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The importance of siderophores in iron nutrition of heterotrophic marine bacteria

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Abstract

Recent studies demonstrate that dissolved iron in seawater is bound to strong organic complexes that have stability constants comparable to those of microbial iron chelates. We examined iron acquisition by seven strains of heterotrophic marine bacteria from a number of siderophore–iron complexes, including desferrioxamine B (DFB) and marine siderophores partially purified from iron-limited cultures. Hydroxamate siderophores were detected in the supernatants of four strains, one of which also produced a catechol. All strains transported iron bound to siderophores regardless of whether or not they produced their own, and the majority took up iron bound to DFB. Uptake rates of Fe siderophores were similar among iron-limited strains and among ligands. Transport of FeDFB by strain Neptune was enhanced 20 times by iron limitation, whereas uptake of unchelated iron (Fe′) did not saturate at the highest concentration tested and was not regulated by the iron nutritional status of the cells. The half-saturation constant for uptake of FeDFB by Neptune was 15 nM, the lowest reported for an Fe siderophore in any microorganism. Iron uptake by the catechol-producing strain, LMG1, differed markedly in two respects from the other strains: LMG1 could not take up iron bound to DFB; furthermore, transport of Fe′ by iron-limited LMG1 was 10 times faster than the other strains and was upregulated 46 times compared to Fe-sufficient cells. Experimental evidence suggests that iron transport by LMG1 may be mediated by surface-associated catechol siderophores that scavenge inorganic ferric species as well as iron bound to weaker complexes, such as EDTA (ethylenediaminetetraacetic acid). The combined results of the study highlight the importance of siderophores in iron transport by heterotrophic marine bacteria and suggest, by inference, that bacteria may rely on siderophores to acquire iron in situ.

Life is contingent on a number of essential elements, among which iron enjoys a status of notable importance. Its biological utility stems from its propensity to form complexes with ligands containing O, N, and S that vary widely in redox potential. Indeed, the redox potentials of iron-containing catalysts span an entire volt, from −0.3 to +0.7 V, depending on the iron ligands and the protein environment (Braun et al. 1998). Organisms have exploited the chemical properties of Fe in a variety of biochemical reactions, including respiratory and photosynthetic electron transport, nitrate reduction, chlorophyll synthesis, nitrogen fixation, and detoxification of oxygen radicals.

In spite of its abundance in the earth's crust, iron is a scarce resource in aerobic environments. The aqueous chemistry of iron is characterized by the exceedingly low solubility of Fe(III), which, at neutral pH, readily forms refractory, particle-reactive hydroxide species. Dissolved-iron concentrations are particularly low in the surface waters of the open ocean. Low external inputs from aeolian deposition (Duce and Tindale 1991) and deep-water upwelling (Coale et al. 1996*a*), compounded with fast removal rates due to particle scavenging, result in concentrations averaging 0.07 nmol kg^{-1} throughout the oceans (Johnson et al. 1997). Iron-enrichment experiments show that these low concentrations limit phytoplankton productivity and biomass in large parts of the Pacific and Southern Oceans (Martin and Fitzwater 1988; Martin et al. 1990, 1994; Price et al. 1991, 1994; Coale et al. 1996*b*).

Recent investigations on the chemical speciation of iron demonstrate that >99.9% of the dissolved iron in the surface ocean is tightly bound to organic ligands. These ligands have been detected in the North Sea (Gledhill and van den Berg 1994), the Western Mediterranean (van den Berg 1995), the North Pacific (Rue and Bruland 1995), the Northwest Atlantic (Wu and Luther 1995), and the equatorial Pacific oceans (Rue and Bruland 1997). They are present at high concentrations in seawater (0.5–15 nmol kg^{-1}) and bind practically all the dissolved iron, leaving <0.05 pmol kg^{-1} as inorganic ferric species. The exact chemical nature of the organic ligands is uncertain, but they possess stability constants similar to those measured for microbial iron chelates (Rue and Bruland 1995).

Under conditions of cellular iron deprivation, many microorganisms release small (ca. 1,000 Da) molecules called siderophores that exhibit extraordinarily high complex formation constants for Fe(III) (Raymond et al. 1984). Siderophores solubilize Fe(III) extracellularly and facilitate its transport into the cell. They typically contain hydroxamates or catecholates that function as Fe-chelating groups; however, a number of other functional groups have also been characterized (Winkelmann 1990). Heterotrophic bacteria isolated from various marine habitats produce siderophores in low iron media (Gonye and Carpenter 1974; Trick 1989), some of which have been isolated and chemically characterized (Takahashi et al. 1987; Jalal et al. 1989; Reid et al.

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1993). Most striking, however, is a planktonic *Vibrio* sp. from the coast of West Africa that excretes aerobactin (Haygood et al. 1993), a well-characterized hydroxamate siderophore produced and utilized by various species of enteric bacteria, such as *Escherichia coli* (Winkelmann 1990).

Heterotrophic marine bacteria account for as much as 50% of the carbon biomass in the sea (Fuhrman et al. 1989) and an equivalent amount of the biogenic iron (Tortell et al. 1996). Recent experiments demonstrate that they are stimulated by Fe enrichments (Pakulski et al. 1996) and that they have high Fe requirements for growth (Tortell et al. 1996). Thus, in low iron waters, bacteria may produce siderophores to alleviate Fe stress and, in doing so, contribute to the pool of organically complexed iron. Little is known, however, of the means by which marine bacteria obtain iron for growth.

Exhaustive research on iron metabolism in terrestrial and pathogenic strains has established that they are equipped with a variety of mechanisms to acquire chelated iron (Byers and Arceneaux 1998). Ferric iron is generally transported as an Fe(III)–siderophore complex that enters the periplasmic space of gram-negative bacteria through specific outer-membrane receptors. Bacteria express outer-membrane receptors that recognize iron bound to their own siderophores as well as receptors specific for siderophores produced by other species. Once in the periplasm, ferrated siderophores are transported to the cytoplasm via nonspecific ferrisiderophore transporters embedded in the bacterial cytoplasmic membrane. Many pathogenic strains, dependent on the highly chelated iron reserves of the host organism, also express outer-membrane receptors that target the iron-containing molecules of their host (Braun et al. 1998). Iron taken up at the outer membrane as an Fe(III) chelate (such as transferrin, lactoferrin, or citrate) may be released as inorganic Fe(III) in the periplasm and subsequently internalized by the inorganic Fe(III) uptake system. No inorganic Fe(III) transporter has been found on the outer bacterial membrane (Zimmermann et al. 1989). These observations suggest that bacteria rely on siderophore uptake mechanisms to obtain iron and that inorganic Fe(III) may not represent a significant nutritional pool in Fe-poor environments.

Marine bacteria produce siderophores, but little is known about their role in iron acquisition. We present here a study on iron transport by heterotrophic marine bacteria. Using siderophores partially purified from marine strains and the fungal siderophore DFB, we show that all the strains surveyed utilize iron bound to siderophores. The results highlight the importance of organic iron transport for bacterial metabolism and provide strong support that heterotrophic marine bacteria may rely on organically bound iron for growth in situ.

Materials and methods

Study organisms—Seven gram-negative bacteria were examined. All strains were planktonic heterotrophs obtained from coastal and offshore waters. Strains PWF3 and *Vibrio natriegens* (PWH3a) were isolated from the Gulf of Mexico, adjacent to the University of Texas Marine Science Institute Pier; strain LMG1 was isolated from Laguna Madre in the Gulf of Mexico, and strains *Pseudomonas* sp. (Isol5) and Jul88 were isolated from Sta. S in the Sargasso Sea (Suttle and Chan 1993). Strains Neptune and P20pac were isolated from the NE subarctic Pacific, at Sta. P19 and P20 (*see* LaRoche et al. 1996). Cultures were initiated from single colonies that grew on seawater agar plates supplemented with 0.5 g liter^{-1} bactopeptone (0.05 g liter^{-1} for Neptune and P20pac). Bacteria were kept in liquid nitrogen in seawater medium containing 20% dimethylsulfoxide and recultured as required.

Growth medium—Bacteria were grown at 20°C in batch culture in the artificial seawater medium Aquil (Price et al. 1988/1989), containing 10 μM phosphate (PO_4^{3-}). Synthetic ocean water and all the nutrient-enrichment stock solutions were purified of trace metals using Chelex 100 ion exchange resin (Bio-Rad Laboratories), according to the procedure of Price et al. (1988/1989). Media were sterilized by microwaving in acid-washed Teflon bottles (Keller et al. 1988) and enriched trace-metal and vitamin (B12, thiamin, and biotin) solutions that were filtered through metal-free 0.2-μm filters (Acrodisc). The organic enrichments were purified of trace metals with Chelex 100 and autoclaved before they were added to sterile media (0.2 g liter^{-1} bactopeptone [Difco] and 0.2 g liter^{-1} casein hydrolysate).

Trace-metal additions were buffered with 100 μM EDTA, so that Cu^{2+}, Mn^{2+}, Zn^{2+}, and Co^{2+} free-ion concentrations were $10^{-13.8}$ M, $10^{-8.3}$ M, $10^{-10.9}$ M, and $10^{-10.9}$ M, respectively. These concentrations were calculated using the chemical equilibrium program MINEQL (Westall et al. 1976). They represent approximations because the affinities of the metals for the organic substrates were unknown and hence, could not be included in the calculation. Total Mo and Se concentrations in the media were 10^{-7} M and 10^{-8} M, respectively. Premixed FeEDTA (1 : 1) was added separately at a total concentration (Fe_T) of 8.4 μM for iron-replete and 12.5 nM for iron-deficient medium. To minimize contamination, all manipulations were performed in a laminar flow hood. Media were allowed to equilibrate chemically overnight and were stored in sterile, acid-washed polycarbonate bottles rinsed with 18.2 Mohm resistivity, Milli-Q water (Millipore).

Siderophore isolation medium—An alternate medium containing high concentrations of organic substrates was used to maximize siderophore production by the bacteria and facilitate siderophore isolation and purification. Aquil was buffered at pH 8.2 with 0.5 M Tris and supplemented with 350 μM NH_4^+, 100 μM PO_4^{3-}, 2 g liter^{-1} glucose, 2 g liter^{-1} bactopeptone (Difco), and 2 g liter^{-1} casein hydrolysate and vitamins. The following concentrations of trace metals were added to the medium: 2 nM Cu, 45 nM Mn, 8 nM Zn, 5 nM Co, 2 nM Mo, 2 nM Se, and 2 nM Fe. EDTA was purposefully omitted from the recipe because it reacted positively with the siderophore assay. Batch cultures were grown at room temperature in 2-liter polycarbonate bottles, bubbled vigorously with sterile air. All solutions were passed through a Chelex 100 column to remove trace metals, and manipulations were performed using sterile, trace-metal clean techniques.

Measurements of siderophores—Cell-free supernatants of stationary-phase cultures grown in the siderophore isolation medium were assayed for the presence of iron-binding compounds with the chrome azurol S (CAS) assay of Schwyn and Neilands (1987). Standard curves relating CAS reactivity to the concentration of iron-binding ligands were determined using the fungal siderophore DFB (Desferal, Ciba-Geigy). The quantity of iron-binding ligands produced by the bacteria is reported in terms of iron-binding equivalents, expressed as moles per gram dry weight of bacteria. Hydroxamate functionalities were detected with the Csaky test for bound hydroxylamine (Gillam et al. 1981) with hydroxylamine as a standard. The concentration of the catechol moiety was measured using two assays: the Arnow (1937) test and the Rioux assay (Rioux et al. 1983), both of which were standardized with 2,3-dihydroxybenzoic acid (2,3-DHBA).

Isolation of siderophores—Iron-free siderophores were obtained following published methods (Wilhelm and Trick 1994, 1995) with minor modifications. Bacterial cultures were grown to late stationary phase ($>10^9$ cells ml^{-1}, ca. 48 h after inoculation) and harvested by centrifugation at 8,000 rpm for 40 min. The supernatant was acidified to pH 3 with concentrated HCl and passed through an Amberlite XAD-16 (Supelco) column that was previously washed with pH 3, Milli-Q water to dissolve adsorbed metals. After extraction of the organics by the XAD resin, the column was rinsed with acidified water to remove metals and sea salts and allowed to dry overnight. Organic compounds, including the siderophores, were desorbed from the resin with two to three washes of methanol (Analar).

The methanol extracts were concentrated to a paste by rotary evaporation in acid-cleaned flasks and resuspended in a small volume (1–5 ml) of buffer: catechol-containing extracts were dissolved in 0.01 M phosphate buffer at pH 7 to minimize irreversible oxidation of the catechol functionality at low pH (Mentasti et al. 1973), while extracts that were not catechol reactive were resuspended in 0.01 M acetate buffer at pH 4. Partial purification of the siderophores was achieved by fractionation of the extracts on a Sephadex LH-20 (Pharmacia) column (50 by 1 cm) in their respective buffers. The eluting buffers were purified with Chelex 100, and metals were removed from the Sephadex by eluting the column with a small volume of EDTA solution (100 μM). The CAS assay-reactive fractions produced by each bacterium were pooled and concentrated fivefold by lyophilization. The concentration of desferrisiderophores in the resulting stock solutions was measured with the CAS assay (i.e., iron-binding equivalents expressed as a molar concentration). The concentration of ferrated siderophores in these solutions was assumed to be negligible.

Determination of sulfoxine-reactive iron (Fe_R)—The iron-binding strengths of the isolated siderophores were determined empirically with sulfoxine (Hudson et al. 1992). This method measures both the equilibrium concentration of inorganic Fe and the FeL that react with sulfoxine. A concentration of 10 nM ^{55}Fe complexed to 1 μM siderophore (i.e., 1 μM iron-binding equivalents) was preequilibrated in the dark for 8 h in synthetic ocean water (pH 8.2). Excess sulfoxine was added and briefly allowed to react for 30 s with the unbound, labile iron in solution, after which the sulfoxine–iron complex was extracted into toluene containing trioctylmethylammonium chloride as an ion-pairing reagent. The fraction of labile iron (inorganic iron plus labile iron complexes) recovered in toluene was used as a relative measure of the iron-binding strength of the siderophores. Sample blanks were determined by omitting sulfoxine from the assay solutions. Organic ligands with known stability constants (EDTA, DFB, and NTA) were used as reference.

Growth rate measurements—Changes in bacterial density were measured spectrophotometrically on a Cary 1E UV-VIS spectrophotometer (Varian) at 600 nm. Cells were acclimated to iron-replete (8.4 μM Fe_T) or iron-deficient (12.5 nM Fe_T) growth medium for approximately eight generations, after which a small inoculum was transferred into fresh medium in polystyrene cuvettes containing a teflon stir bar. The cuvettes and stir bars were previously acid washed and sterilized by microwaving in Milli-Q water. The cuvettes were sealed with parafilm (previously soaked in 70% ethanol) and placed in the spectrophotometer equipped with a temperature-controlled, multisample holder. The temperature was set at 20°C, and automated measurements of absorbance were taken at preprogrammed time intervals. Absolute growth rates were determined from log-linear regressions of absorbance over time during the exponential growth phase.

Measurements of iron quotas—Iron quotas (moles of Fe per cell) were measured using the radiotracer $^{55}FeCl_3$ (specific activity 15–25 mCi mg^{-1}, DuPont Canada). Cells were taken through one transfer in growth medium containing 1 or 10% of total Fe as $^{55}FeCl_3$ (12.5 nM and 8.4 μM Fe medium, respectively) to ensure uniform labeling. Triplicates were then inoculated in fresh radioactive medium. In late exponential phase, samples were filtered (<100 mm Hg pressure) onto 0.2-μm pore-size polycarbonate filters (Poretics) and washed with a titanium(III) EDTA–citrate reducing solution to dissolve ferric species adsorbed to the cell surfaces (Hudson and Morel 1989). Particulate ^{55}Fe was measured by liquid scintillation counting on a Beckman LS 6500 counter. Cell densities were determined by epifluorescence microscopy with acridine orange staining (Hobbie et al. 1977).

Measurements of iron uptake rates—Short-term iron uptake rates (moles of Fe per cell per minute) were measured in the dark at 20°C with ^{55}Fe. Cells were grown in either iron-replete or iron-deplete medium and harvested in late exponential phase (10^7 cells ml^{-1}) by centrifugation at 8,000 rpm for 40 min. Some strains, namely *Pseudomonas* sp., P20pac, and *V. natriegens,* required higher cell densities to obtain pellets during centrifugation. These cultures were harvested when cell densities were 5×10^7 cells ml^{-1}. Cells were resuspended in acid-washed polycarbonate bottles containing uptake medium.

The bottles were subsampled for particulate iron regularly for up to 1 h. Samples were fixed with 4% borate-buffered formalin and then filtered and rinsed as described for the Fe quota measurements. Samples for cell density were obtained

Table 1. Absolute and relative growth rates and intracellular iron quotas of seven bacterial isolates in iron-replete and iron-deficient medium. Maximum growth rates (μ_{max}) and quotas (Q_{max}) were determined in iron-replete medium. Values are means of duplicate measurements ±1 SD.

Strain	Growth rate (d^{-1} ± SD)			Iron quota ($\times 10^{-20}$ mol Fe $cell^{-1}$ ± SD)		
	Iron-replete μ_{max}	Iron-deficient μ	μ/μ_{max}	Iron-replete Q_{max}	Iron-deficient Q	Q_{max} Q^{-1}
Neptune	12.9 ± 0.95	6.74 ± 1.87	0.52	43.4 ± 11.7	0.37 ± 0.06	117
P20pac	4.27 ± 0.32	1.21 ± 0.23	0.28	58.5 ± 12.5	2.61 ± 0.03	22
Pseudomonas sp. (Isol5)	3.59 ± 0.32	3.77 ± 0.18	1.0	5.31 ± 2.27	1.57 ± 0.26	3.4
Jul88	7.37 ± 1.01	2.18 ± 0.23	0.30	16.1 ± 0.01	0.50 ± 0.13	32
LMG1	15.7 ± 0.63	9.95 ± 1.16	0.63	61.9 ± 15.3	5.60 ± 0.98	11
PWF3	11.9 ± 0.08	5.79 ± 2.27	0.49	51.8 ± 10.5	0.72 ± 0.15	72
V. natriegens (PWH3a)	20.9 ± 2.06	5.55 ± 0.10	0.26	54.7 ± 14.9	2.22 ± 0.55	25

at the initial and final sampling times, and the densities were extrapolated log linearly for the intermediate samples. Iron uptake rates were calculated from the slope of the least-squares linear regression between iron quotas and time.

Iron uptake media—Three types of media were used for Fe uptake experiments.

Inorganic Fe uptake medium: Growth medium containing standard additions of trace metals and organic substrates was supplemented with a range of total iron concentrations (Fe_T) at a fixed EDTA concentration (100 μM). Labile inorganic iron concentrations in the resulting media (sulfoxine-reactive iron: Fe_R) were directly proportional to the total iron added, Fe_T: $\log Fe_R = -1.6 + 1.07 \log Fe_T$; $n = 7$; $r^2 = 0.99$; $Fe_T = 12.5$ nM to 8.4 μM. These values are greater than those reported by Sunda and Huntsman (1995) for media containing similar concentrations of iron and EDTA trace metals. The presence of organic substrates in our medium may account for the greater kinetic lability of iron observed here. In one experiment, the concentration of EDTA was varied to establish the potential importance of Fe acquisition from the EDTA complex.

Inorganic Fe uptake medium supplemented with siderophores: Iron-deficient growth medium containing 12.5 nM $^{55}Fe_T$ and EDTA trace metals was supplemented with 5 μM siderophore (i.e., 5 μM iron-binding equivalents). The medium was preequilibrated for at least 8 h to allow chemical equilibrium between the added siderophore and EDTA.

Siderophore Fe uptake medium: Siderophore uptake medium consisted of synthetic ocean water supplemented with PO_4^{3-} and organic substrates preequilibrated with ^{55}Fe siderophores. Siderophores (1 or 10 μM iron-binding equivalents) were added in excess of iron (0.2–55 nM) to ensure complete binding of the iron. Uptake of the marine siderophore nep-L was not investigated in this medium because a large fraction of the added iron remain unchelated (*see Table 3*), making it impossible to measure the effect of the chelated iron exclusively.

Results

Effect of iron on growth rates and quotas—Growth rates of the strains decreased significantly in iron-deficient (12.5 nM Fe_T) relative to iron-replete growth medium (8.4 μM Fe_T; Table 1). *Pseudomonas* sp. was an exception, as it showed no growth reduction under low iron conditions. On average, iron deficiency elicited a 50% reduction in growth rate among the strains: maximum rates with high iron were variable, ranging from 3.6 d^{-1} for *Pseudomonas* sp. to 21 d^{-1} for *V. natriegens,* and iron-limited rates were as slow as 1.2 d^{-1} for P20pac but as fast as 10 d^{-1} for LMG1.

Iron quotas of all the strains were reduced in iron-deficient Aquil growth medium (Table 1). Under high iron conditions, quotas averaged 42 $\times 10^{-20}$ mol Fe $cell^{-1}$ and decreased to an average of 2 $\times 10^{-20}$ mol Fe $cell^{-1}$ as Fe in the medium was reduced to 12.5 nM. The greatest reduction in cellular iron was observed for Neptune, whose quota in iron-replete medium (43 $\times 10^{-20}$ mol Fe $cell^{-1}$) was 120 times greater than in iron-deficient medium (0.37 $\times 10^{-20}$ mol Fe $cell^{-1}$). *Pseudomonas* sp. contained the smallest Fe quota at high Fe (5.3 $\times 10^{-20}$ mol Fe $cell^{-1}$) and showed only a threefold decrease in iron-deficient medium (1.6 $\times 10^{-20}$ mol Fe $cell^{-1}$). Strain LMG1, which maintained the fastest growth rate in iron-deficient medium, also had the highest iron quota in iron-deficient medium (5.6 $\times 10^{-20}$ mol Fe $cell^{-1}$).

Measurements and preliminary characterization of siderophores—Extracellular iron chelators measured with the CAS assay were detected in the supernatants of four of the seven strains surveyed (Table 2). Of the two strains isolated from iron-limited waters of the North Pacific, only Neptune produced detectable concentrations of siderophores. No siderophores were detected in the supernatant of the two Sargasso Sea isolates, but all three isolates from the Gulf of Mexico produced detectable concentrations of ligands under the experimental culture conditions. A mean concentration of 8.8 μmol $gdwt^{-1}$ desferri ligand was detected among the producing strains, ranging from as low as 0.62 μmol $gdwt^{-1}$ for *V. natriegens* to 15.6 μmol $gdwt^{-1}$ for strain LMG1. Production was induced by iron deficiency, because no CAS-reactive substances were detected in the cultures when the growth medium was supplemented with 2 μM Fe (determine for strain Neptune only—Granger 1998). Moreover, the differences in siderophore concentration observed between strains did not result from differences in cell yield, because no relationship was apparent between final cell yield and siderophore concentration (Granger 1998).

The Csaky test revealed that the hydroxamate moiety was

Table 2. Concentration of iron ligands and hydroxamates (expressed in μmoles per gram dry weight of cells) in the supernatants of seven bacteria grown in siderophore isolation medium containing 2 nM total Fe. The CAS assay was used for desferriligands, and, as described in Methods, measures the concentration of iron-binding equivalents relative to DFB. The Csaky test was used for hydroxamate functionalities. Values represent the mean ± 1 SD for three replicate experiments (two replicates per experiment). Dashed lines indicate that the concentration of the substance was below the detection limit of the assay.

Strain	CAS assay (μmol ligands $gdwt^{-1}$)	Csaky test (μmol hydroxamate $gdwt^{-1}$)
Neptune	13.1 ± 5.14	44.2 ± 23.6
P20pac	—	—
Pseudomonas sp. (Isol5)	—	—
Jul88	—	—
LMG1	15.6 ± 5.54	19.4 ± 9.68
PWF3	5.99 ± 2.42	10.0 ± 4.52
V. natriegens (PWH3a)	0.62 ± 0.17	0.66 ± 0.24

present in the supernatants of all the strains that produced CAS-reactive substances (Table 2). None of the CAS-negative supernatants reacted positively with the Csaky test. The ratio of Csaky to CAS reactants ([hydroxamate]/[desferri ligand]) varied from 0.5 to 3.4, indicating that the siderophores may possess one to three hydroxamate functional groups around each iron center.

The supernatant of a single strain, LMG1, reacted positively for both the Arnow (14.2 ± 7.63 μmol $gdwt^{-1}$) and Rioux (25.9 ± 8.76 μmol $gdwt^{-1}$) tests for catechols. A significant reaction was also observed in the supernatant of *V. natriegens* with the Rioux assay (28.4 ± 7.41 μmol $gdwt^{-1}$) yet not with the Arnow test. These tests have different specificities, which may account for these results. The Arnow does not detect catechols sterically hindered at the three or four position, while the Rioux can detect a number of compounds, such as phenolics, that reduce iron at low pH. Because the concentration of catechols detected by the Rioux assay in the supernatant of *V. natriegens* was well in excess of the concentration of CAS ligands (0.62 μmol $gdwt^{-1}$), it is unlikely that the compound(s) was a siderophore.

Isolation of siderophores—The extracellular iron-binding ligands measured in the supernatants of the strains Neptune, PWF3, and LMG1 were isolated and partly purified by gel-permeation chromatography. A single CAS-reactive band was recovered for Neptune from the fractionation of the XAD concentrate on a Sephadex LH-20 column. This siderophore was henceforth referred to as "nep-L." Two CAS-reactive bands were collected and pooled for PWF3 and are collectively called "pwf-L," and two to three CAS-reactive bands were collected and pooled for LMG1 and are referred to as "lmg-L."

Measurements of sulfoxine-reactive iron in seawater containing 10 nM iron and 1 μM ligand showed that >99% of the iron was bound to pwf-L (Table 3). The affinity of this ligand for iron was very high, similar to that measured for the fungal siderophore DFB. Neptune, however, produced a considerably weaker ligand that bound only 80% of the total iron in solution, so that the remaining 20% was sulfoxine reactive. Thermodynamic constants of pwf-L and nep-L, determined empirically by Maldonado and Price (in press), corroborate the relative scale of iron-binding strengths determined by sulfoxine reactivity (Table 3). The binding strength of lmg-L was not determined.

Table 3. Relative Fe binding strengths of ligands. The concentration of sulfoxine-reactive iron (Fe_R) was determined in seawater with 1 μM ligand and 10 nM ^{55}Fe. The percentage of the total iron that was sulfoxine reactive is reported (% Fe_R). Values are the mean of duplicate measurements ± 1 SD.

Ligand	Fe_R nM	% Fe_R	K_{eq}
NTA	10 ± 0.5	100	10^{16}*
EDTA	8.1 ± 0.4	81	10^{25}*
DFB	0.002 ± 0.005	0.02	10^{30}*
Nep-L	1.8 ± 0.8	18	10^{27}†
Pwf-L	0.004 ± 0.002	0.04	10^{29}†

* K_{eq} of Fe–ligand complexes obtained from Martell and Smith 1977, 1982.
† K_{eq} of Fe–ligand complexes obtained from Maldonado and Price unpubl.

Uptake kinetics of inorganic iron—We established that Fe bound to EDTA was either not taken up by the cells, or negligibly so, because uptake rates of iron-limited Neptune at a fixed iron concentration were inversely proportional to the EDTA concentration (Fig. 1). The total concentration of

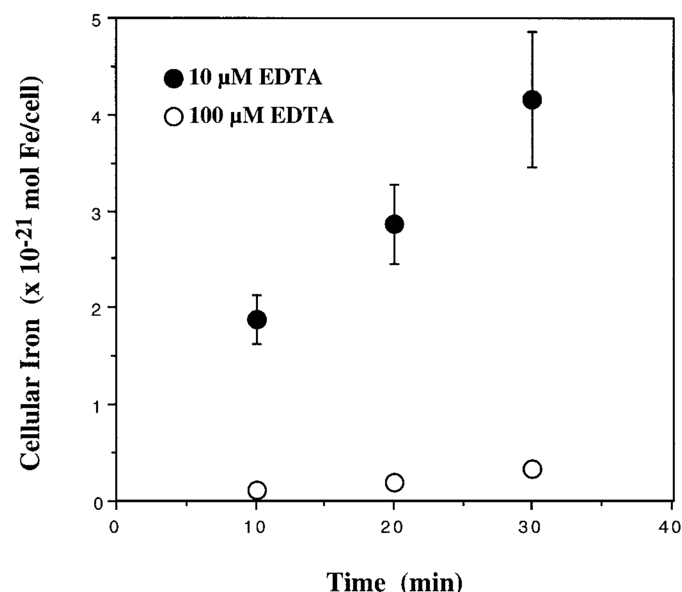

Fig. 1. Short-term iron uptake by iron-limited Neptune. Cells were resuspended in synthetic ocean water enriched with organic substrates and containing 10 nM ^{55}Fe and 100 or 10 μM EDTA. This corresponds to (mean ± SD) 74 ± 13 pM and 600 ± 71 pM Fe_R, respectively. Points represent the mean ± SE of replicate measurements on a single cell culture. Iron uptake rates of iron-limited Neptune (±SD) in 74 pM Fe_R = 0.10 ± 0.01 ($\times 10^{-22}$) mol Fe $cell^{-1}$ min^{-1} and in 600 pM Fe_R = 1.14 ± 0.59 ($\times 10^{-22}$) mol Fe $cell^{-1}$ min^{-1}.

Table 4. Short-term iron uptake rates of bacteria in iron-deficient Aquil growth medium (12.5 nM $^{55}Fe_T$ and 100 μM EDTA: 70 pM Fe_R) in the absence (Aquil) and presence (Aquil + nep-L; Aquil + DFB; Aquil + pwf-1) of 5 μM siderophore (i.e., 5 μM iron-binding equivalents). Cells were preconditioned in iron-deficient growth medium prior to the experiment. Values represent the mean ± SD of at least two experiments (two replicates per experiment).

Strain	Iron uptake rate (±SD) × 10^{-22} mol Fe cell^{-1} min^{-1}			
	Aquil	Aquil + nep-L	Aquil + DFB	Aquil + pwf-L
Neptune	0.21 ± 0.10	1.35 ± 0.20	2.16 ± 0.35	0
P20pac	0.18 ± 0.05	0.07 ± 0.01	2.28 ± 0.08	0
Pseudomonas sp. (Isol5)	0.19 ± 0.05	0.29 ± 0.01	0.32 ± 0.03	0
Jul88	0.05 ± 0.02	0.45 ± 0.06	2.23 ± 0.31	0
LMG1	1.85 ± 0.63	2.05 ± 1.54	2.06 ± 0.62	2.48 ± 0.45
PWF3	0.19 ± 0.03	2.88 ± 0.05	4.77 ± 1.55	2.96 ± 0.20
V. natriegens (PWH3a)	0.12 ± 0.01	0.06 ± 0.01	1.86 ± 0.51	0

iron was identical in the two treatments, whereas EDTA varied 10-fold. An 8.5-fold reduction in Fe_R resulted, roughly, in an 11-fold reduction in the rate of iron uptake by iron-limited Neptune. Cellular uptake in EDTA-buffered growth medium was thus assumed to be restricted to the pool of dissociated, ''inorganic'' iron.

Cells were preconditioned in iron-deficient growth medium and resuspended in fresh iron-deficient Aquil (70 pM Fe_R) to measure short-term iron uptake in growth medium not conditioned by the cells. The uptake rates were similar for five strains, roughly 1.8 × 10^{-23} mol Fe cell^{-1} min^{-1} (Table 4), but they differed for Jul88 and LMG1. Strain Jul88 had a slower uptake rate (0.5 × 10^{-23} mol Fe cell^{-1} min^{-1}), while uptake by LMG1, the sole catechol producer, was approximately 10 times faster (18.5 × 10^{-23} mol Fe cell^{-1} min^{-1}). The experimental strains had similar cell volumes and surface areas (Granger 1998), so these differences in uptake rates were not due to variations in cell size.

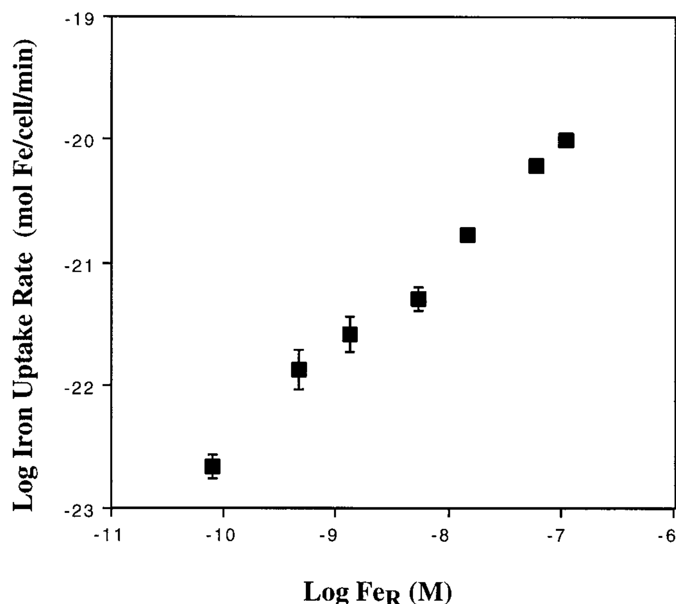

Fig. 2. The relationship between the $\log_{10}$ of sulfoxine-reactive iron, Fe_R, and the $\log_{10}$ of the iron uptake rate of iron-limited Neptune. Cells were resuspended in ^{55}Fe-labeled growth medium, containing total iron concentrations from 12.5 nM to 8.4 μM. The iron was added with 100 μM EDTA to obtain a range of Fe_R from 70 pM to 110 nM. Each point represents the mean ± SE of four to six measurements of uptake rates, made on two to three cell cultures (two replicates per culture), as well as the mean ± SE of two replicate measurements of Fe_R.

The kinetics of inorganic iron uptake were further characterized for strain Neptune. Rates of Fe(III) acquisition by iron-limited cultures were determined over a range of iron concentrations (Fig. 2). Short-term iron uptake rates did not exhibit saturation kinetics characteristic of facilitated or active transport. The increase in uptake rate with respect to sulfoxine-reactive iron remained linear over the entire range of iron concentrations tested. However, a slight inflection is perceptible above 1 nM Fe_R, possibly reflecting the activity of a higher affinity transport system at low Fe_R. Regulation of inorganic iron uptake was examined more closely in Neptune and LMG1 by examining uptake at a higher substrate concentration (5 nM Fe_R) for both iron-replete and iron-limited cells. The uptake rate of iron-limited LMG1 was seven times faster than that observed for Neptune (Fig. 3), corroborating the previous result observed at a lower iron concentration (Table 4). The greatest contrast between the two strains, however, was in their regulation of iron transport under iron stress (Fig. 3). While inorganic iron uptake by iron-limited Neptune was only two times faster than that measured for iron-replete Neptune, uptake by iron-limited LMG1 was enhanced 46 times compared to iron-replete LMG1.

Enhancement of iron uptake by siderophores—Short-term iron uptake rates of the strains measured in the growth medium supplemented with siderophores established that iron acquisition by the strains was enhanced by their presence (Table 4). Uptake rates of four strains (Neptune, *Pseudomonas* sp., Jul88, and PWF3) preconditioned in iron-deficient growth medium were faster in medium supplemented with nep-L than in standard Aquil (Table 4), suggesting that these strains were able to take up iron bound to the siderophore. Iron uptake rates of iron-limited P20pac and *V. natriegens,* however, were slower in Aquil supplemented with nep-L, implying that they could not take up iron bound to the siderophore. Uptake by iron-limited LMG1, the sole catechol producer, was neither repressed nor enhanced in the presence of nep-L.

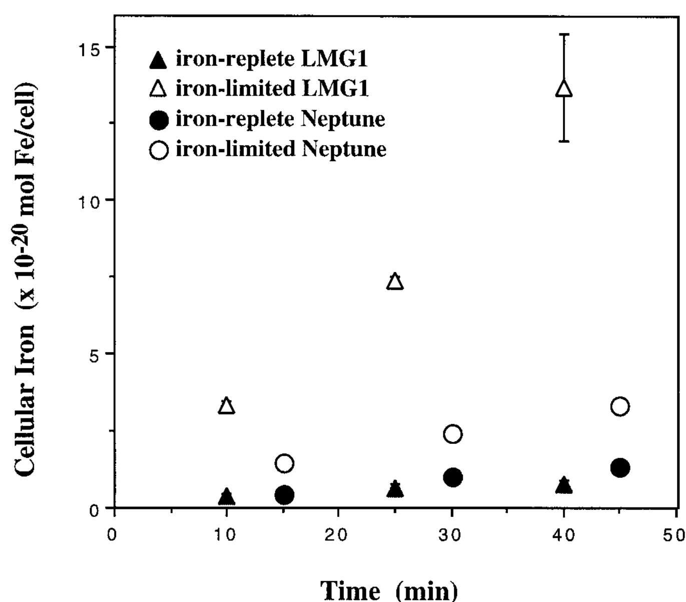

Fig. 3. Short-term iron uptake by iron-replete and iron-limited Neptune and iron-replete and iron-limited LMG1. Cells were resuspended in growth medium containing 685 nM Fe_T: 100 μM EDTA (10% ^{55}Fe), corresponding to 5 nM Fe_R. Each point represents the mean ± SE of replicate bottles for a single experiment. Uptake rates (±SD) in mol Fe cell^{-1} min^{-1} of: Fe-limited Neptune = 6.21 ± 0.8 ($\times10^{-22}$); Fe-replete Neptune = 2.88 ± 0.64 ($\times10^{-22}$); Fe-limited LMG1 = 34.6 ± 7.64 ($\times10^{-22}$); Fe-replete LMG1 = 0.75 ± 0.77 ($\times10^{-22}$).

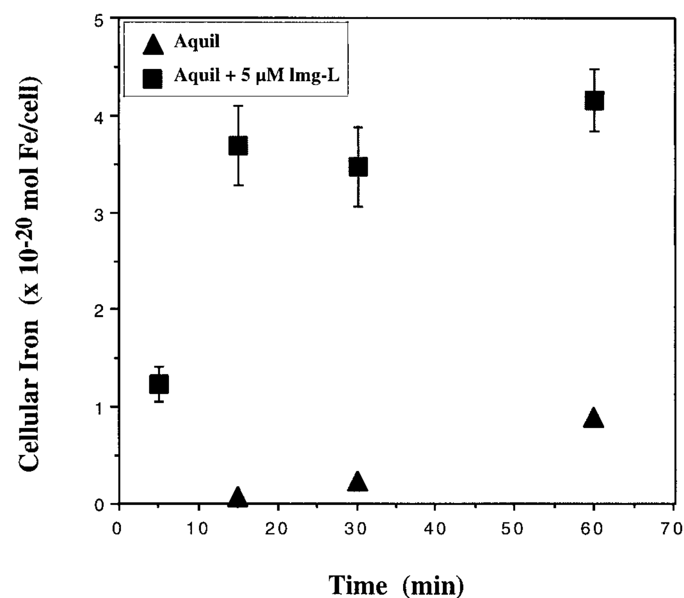

Fig. 4. Short-term iron uptake by iron-limited LMG1. Points represent the mean ± SE of replicate bottles for a single experiment. Cells were resuspended in iron-deficient Aquil growth medium (12.5 nM $^{55}Fe_T$: 100 μM EDTA) and in iron-deficient Aquil growth medium supplemented with 5 μM lmg-L.

Iron bound to the fungal siderophore DFB was taken up by six strains preconditioned in iron-deficient growth medium, as shown by their enhanced uptake rates in medium supplemented with DFB (Table 4). The results were again ambiguous for iron-limited LMG1, because iron uptake was neither reduced nor enhanced by the siderophore.

Iron bound to pwf-L was available only to the producing strain, PWF3 (Table 4). Except for LMG1, iron uptake among the remaining strains was completely repressed in the presence of pwf-L, which presumably bound all the inorganic iron (Table 3). The results confirm that these strains could neither access the pool of iron bound to pwf-L nor that of iron bound to EDTA, as determined previously for Neptune (Fig. 1). Iron uptake by LMG1 was not repressed or enhanced in the presence of pwf-L, as observed with other ligands. When iron-limited LMG1 was resuspended in growth medium enriched with its own ligand (lmg-L), it exhibited an unusually rapid accumulation of Fe over the first 15 min (Fig. 4), roughly 10–15 times faster than the rate of iron accumulation measured for all the strains in growth medium supplemented with the hydroxamate siderophores nep-L, pwf-L, or DFB (Table 4). No additional Fe was taken up by LMG1 after 15 min. The cessation of uptake was likely not due to the depletion of the bioavailable iron in the medium, because <5% of total Fe (12.5 nM) was accumulated by the cells during this time. These observations suggest that ^{55}Fe lmg-L adsorbed to the cell surface during the initial stages of the experiment. Subsequent accumulation of cellular iron may have been masked by the error associated with the measurements.

Uptake rates of iron bound to siderophores—Rates of uptake of iron bound to DFB were similar among five iron-limited strains, ranging between 3 and 8 $\times$ 10^{-22} mol Fe cell^{-1} min^{-1} (Table 5). *Pseudomonas* sp. was an exception, showing a much slower rate of uptake from FeDFB, because it was not iron-limited in the iron-deficient growth medium. Iron bound to DFB was completely unavailable to strain LMG1. The marine siderophore pwf-L was taken up by the producing strain, PWF3, at a rate similar to that measured for FeDFB uptake by the remaining iron-limited strains (6 $\times$ 10^{-22} mol Fe cell^{-1} min^{-1}). Iron bound to pwf-L was also

Table 5. Short-term uptake rates of iron bound to the siderophores DFB and pwf-L. Bacteria were preconditioned in iron-deficient growth medium and resuspended in siderophore uptake medium containing 10 nM Fe bound to 1 μM siderophore as the sole iron source. Values represent the mean ± SD of at least two experiments (two replicates per experiment). ND, not determined.

Strain	Iron uptake rate (± SD) $\times$ 10^{-22} mol Fe cell^{-1} min^{-1}	
	DFB	pwf-L
Neptune	6.81 ± 2.28	0
P20pac	ND	0
Pseudomonas sp. (Isol5)	0.09 ± 0.02	0
Jul88	6.31 ± 0.80	0
LMG1	0	0.11 ± 0.01
PWF3	8.37 ± 0.30	5.85 ± 0.74
V. natriegens (PWH3a)	3.35 ± 1.10	0

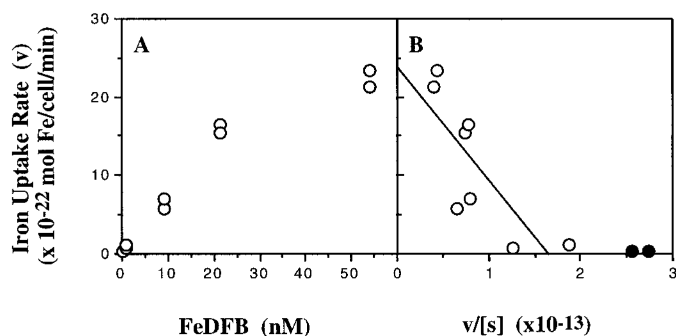

Fig. 5. (A) Uptake rate of iron bound to DFB by iron-limited Neptune. The cells were resuspended in siderophore uptake media containing 0.2–55 nM ^{55}Fe and 10 μM DFB. A single cell culture was resuspended in duplicate bottles for five iron concentrations (duplicates plotted). (B) Uptake kinetics of iron bound to DFB by iron-limited Neptune fitted with the v vs. $v/[S]$ linearization of the Michaelis–Menten equation (Neame and Richards 1972). The iron uptake rates measured at a concentration of 2 nM FeDFB (closed circles) were not included in the least-squares regression. V_{max} = 2.40 ± 1.27 (×10^{-21}) mol Fe cell^{-1} min^{-1}; K_s = 14.6 ± 13 nM FeDFB.

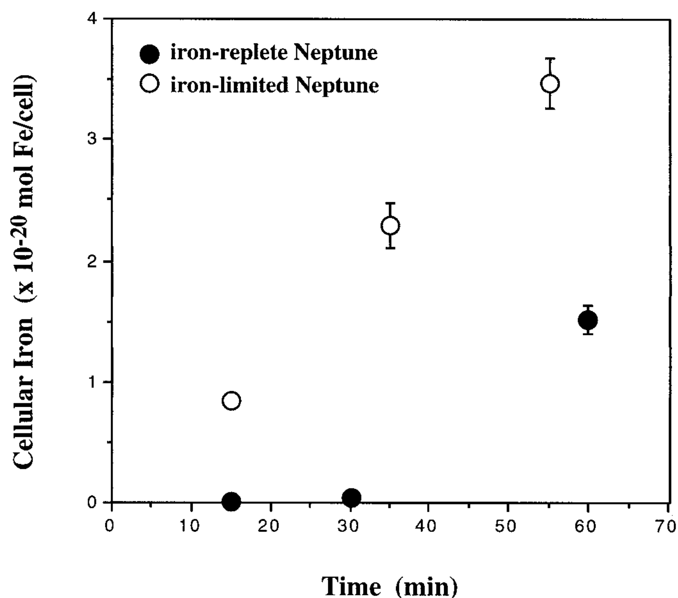

Fig. 6. Short-term uptake of iron bound to DFB by iron-replete and iron-limited Neptune. Points represent the mean ± SE of replicate bottles for a single experiment. Cells were resuspended in siderophore uptake medium containing 10 nM ^{55}Fe and 1 μM DFB. Uptake rate (±SD) in mol Fe cell^{-1} min^{-1} of Fe-limited Neptune = 6.53 ± 0.86 (×10^{-22}); Fe-replete Neptune for initial 30 min = 0.29 ± 0.01 (×10^{-22}).

taken up by strain LMG1 but at a negligible rate compared to iron-siderophore uptake by other iron-limited strains.

The rates of iron-siderophore uptake measured in siderophore uptake medium (Table 5) were consistently faster than the rates observed in growth medium supplemented with the siderophores (Table 4), despite there having been relatively the same amount of total iron added to the media (10 nM Fe in siderophore uptake medium, 12.5 nM Fe in Aquil). The bioavailable iron pools may have been different in the respective media. At chemical equilibrium, most of the iron in the supplemented Aquil should be bound to the DFB or pwf-L because they have considerably greater affinity constants for iron than EDTA. However, the medium may not have achieved chemical equilibrium at the time of the experiments (approximately 8 h after addition of the siderophore), so a significant fraction of the iron may have remained bound to EDTA.

Uptake kinetics of FeDFB by Neptune—Utilization of iron bound to siderophores was further investigated for iron-limited Neptune. Short-term iron uptake experiments were performed using DFB as a model ligand, because it was utilized by six of the seven experimental strains and because it bound all the Fe(III) in solution (*see Table 3*). The rate of uptake exhibited saturation kinetics with respect to substrate concentration, characteristic of facilitated transport (Fig. 5). The data were analyzed with the v vs. $v/[S]$ linearization of the Michaelis–Menten equation (Neame and Richards 1972) and fit the model with an r^2 of 0.62 (n = 8; Fig. 5). The half-saturation constant for iron transport by Neptune was 15 (±13.0) nM, and the maximum uptake rate was 2.4 (±1.27) × 10^{-21} mol Fe cell^{-1} min^{-1}.

Transport of FeDFB by Neptune was upregulated significantly in response to iron stress (Fig. 6). During the initial 30 min, the iron uptake rate was 20 times faster for iron-limited than for iron-replete cells. This result contrasts the regulation of inorganic iron uptake by Neptune, which was enhanced only twofold by iron limitation (Fig. 3). After 30 min, the rate of FeDFB uptake by the iron-replete cells increased gradually (Fig. 6), presumably as they became increasingly Fe deficient.

Siderophore growth assays—To establish that the siderophore-bound iron that was taken up was metabolized by the bacteria, growth assays were performed for Neptune, PWF3, and LMG1 (Fig. 7). The growth rate of Neptune was increased in iron-deficient growth medium enriched with nep-L and DFB, but it was repressed in pwf-L–enriched medium (Fig. 7A). Similarly, the growth rate of PWF3 was enhanced by all three ligands (nep-L, DFB, and pwf-L; Fig. 7B). Strain LMG1 behaved in parallel to the uptake experiments, showing neither repression nor enhancement of growth in medium enriched with nep-L, DFB, or pwf-L (Fig. 7C). A growth rate enhancement was observed in medium enriched with lmg-L, confirming that it can use iron bound to its own siderophore.

Discussion

Three lines of evidence support the general conclusion of this study that heterotrophic marine bacteria utilize iron bound to siderophores. Initial observations of enhanced short-term Fe transport rates demonstrated that siderophores increased the bioavailable pool of Fe for bacteria in the EDTA-buffered growth medium. They showed that in the majority of cases, Fe uptake rates were faster in the presence of the fungal siderophore DFB than with EDTA alone. Par-

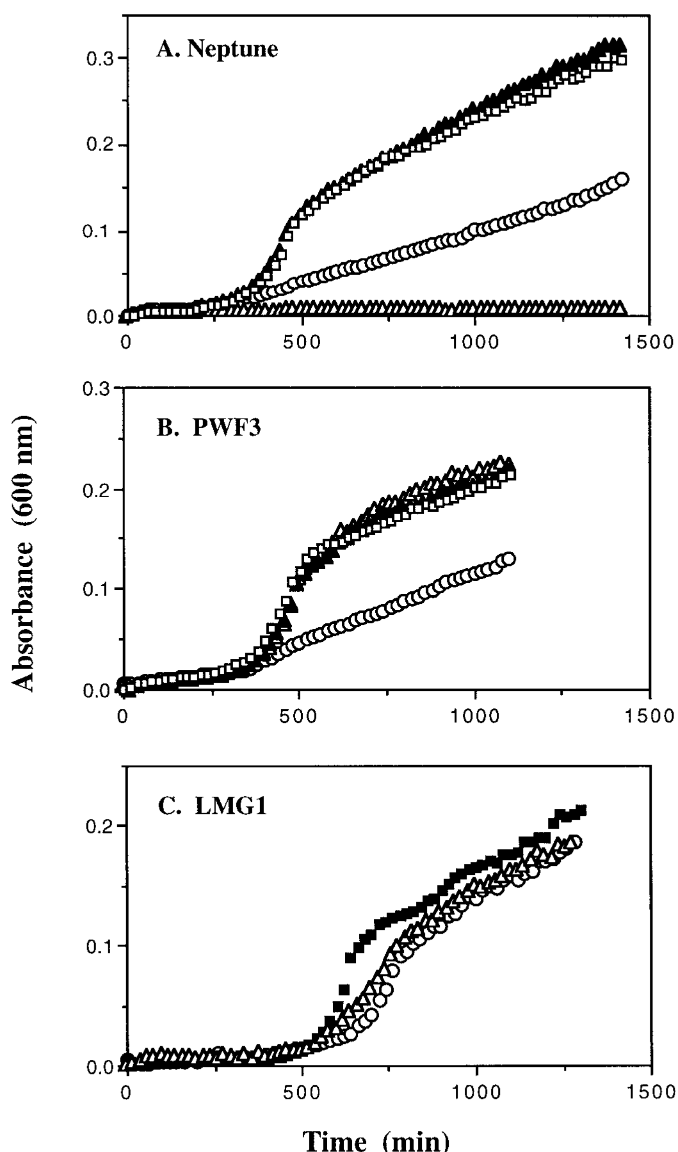

Fig. 7. Growth of Neptune, PWF3, and LMG1 in iron-deficient growth medium containing 12.5 nM Fe_T with 100 μM EDTA and 5 μM siderophore: no siderophore added (○), nep-L (▲), DFB (□), pwf-L (△), or lmg-L (■). Only single replicates are plotted for clarity; growth rates = mean ± SD of triplicate measurements. (A) Growth of iron-limited Neptune (8.3 ± 0.4 d^{-1}) with nep-L (12.8 ± 0.6 d^{-1}), DFB (13.4 ± 0.4 d^{-1}), and pwf-L (nil). (B) Growth of iron-limited PWF3 (7.8 ± 0.7 d^{-1}) with nep-L (14.4 ± 0.5 d^{-1}), DFB (14.2 ± 1.5 d^{-1}), and pwf-L (13.5 ± 0.8 d^{-1}). (C) Growth of iron-limited LMG1 (11.3 ± 0.6 d^{-1}) with lmg-L (16.8 ± 2.2 d^{-1}), nep-L (9.4 ± 2.4 d^{-1}), DFB (11.8 ± 0.3 d^{-1}), and pwf-L (9.6 ± 0.5 d^{-1}); DFB and pwf-L curves not plotted for clarity.

tially purified siderophores from strains Neptune and PWF3 elicited a similar response, although they also inhibited or had no effect on Fe uptake in some conspecifics. Only four of the seven bacteria examined produced iron chelates under the experimental conditions. All strains, however, utilized iron bound to at least one type of siderophore. Subsequent experiments, performed using Fe–siderophore complexes as the sole Fe source in the absence of competing organic ligands, confirmed these results. Uptake rates of hydroxamate-bound Fe were remarkably similar among all strains and were indistinguishable for pwf-L and DFB. Thus, the cell-surface receptors appear to possess similar affinities for these different siderophores and to be similar in a number of marine bacteria. Growth assays established that the transported Fe was metabolized, confirming that the iron–siderophore complexes satisfied the iron nutritional requirements of the bacteria. In toto, the results imply that perhaps all heterotrophic marine bacteria are able to use siderophores, regardless of whether or not they produce their own.

As discussed below, the results of this study also suggest that the uptake of inorganic ferric species (Fe′) by bacteria is generally inefficient and may not be under physiological control, at least in hydroxamate-producing strains. Iron limitation induces a high-affinity siderophore transport system that may be the sole means by which these bacteria acquire Fe from their environment. Moreover, transport kinetics of Fe by LMG1, a catechol producer, were fundamentally different from the other strains, possibly reflecting a habitat-specific adaptation associated with the synthesis of this type of siderophore.

Specificity of iron–siderophore transport—A critical aspect in the design of the Fe–siderophore uptake experiments concerned the chemical forms of Fe present in the medium. With weak ligands, large amounts of Fe would be present in solution in equilibrium with the Fe–siderophore complex. Bacteria could potentially acquire Fe directly from the siderophore, from the inorganic pool (Fe′), or from both, and uptake experiments employing ^{55}Fe would not establish which of these species of Fe were utilized. For two of the siderophores that were examined, however, virtually all of the Fe (>99%) was bound in strong complexes, and the concentration of Fe′ was negligible. Iron uptake from DFB and pwf-L must thus involve transport of the Fe–siderophore complex or an exchange reaction with a transport ligand at the cell surface. In both of these cases, the siderophores play a direct role in Fe acquisition by the bacteria.

Utilization of siderophore-bound Fe was a general characteristic of all the bacteria. What was particularly surprising was that DFB, a siderophore produced by a terrestrial fungus, was taken up by six of the seven strains examined. The marine siderophore nep-L was also utilized by four experimental strains. Cross-utilization of siderophores produced by microorganisms of different provenance suggests that perhaps only a finite number of ligand types are synthesized, allowing strains to specialize in the uptake of particular siderophores. Opportunistic strains may "cheat" by forgoing the expense of synthesizing their own chelates to rely on those provided by others. Some strains, however, may monopolize iron by producing uncommon ligands; the marine siderophore pwf-L, for example, was not recognized by foreign strains and hence repressed their iron uptake and growth.

Utilization of exogenous siderophores by marine bacteria has been reported previously. Trick (1989) showed that some bacteria produced siderophores that promoted growth of unrelated isolates, while other siderophores only satisfied the

iron requirements of the strains that produced them. Recognition of multiple siderophores is common among gram-negative bacteria. For example, five Fe(III) transport systems have been identified in *E. coli,* each of which is specific for a particular iron complex: enterobactin, aerobactin, the fungal hydroxamates, ferrichrome and coprogen, and citrate. Each transport system is characterized by outer-membrane receptor proteins that discriminate among different siderophores (Braun et al. 1998).

Iron transport mediated by catechol siderophores—Under the experimental conditions, strain LMG1 produced siderophores containing the hydroxamate and the catechol functionalities. Transport of iron bound to exogenous hydroxamates was not observed for this strain, however, because uptake was repressed by DFB and pwf-L. Yet short-term iron uptake rates conducted in Aquil growth medium containing FeEDTA were not affected by these siderophores. Because siderophore-bound Fe was not taken up directly by LMG1, it may have utilized iron bound to EDTA. Iron may have been shuttled from the siderophore to the surface receptor by EDTA, allowing LMG1 to sustain uptake and growth in spite of the considerable reduction in the pool of inorganic iron in the medium. Scavenging of iron from EDTA may be mediated by the catechol siderophores produced by LMG1. Such siderophores are generally hydrophobic and thus tend to remain associated with the cell surface (Neilands 1973). Indeed, the rapid saturation of iron uptake from lmg-L is consistent with the hypothesis that the ferrated catechol siderophore adsorbed to the cell surface. Extracellular iron would not be dissolved by the titanium(III) citrate–EDTA rinse because iron bound to catechols has considerably lower redox potentials (e.g., $E_h = -750$ mV for enterobactin; Lee et al. 1985) than titanium(III) citrate ($E_h = -480$ mV; Hudson and Morel 1989) and hence would not be reduced by it. Wilhelm and Trick (1994) actually recovered measurable amounts of catechol siderophores associated with the cell surface of the marine cyanobacterium *Synechococcus* PCC7002 with a brief chloroform wash. The biological function of catechols may thus be to scavenge iron from other ligands or complexes while remaining associated with the cell surface, as they proposed.

Catechol siderophores are characterized by extremely high-stability constants for iron, up to 10^{52} for enterobactin synthesized by *E. coli* (Weilt et al. 1979). Constants reported for marine bacterial and cyanobacterial catechol siderophores are ca. 10^{40} (Butler et al. 1993; Lewis et al. 1995), 10 orders of magnitude higher than those estimated for the hydroxamate siderophores nep-L and pwf-L (10^{27} and 10^{29}, respectively). Catechols are thus very effective in sequestering iron. The rate of iron exchange between competing ligands, however, is not necessarily dictated by their respective stability constants (Albrecht-Gary and Crumbliss 1998). Few studies have examined the kinetics of iron exchange between catechol siderophores and competing ligands. Yet anecdotal evidence suggests that the reaction may be rapid. Schwyn and Neilands (1987) reported that the rate of iron exchange between the CAS assay reagent and siderophores is fastest for catechols, reaching chemical equilibrium within minutes, and slowest for hydroxamates such as DFB, which requires days to equilibrate. Iron bound to weaker ligands such as EDTA ($K_{eq} = 10^{25}$; Martell and Smith 1977) may hence be rapidly scavenged by catechol siderophores and taken up by the cell. Iron exchange between catechols and hydroxamates, such as pwf-L and DFB, may be too slow to sustain measurable short-term uptake. In this scenario, EDTA may thus act as a shuttle, transferring iron from the hydroxamate siderophores to the catechol ligands, much like 5-sulfosalicylic acid acts as an iron shuttle between the CAS assay reagent and kinetically slow siderophores such as DFB (Schwyn and Neilands 1987). The potential role of EDTA as an iron shuttle would explain the observation that iron uptake of LMG1 was not repressed in EDTA-buffered growth medium supplemented with siderophores, while uptake was negligible or nil in medium containing iron siderophores as the sole iron source.

Table 6. Short-term iron uptake rates of seven bacterial stains in iron-deficient growth medium (12.5 nM Fe_T), compared with the steady-state iron uptake rates during the exponential growth of the strains in the same medium. Values for the short-term uptake rates were obtained from Table 4. Steady-state rates were calculated by multiplying the growth rate by the intracellular iron quota of the strains in iron-deficient growth medium (Table 1).

Strain	Short-term uptake rate (±SD) × 10^{23} mol Fe cell^{-1} min^{-1}	Steady-state uptake rate (±SD) × 10^{23} mol Fe cell^{-1} min^{-1}
Neptune	2.01 ± 0.99	1.73 ± 0.08
P20pac	1.79 ± 0.52	2.19 ± 0.01
Pseudomonas sp. (Isol5)	1.92 ± 0.48	4.11 ± 0.03
Jul88	0.51 ± 0.22	0.76 ± 0.02
LMG1	18.5 ± 6.28	38.7 ± 0.79
PWF3	1.87 ± 0.28	2.90 ± 0.24
V. natriegens (PWH3a)	1.15 ± 0.06	8.55 ± 0.04

Steady-state vs. short-term iron uptake rates—Siderophores were detected in the supernatant of a number of bacteria cultures grown to high cell densities in siderophore isolation medium. When they were added to iron-deficient Aquil growth medium, they promoted growth. Because the culture conditions in these two media were not directly comparable, it was uncertain whether the bacteria actually released the siderophores during exponential growth. To evaluate the potential role of siderophore production in steady-state Fe acquisition, steady-state uptake rates (in units of mol Fe cell^{-1} d^{-1}) were computed by multiplying the iron-limited growth rates of the strains in iron-deficient Aquil by their iron quotas. These rates were then compared to the short-term iron uptake rates of the cells resuspended in fresh iron-deficient Aquil (Table 6). Strains that condition their medium by producing siderophores during exponential growth should thus maintain faster steady-state than short-term iron uptake rates in iron-deficient Aquil. This was the case for LMG1, PWF3, and *V. natriegens,* whose steady-state iron uptake rates exceeded short-term uptake rates. Steady-state uptake rates of Jul88 and P20pac did not differ from short-term uptake rates, as expected, because these bac-

teria did not produce siderophores in the isolation medium. The trend, however, was inconsistent for Neptune and *Pseudomonas* sp. Neptune showed no difference between steady-state and short-term uptake, a result that may be because nep-L is a weak ligand that, at a low concentration, is unable to compete with EDTA. Alternatively, induction of siderophore transport may be induced at a different iron concentration than siderophore production, as observed for a marine cyanobacterium (Trick and Wilhelm 1995). *Pseudomonas* sp., which produced no siderophores in the isolation medium, inexplicably showed faster steady-state than short-term iron uptake rates. The mean cell size in the quota experiments of *Pseudomonas* sp. was larger than in the short-term iron uptake experiments (Granger 1998), which may account for the discrepancy.

Uptake kinetics of iron bound to siderophores—Most of the experimental strains (except LMG1) transported iron bound to DFB at a similar rate. Because it was representative of the majority of the strains, Neptune was chosen for further investigation of the uptake kinetics of iron bound to a hydroxamate siderophore, FeDFB. The rate of FeDFB acquisition by iron-limited Neptune was hyperbolic with respect to substrate concentration, indicative of facilitated transport. This is in concordance with studies that have established that uptake of ferrisiderophores in microorganisms requires a membrane-embedded active transport system (Byers and Arceneaux 1998). The saturated rate of FeDFB uptake by iron-limited Neptune was lower than that measured for nonmarine, gram-negative strains. The V_{max} for enterobactin uptake by *E. coli,* for example, is 2.0×10^{-20} mol Fe cell^{-1} min^{-1} (Ecker et al. 1986), 10 times faster than the rate determined here (2.4×10^{-21} mol Fe cell^{-1} min^{-1}). Although the discrepancy between the rates could arise from a comparison of different ligands or physiological states, it may portend a fundamental trade-off in Fe transport by marine bacteria that favors high affinity over maximizing resource acquisition. Indeed, Neptune's estimated half-saturation constant of 15 nM FeDFB is remarkably low. Half-saturation constants for ferrisiderophore transport by other gram-negative bacteria generally range between 0.1 and 2 μM (Winkelmann 1990). The freshwater cyanobacterium *Anabaena* sp. has one of the lowest, with a reported half-saturation constant of 40 nM for schizokinen (Lammers and Sanders-Loehr 1982), which is still higher than what we measured for Neptune. The kinetic parameters for DFB uptake by Neptune characterize a transport mechanism that operates best at relatively low substrate concentrations, one that has a higher affinity for its substrate than analogous receptor molecules of nonmarine strains. Such a difference may reflect habitat-specific adaptations to ambient substrate concentrations. Certainly, concentrations of organically complexed iron in the ocean are among the lowest of any environment, averaging 0.07 nmol kg^{-1} (Johnson et al. 1997). Concentrations of iron siderophores in soil, by comparison, are approximately 78 nM (Powell et al. 1983).

Implicit in the comparison of the kinetic parameters of Neptune with nonmarine strains is the assumption that iron uptake from siderophores occurs by the same mechanism, namely transport of the entire Fe chelate. Uptake of Fe bound to DFB by Neptune could, however, occur via extracellular reduction, as observed for marine phytoplankton (Maldonado and Price in press), or it could be mediated by the exchange of Fe between DFB and a cell-surface Fe(III) transporter. The latter option is improbable, because the iron uptake rates measured for iron-limited Neptune at 10 nM Fe were identical whether 1 or 10 μM DFB was added to the medium (Table 5 and Fig. 5, respectively). An increase in the extracellular concentration of DFB would be predicted to decrease iron uptake, because the uncomplexed DFB would compete for Fe(III) with surface transport sites. Because nonmarine strains take up FeDFB as an intact metal–siderophore complex (Müller and Raymond 1984), we believe this to be the most plausible mechanism for Fe uptake from DFB by Neptune, although the existence of a cell-surface reductase cannot be ruled out.

The iron uptake rates reported here can be compared to those of natural populations of bacteria in the field where FeDFB has been used as a tracer (2 nM ^{55}FeDFB). In the subarctic Pacific Ocean, iron concentrations are low, and bacterioplankton uptake rates during summer average 1.6×10^{-23} mol Fe fl^{-1} min^{-1} (Maldonado and Price in press). Strain Neptune, which was isolated from this part of the sea, took up FeDFB at the fastest rate when grown under Fe-limiting conditions. This rate was 2.5×10^{-23} mol Fe fl^{-1} min^{-1} at a concentration of 1 nM FeDFB, remarkably similar to that measured in the field. These results suggest that indigenous bacteria may be iron limited and possess transport systems like that of strain Neptune.

The regulation of ferrisiderophore transport—Expression of the iron-siderophore uptake mechanism in strain Neptune was regulated by the nutritional iron status of the cells. Iron deficiency reduced growth rate as the intracellular concentration of iron declined. Concomitantly, the uptake rate of iron bound to DFB was enhanced 20 times in iron-limited compared to iron-sufficient cells. The transport system for FeDFB was constitutive and rapidly upregulated following iron starvation. Within 30 min of resuspension in low Fe medium, bacteria that had been grown under Fe-replete conditions showed a dramatic increase in uptake rate of FeDFB. The properties of this transport mechanism in Neptune are reminiscent of Fe(III)-regulated outer-membrane proteins involved in siderophore acquisition, which constitute a characteristic response to Fe stress and are part of the expression of the high-affinity uptake systems of bacteria (Braun et al. 1998). Such a response was observed by Reid and Butler (1991), who showed that the marine bacterium *Alteromonas luteoviolaceans* upregulated the expression of an outer-membrane protein—and produced two new ones—in response to iron stress.

Inorganic iron acquisition—Rates of inorganic Fe transport were determined in media containing FeEDTA. Under these conditions, the majority of the Fe was bound to EDTA, although considerable quantities existed as reactive inorganic species (Fe′). To rule out the possibility that FeEDTA was itself a substrate for transport, uptake rates of Neptune were measured using a constant amount of Fe complexed to 10 or 100 μM EDTA. The results showed that uptake was directly proportional to Fe′ and not related to the concentration

of FeEDTA, validating our use of this protocol to measure inorganic Fe uptake.

Among all the strains except LMG1, inorganic Fe transport rates were similar. Rates of uptake by Neptune, which was chosen for more detailed study of Fe′ uptake kinetics, did not saturate at the highest concentration of iron tested (100 nM Fe_R). This result was surprising in that the same concentration of Fe′ is known to saturate short-term uptake rates of eukaryotic marine phytoplankton. Maximum rates of transport of *Thalassiosira weissflogii* and *Pleurochrysis carterae* are measured at a threshold concentration of approximately 5 nM Fe_R (Hudson and Morel 1990). Diatoms and other eukaryotic algae are thought to satisfy their iron requirements by acquiring dissolved inorganic species, with Fe(III)-specific transport ligands on the cell surface (Anderson and Morel 1982; Hudson and Morel 1990). Considering the range of Fe concentrations tested here, the slope of the relationship between uptake rate and Fe′ was close to 1 (0.86), implying that Fe uptake may not be facilitated by a transporter but may occur by simple diffusion through the outer membrane. Conversely, the inflection above 1 nM Fe_R may indicate saturation of a transporter. This concentration is near the solubility limit of $Fe(OH)_3$. Beyond this point, FeEDTA dissociation maintains a supersaturated level of inorganic iron around the cell, and this iron could accumulate in the periplasm by diffusion.

In stark contrast to uptake of Fe from the FeDFB complex, inorganic iron uptake was not regulated by the iron nutritional status of the cells. Little difference was observed between the uptake rates of iron-limited compared to iron-replete cells at a concentration of 5 nM Fe_R. Such a result differs fundamentally from that observed in eukaryotic phytoplankton, in which transport rates are up to 20 times faster in iron-limited cells (Harrison and Morel 1986). This could imply that inorganic iron transport by Neptune is expressed maximally regardless of the iron status of the cell or that the cell relies exclusively on a siderophore-mediated mechanism to acquire iron under limiting conditions and does not modulate the rate of inorganic iron transport. Comparison of surface-area–normalized iron uptake rates of Neptune and eukaryotic phytoplankton, however, reveals that transport by Neptune is relatively inefficient (Table 7). The surface-normalized rate in iron-limited Neptune was 100 times slower than that measured for iron-limited *T. weissflogii* at the same sulfoxine-reactive iron concentration, although the diffusive flux of iron was almost five times greater for bacteria because of their small size. *T. weissflogii* transported iron at 20% of the diffusive flux of iron to the cell (Table 7). The rate of inorganic iron uptake by iron-limited Neptune, however, was 2,800 times slower than the diffusive flux of iron to the cell! By direct comparison with eukaryotic phytoplankton that are believed to have evolved to utilize inorganic Fe(III), it appears that inorganic iron acquisition is not optimized in Neptune and is in fact inefficient.

Little is known about inorganic Fe(III) transport in gram-negative bacteria, although some species, such as *E. coli,* appear unable to use inorganic chemical forms. In this instance, iron acquisition is restricted to the uptake of organic iron chelates (Braun et al. 1998). A ferric iron transport system was first found and characterized in *Serratia marcescens*

Table 7. Cell-surface–specific short-term inorganic iron uptake rates of two heterotrophic marine bacterial strains (Neptune and LMG1) and a eukaryotic phytoplankton (*T. weissflogii*) at a concentration of 70 pM Fe_R. Values for the cell-surface–specific uptake rates are compared with the diffusive flux of iron to the cell surface, where the diffusive flux $= 4\pi r D \cdot Fe_R\ SA^{-1}$ (Paschiak and Gavis 1974; Hudson and Morel 1990); r, cell radius (assuming a spherical shape for the cells); D, 9×10^{-6} cm^2 s^{-1} is the molecular diffusion constant of inorganic Fe(III) is seawater (Li and Gregory 1974); SA, cell-surface area. Estimates of bacterial cell volumes and surface areas were obtained by confocal microscopy as reported by Granger (1998); bacterial cell radii were computed from cell-volume measurements assuming a spherical shape.

Parameter	Neptune	LMG1	*T. weissflogii**
Radius (μm)	0.98	0.95	5.6
Cell volume (μm^3)	3.97	3.56	735
Uptake rate $\times 10^{-23}$ (mol Fe μm^{-2} min^{-1})	0.14†	1.38†	13.3
Diffusive flux $\times 10^{-23}$ (mol Fe μm^{-2} min^{-1})	385	398	67.5

* Values from Hudson and Morel 1990.
† Values from Table 4 normalized to cell surface area.

(SFU—Zimmermann et al. 1989). The proteins encoded by the *sfuABC* genes are localized in the periplasm and cytoplasmic membrane; no siderophore production and no outer-membrane receptor protein related to the SFU system could be detected. Hence, the mechanism by which iron passes the outer membrane via the SFU system of *S. marcescens* remains unknown, suggesting that it is not specifically designed to scavenge Fe(III) from the environment but rather, to internalize Fe(III) released in the periplasm from organic ligands.

A similar mechanism could be operating in Neptune. Uptake of inorganic Fe(III) at the outer membrane may not be facilitated by a specific receptor but rather, may occur passively via nonspecific porins. In gram-negative cells, most substrates diffuse through open porin channels embedded in the outer membrane that have an exclusion limit of approximately 600 Da (Nikaido 1982). Passive diffusion through these channels would explain why no saturation was observed at the highest concentration tested, allowing iron to accumulate in the periplasm in proportion to its external concentration. It would also explain why no upregulation of inorganic iron transport was observed for iron-limited cells, if diffusion through the porins is the rate-limiting step for translocation of iron from the external medium to the cytoplasm. Regardless of the mechanism by which inorganic Fe(III) was accumulated, it appears that inorganic iron transport in Neptune and, by inference, in all the strains but LMG1, is inefficient and perhaps plays only a secondary role in iron acquisition in situ.

Inorganic iron transport by the catechol-producing strain, LMG1—The short-term uptake rates of inorganic iron observed for iron-limited LMG1, as well as the computed steady-state uptake rates (Table 6), were consistently faster than those observed for the other strains. Accordingly, LMG1 grew fastest and maintained the highest quota of all the experimental strains in iron-deficient medium. Inorganic

iron uptake was enhanced 46 times in iron-limited compared to iron-replete LMG1, demonstrating it was regulated by the iron status of the cell, in sharp contrast to Neptune. Such enhancement may not necessarily reflect the upregulation of an Fe(III) transporter per se, but rather an increase in the concentration of catechols associated with the cell surface under iron stress. It follows that the faster inorganic iron uptake rates of LMG1 may have been mediated by surface-bound catechols that scavenged free iron and, possibly, iron bound to EDTA from the extracellular medium.

The surface-area–normalized Fe′ uptake rate of iron-limited LMG1, although greater than those of the other bacterial strains, was still much slower than the diffusive flux of sulfoxine-reactive iron to the cell surface and slower than the surface-specific iron uptake rate achieved by *T. weissflogii* at the same substrate concentration (Table 7). It appears that, like the other bacterial strains, the inorganic iron uptake mechanism of LMG1 is relatively inefficient. Thus, the biological function of catechol siderophores may not lie specifically in their ability to mediate inorganic iron transport but rather, in their propensity to rapidly scavenge iron from weaker complexes, increasing the instantaneous bioavailable pool of iron for the cell.

Ecological implications—The combined experimental evidence establishes that heterotrophic marine bacteria can acquire iron bound to siderophores. The findings also suggest that the majority of bacteria do not transport inorganic Fe(III) efficiently, implying that the direct uptake of inorganic Fe(III) is not a means by which marine bacteria acquire iron in situ. Dissolved iron in seawater is effectively chelated, so it is highly probable that heterotrophic marine bacteria satisfy their nutritional requirements by using these organically complexed species. Although the chemical structure and origin of the organic ligands remain unknown, bacterial siderophores are the most likely candidate. The stability constants of the natural chelates are in the range of those measured for the marine hydroxamate siderophores, nep-L and pwf-L. Moreover, the observation that marine bacterial isolates produce siderophores in culture and that all the strains examined can utilize iron bound to siderophores begs the question as to why such mechanisms exist if they serve no purpose. Iron-limited bacteria may release siderophores in seawater to increase the solubility of iron and to satisfy their nutritional requirements for this biologically precious metal. Release of siderophores may retard the imminent precipitation of iron and increase its residence time in the water column. Bacteria may thus play a fundamental role in the biogeochemical cycling of iron in the ocean.

Catechol-producing strains may be adapted to habitats where the ability to quickly scavenge ''labile'' iron confers a fitness advantage. In response to a pulse of organic substrates from a phytoplankton bloom or adsorbed onto particulate biogenic debris, the rapid sequestration of iron by such a strain may allow it to maintain a high metabolic rate to capitalize on resources. The marine embayment from which LMG1 was isolated (Laguna Madre) may provide such a habitat. In fact, purple-pigmented bacteria (e.g., LMG1) have been reported to flourish in the Mediterranean, succeeding phytoplankton blooms (Gauthier et al. 1975). Purple pigmentation in marine bacteria is generally characteristic of the genus *Alteromonas* (Gauthier 1976). Furthermore, of the experimental strains, LMG1 was unique in its tendency to stick to the walls of the growth bottles and to form dense mucoid masses at high cell densities, suggesting a predisposition to adsorb onto particles. Catechol siderophores are unlikely, however, to constitute the organic ligands that complex dissolved iron in situ. Their hydrophobicity and short lifetime, because of their propensity to oxidize, preclude them from being a viable strategy to maintain iron dissolved in seawater.

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