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## Identification of a natural desferrioxamine siderophore produced by a marine bacterium

**Abstract**—Desferrioxamine B (DFOB, a terrestrial bacterial siderophore) has been used in experiments on waters from iron-replete areas of the oceans to manipulate the levels of iron available to the biota. Addition of DFOB complexes the available iron (III) and has been suggested to render this complexed iron unavailable to marine microorganisms. We have identified a marine bacterium belonging to the genus *Vibrio* that produces the siderophore desferrioxamine G. Desferrioxamine G, another known terrestrial siderophore, is very similar in structure to DFOB, differing only by the substitution of a terminal methyl group by a propionic acid moiety. These results could affect the interpretation of DFOB iron-removal experiments and further suggest that addition of DFOB might not impose iron stress uniformly across the biological community.

Iron is arguably the most important transition metal ion for living systems. Although iron is one of the most abundant crustal elements, Fe (III) is highly insoluble in aerobic aqueous conditions, making iron acquisition difficult for microorganisms. The difficulty of iron acquisition is further compounded for marine organisms, since total iron concentrations in surface seawater are 20 pM to 1 nM with >99% of the dissolved iron complexed by organic ligands (e.g., Rue and Bruland 1995; Wu and Luther 1995). This low level of iron is known to limit primary production by phytoplankton in regions characterized by high levels of nitrate and low levels of chlorophyll (HNLC regions) such as the subarctic Pacific, equatorial Pacific, Southern Oceans, and certain

coastal upwelling regions (e.g., Coale et al. 1996; Boyd et al. 2000). HNLC regions of the ocean are rich in nutrients for phytoplankton such as phosphate, nitrate, and silica; however, phytoplankton growth is depressed in these areas. To test the hypothesis that the low iron concentration limits primary production, IronEx experiments were carried out by seeding surface waters with low concentrations of dissolved iron (e.g., Coale et al. 1996). These iron addition experiments provided strong evidence for iron limitation of biological productivity in areas with low levels of iron (0.05 to 1 nM). Iron may also influence community structure and primary production in iron-replete waters (Wells 1999). Consequently, iron-removal experiments have been completed by addition of desferrioxamine B (DFOB) to seawater samples to complex Fe (III) (Hutchins et al. 1999; Wells 1999). The addition of desferrioxamine B to seawater from certain iron-replete regions in on-deck experiments causes a decrease in iron availability to both phytoplankton and heterotrophic bacteria and makes the sample artificially iron limited. Iron removal via DFOB addition is a valuable complement to Fe addition experiments because it can induce differing levels of iron stress in natural populations without affecting the availability of other bioactive metals.

Desferrioxamine B is a siderophore commonly produced by terrestrial bacteria (Keller-Schierlein et al. 1965). Siderophores are low molecular weight compounds that typically have very high affinity constants for ferric ion ( $10^{25}$ – $10^{50}$ ). The role of siderophores is to scavenge extracellular iron from the environment and transport it into microbial cells.

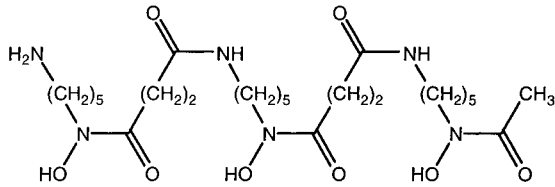
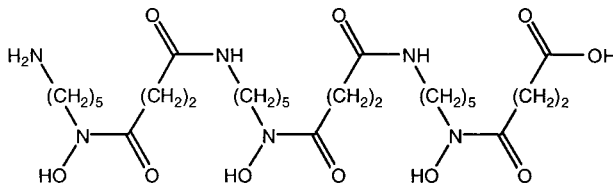
**Desferrioxamine B****Desferrioxamine G**

Fig. 1. Structures of desferrioxamines B and G.

Siderophore production is regulated by iron levels, with repression of siderophore biosynthesis under high iron conditions. Although the structures of many siderophores from terrestrial sources are known, only a few siderophore structures from marine bacteria have been fully characterized (Haygood et al. 1993; Reid et al. 1993; Butler 1998; Martinez et al. 2000). Additional marine organisms (cyanobacteria and heterotrophic bacteria) have been shown to produce siderophores (Wilhelm and Trick 1994; Granger and Price 1999); however, the spectrum of siderophore types and functionalities produced in the ocean is only just beginning to be elucidated.

Initially, it was suggested that DFOB, as a foreign siderophore, imposes iron stress uniformly upon the biological community. However, evidence now exists for specific iron uptake mediated by DFOB in marine phytoplankton and heterotrophic bacteria (Soria-Dengg and Horstmann 1995; Granger and Price 1999; Maldonado and Price 1999). This iron uptake has been monitored for both laboratory cultures and field experiments using natural communities from the subarctic Pacific (including one coastal upwelling area), the Sargasso Sea, and the Gulf of Mexico. Herein we report the isolation and structure determination of a siderophore, desferrioxamine G, produced by a marine bacterium isolated off the coast of Southern California. Desferrioxamine G (DFOG) is very similar in structure to DFOB (Fig. 1). Furthermore, both DFOB and DFOG are recognized and transported by the same outer membrane receptor protein in enteric bacteria (Deiss et al. 1998), suggesting that the use of DFOB for induction of iron stress is not merely addition of an inert iron chelator, but rather addition of a potential nutrient to a subset of marine phytoplankton and bacteria.

**Isolation**—Approximately 1,000 larvae of *Bugula neritina* were released from wild-collected adults (from Torrey Pines Artificial Reef, San Diego) into nonsterile natural seawater. Larvae were rinsed three times with sterile 80% seawater

and homogenized in 5 mL of sterile 80% seawater and plated on SWC medium (Nealson 1978). Strain BLI-41 was purified and maintained on Difco Marine Agar 2216 plates. Since strain BLI-41 was not reisolated in other experiments, it does not appear that there is a specific association between the larvae and strain BLI-41. The larvae do not feed and lack a gut. Thus the strain is likely a seawater bacterium that can nonspecifically attach to particles, including larvae.

**Siderophore assay**—Liquid cultures were grown in artificial seawater glycerol (ASG; Haygood et al. 1993) containing per liter, 15.5 g NaCl, 0.75 g KCl, 12.35 g MgSO<sub>4</sub>·7H<sub>2</sub>O, 1 g NH<sub>4</sub>Cl, 0.1 g glycerophosphate, 3 mL glycerol, 10 mM Hepes pH 7.4, 2 mM NaHCO<sub>3</sub>, and 10 g casamino acids. After 2 d growth, the production of siderophore was assayed using chromazurol S (CAS) shuttle solution containing CAS/Fe(III)/HDTMA (hexadecyl-trimethylammonium) as described in Haygood et al. (1993). The assay was positive using equal volumes of broth culture and CAS shuttle solution.

**16S rRNA gene sequencing**—DNA was extracted from colonies on plates with a Qiagen DNeasy tissue kit according to manufacturers instructions. The 16S rRNA genes were amplified by polymerase chain reaction (PCR) using 27f and 1492r primers as described in Haygood et al. (1993). PCR conditions were: 10 ng DNA, 10 pmoles of each primer, 0.2 mM dNTPs, Boehringer Mannheim buffer, and Taq polymerase. The temperature profile was 94°C for 1 min, 50°C for 1 min, 72°C for 1 min for 30 cycles followed by 7 min at 72°C in an Ericomp Twinblock thermal cycler. The PCR product was sequenced using a BigDye terminator cycle sequencing kit (Perkin Elmer Applied Biosystems) with standard bacterial 16S rRNA primers (Haygood et al. 1993) on an ABI 373 automated sequencer.

**Phylogenetic analysis**—Preliminary analyses using BLAST searches (Altschul et al. 1990) of genbank and sequence match in the ribosomal database project (RDP; Maida et al. 2000)] showed that the organisms most closely related to strain BLI-41 are members of the genus *Vibrio*. Aligned sequences of representative *Vibrios* and relatives were downloaded from RDP, and additional sequences from Genbank were added to the alignment and aligned by eye using Sequencher (Gene Codes). PAUP 4.04b4 (Sinauer) was used for phylogenetic analysis. The final data set contained 36 taxa. Variable regions that could not be aligned with confidence (48 characters) were excluded. 1,421 characters were analyzed, 207 of which were phylogenetically informative. Distance and maximum parsimony analyses gave similar results. Maximum parsimony analyses used a heuristic search algorithm with 10 rounds of random addition to generate starting trees and a transversion cost of two times transitions. Bootstrap analyses used the same conditions with 500 rounds of resampling.

**Isolation of siderophores**—Strain BLI-41 was grown in natural seawater medium containing 1 g NH<sub>4</sub>Cl, 2 g casamino acids, and 0.1 g glycerophosphate per liter of natural seawater (typically 1 liter growth per 4-liter Erlenmeyer

flask). Seawater was obtained from the Pacific Ocean off the coast of the University of California Santa Barbara and aged for 1–5 months at 4°C in the dark prior to use. The culture was grown overnight on a rotary shaker (140 rpm) at ambient temperature. The presence of siderophores was detected with the CAS shuttle solution assay. The siderophore was isolated from the supernatant following centrifugation of the culture (20 min, 4800 × g) and adsorption onto Amberlite XAD-2 resin. The resin was sequentially washed with two column volumes each of doubly deionized water (Nanopure®), 50% and 100% MeOH. Siderophore-containing fractions, corresponding to elution with 50% MeOH, were pooled, dried in vacuo, and applied to a C18 or C4 reversed-phase HPLC column (Vydac 10 mm ID × 250 mm L). The siderophore was purified using a gradient of 100/0 (% A/B) to 0/100 over 35 min [A = 99.95% dH<sub>2</sub>O (Nanopure®) and 0.05% trifluoroacetic acid (TFA) B = 39.95% dH<sub>2</sub>O, 0.05% TFA, and 60% methanol]. The absorbance of the eluent was monitored at 215 nm.

**Structure determination**—Initial mass determination for the siderophore from strain BLI-41 was determined using electrospray mass spectrometry performed using a VG-Fisons (Micromass) Platform II quadrupole mass spectrometer coupled with a Michrom BioResources HPLC unit used for direct injection (UCSB). Exact mass determination was completed on a Q-Star quadrupole time-of-flight mass spectrometer (PE. Biosystems) with all samples infused using a PE SCIEX ion spray source. Tandem mass spectrometric analysis (MS-MS) was completed on a similar instrument. <sup>1</sup>H NMR and <sup>13</sup>C nuclear magnetic resonance (NMR) spectra were recorded on a Varian 400 or 500 MHz instrument using either dimethyl-d<sub>6</sub> sulfoxide or deuterium oxide (*d* = 4.8 ppm) as solvent. NMR spectra of DFOG isolated from strain BLI-41 were compared to published spectra of DFOG and literature assignments of DFOB (Borgias et al. 1989; Reissbrodt et al. 1990).

**Results and discussion**—Desferrioxamines B, D2, E, and G were first isolated from various species of *Streptomyces* (Keller-Schierlein et al. 1965). Although desferrioxamines are generally considered bacterial siderophores from the gram-positive *Actinomycetes* species, many gram-negative bacteria also produce these siderophores (e.g., *Erwinia herbicola*, *Pseudomonas stutzeri*, *Chromobacterium violaceum*, *Hafnia alvei*) (Mucha et al. 1999). Moreover aerobactin, which was first identified in terrestrial and enteric bacteria, has now been found to be produced by some marine *Vibrio* species, suggesting some crossover in siderophore production can occur between terrestrial and marine bacteria (Haygood et al. 1993; Murakami et al. 1998).

The siderophore produced by strain BLI-41 was identified as DFOG by NMR, exact mass, and tandem mass spectrometry. High-resolution peak matching of DFOG, from strain BLI-41, yielded an exact *m/z* value of 619.3647 (*M* + *H*)<sup>+</sup> for the isotopically pure <sup>12</sup>C-containing molecular ion. The mass was within ±3.2 ppm for a calculated elemental composition of (*M* + *H*)<sup>+</sup> of C<sub>27</sub>H<sub>51</sub>N<sub>6</sub>O<sub>10</sub> (*M*<sub>calc</sub> = 619.3667). Further structural characterization was completed by comparing tandem mass spectra for DFOG from BLI-41 (Fig. 2)

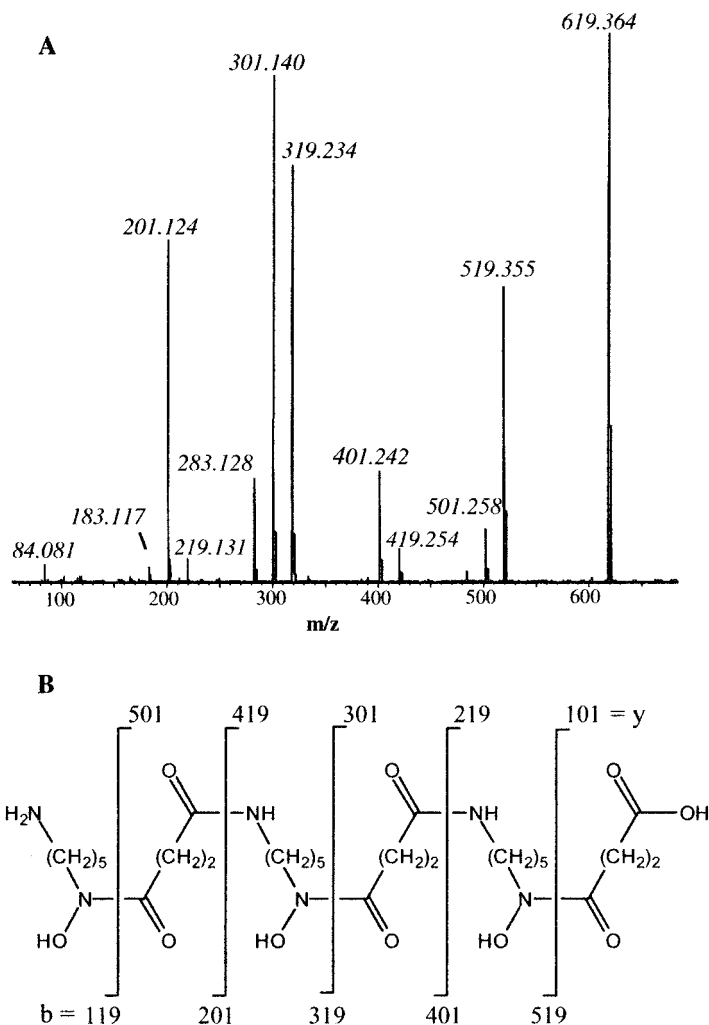


Fig. 2. (A) Tandem mass spectrum for desferrioxamine G from BLI-41. (B) Structure of desferrioxamine G showing the *y* and *b* fragment analysis from tandem mass spectrometry. The *y* and *b* nomenclature refers to the charge when retained by the COOH-terminal fragment or the NH<sub>2</sub>-terminal fragment of the peptide, respectively (Martinez et al. 2000).

to that of *H. alvei* (Reissbrodt et al. 1990). Fragment analysis established the connectivity of the three diaminopentane residues. All corresponding *y* and *b* fragments were found within the tandem mass spectrum except *m/z* 101 and 119, which were observed by ESI-MS at increased cone voltage and temperature sufficient to produce daughter ions. Final structural analysis was completed by comparison of <sup>13</sup>C-NMR and <sup>1</sup>H-NMR spectra for DFOG from BLI-41 (Table 1 and Fig. 3) to known spectra for DFOG and DFOB (Keller-Schierlein et al. 1984; Borgias et al. 1989; Reissbrodt et al. 1990). The NMR analysis also ruled out danoxamine, which is a related siderophore with the same mass as DFOG. Danoxamine contains a primary alcohol in place of the terminal amine moiety of DFOG; the strain BLI-41 siderophore lacks a <sup>13</sup>C NMR signal at 60.5 ppm indicative of the methylene carbon attached to the hydroxyl group of danoxamine (Keller-Schierlein et al. 1984).

Table 1.  $^{13}\text{C}$ -NMR chemical shifts for DFOG from strain BLI-41 in deuterium oxide. Refer to Fig. 3 for corresponding structure. Assignment according to Borgias (1989) and Reissbrodt (1990).

Carbon number	Chemical shift (ppm)
1	38.31
1', 1''	38.63, 38.75
2	25.74
2', 2''	27.28, 27.31
3, 3', 3''	22.09, 22.36
4, 4', 4''	24.71, 24.87
5, 5', 5''	47.05, 47.30
6, 6', 6''	173.33, 173.38, 173.43
7, 7', 7''	26.97, 27.05
8, 8'	29.72, 29.74
8''	29.86
9, 9'	174.30, 174.39
9''	176.77

Strain BLI-41 is a member of the genus *Vibrio*. Although the members of the genus *Vibrio* are too closely related for 16S rRNA sequence to permit statistically significant resolution of the internal topology of the group, bootstrap support for inclusion of strain BLI-41 within the genus *Vibrio* is strong (Fig. 4). Based on rRNA sequencing, strain BLI-41 does not fall within any of the described *Vibrio* species that have sequences deposited in public databases. *Vibrio* sp. BLI-41 appears to be most closely related to *Vibrio* sp. DS40M5 and *V. nigripulchritudo*, although this relationship was not strongly supported by bootstrap analysis. Both *Vibrio* sp. DS40M5 and *V. nigripulchritudo* are seawater isolates (Haygood et al. 1993; Farmer and Hickman-Brenner 1998). Although strain BLI-41 was isolated from an invertebrate larva, this strain is likely a seawater bacterium that can nonspecifically attach to seawater particles, including larvae.

In summary, excess DFOB has been used by a number of investigators to create iron-stressed conditions in iron-replete marine water samples (i.e., by complexing Fe[III]) (Hutchins et al. 1999; Wells 1999). Although DFOB is typically added under iron-replete conditions, it is clear that subpopulations of marine phytoplankton and heterotrophic bacteria are able to make use of Fe (III) bound to DFOB when added under iron deficient conditions (Soria-Dengg and Horstmann 1995; Granger and Price 1999; Maldonado and Price 1999). We have shown that marine *Vibrio* sp. BLI-41 produces DFOG, a linear desferrioxamine differing from DFOB only in the substitution of the terminal methyl group by propanoic acid. Given that DFOB and DFOG are recognized and transported by the same outer membrane receptor protein in certain enteric terrestrial bacteria, subpopulations of marine microorganisms may have evolved specific uptake systems for Fe(III)-chelating compounds similar in structure to DFOG. This could be an example of siderophore piracy by phytoplankton and heterotrophic bacteria similar to that observed in certain enteric bacteria (Granger and Price 1999). Therefore, caution should be exercised in interpreting the results

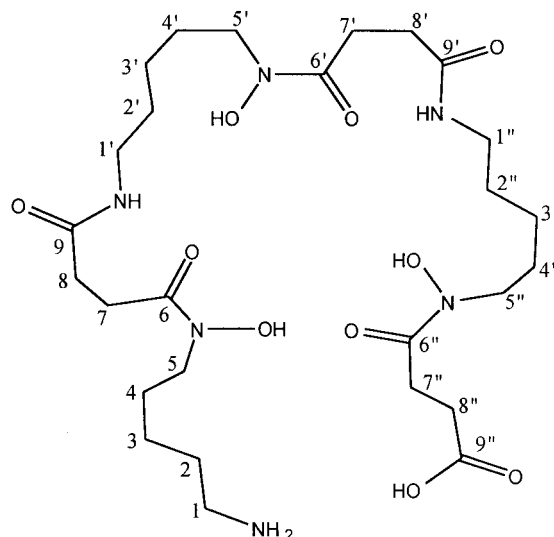


Fig. 3. Structure of desferrioxamine G. See Table 1 for  $^{13}\text{C}$ -NMR chemical shifts of corresponding carbon atoms. Numbering according to Reissbrodt (1990).

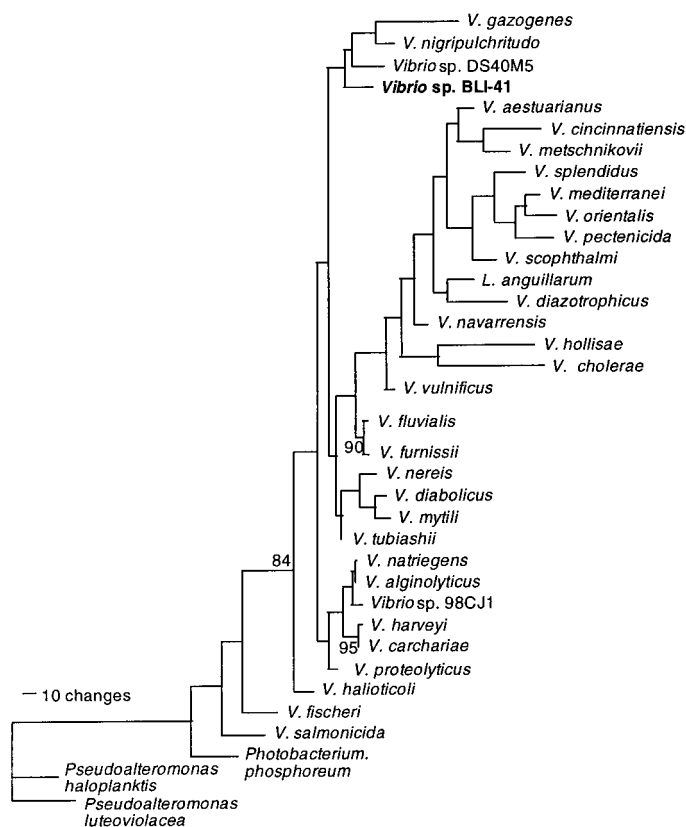


Fig. 4. Phylogenetic tree of *Vibrio* species based on maximum parsimony analysis of 16S rRNA gene sequences. The two *Pseudoalteromonas* species were set as the outgroup. Tree shown is one of 14 equally parsimonious trees, all of which have the group containing strain BLI-41 shown here. Bootstrap values greater than 75% are shown.

of DFOB addition experiments with natural populations of marine bacteria and phytoplankton.

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