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Authors

Kem, Michelle P
Naka, Hiroaki
Iinishi, Akira
et al.

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Fatty acid hydrolysis of acyl-marinobactin siderophores by *Marinobacter* acylases

Michelle P. Kem,[†] *Hiroaki Naka,*[‡] *Akira Iinishi,*[†,ǂ] *Margo G. Haygood,*[‡] *Alison Butler*[†]*

[†]Department of Chemistry and Biochemistry, University of California, Santa Barbara, California 93106-9510, United States

[‡]Institute of Environmental Health, Oregon Health & Science University, Portland, Oregon 97239-3098, United States

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Present Addresses

[ǂ]Rohto Pharmaceutical Co., Ltd., 1-8-1 Tatsumi-nishi Ikuno- Ku, Osaka, Japan.

***Corresponding Author**

Tel: 805-893-8178. Fax: 805-893-4120. E-mail: butler@chem.ucsb.edu.

Abbreviations

HSL, homoserine lactone; AHL, acyl homoserine lactone; IPTG, isopropyl β-D-thiogalactopyranoside; O.D., optical density; SDS-page, sodium dodecanoyl sulfate polyacrylamide gel electrophoresis; Ni-NTA, nickel nitrilotriacetic acid; RP-HPLC, reverse phase high performance liquid chromatography; MWCO, molecular weight cut off; Val, valine; Asn, asparagine; His, histidine.

ABSTRACT. The marine bacteria *Marinobacter* sp. DS40M6 and *Marinobacter nanhaiticus* D15-8W produce a suite of acyl peptidic marinobactin siderophores to acquire iron under iron-limiting conditions. During late-log phase growth, the marinobactins are hydrolyzed to form the marinobactin headgroup with release of the corresponding fatty acid tail. The *bntA* gene, a homologue of the *Pseudomonas aeruginosa* pyoverdine acylase gene, *pvdQ,* was identified from *Marinobacter* sp. DS40M6. A *bntA* knockout mutant of *Marinobacter* sp. DS40M6 produced the suite of acyl marinobactins A-E, without the usual formation of the marinobactin headgroup. Another marinobactin-producing species, *Marinobacter nanhaiticus* D15-8W, is predicted to have two *pvdQ* homologues, *mhtA* and *mhtB*. MhtA and MhtB have a 67% amino acid sequence identity. MhtA catalyzes hydrolysis of the apo-marinobactin siderophores, as well as, the quorum sensing signaling molecule, dodecanoyl-homoserine lactone. In contrast to hydrolysis of the suite of apo-marinobactins by MhtA, hydrolysis of the iron(III)-bound marinobactins was not observed.

Bacterial growth and colonization is often limited by the availability of iron in aerobic environments. Faced with a paucity of available iron, many bacterial species up-regulate the production of siderophores, high affinity iron (III) ligands to facilitate iron uptake. One class of siderophores is distinguished by having a fatty acid appended to a peptidic headgroup, as seen with many structurally characterized marine siderophores, resulting in an amphiphilic compound.[1,2,3] The marinobactins (Figure 1A) from *Marinobacter* sp. DS40M6 were among the first amphiphilic marine siderophores to be structurally characterized.[4,5]

Lipopeptidic siderophores are produced among many genera of bacteria, and are not limited to marine bacteria. In addition to the suites of marine acylated peptidic siderophores (e.g., aquachelins, amphibactins, amphi-enterobactins, loihichelins, moanachelins, etc.),[4,2,6,7] and the mycobactins produced by many species of *Mycobacteria*,[8] many newly reported non-marine acyl peptidic siderophores (e.g. cupriachelins, serobactins, taiwachelins, etc.)[9,10,11] are being discovered. Recently, the opportunistic human pathogen *Pseudomonas aeruginosa* was also found to produce a fatty acid-containing precursor of pyoverdine.[12] Like the marinobactins, pyoverdine is a peptide-based siderophore synthesized as a lipopeptide. Pyoverdine, however, is found in culture supernatants as a non-acylated species due to expression of the Ntn-hydrolase, PvdQ, an acylase, which hydrolyzes the fatty acid prior to excretion from the cell.[12] A *pvdQ* deletion results in the abolition of pyoverdine production and a decrease in virulence factors, such as, swarming motility and biofilm formation.[13]

PvdQ is a proenzyme that forms a heterodimeric protein with an αββα-fold, characteristic of the Ntn-hydrolase family, and shows promiscuity in its ability to also hydrolyze long chain acyl homoserine lactones used for quorum sensing.[14,15] The location of the *pvdQ* gene in the pyoverdine synthetic operon, however, suggests PvdQ is also involved in the enzymatic tailoring of pyoverdine during biosynthesis.[16,17] Interestingly, PvdQ is conserved among fluorescent *Pseudomonas* species, yet the *pvdQ* gene is not always located in the pyoverdine synthetic operon. Even with this difference in genomic organization of *pvdQ* orthologues, expression of *pvdQ* is iron regulated and important for pyoverdine production among all tested species.[18]

Enzymes similar to PvdQ, such as, AiiC from *Anabaena* sp. PCC 7120, QuiP from *P. aeruginosa*, AhlM from *Streptomyces* sp. M664 and AiiD from *Ralstonia* sp. XJ12B have been reported in the literature for their ability to act as quorum quenchers.[19,20,21,22] Quorum sensing is the cell density-dependent regulation of gene expression in bacterial communities controlled by small signaling molecules termed autoinducers.[23] One well-studied class of autoinducers are the N-acylhomoserine lactones which have fatty acids ranging from 4 to 16 carbons, many of which have an oxo or hydroxyl group at the 3' position and unsaturated bonds.[24,25] These aforementioned quorum quenchers work by hydrolyzing the fatty acid from the homoserine lactone, destroying the quorum signaling function (Figure 1B). Interest in the nature of PvdQ for its involvement in *P. aeruginosa* virulence is increasing.[16,26,27] Further insight into the biological significance of these Ntn-hydrolases would aid in understanding how these acylases affect bacterial iron acquisition and survival.

Recently, the hydrolysis of the marinobactin siderophores in *Marinobacter* sp. DS40M6 has been seen, producing the marinobactin head group (M_{HG}, Figure 1), following export of the marinobactins from the cell.[28] We have now identified the acylase, *bntA*, from a representative cosmid library of *Marinobacter* sp. DS40M6 required for the deacylation of the marinobactins during bacterial growth. Another marinobactin-producing strain, *Marinobacter nanhaiticus* D15-8W, whose genome has been sequenced, was also determined to produce the marinobactin headgroup. Two BntA- and PvdQ-like enzymes are encoded in the genome of *M. nanhaiticus*

D15-8W, MhtA and MhtB. We report hydrolase activity of heterologously expressed MhtA with apo-marinobactins and acyl homoserine lactones. Interestingly, however, MhtA does not catalyze fatty acid hydrolysis of Fe(III)-marinobactins, which may suggest a regulatory role on iron uptake and growth.

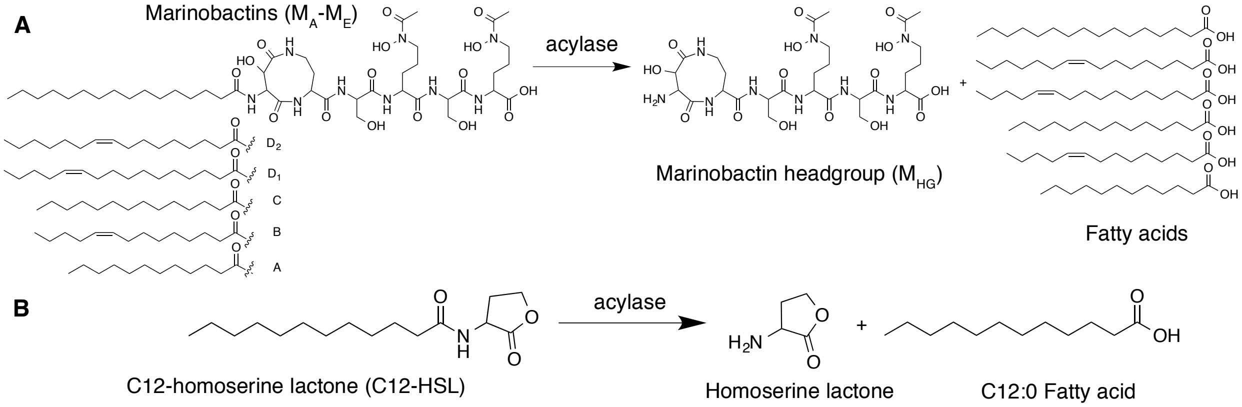

Figure 1. (A) The marinobactin siderophores produced by *Marinobacter* sp. DS40M6 and *Marinobacter nanhaiticus* D15-8W. Both *Marinobacter* species are proposed to produce an acylase that hydrolyzes the fatty acid tail, producing M$_{HG}$. (B) Hydrolysis of the quorum sensing autoinducer, C12:0-HSL, by quorum quenching acylases.

Materials and methods

Bacterial strains, media and growth conditions

The bacterial strains and plasmids we used are listed in Table S1. Primers used in this study for gene amplification and mutant construction are listed in Table S2. *Marinobacter* sp. DS40M6 was isolated off the coast of west Africa from open ocean water.[4] *M. nanhaiticus* D15-8W was a generous gift from Professor Li Zheng (First Institute of Oceanography, State Oceanic Administration, China).[29] *Marinobacter* sp. DS40M6 and *M. nanhaiticus* D15-8W were cultured in a 4 L flask in artificial seawater (ASW) media (15.5 g/L NaCl, 0.75 g/L KCl, 0.2 g/L $MgSO_4 \cdot 7H_2O$, 0.1 g/L $CaCl_2 \cdot 2H_2O$, 1.0 g/L NH_4Cl, 5 g/L succinic acid, 3 g/L Na_2HPO_4, pH

7.0) on a rotary shaker at 180 rpm and ambient temperature. *Escherichia coli* strains were cultured in Luria Bertani (LB) broth or on LB agar plates at 37°C with shaking at 225 rpm unless specified otherwise. When required, the LB broth was supplemented with 100 µg/mL ampicillin, 50 µg/mL kanamycin or 50 µg/mL spectomycin.

Identification of marinobactin biosynthetic genes in Marinobacter species

The publicly available BLAST (Basic Local Alignment Search Tool) algorithm and NRPS/PKS predictor software (http://nrps.igs.umaryland.edu/nrps/)[30] was used to search for marinobactin biosynthetic genes in sequenced genomes based on the published structure.[4]

Identification of a putative marinobactin acylase in Marinobacter sp. DS40M6

The *pvdQ*-like genes from sequenced *Marinobacter* species were aligned with Cobalt alignment software and degenerate primers were designed using the CODEHOP (COnsensus-DEgenerate Hybrid Oligonucleotide Primer) PCR primer design strategy.[31] Amplification from *Marinobacter* sp. DS40M6 genomic DNA was performed using the degenerate primers M6F1 and M6R1 along with touchdown PCR (TD-PCR).[32]

Cosmid library construction and screening of Marinobacter sp. DS40M6

Construction of the *Marinobacter* sp. DS40M6 cosmid library was performed using the SuperCos1 kit (Agilent). Briefly, *Marinobacter* sp. DS40M6 genomic DNA was partially digested with the restriction enzyme, Sau3AI. DNA fragments between 24 kb and 48 kb were ligated into the dephosphorylated BamHI sites of the SuperCos1 vector. *In vitro* packaging was performed using Gigapack III XL Packaging Extract (Agilent). The cosmid library was screened for the gene of interest using the Digoxigenin-11-dUTP (DIG) High Prime DNA labeling kit (Roche). A probe was designed from a 238 bp fragment of the acquired 416 bp gene fragment using primers M6P-F1 and M6P-R1. Cosmids with a positive hit were isolated and rescreened by colony PCR using the M6P-F1/R1 primers. The insert from the cosmid-containing the gene of interest was subcloned into the pET24a(+) vector following digestion with BamHI and rescreened using the M6P-F1/R1 primers. Sequence analysis of the positive subclone was performed at the UC Berkeley sequencing facility.

Cloning and heterologous expression of mhtA

The *mhtA* gene, including the coding sequence for the periplasmic signal sequence, from *M. nanhaiticus* D15-8W was cloned into the pET-22b(+) vector at the NdeI and XhoI sites using primers *mhtA*-NdeI-F and *mhtA*-XhoI-R to produce a construct with a 6X C-terminus His-tag. The resulting plasmid, p22-MhtA-His_6, was transformed into *E. coli* BL21-CodonPlus(DE3)-RIPL cells for protein expression. The culture was grown at ambient temperature and induced with 0.4 mM IPTG at an O.D. of 0.4 overnight at 16°C. Cells were harvested at 6,000 rpm for 10 minutes and lysed by sonication in 20 mM sodium phosphate buffer pH 7.8, 0.1 M NaCl, 5 mM imidazole, and 1% triton x-100. The crude lysate was centrifuged at 10,000 x g for 15 minutes followed by filtration through a 0.45-μm filter. The clarified lysate was incubated with Ni-NTA resin (pre-equilibrated with 20 mM sodium phosphate pH 7.8, 0.1 M NaCl, 5 mM imidazole) for 2 hours at 4°C. The resin was loaded onto a gravity column and washed with 5 column volumes of wash buffer (20 mM sodium phosphate pH 7.8, 0.1 M NaCl, 20 mM imidazole). The His-tagged protein was eluted with 3 column volumes of elution buffer (20 mM sodium phosphate pH 7.8, 0.1 M NaCl, 500 mM imidazole, 0.1% triton x-100) and the eluted fractions were concentrated and buffer exchanged into activity buffer (20 mM Tris pH 8, 50 mM NaCl, 2 mM $CaCl_2$) using a 50 MWCO spin column (2 mL, Millipore).

Construction and complementation of Marinobacter sp. DS40M6 deletion mutants

The deletion fragment of the *bntA* gene was constructed by splicing by overlap extension (SOE) PCR[33] as previously described.[6] First PCR was performed using primers *bntA*-mut-F-*XhoI* and bntA-mut-1-*Eco47III and bntA*-mut-2-*Eco47III* and *bntA*-mut-R-*SpeI,* in separate tubes, to amplify upstream and downstream regions of the *bntA* gene, respectively. The obtained DNA fragments were mixed and used as a DNA template of second PCR performed using primers *bntA*-mut-F-*XhoI* and *bntA*-mut-R-*SpeI*, and the generated deletion fragment was gel-purified and ligated into pGEM-T easy. The inserted DNA was subsequently excised by *XhoI* and *SpeI* digestion, and ligated into the corresponding sites of the suicide vector pDM4.[34] The obtained plasmid was further modified by ligating the *SmaI*-digested spectinomycin (Sp) resistance cassette from pIC156,[35] into its corresponding site, generating pHN26. Then, pHN26 was conjugated from *E. coli* S17-1λpir into *Marinobacter* sp. DS40M6 by diparental mating, and first recombinants were selected by plating exconjugants on Marine Agar supplemented with 100μg/ml Sp and 10 μg/ml gentamicin. First recombinants were grown in Marine Broth without antibiotics overnight, and streaked on Marine Agar containing 15% sucrose to select second recombinants. Then, the Δ*bntA* mutant was screened by colony PCR, and the mutation was further confirmed by PCR using primers constructed outside or inside the *bntA* gene.

To complement the deletion, the Sp resistance cassette was PCR-amplified using primers, Sp-*SacI*-F and Sp-*EcoRI*-R, and pIC156 as a template. The amplified DNA fragment was ligated into pGEM-T easy then subcloned into the *SacI* and *EcoRI* sites of pMMB208,[36] generating pHN27. The *bntA* gene with the Shine-Dalgarno sequence was PCR-amplified using primers *bntA*-com-*SphI*-F and *bntA*-com-*XbaI*-R, and ligated into pGEM-T easy then subcloned into the

SphI and *XbaI* sites of pHN27, generating pHN28. pHN27 and pHN28 were conjugated into *Marinobacter* sp. DS40M6 wild type and/or Δ*bntA* as described above.

Analysis of siderophore production by bntA knockout mutant

Wild type *Marinobacter* sp. DS40M6 + pHN27, Δ*bntA Marinobacter* sp. DS40M6 + pHN27 and Δ*bntA Marinobacter* sp. DS40M6 + pHN28 were grown in 1 L of ASW media as stated above and siderophores were isolated as previously described.[4,5] Siderophores were purified by RP-HPLC going from 0% acetonitrile + 0.05% TFA (trifluoroacetic acid) in water to 100% acetonitrile + 0.05% TFA over 50 minutes on a C18 analytical column (Vydac). Electrospray ionization mass spectrometry (ESI-MS) in combination with tandem mass spectrometry (ESI-MS/MS) using a Micromass QTOF-2 tandem mass spectrometer was used to verify the identity of the isolated siderophores.

Activity analysis of MhtA-His$_6$ with apo- and Fe(III)- marinobactins

Siderophores used as substrates were isolated and purified as previously described.[4] The concentration of apo-marinobactins was determined by spectroscopic titration at 400 nm with a standardized stock solution of Fe(III). Iron(III)-bound marinobactins were obtained by adding 1.5 equivalents of $FeCl_3$ (1.8 mM in water) for 15 minutes at ambient temperature. *In vitro* activity analysis of MhtA-His$_6$ was performed using 50 μM substrate in 20 mM tris pH 8, 50 mM NaCl, 2 mM $CaCl_2$. As a control, *E. coli* BL21-CodonPlus(DE3)-RIPL cells without the p22-MhtA-His$_6$ plasmid were lysed and purified by affinity chromatography on Ni-NTA resin in tandem with *E. coli* BL21-CodonPlus(DE3)-RIPL cells containing the MhtA expression plasmid. The Ni-NTA eluents were used in equal volumes for activity analysis. The reactions were performed in the presence of 10% methanol to increase the solubility of apo- and Fe(III)-marinobactins and to prevent micelle formation. All reactions were performed in 200 μl volumes and incubated at 30°C for 72 hours. Reactions were quenched by the addition of 0.5 volume of 2.5 N HCl. Protein was precipitated out by adding 5 volumes ice cold ethanol and incubated at -20°C for 1 hour to overnight followed by centrifugation at 12,000 rpm, 4°C, for 15 minutes. The supernatant was removed, diluted 1:5 in water and lyophilized to dryness. The samples were resuspended in 200 μl of water and analyzed on a C18 analytical column as described above.

Activity analysis of MhtA-His$_6$ with C12:0-HSL

The activity of MhtA-His$_6$ with C12:0-HSL was monitored under the same conditions as the marinobactins with the exception that 1 mM substrate was used in a 300 μl reaction volume. A 50 μl aliquot of the reaction mixture was removed at various time points and quenched by the addition of 0.5 volume of 2.5 N HCl. Protein was removed and samples were prepared to dryness as described in the previous section. The reaction was monitored by following the formation of the hydrolysis product, homoserine lactone, which was detected by derivatization with dansyl chloride (5-dimethylamino-1-naphthalenesulphonyl chloride). The dried sample was resuspended in 100 μl of 50 mM sodium bicarbonate and adjusted to pH 9-10 with 3M NaOH. Dansyl chloride (20 μl of 50 mM in acetonitrile) was added to the sample and incubated for 30 minutes in the dark. Reactions were quenched by the addition of HCl, filtered through a 0.22 μM spin filters and analyzed by RP-HPLC at 254 nm using ddH$_2$O + 0.05% TFA and acetonitrile + 0.05% TFA as mobile solvents.

Results

Identification of a putative marinobactin acylase in Marinobacter sp. DS40M6

A BLASTp analysis of sequenced *Marinobacter* species using PvdQ as a query was performed to identify a potential marinobactin acylase. Six *Marinobacter* species were found to contain PvdQ-like proteins and all were predicted to be part of the Ntn-hydrolase superfamily based on homology (Table 1). Using degenerate primers designed from the amino acid sequences of PvdQ-like proteins in Table 1, a 416 bp gene fragment was amplified from *Marinobacter* sp. DS40M6 genomic DNA (Figure S1) indicating *Marinobacter* sp. DS40M6 also has an acylase related to PvdQ. Cosmid library construction followed by hybridization screening resulted in the discovery of a 2646 bp ORF (named *bntA*), encoding a protein of 882 amino acids with a molecular weight of 94 kDa. The protein has a 96% amino acid identity to a protein annotated as a peptidase from *Marinobacter algicola* DG893 and 59-66% similarity to proteins annotated as acylases from other sequenced *Marinobacter* species. The protein has a 33% sequence identity to AaC, the quorum quencher from *Ralstonia solanacearum,* and 32% sequence identity to PvdQ from *Pseudomonas aeruginosa* PAO1. The nucleotide sequence of *bntA* has been deposited in the GenBank database under accession number KM670457.

Ntn-hydrolases are characterized by a distinct αββα core structure following a post-translational processing of the propeptide into a heterodimeric form. [37] An amino acid alignment surrounding the active site region of PvdQ, BntA, and the other putative *Marinobacter* acylases, was performed to look for sequence conservation (Figure S2). The crystal structure of PvdQ from *P. aeruginosa* PAO1 was used to determine the nature of the highlighted residues.[14, 38] A well-conserved glycine-serine-alanine sequence necessary for the post-translational processing of Ntn-hydrolases is closely conserved among PvdQ, BntA and the putative *Marinobacter* acylases.[22; 39; 40] Residues important for catalysis in PvdQ, including the N-terminal nucleophilic serine, and residues lining the substrate-binding site are conserved among all species. Conservation of amino acids, Val330 and Asn540, responsible for stabilization of the transient oxyanion transition state[14] are also present.

Table 1. Putative PvdQ-like Ntn-hydrolases in sequenced *Marinobacter* species.

Accession #	**ORF predicted function (species)**	**Size (AA)**	**% identity to PvdQ (*P. aeruginosa* PAO1)**
ADP96903	Acyl-homoserine lactone acylase (*Marinobacter adhaerens* HP15)	898	32%
EON91289	Aculeacin A acylase (*Marinobacter lipolyticus* SM19)	829	35%
EHJ0588	Penicillin amidase (*Marinobacter manganoxydans* MnI7-9)	890	33%
EDM49623	Peptidase S45 (*Marinobacter algicola* DG893)	882	32%
ERP91591	Peptidase S45 (*Marinobacter* sp. ES-1)	888	31%
ENO13542	Acyl-homoserine lactone acylase (*Marinobacter nanhaiticus* D15-8W)	894	30%
ENO13543	Acyl-homoserine lactone acylase (*Marinobacter nanhaiticus* D15-8W)	915	31%

Siderophore production by bntA knockout mutants in Marinobacter sp. DS40M6

To investigate the function of *bntA* with the marinobactins, a knockout mutant of *bntA* was constructed and analyzed for siderophore production and marinobactin headgroup formation under low-iron growth conditions. Wild type *Marinobacter* sp. DS40M6 and Δ*bntA Marinobacter* sp. DS40M6 were grown in ASW media for 5 days and siderophores were isolated and analyzed by RP-HPLC. An analysis of siderophore production by wild type *Marinobacter* sp. DS40M6 shows the appearance of M_{HG} around 11 minutes, which is not seen in the culture supernatant of Δ*bntA Marinobacter* sp. DS40M6 (Figure 2). Complementation of the *bntA* gene restored the production of M_{HG} (Figure 2C) as seen by the appearance of a peak at 11 minutes. An analysis of Δ*bntA Marinobacter* sp. DS40M6 + pHN28-*bntA* resulted in the appearance of mostly M_{HG} after 5 days of growth due to the majority of the full-length marinobactins being hydrolyzed by BntA. It is believed that the acylated marinobactins disappear in the complement strain since the expression level of BntA in the complement strain is likely greater than in the WT strain, which results in the complete conversion of full-length marinobactins to M_{HG} when grown for 7 days. ESI-MS/MS was used to verify that the compound eluting at 11 minutes is

M_{HG}, m/z 750 $[M+H]^+$ (Figure S3).[28] This result indicates that expression of the identified *bntA* gene is required for hydrolysis of the marinobactin siderophores during bacterial growth. Expression of recombinant BntA in *E. coli* resulted in the production of a 100 kDa protein observed by SDS-page analysis corresponding to the insoluble, pre-processed form of the enzyme, which lacks activity. The addition of solubility tags and expression in various *E. coli* expression systems did not produce soluble, active protein (data not shown). Similar difficulties expressing other AHL-acylases in *E. coli* have been reported.[20,40]

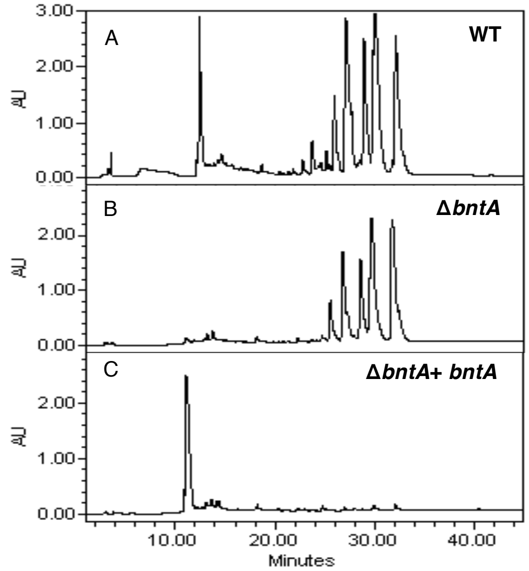

Figure 2. RP-HPLC trace of culture supernatants from (A) WT *Marinobacter* sp. DS40M6 + pHN27 (B) Δ*bntA Marinobacter* sp. DS40M6 + pHN27 and (C) Δ*bntA Marinobacter* sp. DS40M6 + pHN28. The peak around 11 minutes corresponds to M_{HG} and the five peaks from 25-32 minutes represent the suite of marinobactins.

Marinobactins produced by Marinobacter nanhaiticus D15-8W

To determine if other bacterial species produce the marinobactins and a marinobactin hydrolase, the genomes of sequenced *Marinobacter* species were screened for putative non-ribosomal peptide synthetases (NRPS) involved in peptide siderophore biosynthesis and a *bntA*-

like gene. NRPSs are large multi-modular synthetases comprised, at a minimum, of a condensation, adenylation, and thioesterase domain to incorporate amino acids into a growing peptide chain in an assembly line fashion.[41] *Marinobacter nanhaiticus* D15-8W contains two genes, ENO16762 and ENO16763, annotated as putative NRPSs. Genes surrounding ENO16762 and ENO16763 encode for putative siderophore uptake, transport and tailoring enzymes (Table S3). Publicly available software (http://nrps.igs.umaryland.edu/nrps/) was used to predict the amino acid specificity of each adenylation domain.[30]

The predicted biosynthetic scheme for marinobactin biosynthesis in *M. nanhaiticus* D15-8W is shown in Figure 3. The first module of gene ENO16763 has high similarity to acyl CoA ligases as predicted by BLASTp analysis. Similar NRPS domains are present in the first module of other lipopeptide-producing NRPSs, such as, PvdL from *P. aeruginosa,* which starts the biosynthesis of pyoverdine.[42] The adenylation (A) domain in module 2 (M2) is predicted to load L-aspartic acid, which would be epimerized to D-aspartic acid. The hydroxylation of aspartic acid may be accomplished by gene ENO16757, which encodes a taurine catabolism dioxygenase/aspartyl hydroxylase, based on homology. The third unit, second amino acid in module three, in the marinobactins is L-diaminobutyric acid, however, no prediction was made for the A domain of module 3 (M3). BLASTp analysis shows M3 to have homology to the pyoverdine biosynthetic protein, PvdL, which has an A domain specific for diaminobutyric acid. The A domain specific for diaminobutyric acid in PvdL has an 8 letter code of DIWELTXX similar to the 8 letter code of the A domain in M3 of *M. nanhaiticus* D15-8W, DIWELTA-, suggesting this domain could be specific for diaminobutyric acid.[42,30] It is not known what enzyme might be responsible for the cyclization of aspartic acid and diaminobutryic acid, however, condensation and condensation-like cyclization (cyc) domains are known to catalyze cyclization of cysteine, serine, and threonine residues forming heterocyclic ring moieties.[43]

NRPS protein ENO16762 was predicted to have adenylation domains specific for L-serine, L-N^5-hydroxyornithine, L-serine, and L-N^5-hydroxyornithine followed by a thioesterase domain. Two epimerases are predicted to convert the first added serine and last N^5-hydroxyornithine to the D-configuration. These predictions correspond to the amino acids present in the marinobactin headgroup attached to a N-terminal fatty acid appendage.

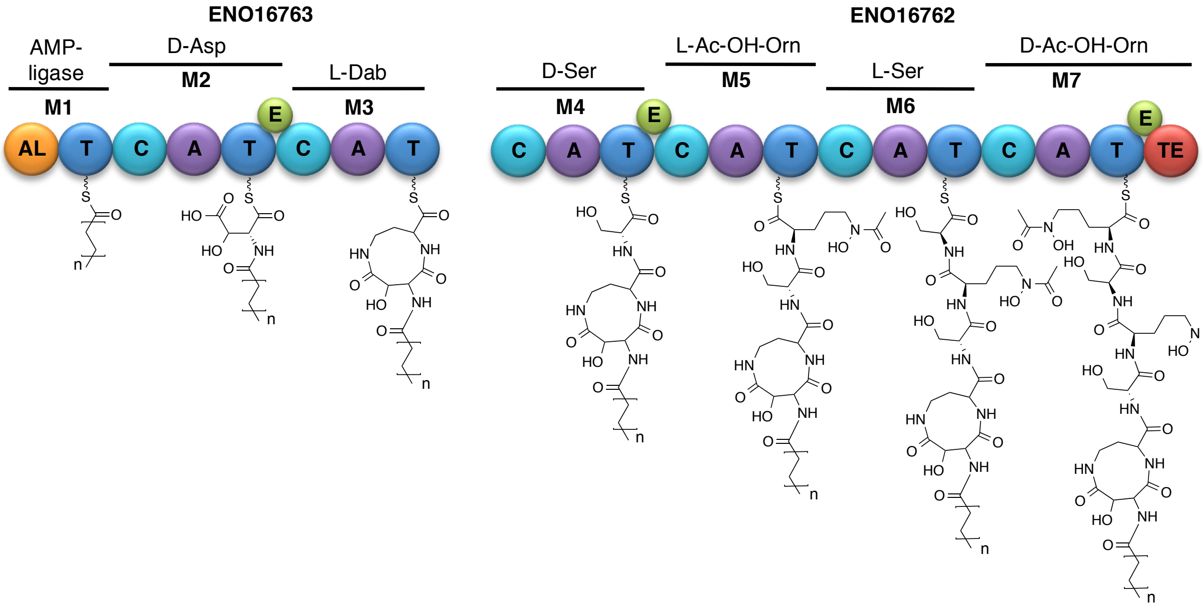

Figure 3. Predicted biosynthetic scheme for marinobactins in *M. nanhaiticus* D15-8W (n = 5-7). Biosynthesis is predicted to begin with acetylation by the AL (Acyl-CoA ligase) domain. Biosynthesis is predicted to occur in a linear fashion using traditional NRPS logic (T = thiolation domain, C = condensation domain, A = adenylation domain, E = epimerase domain, TE = thioesterase domain).

To determine whether *M. nanhaiticus* D15-8W produced the marinobactin siderophores as predicted, siderophores were isolated from a culture of *M. nanhaiticus* D15-8W grown in iron-limited ASW media. Five major peaks were seen in the HPLC profile and subjected to mass spectrometry analysis (Figure S4A). Compounds were identified based on mass and the ESI-MS/MS fragmentation pattern. Peak 1 was identified as M_{HG} (*m/z* 750) suggesting *M. nanhaiticus* D15-8W produces the marinobactins and an acylase able to hydrolyze the fatty acid tail from the marinobactin headgroup (Figure S4B). Peak 2 was identified as marinobactin A (M_A; *m/z* 932 $[M+H]^+$) with a C12:0 fatty acid. Peaks 3, 4 and 5 were identified as M_C (C14:0; *m/z* 960 $[M+H]^+$), M_D (C16:1; *m/z* 986 $[M+H]^+$), and M_E (C16:0; *m/z* 988 $[M+H]^+$), respectively (Figure S5). Production of marinobactin B (C14:1) is not observed in the cultures of *M. nanhaiticus* D15-8W, although the other members of the suite of acyl marinobactins are produced by *M. nanhaiticus* D15-8W, as well as the marinobactin headgroup, consistent with what has been previously seen with *Marinobacter* sp. DS40M6.[28]

Screening the M. nanhaiticus D15-8W genome for potential marinobactin acylases

The *M. nanhaiticus* D15-8W genome was screened for homologues of *bntA* from *Marinobacter* sp. DS40M6 and *pvdQ* from *P. aeruginosa* PAO1 using BLASTp analysis. Two genes, ENO13542 (named *mhtA*) and ENO13543 (named *mhtB*), were annotated as acyl-homoserine lactone acylases and have a 30% and 31% identity to PvdQ from *P. aeruginosa*, respectively, and a 67% identity to each other. Publicly available software (http://www.psort.org/psortb/index.html) was used to predict the cellular location of each protein with MhtB predicted to be an outer membrane-associated protein and MhtA predicted to be a periplasmic protein. Using the same software program, BntA, from *Marinobacter* sp. DS40M6 was also predicted to be membrane-associated and has a slightly higher percent identity to the predicted outer membrane protein, MhtB, at 63% versus 58% with MhtA. However, due to solubility issues with the recombinant expression of BntA, the predicted periplasmic protein, MhtA, from *M. nanhaiticus* D15-8W was selected for our initial investigation; we cloned and overexpressed the *mhtA* gene to investigate fatty acid hydrolysis with the marinobactins and acyl-homoserine lactones.

Expression of MhtA from *M. nanhaiticus D15-8W*

Expression of MhtA-His$_6$ resulted in the appearance of a 100 kDa band (lane 3, Figure S6) on an SDS-page gel when compared to the *E. coli* without plasmid control. By comparison to other Ntn-hydrolases (e.g. PvdQ), this is the correct mass for the unprocessed polypeptide prior to the self-activation that all Ntn-hydrolases undergo,[37] and the same was observed with overexpression of BntA-His$_6$ from *Marinobacter* sp. DS40M6 (Figure S7). While the expression level of MhtA-His$_6$ was too low to identify an α-subunit (~25 kDa) and a β-subunit (~70 kDa) of the active form of the enzyme unambiguously on a gel, active enzyme was found to be present through an activity assay, namely, the hydrolysis of octanoyl-*p*-nitroaniline, which produces the yellow *p*-nitroanaline product (Figure S8).[44] This preparation of MhtA-His$_6$ was used to investigate hydrolysis of the acyl marinobactins *in vitro*.

Reactivity of MhtA-His$_6$ with apo- and Fe(III)- bound marinobactins and C12-HSL

Hydrolysis of the marinobactins by the putative periplasmic *M. nanhaiticus* D15-8W Ntn-hydrolase, MhtA, was monitored *in vitro*. Incubation of MhtA-His$_6$ with 50 μM apo-M$_A$ resulted in the appearance of a peak around 11 minutes (Figure 4) that was not detected in the *E. coli* only control (Figure S9). ESI-MS analysis of this peak showed a parent ion of *m/z* 750 ([M+H]$^+$) with fragmentation ions of *m/z* 560, *m/z* 473, and *m/z* 273 as previously reported for M$_{HG}$ (Figure S10).[28] Incubation of MhtA-His$_6$ with 50 μM apo-M$_E$ also produced M$_{HG}$; however, M$_E$ with a

C16:0 fatty acid tail appears to be hydrolyzed at a slower rate than M_A with a C12:0 fatty acid tail (data not shown).

To explore the activity of MhtA-His$_6$ with the Fe(III)-bound marinobactins, recombinant MhtA-His$_6$ was incubated with 50 µM Fe(III)-M_A under the same conditions as the apo-marinobactins. After 72 hours of incubation no hydrolysis of the iron-bound siderophore was observed (Figure 4). Likewise, Fe(III)-M_E was not hydrolyzed.

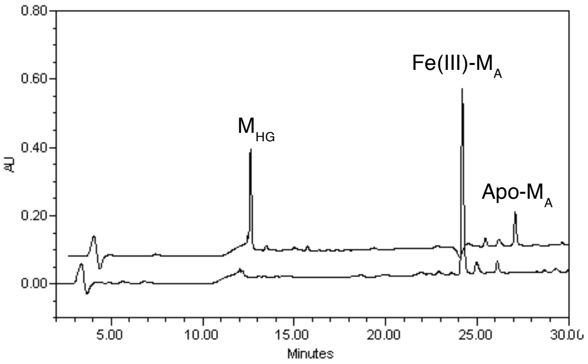

Figure 4. RP-HPLC at 215 nm of 50 µM apo-M_A (blue) and 50 µM Fe(III)-M_A (black) after incubation with MhtA-His$_6$ for 72 hours at 30°C.

PvdQ and other quorum quenching acylases hydrolyze the fatty acids of acyl-homoserine lactones;[15, 20, 19; 21; 22] thus, the reactivity of MhtA-His$_6$ with C12:0-HSL was also tested. The hydrolysis product, homoserine lactone, was monitored by derivatization with dansyl chloride for RP-HPLC analysis. A peak around 16.3 minutes was observed to increase over time indicating that homoserine lactone was being formed (Figure 5A). ESI-MS analysis of this peak showed an *m/z* value of 353 corresponding to the $[M+H]^+$ of dansyl homoserine (Figure 5B). The high pH (pH 9-10) for the derivatization reaction with dansyl chloride caused the hydrolysis of the ester bond of the homoserine lactone ring, resulting in the formation of acyclic dansyl L-homoserine, an increase of 18 mass units from the predicted dansyl homoserine lactone product.

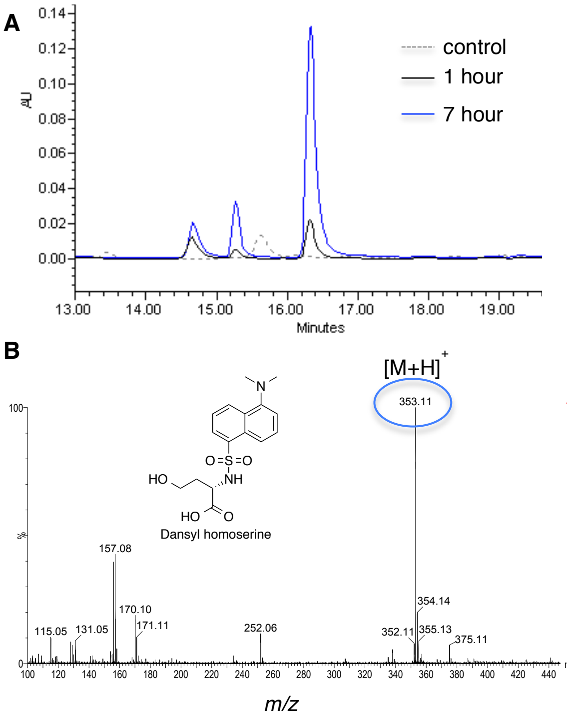

Figure 5. (A) RP-HPLC at 254 nm of C12:0-HSL hydrolysis product derivatized with dansyl chloride following incubation with MhtA-His$_6$. (B) ESI-MS of peak at 16.3 minutes corresponding to the [M+H]$^+$ of dansyl homoserine.

Discussion

A marinobactin acylase that is required for hydrolysis of the fatty acid appendage from the suite of amphiphilic marinobactin siderophores was identified in *Marinobacter* sp. DS40M6. Based on sequence similarity to well characterized enzymes, we propose it is a marinobactin hydrolase, and that the effect of the gene deletion is due to loss of this activity, in accord with the gene deletion effect of *pvdQ* in the biosynthesis of pyoverdine. Although we do not yet know the identity of the compounds *Marinobacter* sp. DS40M6 uses for quorum sensing, an alternative explanation for the effect of the hydrolase gene deletion is that the hydrolase plays a regulatory role through a quorum sensing pathway, or a competitive role partitioned between hydrolysis of the acyl marinobactins and acyl homoserine lactones. The *bntA* gene, identified as the candidate gene, has a 32% amino acid sequence identity to the siderophore acylase, PvdQ, from *P. aeruginosa* and 31-33% identity to the acyl-homoserine lactone acylases, Aac from *R. solanacearum* GM11000, AiiD from *Ralstonia* sp. XJ12B, and HacA from *P. syringae*. All of the aforementioned proteins are propeptides, which proceed through the same post-translational processing into a heterodimeric, active form characteristic of Ntn-hydrolases.

The Δ*bntA Marinobacter* sp. DS40M6 knockout mutant produced the marinobactins but did not produce M_{HG}. Complementation of the *bntA* gene restored the production of M_{HG} in Δ*bntA Marinobacter* sp. DS40M6. Attempts to overexpress BntA in *E. coli* resulted in insoluble, inactive protein, as has been observed with the expression of other Ntn-hydrolases in *E. coli*.[20,40] Previous work by our group, however, has shown the ability of *Marinobacter* sp. DS40M6 to hydrolyze structurally similar lipopeptide siderophores and acyl-homoserine lactones suggesting this enzyme has a broad substrate range like PvdQ.[16,28] To our knowledge PvdQ is the only other enzyme known to hydrolyze a fatty acid from an acylated siderophore. PvdQ, however, hydrolyzes its substrate, acylated pyoverdine precursor, prior to siderophore excretion from the cell, whereas BntA appears to hydrolyze the marinobactins after they are released into the extracellular medium.[28,12]

Marinobacter nanhaiticus D15-8W was shown to produce the marinobactin siderophores, which are also converted into M_{HG} during bacterial growth. *M. nanhaiticus* D15-8W has two BntA-like acylases, MhtA and MhtB, with MhtA predicted to be periplasmic and MhtB membrane associated. To avoid the potential insolubility of the membrane-associated BntA, we chose to investigate the predicted periplasmic MhtA. Incubation of MhtA-His_6 with the marinobactins produced M_{HG} as detected by RP-HPLC and ESI-MS/MS, where the hydrolysis of M_A appeared to be favored over M_E under the experimental conditions used. This preference of substrate based on the length of the fatty acid was also seen with PvdQ and other quorum quenching acylases.[16,20,45,40] In PvdQ, bulky, hydrophobic residues create a hydrophobic binding site for the acyl substituent of the bound substrate.[38, 14] The size of the hydrophobic binding pocket, as well as the hydrophilicity of the opening of this binding pocket, contributes to the selectivity of the substrate based on fatty acid length.[38] Many of these residues are conserved or closely conserved between PvdQ, the putative *Marinobacter* acylases, and BntA from *Marinobacter* sp. DS40M6.

Interestingly, hydrolysis of the Fe(III)-bound marinobactins to produce Fe(III)-M_{HG} did not occur. The geometry and conformation of the apo-marinobactins change when bound to Fe(III), possibly blocking access of the amide bond to the serine nucleophile.[46] A change in marinobactin conformation could also prevent access of the substrate at the active site all together. The inability of MhtA to hydrolyze ferric marinobactins is of interest due to the potential regulatory role it might serve with respect to marinobactin hydrolysis and iron uptake, experiments which are under investigation.

Like PvdQ, MhtA is able to hydrolyze acyl homoserine lactones involved in quorum sensing. Cell-free extracts of *Marinobacter* sp. DS40M6 were shown to catalyze hydrolysis of C8-HSL suggesting BntA could possibly catalyze this reaction too.[28] The majority of sequenced *Marinobacter* species are predicted to have acylases similar to BntA and MhtA, however, most of these species do not have the biosynthetic genes for marinobactin production. Also, *M. nanhaiticus* D15-8W does not have any LuxI homologues, yet some bacteria that do not express any proteins homologous to LuxI can still synthesize acyl homoserine lactones.[47,48,49] *M. nanhaiticus* D15-8W does, however, have putative homologues to LuxR from *Vibrio fischeri* suggesting the bacterium might respond to these autoinducer signals. Previous studies have shown that some bacteria with acyl homoserine lactone acylases can use the fatty acid products as sole carbon sources during growth, as in the case of QuiP from *P. aeruginosa* and AiiD from *Ralstonia* sp. XJ12B.[20,22] *Marinobacter* species are well known for their importance in bioremediation of oil in the ocean due to their abilities to degrade and utilize hydrocarbons.[50] It is possible that both *Marinobacter* sp. DS40M6 and *M. nanhaiticus* D15-8W utilize the fatty acid products of marinobactin hydrolysis as an energy source, while simultaneously recycling the marinobactins into a more hydrophilic Fe(III)-chelator. Investigations to determine if the efficiency of iron uptake differs between the full-length marinobactins and M_{HG} are currently in progress.

It can be hypothesized that the marinobactins are the biological substrate of BntA based on the mutant studies of Δ*bntA*; however, it is not known how MhtA and MhtB might be involved in this process for *M. nanhaiticus* D15-8W. The predicted outer membrane association of BntA and MhtB correspond to the hypothesis that the marinobactins are hydrolyzed following excretion into the extracellular milieu; however, it is unclear how the predicted periplasmic localization of MhtA allows access to excreted marinobactins. It is possible that MhtA can process acyl marinobactins taken up by the cell as a form of siderophore recycling. Hydrolysis of the fatty acid from the amphiphilic marinobactins would produce a more hydrophilic siderophore without altering the iron chelating properties. The fatty acid tail provides a way to keep the siderophore close to the bacterial membrane so it is not lost through diffusion. However, at high bacterial populations, a more hydrophilic siderophore could be released and used by nearby bacteria while providing a fatty acid hydrocarbon source.

In sum, we propose that the putative Ntn-hydrolases, BntA from *Marinobacter* sp. DS40M6 and MhtA from *M. nanhaiticus* D15-8w, are responsible for hydrolyzing the fatty acid appendage from the marinobactins during bacterial growth, releasing the marinobactin head group. Further investigations are in progress on the significance of the difference in reactivity of the iron(III)-bound versus apo-marinobactin and the potential regulatory role differential processing may play in the iron uptake process.

Supporting Information. Bacterial strains, plasmids, and primers used; Marinobactin acylase gene fragment amplification; Marinobacter Ntn-hydrolases sequence alignment with PvdQ; ESI-MS/MS of M_{HG} from Δ*bntA Marinobacter* sp. DS40M6 + pMMB208-*bntA*; Predicted marinobactin biosynthetic genes from *M. nanhaiticus* D15-8W; RP-HPLC chromatogram of marinobactins and ESI-MS/MS of isolated siderophores; SDS-page gel of MhtA and BntA expression in *E. coli*; ESI-MS/MS of M_{HG}. This material is available free of charge via the Internet at http://pubs.acs.org.

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Fatty acid hydrolysis of acyl-marinobactin siderophores by *Marinobacter* acylases

Michelle P. Kem,[†] *Hiroaki Naka,*[‡] *Akira Iinishi,*[†][ǂ] *Margo G. Haygood,*[‡] *Alison Butler*[†]*