

Identification of new members within suites of amphiphilic marine siderophores

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Abstract Marine bacterial isolates *Vibrio* sp. HC0601C5 and *Halomonas meridiana* str. HC4321C1 were isolated off the coast of southern California and were found to produce an expanded suite of previously identified amphiphilic siderophores. Specifically two new members of the amphibactin family, amphibactins S and T, which have a C14:1 ω -7 fatty acid and a saturated C12 fatty acid, respectively, were produced by *Vibrio* sp. HC0601C5. These siderophores are produced in addition to a number of previously described amphibactins and are excreted into the culture supernatant. Two new members of the aquachelin family of siderophores, aquachelins I and J, which have an hydroxylated C12 fatty acid and a saturated C10 fatty acid, respectively, were produced by *Halomonas meridiana* str. HC4321C1. These four new siderophores are more hydrophilic than their previously reported relatives, aquachelins A–D and the amphibactin suite of siderophores.

Keywords Marine siderophores · Amphiphilic siderophores · Amphibactins · Aquachelins

Introduction

Iron(III) is found at extremely low concentrations, 0.02–1 nM, over much of the World's oceanic surface waters in part due to its low solubility in aqueous aerobic conditions (Rue and Bruland 1995; Wu and Luther 1995). Unless bound by strong chelators, Fe(III) will form oxyhydroxides and precipitate out of the water column, thereby reducing the bioavailable pool of iron. The vast majority of Fe(III) in surface seawater is complexed to a class of organic ligands characterized by distinct stability constants (Gledhill and Van den Berg 1994; Rue and Bruland 1995; Wu and Luther 1995). Siderophores may comprise a portion of the in situ iron-binding ligands in surface ocean waters (Gledhill et al. 2004; Lewis et al. 1995; Macrellis et al. 2001; Rue and Bruland 1995). Siderophores are low molecular weight iron chelators, whose biosynthesis is stimulated by low iron concentrations (Sandy and Butler 2009). Despite the increasing number of siderophores identified from marine bacteria, far fewer marine siderophores have been characterized compared to siderophores isolated from terrestrial bacteria (Vraspir and Butler 2009). Nevertheless, a defining characteristic of many marine siderophores is amphiphilicity. Marine amphiphilic siderophores have been found to be produced in suites, where each member within the suite has a conserved iron(III)-binding polar head group appended by one or two fatty acids, which can vary by length, degree

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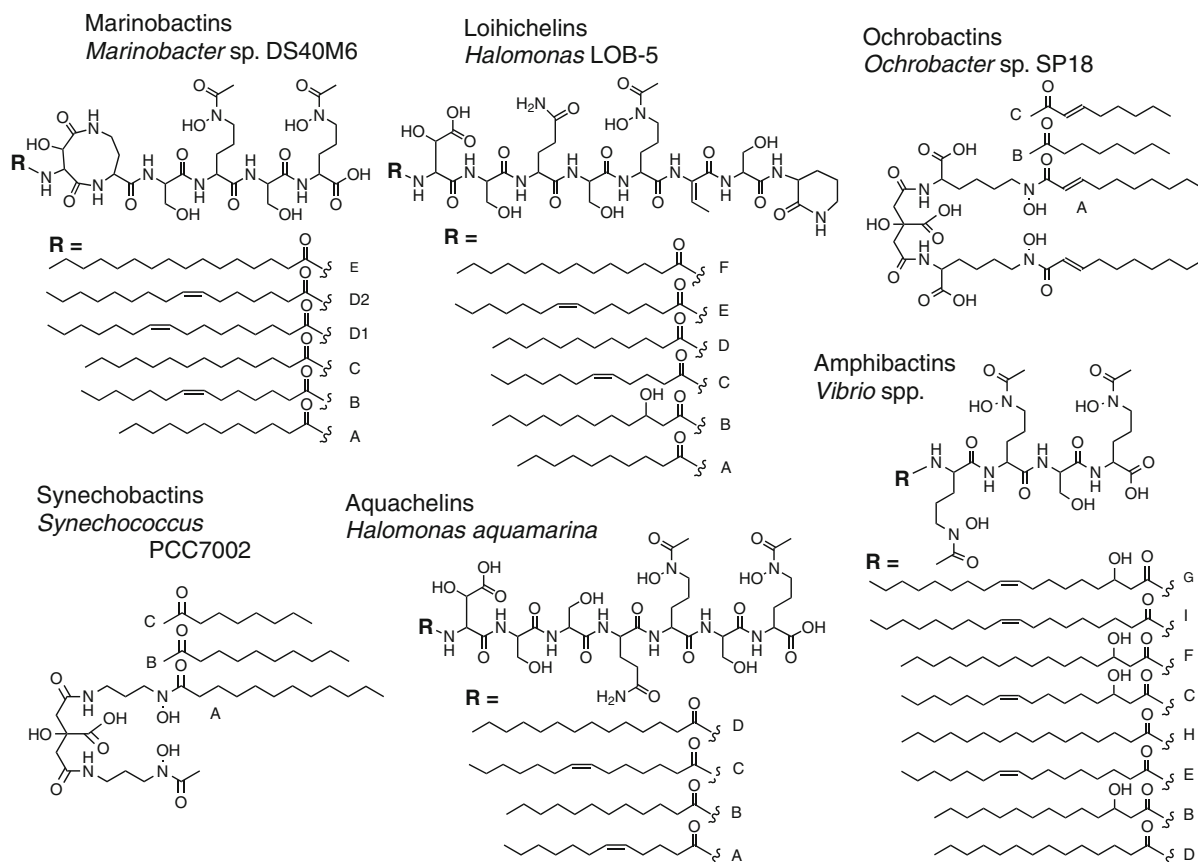


Fig. 1 Amphiphilic marine siderophores including marinobactins (Martinez et al. 2000), loihichelins (Homann et al. 2009), synechobactins (Ito and Butler 2005), aquachelins (Martinez et al. 2000), amphibactins (Martinez et al. 2003), and

ochrobactins (Martin et al. 2006). The amphibactins and ochrobactins were isolated primarily from the cell pellet. The letter designations derive from the order the siderophores elute by the HPLC

of unsaturation and hydroxylation. The chemical structure of the polar head group subdivides the marine amphiphilic siderophores into two groups: peptide-based siderophores and citrate-based siderophores (see Fig. 1).

Several suites of marine amphiphilic siderophores have been identified and characterized (Fig. 1). The amphibactins (Fig. 1) and ochrobactins (Fig. 1) are isolated by extraction of the bacterial cell pellet after centrifugation of the bacterial culture medium in the siderophore isolation process (Martin et al. 2006; Martinez et al. 2003), indicating that they are quite hydrophobic, whereas the aquachelins (Fig. 1) are isolated from the supernatant after pelleting the bacterial cells, and thus are more hydrophilic. The combination of a smaller peptidic head group with

longer fatty acids makes the amphibactins much more hydrophobic than the aquachelins (Martinez et al. 2003). We report herein the identification of four novel siderophores from bacteria isolated from the Santa Barbara basin. *Halomonas meridiana* str. HC4321C1 produces the previously identified aquachelins A–D and two new members of the aquachelin suite of siderophores. *Vibrio* sp. HC0601C5 produces a portion of the known suite of amphibactins, as well as two new amphibactins with novel fatty acid appendages. In contrast to the previous discovery of membrane bound amphibactins by *Vibrio* sp. R10 (Martinez et al. 2003), the new, as well as known, amphibactins produced by *Vibrio* sp. HC0601C5 are secreted into and extracted from the culture supernatant.

Materials and methods

Microorganisms

Halomonas meridiana str. HC4321C1 and *Vibrio* sp. HC0601C5 (accession numbers HQ111426 and HQ111425, respectively) were isolated from ocean water collected on July 15, 2007 at 34°13.7' N, –120°02.4' W and July 4, 2007 at 34°20.9' N, –120°13.7' W, respectively, while aboard the R/V Atlantis in the Santa Barbara basin. To identify bacterial isolates that produce siderophores, 200 µl seawater was spread onto agar plates (0.5 g bacto yeast extract, 5 g bacto peptone, and 15 g bacto agar (BD), per liter of aged natural seawater) containing chrome azurol S (Schwyn and Neilands 1987). Based on halo-producing colonies, putative siderophore-producing bacteria were picked from the indicator plates and streaked onto maintenance media plates (Schwyn and Neilands 1987). Maintenance media plates contain 0.5 g bacto yeast extract, 5 g bacto peptone, and 15 g bacto agar (BD), per liter of aged natural seawater.

Siderophore isolation

Halomonas meridiana str. HC4321C1 and *Vibrio* sp. HC0601C5 were treated the same for siderophore isolation unless specified. For siderophore isolation, the strains were cultured in an artificial seawater medium without added iron for approximately 4–6 days (HC4321C1) and 1–2 days (HC0601C5) on a rotary shaker (200 rpm) (Martin et al. 2006). The cultures were harvested by centrifugation at 6,000 rpm for 30 min at 4°C. Amberlite XAD-2 resin (Aldrich) was added to the cell-free supernatant to adsorb the siderophores, after which the mixture of XAD-2 and supernatant was washed with doubly deionized water to remove salts. The XAD-2 resin was then packed in a column and the siderophores were eluted with 100% methanol. Siderophore-containing fractions were pooled and concentrated via rotary vacuum evaporation. The concentrated solution was further purified by reversed-phase high-performance liquid chromatography (RP-HPLC) using a preparative Higgins Analytical C₄ column (250 mm length × 20 mm diameter). Compounds isolated from *H. meridiana* HC4321C1 were eluted with a linear gradient from 100% solvent A [0.05% trifluoroacetic acid (TFA) in doubly deionized

water (Barnstead Nanopure II)] to 100% B (0.05% TFA in acetonitrile) over 35 min. Compounds isolated from *Vibrio* sp. HC0601C5 were eluted with a linear gradient from 50% solvent A [0.05% TFA in doubly deionized water (Barnstead Nanopure II)] to 100% solvent B (0.05% TFA in 80% methanol) over 15 min, holding at 100% solvent B for an additional 10 min. The eluents were monitored at 215 nm and peaks were collected by hand and stored on dry ice. If necessary, fractions were ultrapurified on the same preparative C₄ column using the same program. Purified siderophores were lyophilized and stored at –20°C.

Mass spectrometry

Electrospray ionization mass spectrometry (ESI-MS) and tandem mass spectrometry using argon as a collision gas were carried out on a Micromass Q-TOF2 (Waters Corp.).

Structural determination of fatty acid appendages

Siderophore fatty acids were converted to methyl esters with 3 N methanolic HCl (Sigma) in glass vials with Teflon-lined screw-caps. The vials were tightly capped and incubated for 3 h at 110°C to generate the methyl esters of the fatty acid appendages. The methyl esters were extracted into hexanes, dried over anhydrous MgSO₄, and analyzed by GC-MS (Varian, Saturn 2100T GC-MS). Identification of the methyl esters was accomplished by comparison with authentic fatty acid methyl ester standards (Supelco); the observed set of fatty acids is the same as observed in the loihichelins (Homann et al. 2009). The position of the double bond in the unsaturated fatty acid of amphibactin S was determined by reductive work-up, using dimethyl sulfide, of the ozonolysis products followed by analysis by GC-MS.

16S rRNA gene amplification and phylogenetic analysis

Strain identity was determined by first isolating the bacteria by multiple rounds of streaking on maintenance media plates, followed by 16S rDNA sequencing. Each bacterium was inoculated into liquid autoclaved DifcoTM Marine Broth 2216 (BD) and incubated on a rotary shaker (200 rpm) at room temperature overnight. Extraction of nucleic acids

was carried out using a Qiagen DNeasy Blood and Tissue Kit. Extracted DNA was amplified by using the universal bacterial 16S rDNA primers, 27F (5'-AGR GTT YGA TYM TGG CTC AG-3') and 1492R (5'-GGY TAC CTT GTT ACG ACT T-3'). Amplification conditions were as follows: 10X Taq buffer, 2 mM dNTP, 20 μ M 27F and 1492R, 25 mM $MgCl_2$, 50% acetamide, Taq DNA polymerase. Reactions were started at 90°C for 3 min and then run for 35 cycles (30 s at 94°C, 1 min at the annealing temperature of 50°C, and 100 s at 72°C), followed by a final 10-min extension at 72°C on a BioRad MJ Mini thermal cycler. Each PCR reaction was directly loaded onto 1% agarose gels stained with ethidium bromide, and visualized under UV illumination. PCR products were then ligated into plasmids using the Champion pET 101 Direction TOPO Expression Kit (Invitrogen). Next, one shot TOP10 competent cells were transformed with 2 μ L of the TOPO cloning reaction. A small amount from each transformation was spread onto prewarmed LB plates (Difco™ LB Broth, Miller; BD) with ampicillin, and incubated overnight at 37°C. Single colonies were then picked and inoculated into liquid LB with ampicillin and incubated at 37°C on a rotary shaker (200 rpm). The plasmids were then extracted using a Molbio mini-

plasmid prep kit. The extracted plasmids were then restriction digested with EcoRI (Promega), for 1 h, according to the conditions specified by the manufacturer (but without acetylated BSA). Samples with inserts were then sequenced using both the M13 reverse (-24)(16-mer) and M13 (18-mer) primer (UC Berkeley DNA Sequencing Facility). Sequences were then aligned using a biological sequence alignment editor (BioEdit). Phylogenetic analysis was based on maximum likelihood analysis of 16S rRNA gene sequences (Dereeper et al. 2008; Edgar 2004; Castresana 2000; Guindon and Gascuel 2003; Anisimova and Gascuel 2006; Chevenet et al. 2006).

Results

Growth of *H. meridiana* str. HC4321C1 under iron limited conditions resulted in the production of aquachelins A–D, I and J (Fig. 2). Aquachelins I and J, which are more hydrophilic and elute earlier than aquachelins A–D, are two new additions to the previously characterized suite of aquachelin siderophores produced by *H. aquamarina* str. DS40M3 (Fig. 1). Growth of *Vibrio* sp. HC0601C5 under similar conditions resulted in the production of

Fig. 2 Structures of amphiphilic siderophores produced by *Halomonas meridiana* str. HC4321C1 and *Vibrio* sp. HC0601C5

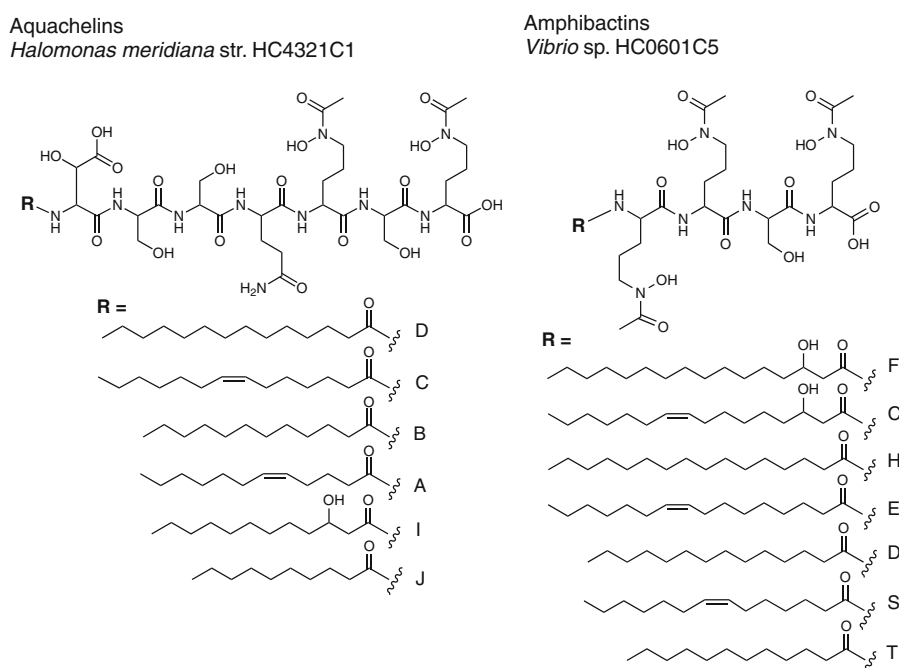
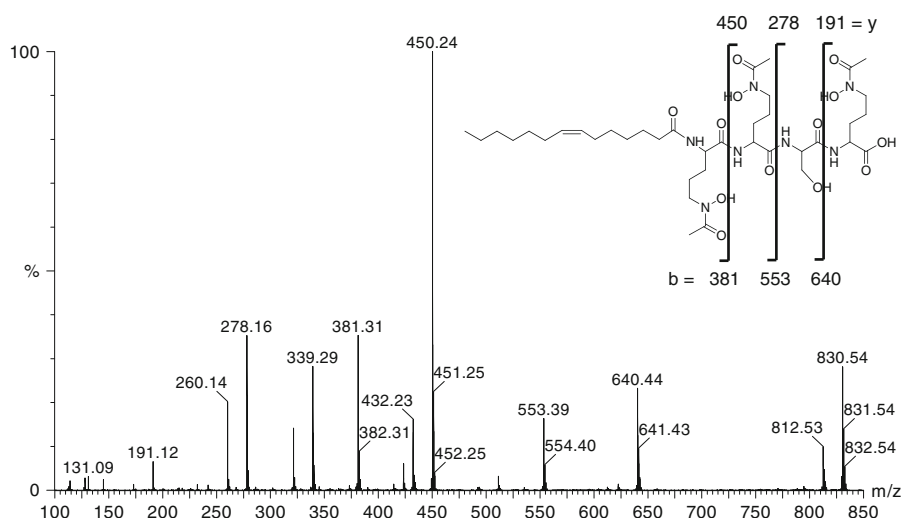


Fig. 3 ESI-tandem mass spectrum of amphibactin S (C14:1). *Inset:* Structure of amphibactin S. The vertical lines through the structure show the mass-to-charge ratio values for the ‘y’ and ‘b’ fragments (Roepstorff and Fohlmann 1984) from the tandem mass spectrum



amphibactins S and T, two new members of the amphibactin family (Fig. 2). In addition, five of the original eight members of the amphibactin suite of siderophores were also synthesized by *Vibrio* sp. HC0601C5 (Figs. 1, 2).

The $[M + H]^+$ molecular ions of amphibactin S and T are 830.54 and 804.55 m/z, respectively. The $[M + H]^+$ molecular ions of aquachelin I and J are 1081.68 and 1037.65, respectively. Tandem mass spectrometry reveals the majority of the $y + 2H^+$ and b fragments expected for these structures (Figs. 3, 4, 5, 6). The y fragments 191, 278, and 450 m/z observed for amphibactin S and amphibactin T are also observed for the suite of amphibactins produced by *Vibrio* sp. R10 (Martinez et al. 2003). The identical y fragments but different b fragments for each amphibactin indicate that the difference in the amphibactins is a result of distinct fatty acid

appendages. Similarly, the conserved y fragments for the aquachelins, 191, 278, 450, 578, 665, 752 m/z, are seen in aquachelin I (Fig. 5) and aquachelin J (Fig. 6), as well as aquachelins A–D, also indicating that the difference in structure lies in the fatty acid appendages (Martinez et al. 2000).

Fatty acid analysis of amphibactins S and T gave major peaks in the GC-MS with the characteristic retention times and mass fragmentation patterns of methyl tetradecenoate (C14:1), and methyl dodecanoate (C12), respectively. The methyl tetradecenoate (C14:1) of amphibactin S had a mass-to-charge ratio of 241.4, two mass units lower than the standard, methyl tetradecanoate (C14:0). The methyl dodecanoate of amphibactin T had a mass-to-charge ratio of 215.5, correlating with the standard for methyl dodecanoate. Fatty acid analysis of aquachelins I and J gave major peaks in the GC-MS with the

Fig. 4 ESI-tandem mass spectrum of amphibactin T (C12:0). *Inset:* Structure of amphibactin T showing the ‘y’ and ‘b’ fragmentation (Roepstorff and Fohlmann 1984) from the tandem mass spectrum

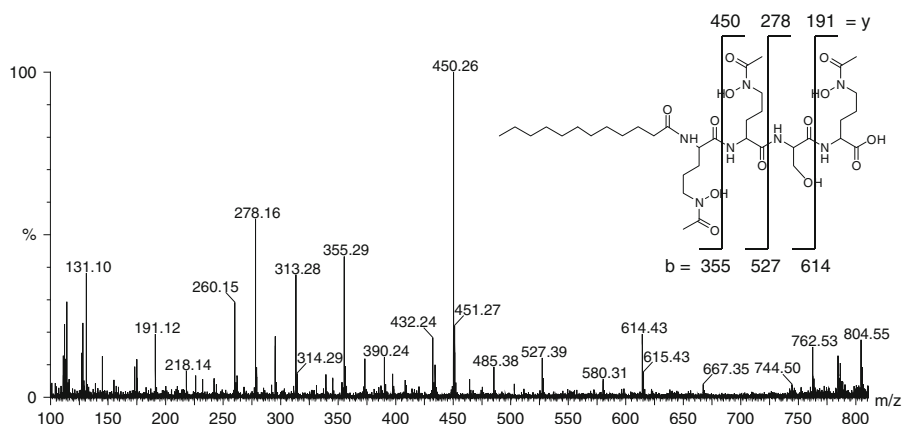


Fig. 5 ESI-tandem mass spectrum of aquachelin I. *Inset:* Structure of aquachelin I showing the 'y' and 'b' fragmentation (Roepstorff and Fohlmann 1984) from the tandem mass spectrum

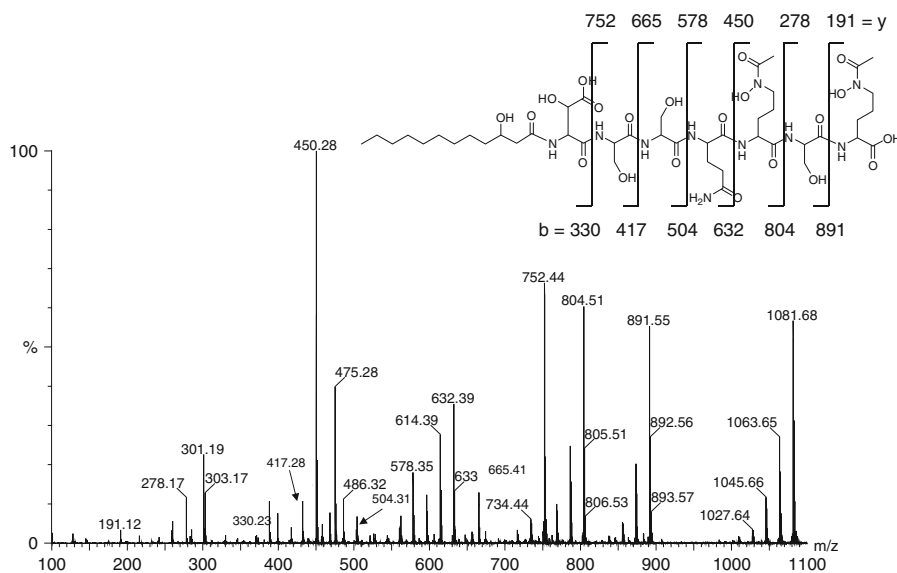
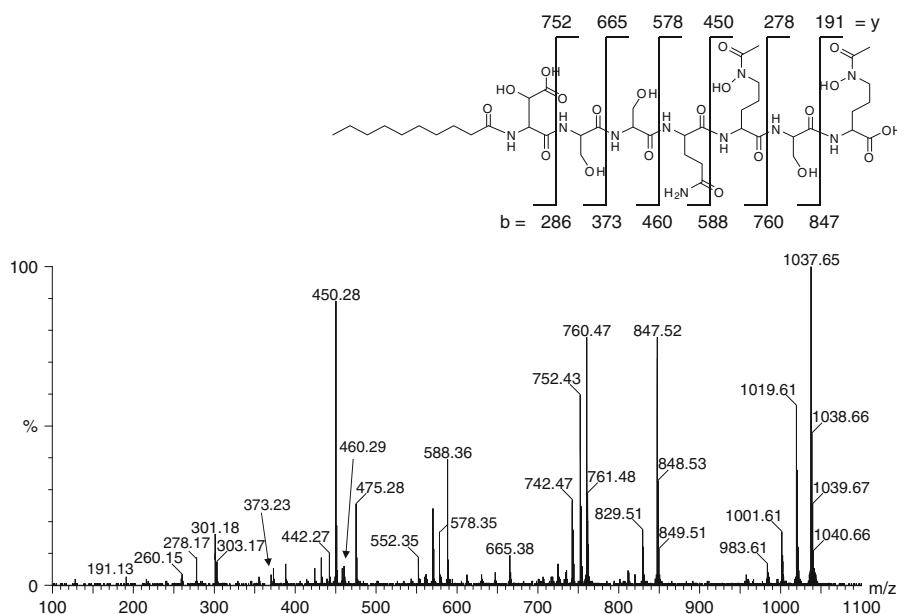


Fig. 6 ESI-tandem mass spectrum of aquachelin J. *Inset:* Structure of aquachelin J showing the 'y' and 'b' fragmentation (Roepstorff and Fohlmann 1984) from the tandem mass spectrum



characteristic retention times and mass fragmentation patterns of 3-hydroxy methyl dodecanoate (C12:OH), and methyl decanoate (C10), respectively. The 3-hydroxy methyl dodecanoate of aquachelin I had a mass-to-charge ratio of 231.2. The hydroxyl group is determined to be on carbon 3 due to the characteristic mass of 3-hydroxyalkanoate methyl ester (103.0 m/z), which is the major fragment in the GC-MS spectrum (Huijberts et al. 1994). The methyl decanoate of aquachelin J had a mass-to-charge ratio

of 187.3, correlating with the standard for methyl decanoate. The identification was confirmed by co-injection with fatty acid methyl ester standards (Supelco). Therefore, the fatty acid portions of aquachelins I and J and amphibactins S and T were determined to be 3-hydroxy dodecanoic acid, decanoic acid, tetradecenoic acid, and dodecanoic acid. The position of the double bond in the tetradecenoic acid of amphibactin S was determined by ozonolysis. GC-MS analysis detected methyl 7-oxoheptanoate

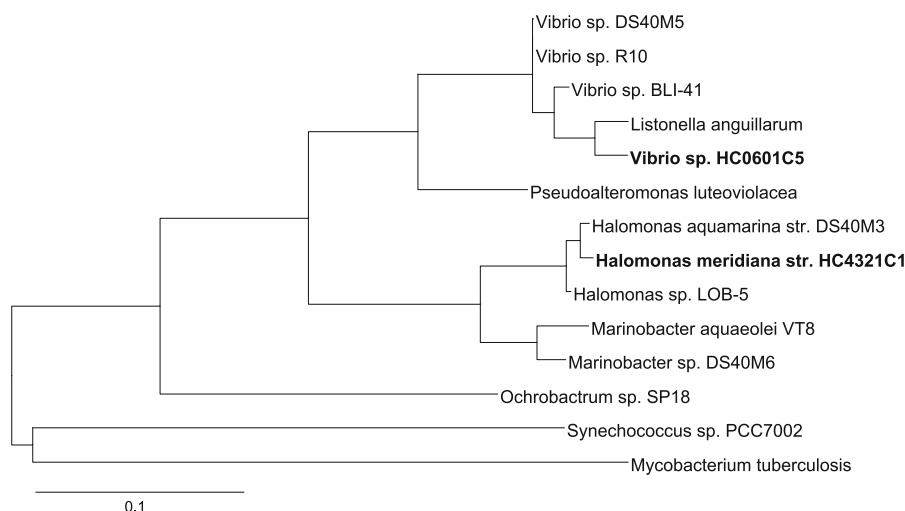


Fig. 7 Phylogenetic tree of siderophore-producing bacteria based on maximum likelihood analysis of SSU rRNA DNA sequences (Dereeper et al. 2008; Edgar 2004; Castresana 2000; Guindon and Gascuel 2003; Anisimova and Gascuel 2006;

Chevenet et al. 2006; Huson et al. 2007). All isolates are marine with the exception of *Mycobacterium tuberculosis*. Scale for branch length indicates the number of substitutions per site

(m/z of 159), indicative of the double bond at the position $\omega 7c$. Therefore amphibactin S contains a *cis*-7-tetradecenoic fatty acid (C14:1 ω -7; Fig. 2).

16S rDNA sequence analysis identifies the two siderophore-producing bacterial strains used in this study as *Halomonas meridiana* str. HC4321C1 and *Vibrio* sp. HC0601C5. *Halomonas meridiana* str. HC4321C1 has a 99% sequence identity with *Halomonas meridiana* str. DSM 5425. *Vibrio* sp. HC0601C5 has a 98% sequence identity with its closest match in a BLAST search (*Vibrio* sp. R117) and a 96% sequence identity with *Vibrio splendidus* ATCC 33125T. The phylogenetic relationships of these strains based on 16S rDNA sequences to other siderophore-producing bacteria is shown in Fig. 7.

Discussion

The new siderophore structures, amphibactin S, amphibactin T, aquachelin I, and aquachelin J belong to previously characterized suites of siderophores. The amphibactins found in this study from strain *Vibrio* sp. HC0601C5 were secreted into the culture medium, in contrast to the amphibactins produced by *Vibrio* sp. R10 (Martinez et al. 2003), which were extracted primarily from the cell pellet. Due to the cell-associated nature of the amphibactins produced

by *Vibrio* sp. R10, we also probed the cell pellet extract for additional siderophores. We found only trace amounts of the amphibactins identified in the culture supernatant in the cell pellet extract of *Vibrio* sp. HC0601C5. The lipid composition of the two *Vibrio* species may be sufficiently different, thus impacting the extent of the amphibactin partitioning and favoring relatively more hydrophilic siderophores in *Vibrio* sp. HC0601C5. It is interesting to note that amphibactins S and T belong to a set of previously reported but structurally uncharacterized siderophores seen in nutrient-enriched seawater incubations in several oceanic regions (Gledhill et al. 2004; Mawji et al. 2008; Vraspir and Butler, unpublished data).

The predominance of amphiphilic siderophore-producing bacteria has been isolated from the marine environment. The number, size, and composition of suites of siderophores are changing as new bacteria are isolated and screened for siderophore production. Initial experiments indicate that modifying growth conditions such as the composition of the growth medium and the temperature at which bacteria are grown changes the relative quantities of siderophores produced. In fact, after discovering aquachelins I and J from *H. meridiana* str. HC4321C1, our analysis of *H. aquamarina* str. DS40M3 showed small quantities of aquachelins I and J in addition to aquachelins

A–D. The detection of the amphibactins and aquachelins from bacteria isolated from the Santa Barbara basin expands the geographic range from which siderophore-producing bacteria have been isolated. The biological importance for marine bacteria of such large and wide spread suites of siderophores is intriguing and under current investigation.

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References

- Anisimova M, Gascuel O (2006) Approximate likelihood ratio test for branches: a fast, accurate and powerful alternative. *Syst Biol* 55(4):539–552
- Castresana J (2000) Selection of conserved blocks from multiple alignments for their use in phylogenetic analysis. *Mol Biol Evol* 17(4):540–552
- Chevenet F, Brun C, Banuls AL, Jacq B, Chisten R (2006) TreeDyn: towards dynamic graphics and annotations for analyses of trees. *BMC Bioinformatics* 7:439
- Dereeper A, Guignon V, Blanc G, Audic S, Buffet S, Chevenet F, Dufayard JF, Guindon S, Lefort V, Lescot M, Claverie JM, Gascuel O (2008) Phylogeny.fr: robust phylogenetic analysis for the non-specialist. *Nucleic Acids Res* 1:36 (Web Server issue):W465–9. Epub 19 Apr 2008
- Edgar RC (2004) MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res* 32(5):1792–1797
- Gledhill M, Van den Berg CMG (1994) Determination of complexation of iron(III) with natural organic complexing ligands in seawater using cathodic stripping voltammetry. *Marine Chem* 47:41–54
- Gledhill M, McCormack P, Ussher S, Achterberg EP, Mantoura RFC, Worsfold PJ (2004) Production of siderophore type chelates by mixed bacterioplankton populations in nutrient enriched seawater incubations. *Marine Chem* 88:75–83
- Guindon S, Gascuel O (2003) A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. *Syst Biol* 52(5):696–704
- Homann VV, Sandy M, Tincu JA, Templeton AS, Tebo BM, Butler A (2009) Loihechelins A–F, a suite of amphiphilic siderophores produced by the marine bacterium *Halomonas* LOB-5. *J Natl Prod* 72:884–888
- Huijberts GNM, de Rijk TC, de Waard P, Eggink G (1994) ¹³C nuclear magnetic resonance studies of *Pseudomonas putida* fatty acid metabolic routes involved in poly(3-hydroxyalkanoate) synthesis. *J Bacteriol* 176:1661–1666
- Huson DH, Richter DC, Rausch C, DeZulian T, Franz M, Rupp R (2007) Dendroscope: an interactive viewer for large phylogenetic trees. *BMC Bioinformatics* 8(1):460
- Ito Y, Butler A (2005) Structure of synechobactins, new siderophores of the marine cyanobacterium *Synechococcus* sp. PCC 7002. *Limnol Oceanogr* 50:1918–1923
- Lewis BL, Holt PD, Taylor SW, Wilhelm SW, Trick CG et al (1995) Voltammetric estimation of iron(III) thermodynamic stability constants for catecholate siderophores isolated from marine bacteria and cyanobacteria. *Marine Chem* 50:179–188
- Macrellis HM, Trick CG, Rue EL, Smith G, Bruland KW (2001) Collection and detection of natural iron-binding ligands from seawater. *Marine Chem* 76:175–187
- Martin JD, Ito Y, Homann VV, Haygood MG, Butler A (2006) Structure and membrane affinity of new amphiphilic siderophores produced by *Ochrobactrum* sp. SP18. *J Biol Inorg Chem* 11:633–641
- Martinez JS, Zhang GP, Holt PD, Jung HT, Carrano CJ et al (2000) Self-assembling amphiphilic siderophores from marine bacteria. *Science* 287:1245–1247
- Martinez JS, Carter-Franklin JN, Mann EL, Martin JD, Haygood MG, Butler A (2003) Structure and membrane affinity of a suite of amphiphilic siderophores produced by a marine bacterium. *Proc Natl Acad Sci USA* 100:3754–3759
- Mawji E, Gledhill M, Minto JA, Tarran GA, Ussher S, Thompson A, Wolff GA, Worsfold PJ, Achterberg EP (2008) Hydroxamate siderophores: occurrence and importance in the Atlantic Ocean. *Environ Sci Technol* 42:8675–8680
- Roepstorff P, Fohlman J (1984) Proposal for a common nomenclature for sequence ions in mass spectra of peptides. *Biomed Mass Spectrom* 11(11):601
- Rue EL, Bruland KW (1995) Complexation of iron(III) by natural ligands in the Central North Pacific as determined by a new competitive ligand equilibration/adsorptive cathodic stripping voltammetric method. *Marine Chem* 50:117–138
- Sandy M, Butler A (2009) Microbial iron acquisition: marine and terrestrial siderophores. *Chem Rev* 109:4580–4595
- Schwyn B, Neilands JB (1987) Universal chemical assay for the detection and determination of siderophores. *Anal Biochem* 160:47–56
- Vraspir JM, Butler A (2009) Chemistry of marine ligands and siderophores. *Ann Rev Marine Sci* 1:43–63
- Wu J, Luther GW (1995) Complexation of Fe(III) by natural organic ligands in the Northwest Atlantic Ocean by a competitive ligand method and a kinetic approach. *Marine Chem* 50:159–177