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Boron Binding by a Siderophore Isolated from Marine Bacteria Associated with the Toxic Dinoflagellate *Gymnodinium catenatum*

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Phytoplankton blooms, including toxic species such as the diatom genus *Pseudo-nitzschia* or the dinoflagellate *Gymnodinium catenatum*, are a characteristic phenomenon in coastal and upwelling regions of the world's oceans. Blooms of harmful algal species have deleterious effects on marine ecosystems, and their impact on public health and local economies can be large and has been increasing globally.[1] This increasing incidence has sparked interest in species of bacteria closely associated (or symbiotic) with toxic dinoflagellates. Thus, axenic (bacteria-free) dinoflagellate cultures often cannot be established, and even when they can, they require the addition of vitamins and artificial metal complexes for satisfactory viability.[2] It can be inferred that under natural conditions, these heterotrophic bacteria contribute some critical factor(s) to the nutrition of the relevant marine algae.

A broad hypothesis that links these bacterial "symbionts" to the growth of toxic dinoflagellates is in their control of the supply of iron since this element, while the fourth most abundant in the Earth's crust, is present under aerobic conditions at neutral pH only in the form of extremely insoluble minerals that severely restrict its bioavailability. The iron level in open ocean waters is even lower than in most terrestrial environments.[3,4] While the iron uptake systems of phytoplankton are largely unknown, bacteria on the other hand have evolved sophisticated systems based on high-affinity iron specific binding compounds called siderophores to acquire, transport, and process this essential metal ion. Several hundred siderophores, whose biosyntheses are repressed by high iron levels, are known, and extensive studies of their isolation, structure, transport, and molecular genetics have been undertaken in the last two decades.[5] The structural variety of siderophores, both terrestrial and marine, has been comprehensively reviewed.[6] Considered essential to their role as iron transporters is the fact that siderophores have both high affinity and high specificity for Fe(III) over other biologically significant cations.

In the process of searching for new siderophores from *Marinobacter* sp. DG870, 893, and 979, marine bacteria associated with *G. catenatum*,[2] we isolated by BioGel *P*2 size exclusion chromatography from extracts of spent culture medium, one CAS[7] positive fraction. Negative ion ESI-MS revealed two major mass peaks in this fraction near 400 amu. Further purification via RP-HPLC chromatography followed by a second round of HPLC produced two clean fractions, one with a mass of 433 amu that was CAS positive and a second with mass 441 amu that was CAS negative. Subsequent high-resolution MS and a variety of 1 and 2-D NMR experiments unequivocally demonstrated that the CAS positive, 433 amu fraction was the known siderophore vibrioferrin (VF), Figure

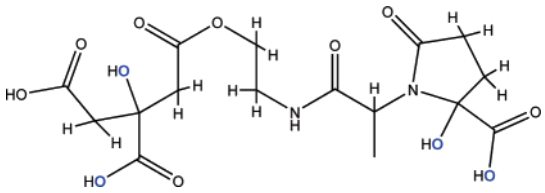

Figure 1. Vibrioferrin.

1. Vibrioferrin is a member of the carboxylate class of siderophores and contains two α-hydroxy acid groups. It was previously isolated from *Vibrio parahemeolyticus*, an enteropathogenic estuarine bacterium often associated with seafood-borne gastroenteritis and has been extensively studied by Yamamoto et al. [8,9] Because of its close association with the CAS positive fraction, we also investigated the nature of the CAS negative species with molecular mass 441 amu. A curious isotope distribution pattern suggested the presence of an element other than the expected C, H, N, or O. In fact a search through the periodic table produced a match only for the unexpected element boron. The presence of boron was rapidly confirmed by ^{11}B NMR and this, coupled with high-resolution MS and a variety of other 1- and 2-D ^{1}H and ^{13}C NMR experiments, revealed that the CAS negative, 441 amu, fraction is boronylated vibrioferrin! Since boron was not a component of the artificial seawater media used in culturing the bacteria, the boron incorporated into VF could only have been derived from the borosilicate glass flasks, suggesting a strong sequestering ability of VF for B. Indeed when we added boric acid to the media in the concentration reported to be present in seawater (0.4 mM)[10] boronylated vibrioferrin was the predominant species observed. A comparison between the ^{13}C NMR peaks of free VF and its boronylated analogue reveal significant coordination induced shifts (CIS) only for the carbon atoms assigned to the two citryl groups demonstrating that the binding of boron by VF is through those four oxygens. This was confirmed by the position of the peak in the ^{11}B NMR at 8.5 ppm that is consistent with a spiro-borate diester.[11] The structure of a variety of salts of "borodicitrate" have been reported where the boron is bound in a tetrahedral fashion through the carboxylate and α-hydroxyl groups from each of two citrates exactly as predicted from the CIS NMR data for B-VF.[12]

Using the structural data for borocitrate binding from the crystal structures as a starting point, we determined the ten lowest energy conformers of the complex using MMFF conformational searching algorithms as implemented in Spartan 2000. The four lowest energy conformers were then geometry-optimized at the DFT (B3LYP) level using Jaguar (Schrödinger), the lowest energy structure of which is shown (Figure 2). It is clear from this structure that it is easy for VF to bind to a tetrahedral borate through the citrate groups in a strain-free way. The presence of several internal hydrogen

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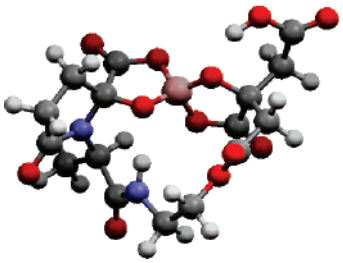

Figure 2. The DFT geometry-optimized structure of the lowest energy MMFF derived conformer of boron-vibrioferrin.

bonds also appears to stabilize the structure. Further experiments show that some, (i.e., rhizoferrin and petrobactin), but not all (i.e., any of the hydroxamate) siderophores also bind boron to varying degrees (data not shown).

While boron is a known dietary requirement for various phytoplankton[13,14] structurally characterized boron-containing natural products are confined to just a few macrolide antibiotics[15] and a bacterial quorum-sensing molecule.[16] The latter observation opens many intriguing possibilities. Quorum sensing molecules have been reported to be involved in siderophore production in some species[17,18] and a naturally occurring derivative of a non-boron containing quorum-sensing molecule has been reported to possess siderophore-like iron binding abilities.[19] Conversely, there is evidence that siderophores, in addition to their iron binding role, may function as signaling molecules.[17] These observations suggest a role for siderophores in inter/intraspecies communication and growth control. Another possibility is that VF could be functioning as a "boronophore", that is, a boron-transporting molecule for the phytoplankton. The presence of a high concentration of boron in seawater does not a priori eliminate the need for an extracellular transporter. Indeed membrane-associated boron transporters have recently been described in plants although an extracellular boron scavenger is unknown.[20] The details of these intriguing and potentially highly significant findings await further investigation, but it is now clear, in the marine environment at least where the concentration of boron is high, that the role of siderophores functioning exclusively as iron binding agents for their producing bacteria has to be reconsidered.

Despite the fact that VF has been extensively studied from a biological perspective, its metal binding characteristics remain largely unknown. Although we have not yet measured accurate Fe binding constants (these will be reported in due course) there is evidence that the interaction is weaker than for most other siderophores. Attempts to determine the overall formation constant by EDTA competition at near neutral pH, as is standard methodology for siderophores, failed in the case of VF as a single equivalent of EDTA effectively removes all of the Fe from FeVF. This sets an upper limit of 10^{23-25} for K_{ML}. This relatively weak Fe binding is not entirely unexpected as VF has only five potential donor groups, insufficient to satisfy the six coordination desired by Fe^{3+}. The low binding constant also suggests a less negative reduction potential for the iron complex (as there is typically a strong correlation between redox potential and the Fe(III) binding constant). Such a redox potential would likely be well within the range of cell surface reductases reported to be present in some phytoplankton.[21] In addition to its unexpected property of binding boron and its relatively weak iron binding, vibrioferrin, when complexed with Fe, is like other marine siderophores that contain an α-hydroxy acid moiety, photoreactive.[22] However despite these properties, bioassays in media rendered iron limiting by the presence of chelators, 2,2′bipyridyl, and EDDHA, revealed that VF is able to relieve iron induced growth inhibition in DG870, 893, and 979 as expected of a true siderophore.

Marine bacteria, including DG870, 893, and 979, specifically associated with toxic, bloom-forming dinoflagellates, represent a unique subset of marine bacteria which share the ability to produce and use vibrioferrin unlike numerous closely related organisms. This functional conservation is interpreted as indicating that there are specific selective processes operating between the bacterium and dinoflagellate.[23] Why are dinoflagellates selecting for such a phylogenetically and functionally specific group of marine bacteria? The unique features of vibrioferrin may provide important clues as to the nature of this and related algal-bacterial relationships.

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Supporting Information Available: Experimental details and NMR spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

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