacteria were grown in acid-cleaned 2-liter polycarbonate bottles containing 1.5 liters of medium, bubbled with acid-cleaned sterile air, and incubated at room temperature. The medium was based on the synthetic seawater formulation, Aquil, containing phosphate and silicate (Price et al. 1988/1989). Vitamins, trace metals (buffered with 100 μ mol L⁻¹ edetic acid [EDTA]), and Fe (8.4 μ mol L⁻¹ for high Fe and 12.5 nmol L⁻¹ for low Fe) additions were made after the medium was sterilized, as previously described (Granger and Price 1999). Nitrate (final concentration 0.3 mmol L⁻¹), bactopectone (final

concentration 0.4 g L^{-1} ; Difco) and casein hydrolysate (final concentration 0.4 g L^{-1}) were purified of Fe and other trace metal contaminants by Chelex 100 ion exchange resin (Bio-Rad) and sterilized prior to addition. Experimental cultures were inoculated with 10 ml of bacteria from cultures acclimated to high- or low-Fe medium for approximately eight generations. Optical density (OD_{600}) was measured periodically to monitor growth.

Membrane preparation—Membrane preparations were made according to published methods (Filip et al. 1973; Cornelis et al. 1989; Reid and Butler 1991) with some modifications. Cells were harvested during late exponential growth (OD_{600} 0.5–0.6 for high-Fe cultures, 0.5 for low-Fe *Pseudoalteromonas rubra* [LMG1], and 0.3–0.4 for the other low-Fe cultures) by centrifugation at $6,000 \times g$ at 4°C for 50 min. After washing with sterile Aquil, bacteria were re-suspended in 25 ml Tris buffer (10 mmol L^{-1} , pH 7.2) containing $100 \mu\text{mol L}^{-1}$ phenylmethylsulfonyl fluoride (PMSF). The cell suspensions were sonicated on ice for 30 s 10 times with at least 30 s on ice between sonication bursts. Following five freeze–thaw cycles, cell debris was removed by two cycles of centrifugation at $14,000 \times g$ for 15 min. Whole membranes were isolated from the supernatant by centrifugation at $100,000 \times g$ for 1 h. To prepare outer-membrane fractions, the whole membrane pellet was incubated on ice in 5 ml 1% *N*-lauroylsarcosine (Sarkosyl) in Tris buffer for 50 min to solubilize the inner membranes. The outer membranes were then pelleted by centrifugation at $100,000 \times g$ for 1 h. Both whole and outer-membrane fractions were washed in 5 ml Tris buffer, re-pelleted by centrifugation at $100,000 \times g$ for 1 h, and stored at -30°C in fresh Tris buffer. Protein concentrations were measured with the Biorad D_c Protein Assay, a modified Lowry assay.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)—Membrane proteins ($20 \mu\text{g}$ per sample, except where stated) were separated by SDS-PAGE (Laemmli 1970) with 3.5% stacking gels and 10% separating gels. Protein bands were visualized with Coomassie Brilliant Blue R-250 (BDH).

Nondenaturing PAGE and ^{55}Fe -ferrioxamine B binding assay—Iron siderophore receptors were localized on non-denaturing gels with ^{55}Fe -ferrioxamine B (^{55}Fe -FB) as substrate following the procedures of Yamamoto et al. (1995) with some modifications. The ^{55}Fe -FB complex was prepared in a 1:10 molar ratio by adding ^{55}Fe (Perkin Elmer) to desferrioxamine mesylate (DFB) dissolved in Aquil at pH 3.3 (Maldonado and Price 1999). After complexation, the ^{55}Fe -FB complex was diluted in Aquil to a final concentration of $1.15 \times 10^{-7} \text{ mol L}^{-1}$ Fe and stored at -30°C . To initiate the Fe-siderophore binding assay, ^{55}Fe -FB was added to the membrane protein fractions ($10 \mu\text{l}$ ^{55}Fe -FB per $20 \mu\text{g}$ protein) and incubated on ice for 30 min. The samples were then mixed with six times sample buffer (7 ml 0.5 mol L^{-1} Tris [pH 6.8], 3 ml glycerol, 1 ml Triton X-100, 0.66 ml *n*-octyl β -D-glucopyranoside, 1.2 mg bromophenol blue) and incubated on ice for a further 15 min to solubilize the proteins. For each set of proteins, two gels were run simulta-

neously at 65 V at room temperature. Triton X-100 (BDH) was added in place of SDS to the resolving (6%) and stacking gels (3.5%). After electrophoresis, one of the gels was dried and exposed to an autoradiogram (Kodak BMS paper) with an LE intensifying screen (Kodak) for 3–4 d at -70°C . The other gel was stained with Coomassie Brilliant Blue. Any evidence of radioactivity on the developed autoradiogram was due to binding of ^{55}Fe -FB by proteins in the dry gel. There was no evidence of unbound ^{55}Fe -FB on control autoradiograms. The proteins responsible for this binding were then identified on the Coomassie-stained gel.

Binding of other ferrated ligands was tested by the same methods. Rhodotorulic acid, maltol, EDTA (BDH), ferri-chrome, and dipyriddy were mixed with ^{55}Fe in a 1:10 (Fe: ligand) molar ratio, as described for Fe-FB, and added to the membrane proteins at the same Fe concentration as ^{55}Fe -FB. None of these compounds were deferrated prior to complexation with ^{55}Fe and it is unknown whether the conditions for complexation were optimal for all the compounds. Membrane fractions were also incubated with $^{55}\text{FeCl}_3$ prior to electrophoresis. The influence of DFB on the binding of ^{55}Fe -FB to outer-membrane proteins was examined by preparing the ^{55}Fe -FB complex in 1:100 and 1:1,000 molar ratios of Fe to DFB. Binding assays were conducted as described above.

Gentle SDS-PAGE—An alternative method to identify ^{55}Fe -FB binding proteins employed the use of modified SDS-PAGE, in which reducing agents were omitted from the sample buffer and the sample was not heated prior to electrophoresis. Membrane fractions were incubated with ^{55}Fe -FB as above but were loaded onto the gel immediately after adding the sample buffer. All stacking gels were 3.5% and resolving gels were 6.5% for *Pseudoalteromonas haloplanktis* (Neptune) and 10% for *Alteromonas macleodii* (Jul88). Gels were run and processed as described for non-denaturing PAGE.

Identification of ^{55}Fe -FB binding proteins—Nondenaturing and gentle SDS-PAGE of outer-membrane proteins was conducted without the addition of ^{55}Fe -FB. The gels were fixed and stained with Coomassie Brilliant Blue. The protein bands responsible for ^{55}Fe -FB binding, identified as described above, were excised and electroeluted from the gel with the use of a 50 mmol L^{-1} ammonium bicarbonate buffer containing 0.1% SDS (pH 8.4). The samples were lyophilized, resuspended in Tris buffer, and then examined by SDS-PAGE.

Cellular localization of ^{55}Fe -FB binding proteins—Proteinase K (Invitrogen) was used to digest proteins on the outer membrane of Fe-deficient *P. haloplanktis* (Neptune) following the methods of Yamamoto et al. (1995). Bacteria were harvested and resuspended at an OD_{600} of ~ 1.5 in 10 mmol L^{-1} Tris-HCl buffer (pH 8.0) containing 2% NaCl and 10 mmol L^{-1} MgCl_2 . The suspension was split into three samples and incubated in a water bath at 37°C . Proteinase K was added to two of the samples at a concentration of 0.5 mg ml^{-1} . Samples were removed from the water bath after 30- and 60-min incubations. The third control tube that

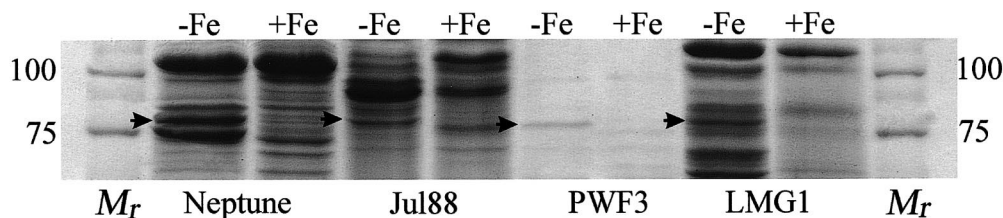


Fig. 1. Coomassie-stained outer-membrane proteins (10 μ g) separated by SDS-PAGE from four strains of heterotrophic marine bacteria grown in low- (-Fe) and high-iron (+Fe) medium. Arrows indicate the position of the \sim 79 kDa protein of the -Fe bacteria. Molecular mass markers (M_r , kDa) are shown.

lacked proteinase K was incubated for 60 min. Bacteria were harvested by centrifugation and resuspended in 20 ml of the above buffer supplemented with 1 mmol L⁻¹ PMSF. The cells were washed twice in this buffer and resuspended in 10 ml of the buffer and then disrupted by sonication. Outer-membrane proteins were prepared as described above and examined by SDS-PAGE and nondenaturing PAGE.

Nucleotide sequence accession numbers—The GenBank accession numbers for the sequences of *P. haloplanktis* (Neptune), *Pseudoalteromonas piscicida* (PWF3), *P. rubra* (LMG1), and *A. macleodii* (Jul88) are AY082669, AY082667, AY082666, and AY082664, respectively.

Results

Bacterial identification—All four of the bacterial strains we examined were γ -proteobacteria. Strain Neptune was most closely related to strain 12-13 in the *P. haloplanktis* subgroup, with a similarity score of 0.998 (GenBank accession No. AF069666; Wichels et al. 1998). The sequence of strain Jul88 was the same (score 1.00) as strains of the *A. macleodii* group from the northwest Pacific Ocean (GenBank entries U86468 and U86469; Fuhrman and Davis 1997). Strain PWF3 was most closely related to *Pseudoalteromonas piscicida* strain 0-7 (similarity score 0.999; DNA database of Japan [DDBJ] accession Nos. AB090232 and AB090233; H. Tsujibo and K. Miyamoto unpubl. data) and strain LMG1 was most closely related to *P. rubra* ATCC 29570T (similarity score 0.995; EMBL entry x82147; Gauthier et al. 1995).

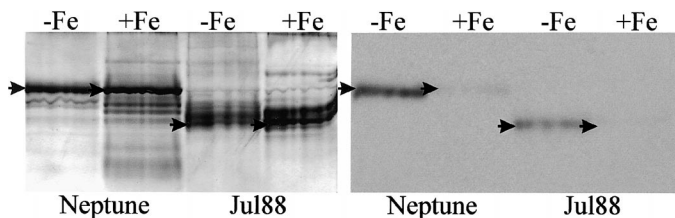


Fig. 2. Outer-membrane proteins of low- (-Fe) and high-iron (+Fe) *P. haloplanktis* (Neptune) and *A. macleodii* (Jul88) separated by nondenaturing PAGE after incubation with ⁵⁵Fe-FB. The left panel shows the Coomassie-stained gel and the right panel the corresponding autoradiogram. Protein bands that bound ⁵⁵Fe-FB are marked by arrows.

Outer-membrane proteins of Fe-replete and Fe-deplete bacteria—SDS-PAGE revealed differences in the types and amounts of outer-membrane proteins synthesized by bacteria in high- and low-Fe media (Fig. 1). A number of unique proteins were observed within and among the strains. In particular, a protein of approximately 79 kDa produced by all of the bacteria was more highly expressed under low- than high-Fe conditions.

Binding of ⁵⁵Fe-FB by outer-membrane proteins—On nondenaturing gels, the ⁵⁵Fe-FB binding assay detected a single protein band in *P. haloplanktis* (Neptune) and *A. macleodii* (Jul88) that was most apparent in the low-Fe bacteria (Fig. 2). Migration of the two ⁵⁵Fe-FB binding proteins on the gels differed between the species. Very small amounts of ⁵⁵Fe-FB bound to the outer-membrane proteins extracted from *P. haloplanktis* (Neptune) and *A. macleodii* (Jul88) when they were cultivated under high-Fe conditions. We were unable to demonstrate ⁵⁵Fe-FB binding by the outer-membrane proteins of *P. piscicida* (PWF3) or *P. rubra* (LMG1). *P. rubra* (LMG1) grew to a similar OD₆₀₀ in low-Fe and high-Fe media, suggesting it was not strongly Fe limited under these conditions. Greater Fe limitation was achieved when the Fe chelator, 2,2'-dipyridyl, was added to a final concentration of 200 μ mol L⁻¹ to a low-Fe culture once it reached an OD₆₀₀ of 0.1 (after 8 h incubation). The final OD₆₀₀ after dipyridyl treatment was 30% less than the untreated control. According to SDS-PAGE, different proteins were present in the *P. rubra* (LMG1) outer membrane in low-Fe medium with and without dipyridyl, but none of these were capable of binding ⁵⁵Fe-FB.

Identification of ⁵⁵Fe-FB binding proteins—Excision and electroelution of the ⁵⁵Fe-FB binding protein bands of *P. haloplanktis* (Neptune) and *A. macleodii* (Jul88) from nondenaturing gels yielded multiple protein bands on SDS-PAGE, so outer-membrane proteins were separated by gentle SDS-PAGE. Two protein bands in the low-Fe sample of *P. haloplanktis* (Neptune) bound ⁵⁵Fe-FB, whereas only a single band was observed with high Fe (Fig. 3). The faster migrating protein detected under high-Fe conditions was more highly expressed in the low-Fe cells. When both proteins were electroeluted from these gels and run on denaturing SDS-PAGE gels, the slower migrating band of the low-Fe sample corresponded to the 79-kDa protein (Fig. 3B) and the faster migrating band had a molecular mass of approx-

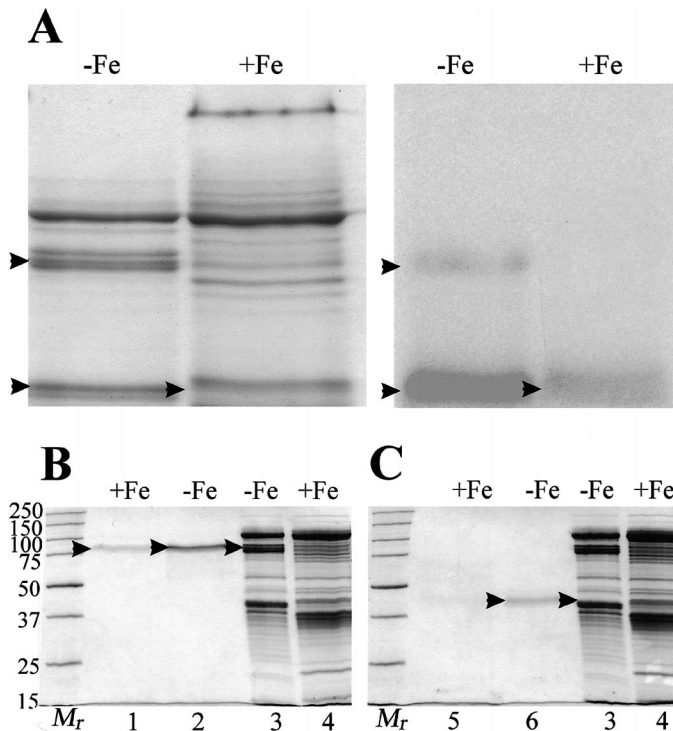


Fig. 3. (A) Outer-membrane proteins of low- ($-Fe$) and high-iron ($+Fe$) *P. haloplanktis* (Neptune) separated by gentle SDS-PAGE after incubation with ^{55}Fe -FB. The left panel shows the Coomassie-stained gel and the right panel the corresponding autoradiogram. Protein bands that bound ^{55}Fe -FB, marked by arrows, were electroeluted from the gel matrix and denatured for molecular mass analysis. (B) SDS-PAGE of the slow-migrating ^{55}Fe -FB binding band from low- ($-Fe$) and high-iron ($+Fe$) *P. haloplanktis* (Neptune). (C) SDS-PAGE of the fast-migrating ^{55}Fe -FB binding band from low- ($-Fe$) and high-iron ($+Fe$) *P. haloplanktis* (Neptune). Outer-membrane proteins of $-Fe$ (lane 3) and $+Fe$ (lane 4) *P. haloplanktis* (Neptune) are included for comparison. Molecular mass markers (M_r , kDa) are shown.

imately 40 kDa (Fig. 3C). When the active band of *A. macleodii* (Jul88) was electroeluted from a gentle SDS-PAGE gel and run on standard SDS-PAGE, its molecular mass was 100 kDa (Fig. 4).

Cellular location of ^{55}Fe -FB binding proteins.—An ^{55}Fe -FB binding assay with proteins extracted from all cellular membranes of *P. haloplanktis* (Neptune) revealed only a single band on the autoradiogram in the same place as with the outer-membrane proteins alone (data not shown). Proteinase K was used to degrade labile extrinsic proteins on the outer membranes of intact cells of strain *P. haloplanktis* (Neptune). After 30 and 60 min of treatment, the abundance of a number of proteins between 75 and 100 kDa decreased compared to controls (Fig. 5A). Binding activity of the putative FB receptor was completely eliminated by this treatment (Fig. 5B).

Fe-siderophore binding assay using different ligands and different concentrations of DFB—We tested the ability of outer-membrane proteins of *P. haloplanktis* (Neptune) and

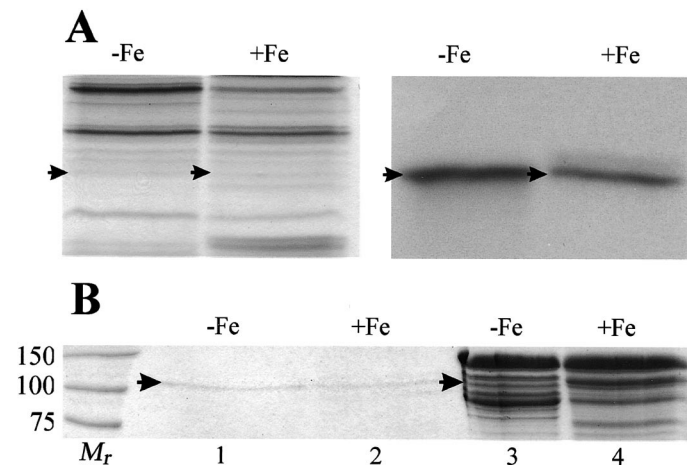


Fig. 4. (A) Outer-membrane proteins of low- ($-Fe$) and high-iron ($+Fe$) *A. macleodii* (Jul88) separated by gentle SDS-PAGE after incubation with ^{55}Fe -FB. The left panel shows the Coomassie-stained gel and the right panel the corresponding autoradiogram. Protein bands that bound ^{55}Fe -FB are marked by arrows. (B) SDS-PAGE of electroeluted ^{55}Fe -FB binding bands from low- ($-Fe$, lane 1) and high-iron ($+Fe$, lane 2) *A. macleodii* (Jul88). Outer-membrane proteins of $-Fe$ (lane 3) and $+Fe$ (lane 4) *A. macleodii* (Jul88) are included for comparison. Molecular mass markers (M_r , kDa) are shown.

A. macleodii (Jul88) to bind other ^{55}Fe -labeled ligands; only binding of ^{55}Fe -ferrichrome was observed (Fig. 6). This siderophore appeared to be bound by the same outer-membrane protein on nondenaturing PAGE that bound ^{55}Fe -FB. There was no evidence of binding of $^{55}FeCl_3$ or ^{55}Fe -rhodotorulic acid, maltol, EDTA or 2,2'-dipyridyl. The ^{55}Fe -EDTA com-

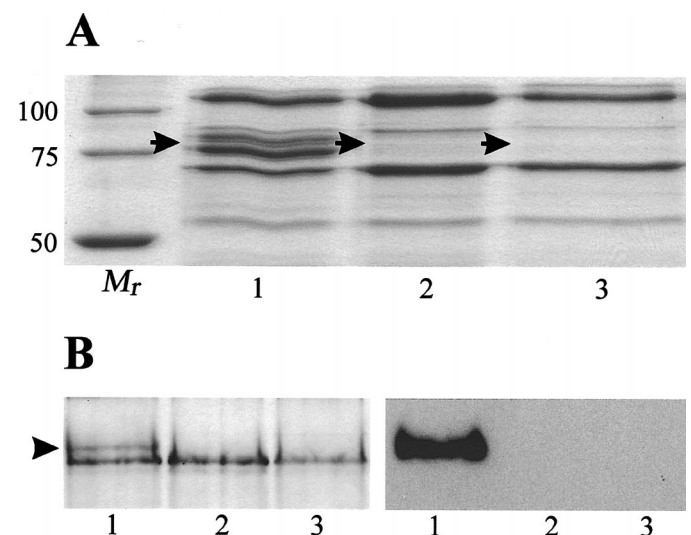


Fig. 5. (A) SDS-PAGE gel of outer-membrane proteins of iron-limited *P. haloplanktis* (Neptune) following exposure of intact cells to proteinase K for 30 min (lane 2) or 1 h (lane 3) at $37^\circ C$. Control cells (lane 1) were heated at $37^\circ C$ for 1 h without proteinase K. Arrows designate proteins of approximately 79 kDa. Molecular mass markers (M_r , kDa) are shown. (B) Nondenaturing PAGE (left panel) of the same proteins after incubation with ^{55}Fe -FB and the corresponding autoradiogram (right panel).

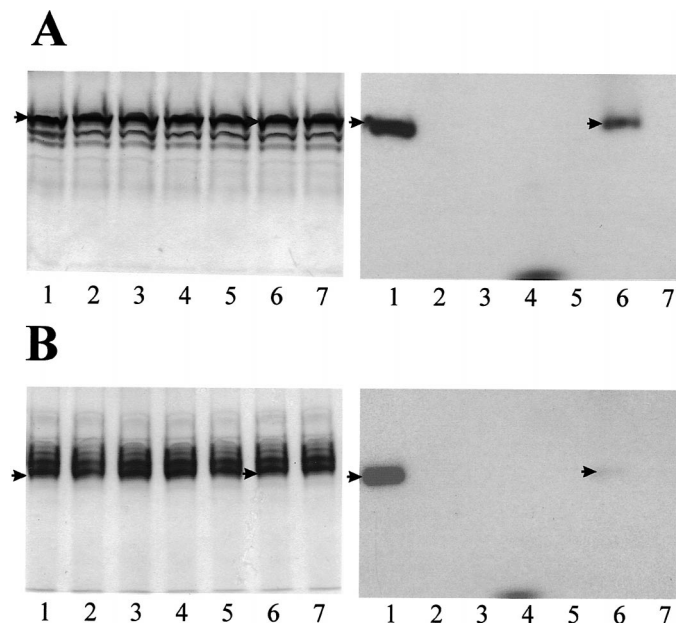


Fig. 6. Siderophore-binding assay of outer-membrane proteins of (A) iron-limited *P. haloplanktis* (Neptune) and (B) iron-limited *A. macleodii* (Jul88) on nondenaturing PAGE. ^{55}Fe was bound to siderophores and siderophore analogs in a 1:10 molar ratio prior to incubation with the proteins. The left panels show the Coomassie-stained gels and the right panels the corresponding autoradiograms. Lane 1, FB; lane 2, rhodotorulic acid; lane 3, maltol; lane 4, EDTA; lane 5, inorganic Fe (FeCl_3); lane 6, ferrichrome; and lane 7, dipyrldyl.

plex migrated at the gel front. When the concentration of DFB added in the binding assay was increased relative to ^{55}Fe (i.e., as the Fe:DFB molar ratio declined from 1:10 to 1:100 and 1:1,000), the amount of ^{55}Fe -FB bound by the receptors decreased (Fig. 7). This result was most pronounced for *A. macleodii* (Jul88), although significantly less binding was seen by the *P. haloplanktis* (Neptune) receptor with ^{55}Fe -FB in a 1:1,000 (Fe:DFB) ratio compared to a 1:10 ratio. All of the ^{55}Fe would have been complexed by DFB at the pH we conducted these assays ($\sim\text{pH } 7$), and most of the DFB would have been uncomplexed.

Discussion

Bacterial strains and environmental prevalence—The four bacterial species examined here were members of the γ -proteobacteria, a group well represented in marine communities. Sequence analysis of cloned polymerase chain reaction product from the Atlantic and Pacific Oceans, for example, has identified the prevalence of the so-called SAR 86 clade within this group, which comprises 10–15% of clones analyzed (Giovannoni and Rappe 2000). Recent reports document the widespread distribution of microbes closely related to *A. macleodii* (Acinas et al. 1999; Garcia-Martinez et al. 2002), one of the γ -proteobacteria we studied, and show that SAR 86 cells can account for 10% of the total bacteria (Eilers et al. 2000). Many of these γ -proteobacteria are attached to particles in the sea.

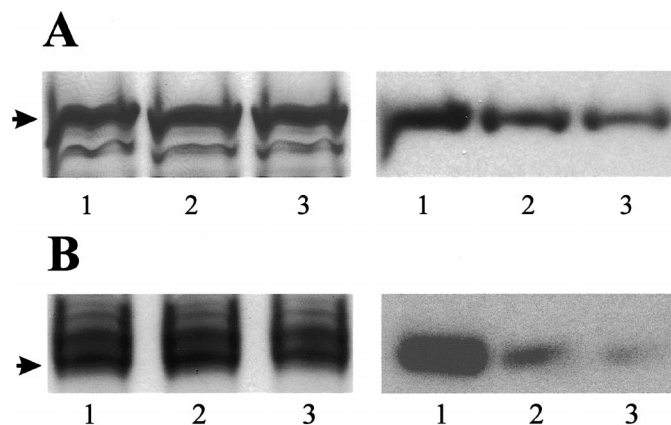


Fig. 7. Nondenaturing PAGE gel of outer-membrane proteins of (A) iron-limited *P. haloplanktis* (Neptune) and (B) iron-limited *A. macleodii* (Jul88). Proteins were incubated with 115 nmol L^{-1} ^{55}Fe and varying concentrations of DFB in the following molar ratios: lane 1, 1:10 Fe:DFB; lane 2, 1:100 Fe:DFB; and lane 3, 1:1,000 Fe:DFB. The left panels show the Coomassie-stained gels and the right panels the corresponding autoradiograms.

Some observations from enclosure experiments conducted in the waters of the subarctic Pacific and of coastal California are relevant to the results of our study. They show that γ -proteobacteria are greatly stimulated by Fe enrichment, possibly because of their greater sensitivity to changes in the supply of dissolved organic C from phytoplankton (Hutchins et al. 2001). An alternative hypothesis to account for their growth under these conditions is that γ -proteobacteria are better able than other groups to acquire Fe following enrichment and deprive competitors of this important resource. As described below, utilization of different types of siderophore complexes by some γ -proteobacteria could allow them to acquire Fe in many forms and be an important adaptation for their existence in Fe-poor parts of the sea.

Identification of ^{55}Fe -FB receptor proteins—Iron deficiency resulted in changes in the outer-membrane protein profiles of all four γ -proteobacteria. In each case, a 79-kDa protein was expressed in low-Fe cells that was either present in small amounts or absent from profiles of Fe-replete cells (Fig. 1). These proteins were of similar molecular mass to siderophore receptors identified from pathogenic bacteria (71–88 kDa; van der Helm 1998) and to the 85-kDa proteins expressed by *A. luteoviolaceus* and *Vibrio* V0210 in low-Fe medium (Reid and Butler 1991; Guan et al. 2001).

The presence of Fe-siderophore receptors on outer membranes of *P. haloplanktis* (Neptune) and *A. macleodii* (Jul88) grown in Fe-deplete media was initially detected on nondenaturing gels with ^{55}Fe -FB as substrate and an autoradiogram detection system (Fig. 2). Our previous work showed that both of these oceanic species were able to take up Fe from FB during short-term experiments (Granger and Price 1999). Transport rates measured over the first 30 min of incubation were 20 times faster in Fe-limited than in Fe-sufficient *P. haloplanktis* (Neptune).

This result correlates well with our observation that expression of the FB receptor is iron regulated: Fe deficiency

induces the receptor and Fe sufficiency represses it. Collectively, the results suggest that the receptors are indeed part of the Fe-siderophore uptake systems of these species. The presence of low levels of the receptors in the Fe-replete bacteria implies that they might be constitutively expressed. We note however, that Fe availability in the high-Fe cultures declines rapidly near the end of exponential growth. The bacteria might thus have already begun to synthesize the FB receptors in response to declining Fe concentrations when we harvested the cultures for analysis. Such a proposal is consistent with the high iron requirements of marine bacteria (Tortell et al. 1996) and the rapid induction of the Fe-FB transport upon transfer of Fe-replete bacteria to Fe-free medium (Granger and Price 1999).

Purification of the *P. haloplanktis* (Neptune) ^{55}Fe -FB binding protein confirmed it was 79 kDa (Fig. 3), like the protein identified initially (Fig. 1). On nondenaturing PAGE, only a single ^{55}Fe -FB binding protein band was detected (Fig. 2), but an additional band was also observed when the outer-membrane proteins were separated by gentle SDS-PAGE (Fig. 3). This smaller protein (~40 kDa) could represent a unique receptor not solubilized by the detergents used in nondenaturing PAGE (Triton X-100 and octyl glucopyranoside). However, we think that this is unlikely because we saw no evidence for such a receptor on the autoradiograms of the nondenaturing gels. In a few experiments in which the membrane proteins were not centrifuged prior to electrophoresis, nonsoluble receptors would have remained in the sample well of the stacking gel and been visible on the autoradiograms. It is more likely that the 40-kDa protein was a subunit or breakdown product of the 79-kDa protein that retained ^{55}Fe -FB binding activity.

The location of the FB receptor on the outer membrane of *P. haloplanktis* (Neptune) was verified by detecting a single ^{55}Fe -FB receptor in the whole membranes, as observed for the outer membranes. The elimination of ^{55}Fe -FB binding ability after intact cells were exposed to proteinase K for 30 min (Fig. 5) confirmed that the portion of the outer-membrane receptor responsible for siderophore binding was externally oriented.

The *A. macleodii* (Jul88) ^{55}Fe -FB binding protein proved to be larger than typical siderophore receptors, having a molecular mass of 100 kDa (Fig. 4). Although outer-membrane proteins of approximately 100 kDa have been shown to be affected by Fe availability in other bacterial species, none of them have been functionally identified (Deneer and Potter 1989*a,b*). The 79-kDa protein originally identified as a putative siderophore receptor in *A. macleodii* (Jul88) might also be a receptor, but for another type of siderophore. Large variation in the size of receptor proteins (79 vs. 100 kDa) for a single siderophore, such as FB, is not commonly observed (cf. van der Helm 1998). Interestingly, under the growth conditions reported here, *A. macleodii* (Jul88) expressed a ferric siderophore receptor but did not produce its own siderophores (Granger and Price 1999). This species could thus be opportunistic, relying on siderophores produced by other species to obtain Fe for growth.

Both *P. haloplanktis* (Neptune) and *A. macleodii* (Jul88) also bound ferrichrome, which, like FB, is a trihydroxamate siderophore (Fig. 6). The apparent lack of strict receptor

specificity and the ability to take up siderophores released by other bacteria would be extremely advantageous to microbes living in environments where the likelihood of encountering their own siderophores is reduced. Although neither ferrichrome nor FB have been identified from bacteria in the ocean, a marine *Vibrio* strain has been shown to produce desferrioxamine G (Martinez et al. 2001), a siderophore previously known from bacteria of terrestrial environments. Preliminary characterization of the siderophore produced by *P. haloplanktis* (Neptune) suggests it might also be a trihydroxamate (Granger and Price 1999). In addition, field experiments demonstrate that prokaryotes and eukaryotes can take up Fe from FB (Hutchins et al. 1999; Maldonado and Price 1999), suggesting that siderophores such as those used in this study might be found in nature.

In contrast to the oceanic (Neptune and Jul88) species, the coastal species *P. piscicida* (PWF3) and *P. rubra* (LMG1) did not bind ^{55}Fe -FB despite repeated attempts. Although both of these strains were known to release hydroxamate-type siderophores under these culture conditions, initial characterization would suggest they were not trihydroxamate structures (Granger and Price 1999). Because *P. piscicida* (PWF3) takes up Fe from ^{55}Fe -FB (Granger and Price 1999), we surmise that either the receptor was expressed at a level below the limit of detection of our assay or that the bacterium uses an alternative mechanism for Fe acquisition from FB. No ^{55}Fe -FB receptor was detected in *P. rubra* (LMG1), consistent with the lack of ^{55}Fe -FB uptake by this species (Granger and Price 1999). *P. rubra* (LMG1) also produces one or more catechol siderophores and might therefore have receptors with very different specificities than the ones we have characterized in the oceanic (Neptune and Jul88) species. Application of the nondenaturing gel assay to field samples, with the use of a variety of ^{55}Fe -labeled siderophores with both catecholate and hydroxamate functionalities, might be useful in identifying whether bacterial populations in the sea possess ferric siderophore receptors and hence use siderophores to acquire iron.

Mechanism of Fe-siderophore uptake—Results from the binding experiments that used a fixed concentration of ^{55}Fe -FB and varying concentrations of DFB showed that the amount of ferri-siderophore binding decreased as the concentration of Fe-free siderophore increased (Fig. 7). This suggests that DFB was bound by the receptor and competed with FB for the active site. At this time, we have not yet evaluated the relative affinities of the receptors for both substrates or independently confirmed the hypothesis that the Fe-free siderophore binds to the receptor. However, judging from the autoradiogram, there is roughly a 10-fold decrease in binding of ^{55}Fe -FB by *A. macleodii* (Jul88) with a 10-fold increase in DFB, implying that the receptor has a similar affinity for the Fe-loaded and Fe-free siderophore. A decrease in labeling of the *P. haloplanktis* (Neptune) receptor was also observed when the proportion of DFB/FB increased, although the result was less pronounced than in *A. macleodii* (Jul88). Receptor binding of apo-siderophores has been observed in other bacteria. Schalk et al. (1999, 2001) found the pyoverdine receptor, FpvA, of *Pseudomonas aeruginosa* bound its Fe-free siderophore, and the siderophore

receptor of *Aeromonas hydrophila* was also shown to bind an Fe-free siderophore analog with the same affinity as its ferric complex (Stintzi et al. 2000).

Outer-membrane receptors were thought until recently to bind only Fe-loaded siderophores, transport them across the outer membrane, and then release them into the periplasmic space. An alternative pathway for Fe-siderophore transport has recently been described (Stintzi et al. 2000) that involves an Fe exchange mechanism in which Fe is transferred from an Fe-siderophore complex to an Fe-free siderophore bound to its receptor and then subsequently internalized. Excretion of siderophores might thus function to prime bacterial receptors, allowing them to acquire Fe from a wide spectrum of organic complexes, including the siderophores of conspecifics. Because their receptors bind Fe-free siderophores, some marine γ -proteobacteria might also be able to use a similar type of exchange mechanism to acquire Fe.

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