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Authors
Gauglitz, Julia M
Iinishi, Akira
Ito, Yusai
et al.

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Microbial tailoring of acyl peptidic siderophores

Julia M. Gauglitz[‡†◊], Akira Iinishi[‡†#], Yusai Ito[‡], and Alison Butler[†]*

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

[‡]Authors contributed equally to this work

[#]Current address: Rohto Pharmaceutical Co., Ltd., 1-8-1 Tatsumi-nishi Ikuno-Ku, Osaka, Japan

[◊]Current address: Earth Research Institute, University of California, Santa Barbara, California 93106-9510

[‡]Current address: National Institute of Health Sciences, Kamiyoga 1-18-1, Setagaya-ku, Tokyo 158-8501, Japan

*To whom correspondence should be addressed. Tel: 805-893-8178. Fax: 805-893-4120.
E-mail: butler@chem.ucsb.edu

Abstract

Marine bacteria produce a predominance of suites of acylated siderophores characterized by a unique, species dependent head group that binds iron(III) and one of a series of fatty acid appendages. *Marinobacter* sp. DS40M6 produces the suite of seven acylated marinobactins, ranging from saturated and unsaturated C12-C18 fatty acids. In the present study we report that in late log phase of growth, the fatty acids are hydrolyzed by an amide hydrolase producing the peptidic marinobactin head group. *Halomonas aquamarina* str. DS40M3, another marine bacterium isolated originally from the same sample of open ocean water as *Marinobacter* sp. DS40M6, produces the acyl aquachelins, also as a suite comprised of a peptidic head group distinct from that of the marinobactins. In contrast to the acyl marinobactins, hydrolysis of the suite of acyl aquachelins is not detected, even when *H. aquamarina* str. DS40M3 is grown into stationary phase. The *Marinobacter* cell-free extract containing the acyl amide hydrolase is active towards exogenous acyl-peptidic siderophores (e.g., aquachelin C, loihichelin C, as well as octanoyl homoserine lactone used in quorum sensing). Further, when *H. aquamarina* str. DS40M3 is cultured together with *Marinobacter* sp. DS40M6, the fatty acids of both suites of siderophores are hydrolyzed and the aquachelin head group is also produced. The present study demonstrates that co-culturing bacteria leads to metabolically tailored metabolites compared to growth in a single pure culture, which is interesting given the importance of siderophore-mediated iron acquisition for bacterial growth, and that *Marinobacter* sp. DS40M6 and *Halomonas aquamarina* str. DS40M3 were isolated from the same sample of seawater.

Introduction

Many bacteria secrete siderophores to facilitate iron acquisition when growing in low-iron aerobic conditions (*1*). Siderophores are low molecular weight, high-affinity iron(III) ligands that can solubilize colloidal iron or sequester iron from iron-bound proteins of a host organism. A distinct characteristic of some siderophores is the presence of a fatty acid that confers amphiphilic character to the siderophore, the most numerous of which are acylated peptides (Figure 1), although citrate-derived amphiphilic siderophores are also well known (*2-4*).

Many marine bacteria produce large suites of acyl peptidic siderophores in which a species-dependent head group is appended by a fatty acid that varies in chain length, degree of unsaturation and hydroxylation (Figure 1A) (*5-9*). Suites of amphiphilic siderophores are also produced by pathogens (*10*), and most recently reported from numerous environmental isolates (*11-15*) (Figure 1B). Based on a genomics analysis, an acyl siderophore precursor has recently been discovered as an intermediate in the biosynthesis of pyoverdine in the human pathogen, *Pseudomonas aeruginosa* (*16-23*).

The functional advantage conferred by amphiphilic siderophores in the process of iron acquisition remains an open question. One advantage could be to prevent or limit siderophore diffusion through membrane association. The membrane partitioning of peptide-derived amphiphilic siderophores (e.g., the marinobactins (*24*), the amphibactins (*24*), the mycobactins (*25, 26*)) is greater for longer chain acyl-siderophores than those with shorter fatty acids or those with unsaturated and hydroxylated fatty acids. For example, the partition coefficients for the suite of marinobactins A-E (36 M^{-1} to 5818 M^{-1}) varies widely with the length of the fatty acid, as well as the extent of unsaturation in the fatty acid (*24*). With differential membrane partitioning, a suite of siderophores could create a concentration gradient of siderophore emanating from the bacterium; such a strategy has been proposed in the bucket brigade model to iron acquisition (*5*).

A variation on the large suites of amphiphilic marine siderophores is seen in *Mycobacteria*, which secrete a pair of related amphiphilic siderophores, the mycobactins and the carboxymycobactins (Figure 1B). Mycobactins are cell-associated, hydrophobic siderophores composed of a 2-hydroxyphenoloxazoline-containing peptidic head group and a long fatty acid (C16-C21) appendage. Carboxymycobactins are relatively hydrophilic and comprised of the same peptidic head group, although appended by a shorter fatty acid that is also carboxylated at the end. The carboxymycobactins and mycobactins are thought to cooperate in iron sequestration, whereby carboxymycobactin scavenges iron from host iron-binding proteins, such as transferrin, and then transfers Fe(III) to the bacterium's membrane-bound apo-mycobactin (*27, 28*).

As mentioned above, an amphiphilic siderophore precursor was discovered in the biosynthetic pathway of pyoverdine in *P. aeruginosa* (*17, 21*). From a bioinformatics analysis, it was

predicted and then confirmed that the biosynthesis of these siderophores begins with acylation of L-glutamic acid in the first module of the nonribosomal peptide synthetase (NRPS). Prior to formation of the fluorescent chromophore of pyoverdine, the acyl peptide precursor is hydrolyzed in the periplasm by the NTN (N-terminal nucleophile) hydrolase, PvdQ (*29*). Interestingly, the major quorum sensing molecule of *P. aeruginosa*, N-dodecanoic homoserine lactone (3-oxo-C12-HSL), is also hydrolyzed by PdvQ, providing a playoff between quorum quenching and pyoverdine biosynthesis (*30*).

We have observed that the marine bacterium *Marinobacter* sp. DS40M6 can alter its own amphiphilic siderophores during growth by hydrolyzing the fatty acids, as well as hydrolyzing fatty acids from exogenous acylated siderophores. We report herein the fatty acid hydrolysis of the suite of marinobactins A-E during growth of *Marinobacter* sp. DS40M6, producing the marinobactin head group (marinobactin HG or M_{HG}), the structural analysis of this head group, as well as fatty acid hydrolysis of the aquachelin siderophores when *Halomonas aquamarina* str. DS40M3 is grown in culture with *Marinobacter* sp. DS40M6. A cell-free extract of *Marinobacter* sp. DS40M6 containing an amide hydrolase carries out the fatty acid hydrolysis of amphiphilic siderophores, as well as acyl homoserine lactones.
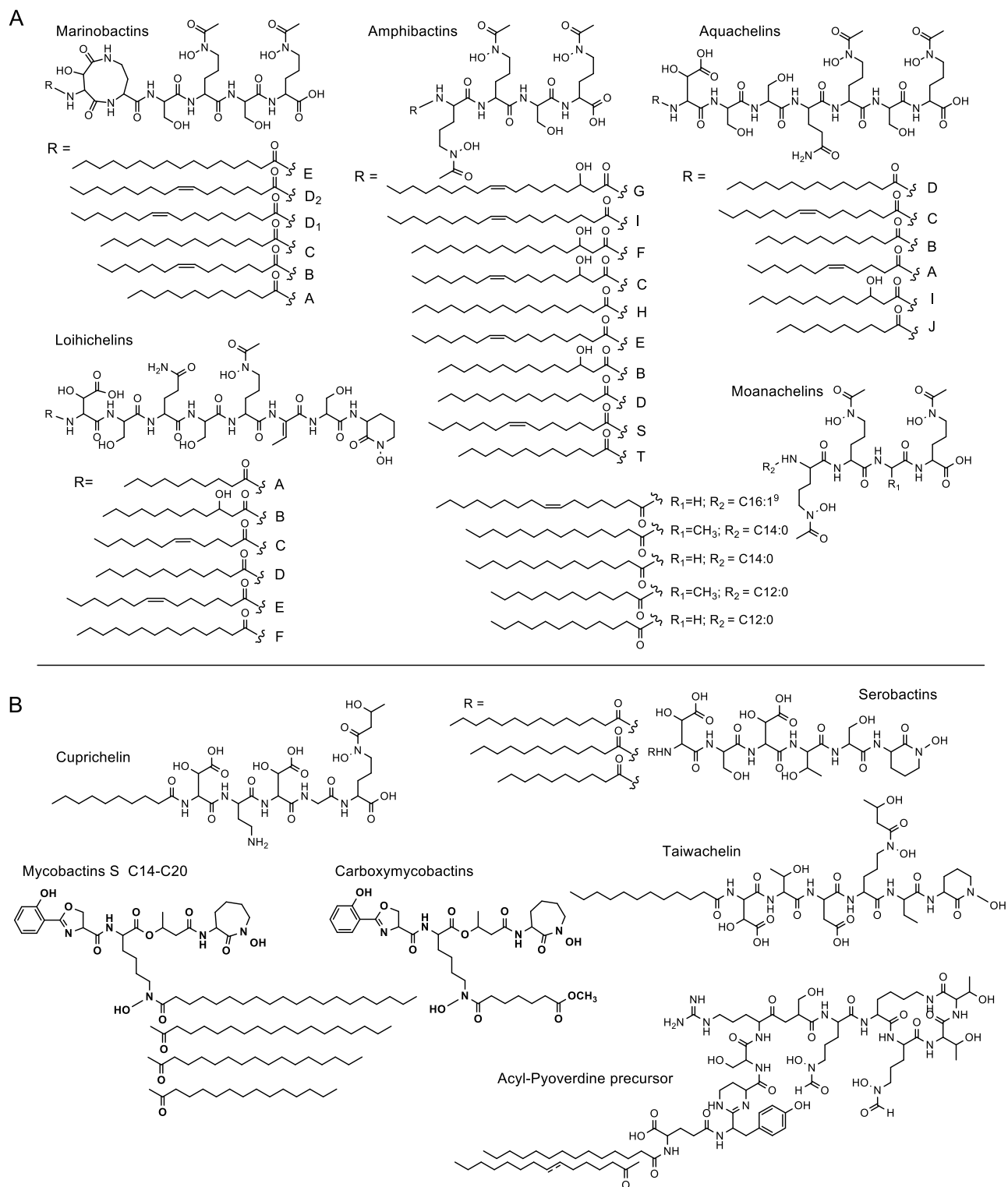
Figure 1. Structures of selected amphiphilic siderophores: **A.** Suites of marine siderophores include the marinobactins (*5*), aquachelins (*5, 8*), amphibactins (*6, 8*), loihichelins (*7*), and moanachelins (*9*); **B.** Siderophores from other environmental isolates include cuprichelin (*12*), taiwachelin (*13*), and the serobactins (*11*); siderophores from pathogens include mycobactins (*26*), carboxymycobactins (*26*), and the acyl precursor to pyoverdine (*17, 21*) are also shown,

along with pyoverdine. (Note: For moanachelin gly-C14:1 (R_1 = H; R_2 = C14:1) (*9*) and marinobactin F (C18:1) (*31*), not shown, the position and E/Z orientation of the double bond has not been determined.)

Experimental Procedures

Microorganisms

Marinobacter sp. DS40M6 and *Halomonas aquamarina* str. DS40M3 were isolated from open ocean water off the west coast of Africa (*5, 32*) and were grown in liquid M6 media. M6 media contains 31 g NaCl, 6 g $Na_2HPO_4 \cdot 7H_2O$, 1.5 g KCl, 2 g NH_4Cl, 10 g Na_2 succinate, 0.4 g $MgSO_4 \cdot 7H_2O$, 0.2 g $CaCl_2 \cdot 2H_2O$, per 2 L doubly deionized water (ddH_2O; Barnstead Nanopure II), adjusted to pH 7.

For inoculation into liquid media, bacteria were grown to confluence on maintenance media plates containing 0.5 g yeast extract, 5 g peptone, and 15 g agar (bacto, BD), per liter of aged natural seawater. The colonies were then suspended in an aliquot of sterile M6 media, and the suspension was inoculated into the sterilized culture medium. The bacterial cultures were prepared in triplicate. All cultures were shaken on a rotary shaker at 200 rpm at ambient temperature. Cultures were regularly tested for a positive response to the CAS assay (*33*), indicating the presence of apo-siderophores.

For bacterial growth in mixed culture, 4 mL of growth media were pipetted onto each confluent plate (preparation as described above), and 2 mL of resuspended *Marinobacter* sp. DS40M6 bacterial cells and 1 mL of resuspended *H. aquamarina* str. DS40M3 cells were inoculated into the autoclave-sterilized M6 culture medium. Experiments were performed in triplicate in 1L or 2L Erlenmeyer flasks. A greater concentration of *Marinobacter* cells was initially required, due to its slower growth rate, in order for both species to concomitantly reach a similar phase in growth, so that siderophore production was detected for both species, in addition to the presence of enzymatic activity in *Marinobacter*.

Siderophore isolation

The marinobactin head group siderophore (marinobactin HG, or M_{HG}) was isolated and purified from the supernatant of a culture of *Marinobacter* sp. DS40M6 as previously described for marinobactins A-E (*5*). M_{HG} was purified by RP-HPLC with a preparative C_{18} column (22 mm ID × 250 mm L, 201SP1022, GraceVydac), using a gradient 97/3 (% A/B) to 90/10 (% A/B) over 40 min, where solvent A is 99.95% ddH_2O with 0.05% trifluoroacetic acid (TFA) and solvent B is 99.95% acetonitrile with 0.05 % TFA. The eluent was monitored at 215 nm.

Time-dependent monitoring of siderophore production and peptidic head group formation

For monoculture growth:

An aliquot of the either *Marinobacter* sp. DS40M6 or *H. aquamarina* str. DS40M3 culture was removed daily, centrifuged to pellet the bacterial cells and the supernatant was filtered

through a sterile 0.22 μm filter, and frozen at -20°C until analysis by RP-HPLC. The siderophore content of the culture supernatant was monitored using an analytical C$_4$ RP-HPLC column (5 mm ID × 250 mm L, 214TP54, Grace Vydac) using a gradient from 100 % solvent A (99.95 % ddH$_2$O, 0.05 % TFA) to 100 % solvent B (99.95 % acetonitrile, 0.05 % TFA) over 100 min, after first holding the column flow at 100 % solvent A before starting the gradient. The absorbance of the eluent was monitored at 215 nm with a photodiode array detector. The peak area integrations for each marinobactin were performed using an integral function of the HPLC software (Empower Pro, Waters Inc.).

For mixed culture growth

Mixed bacterial cultures were treated the same as control cultures of only *H. aquamarina* str. DS40M3 or *Marinobacter* sp. DS40M6. Starting at day 0 and continuing up to day 30, depending on the experiment, supernatant aliquots were prepared as described above for M$_{HG}$ production and analyzed by RP-HPLC using an analytical C$_4$ column with the following modified program: gradient of 100 % solvent A (99.95 % H$_2$0, 0.05 % TFA), held for 10 minutes, to 100% solvent B (50 % acetonitrile, 49.95 % H$_2$0, 0.05 % TFA) over 30 minutes, followed by a 5 minute transition back to solvent A. Peak areas for the marinobactin and aquachelin siderophores overlap, preventing peak area integrations for individual siderophores or suites.

Structure determination

Electrospray ionization mass spectrometry (ESI-MS) and tandem mass spectrometry using a Micromass QTOF-2 mass spectrometer (Waters Corp.) in positive ion mode with argon as a collision gas was used to determine the mass and partial amino acid connectivity of the marinobactin and aquachelin head groups. For M$_{HG}$, ^{1}H, ^{13}C, and various two-dimensional nuclear magnetic resonance (NMR) techniques, including ^{1}H-^{1}H correlation spectroscopy (COSY), heteronuclear multiple quantum coherence (HMQC), and heteronuclear multiple bond correlation (HMBC) were recorded on a 800 MHz JEOL DELTA2 ECA800 spectrometer. A ^{1}H-^{15}N heteronuclear single quantum coherence (^{15}N-HSQC) NMR spectrum of the ^{15}N-incorporated M$_{HG}$ was recorded on a 500 MHz JEOL DELTA2 ECA500 instrument. M$_{HG}$ was dissolved in d_6-dimethylsulfoxide (d_6-DMSO, 99.9 %, Cambridge Isotope, Inc.) (d = 2.46 ppm) and experiments were run at 23.5 °C at the National Institute of Health Sciences in Tokyo, Japan.

^{15}N labeled marinobactins

To prepare ^{15}N-isotopically labeled marinobactins (^{15}N-marinobactins A-E and M$_{HG}$), *Marinobacter* sp. DS40M6 was grown in M6 medium as described above, except that ^{15}N-ammonium chloride (Cambridge Isotope Lab. Inc.) was used in the culture medium as the sole nitrogen source. The culture was grown for 4 to 5 days for isolation of ^{15}N-marinobactins A-E, and approximately 12 days for isolation of ^{15}N-M$_{HG}$. ^{15}N-M$_{HG}$ and ^{15}N-marinobactins A-E were purified as described above. The masses of ^{15}N-isotopically labeled marinobactins were determined by ESI-MS.

To investigate fatty acid amide hydrolysis, 20 μM ^{15}N-marinobactin E (final concentration) was added to a culture of *Marinobacter* sp. DS40M6 which had been grown for 4 to 5 days. The cells were pelletted by centrifugation, washed and resuspended in fresh M6 media of an OD of 0.35 at 600 nm. These cultures, which were set up in triplicate or quadruplicate, were incubated on a rotary shaker (200 rpm) at ambient temperature. A 1 mL aliquot from each culture was removed at 15 minutes and at 4 days after addition of ^{15}N-marinobactin E and analyzed for the presence of marinobactins and M_{HG}. The masses of the peaks eluted from the HPLC were determined by ESI-MS of the hand-collected sample using a Micromass QTOF-2 mass spectrometer, specifically tracking the ^{15}N-marinobactins.

Preparation of cell-free extract

Marinobacter sp. DS40M6 was grown in M6 medium as previously described and typically required four to five days to reach stationary phase. The optical density of the culture at 600 nm was allowed to reach approximately 0.4 to 0.6 to maximize the cell mass harvested from liquid culture. The cells were pelletted by centrifugation and re-suspended by gentle pipetting in a buffer containing 50 mM tris-HCl pH 8.0 (buffer-A). The suspension was placed in an ice bath and sonicated with a probe tip ultra-sound sonicator (Sonifier 250, Branson). The sonicated homogenate was centrifuged at 7,800 x g for 10 min to remove large cell debris. The supernatant was ultra-centrifuged without detergent at 108,000 x g for 90 min at 4 °C (Discovery M120SE, Sorvall, Hitachi) to obtain a pellet containing the membrane fraction. This pellet was re-suspended in buffer-A with 2 % Triton X-100 by gentle pipetting and allowed to equilibrate for 30 minutes in an ice bath. The final ultra-centrifugation of the suspension at 108,000 x g for 60 min at 4 °C resulted in enzymatic activity in the supernatant. The enzyme-detergent extract was concentrated by ultra-filtration using a Centriprep YM30 (Amicon, Millipore) as necessary. Subsequent steps to further purify the enzyme (size exclusion, DEAE, MonoQ chromatography) resulted in diminished activity or complete loss of activity.

Acyl amide hydrolase reactivity

Fatty acid hydrolysis of acyl siderophores (marinobactins A-E, aquachelin C, loihichelin C) and N-octanoyl-homoserine lactone (C8-HSL) was investigated using the detergent-extracted acyl amidase preparation described above, with incubations ranging from 2-5 days. The reaction products were separated from the reaction mixture by RP-HPLC on an analytical C4 column (Grace Vydac, 214TP54) or an analytical C18 column (Grace Vydac, 218TP54), with a reverse phase analytical column at a flow rate of 1 mL/min. The gradients were programmed at 99.95 % ddH$_2$O with 0.05 % TFA for 3 minutes, followed by a linear increase of 1% per minute of 99.95% acetonitrile with 0.05 % TFA. The eluent was monitored by a PDA and peaks were collected manually and analyzed by ESI-MS.

Results

Time dependence of fatty acid hydrolysis of marinobactins A-E

Marinobacter sp. DS40M6 produces the suite of amphiphilic marinobactin siderophores A-E and the membrane-associated marinobactin F (Figure 1) (*5, 31*). Beginning in late log phase of growth and continuing into stationary phase, a new hydrophilic siderophore is produced, concomitant with a decrease in the amount of marinobactins A-E in the culture supernatant (Figure 2, Figure 3) (marinobactin F is membrane-associated and thus not found in the culture supernatant; thus the suite will be referred to as marinobactins A-E). ESI-MS analysis of this new siderophore gives an intense molecular ion peak at m/z 750.3 $(M+H)^+$, a doubly charged ion peak at m/z 375.6 $(M+2H)^{2+}$ and a minor ion peak at m/z 803, which is consistent with Fe(III) coordination, $([M-3H+Fe(III)]+H)^+$ (Supporting Information Figure S1). The mass at m/z 750 matches the mass of the marinobactin peptide head group. The exact mass of M_{HG}, m/z 750.3240 for $(M+H)^+$ is consistent with $C_{28}H_{48}N_9O_{15}$. The shorter retention time of M_{HG}, compared with the amphiphilic marinobactins A-E, is consistent with a more hydrophilic compound, such as one lacking a fatty acid appendage (Figure 3). M_{HG} produces a positive ninhydrin test, consistent with the presence of a primary amine, whereas the ninhydrin test with apo marinobactin E, which has the fatty acid amide in place of a primary amine, is negative. M_{HG} retains the ability to coordinate Fe(III) similarly to the acyl-marinobactins.
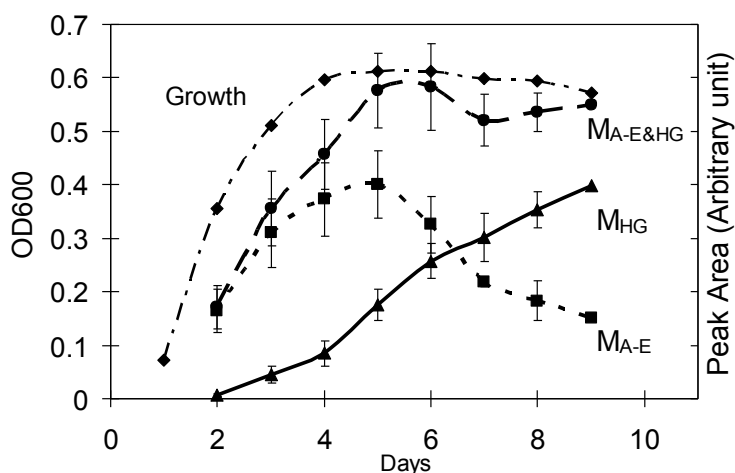

Figure 2. *Marinobacter* sp. DS40M6 siderophore production. Data points represent the mean values of triplicate cultures; error bars indicate standard deviation. Solid line with triangle data points: M_{HG} production; dotted line with square: the sum of the marinobactin A to E siderophores; dashed-line with circle: the sum of the all marinobactins (A – E and HG). The left y-axis denotes the optical density at 600nm and the right denotes HPLC peak areas in arbitrary units from Figure 3.

The time-dependence of marinobactin production in the culture supernatant of *Marinobacter* sp. DS40M6 (Figures 2 and 3) shows that 1) marinobactins A-E reach a maximum at about day

five of microbial growth and then decrease; and 2) that M_{HG} production increases steadily during growth, becoming the predominant siderophore in the culture medium in stationary phase.
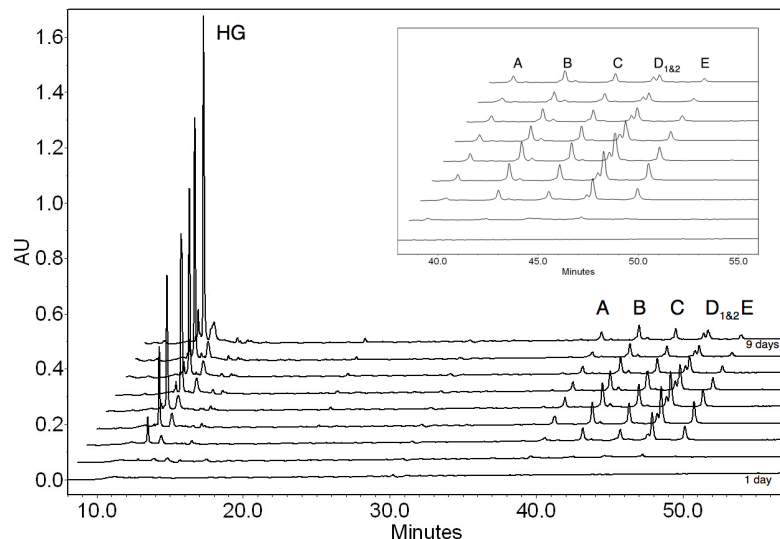

Figure 3. Marinobactins A-E and M_{HG} production over time: RP-HPLC chromatograms of *Marinobacter* sp. DS40M6 culture supernatant from day 1 (bottom) to day 9 (top). The peak growing in with a retention time of ~12 minutes is M_{HG}; the retention times of marinobactins A-E are typically between 35-50 min. Marinobactin F is not observed here because it is membrane associated and not released from the bacterium to the supernatant.
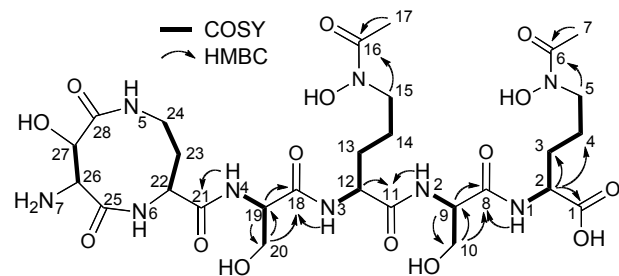
Marinobactin HG structure determination

Fragmentation analysis of the new hydrophilic siderophore, M_{HG}, by tandem mass spectrometry establishes the partial connectivity of the amino acids (Figure S2), consistent with marinobactins A-E.

NMR characterization of M_{HG} confirms that it is the peptidic head group moiety of the acyl marinobactin siderophores. The ^{13}C and ^{1}H NMR spectral values of M_{HG} in d_6-DMSO are summarized in Table 1 (Figures S3 and S4). M_{HG} has two significant structural differences from the acylated marinobactins, which are reflected in the NMR spectra. One is the absence of a fatty acid appendage, and the other is the presence of a terminal amine. Thus, ^{1}H resonances at δ_H 0.85 and 1.23-1.26 (-CH_3 and -CH_2, respectively) observed for marinobactin E are not observed in the ^{1}H NMR spectrum of M_{HG} (Figure S3) as a result of the absence of a fatty acid. Moreover, the number of characteristic amide proton resonances decrease from seven (i.e., five at *ca.* δ_H 8 ppm and two in the nine-membered ring at *ca.* δ_H 9.85 ppm) in marinobactin E to six in M_{HG}, consistent with the conversion of the fatty acid amide of marinobactin E to an amine in M_{HG}. The two amide proton signals in the nine-membered ring of M_{HG} were broad and overlapped at δ_H 9.68 ppm. The two amide proton signals of marinobactins C and E were also broad, but clearly separated (*34*). This change shows the increase in symmetry of the nine-membered ring of M_{HG} on fatty acid hydrolysis. Additionally, the proton signal at C26 in marinobactin E at δ_H 5.05 is

shifted to higher magnetic field for M_{HG} at δ_H 4.06 for M_{HG}. The structure elucidation of M_{HG} is also consistent with the positive ninhydrin test for a free amine in M_{HG} but not the acylated marinobactins A-E.

Table 1: NMR assignments. ^{13}C, and ^{1}H NMR chemical shifts (δ, ppm) of M_{HG} in d_6-DMSO. [See nmr spectra in Figures S5-S7] (br, broad; nd, not detected)

		δ_C	δ_H	type	COSY	HMBC
(N-Ac,	C1	174.0				2
N-OH)-Orn	C2	52.2	4.14	C	3, NH1	
	C3	29.0	1.65,1.50	CH$_2$	2, 4	2
	C4	23.4	1.5	CH$_2$	3, 5	2
	C5	47.0	3.42	CH$_2$	4	
	C6	170.8		C		5, 7
	C7	20.9	1.93	CH$_3$		
	N1		8.02	NH	2	
Ser	C8	170.2		C		NH1, 9, 10
	C9	55.4	4.30	CH	10, NH2	10
	C10	62.3	3.51	CH$_2$	9	9
	N2		7.85	NH	9	
(N-Ac,	C11	171.9		C		NH2, 12
N-OH)-Orn	C12	52.8	4.30	CH	13, NH3	
	C13	29.8	1.42,1.63	CH$_2$	12, 14	
	C14	23.4	1.5	CH$_2$	13, 15	
	C15	47.2	3.42	CH$_2$	14	
	C16	170.9		C		15, 17
	C17	20.9	1.94	CH$_3$		
	N3		8.19	NH	12	
Ser	C18	170.1		C		NH3, 19, 20
	C19	55.9	4.35	CH	20, NH4	20
	C20	62.4	3.54	CH$_2$	19	19
	N4		8.35	NH	19	
DHBA	C21	169.7		C		NH4
	C22	51.3	4.32	CH	23	
	C23	22.0	1.91	CH$_2$	22	
	C24	36.9	3.27,3.35	CH$_2$	23	
	N5		9.68 br	NH	24	

	N6		9.68 br	NH	
(α-OH)-Asp	C25	172.7		C	
	C26	54.9	4.06	CH	27
	C27	71.7	4.37	CH	26
	C28	172.7		C	
	N7		nd	NH$_2$	

Table Footnote: The orientation of β-hydroxyaspartic acid and diaminobutyric acid in the 9-membered ring was established by HMBC on marinobactins C and E *(34)*. The C$_\alpha$ of β-hydroxyaspartate, C26, couples to the N$_6$ ^{1}H at 9.8 ppm and not with the N$_5$ ^{1}H located further away at 9.72 ppm, consistent with the orientation of the 9-membered ring that is shown here (*34*). The structure above shows the ^{1}H-^{1}H correlation (bold lines) and key HMBC (H→C) correlations.

Microbial hydrolysis of the fatty acid amide of marinobactin E to M$_{HG}$

M$_{HG}$ production was further investigated by adding ^{15}N-labeled marinobactin E back to a culture of *Marinobacter* at day 4 of growth, which is in the range of late log phase or the beginning of the stationary phase of growth. The cells at 4 days of growth were washed and resuspended in fresh media. After addition of apo-^{15}N-M$_E$ to the bacterial culture (HPLC bottom trace in Figure S8), the intensity of the M$_{HG}$ HPLC peak increased significantly. ESI-MS revealed that the M$_{HG}$ peak in Figure S8 consisted of molecular ions at both m/z 759, which is the ^{15}N-isotopically labeled M$_{HG}$, as well as m/z 750, which is the non-isotopically labeled (natural abundance) M$_{HG}$, produced during growth of *Marinobacter* sp. DS40M6 over the four-day incubation period. The HPLC chromatogram shows that the ^{15}N-marinobactins A-E and ^{15}N-M$_{HG}$ elute off the XAD-2 resin nearly identically to the purified non-isotopically labeled marinobactins (Figure S8). Thus, the conversion of exogenously added ^{15}N-labeled marinobactin E by the bacterial culture demonstrates that the bacteria hydrolyze the acyl amide bond of marinobactin E to produce M$_{HG}$, as opposed to a hydrophilic head group siderophore being produced independently or as a precursor to the acyl-marinobactins.

If instead of using a *Marinobacter* culture resuspended in fresh media, ^{15}N-marinobactin E is added to cells resuspended in an isotonic salt medium (thus lacking a carbon or nitrogen source for bacterial growth), then only the m/z 759 M$_{HG}$ is detected. It was also observed on addition of ^{15}N-marinobactin E to the culture supernatant from the *Marinobacter* growth at 4 days that some conversion to the ^{15}N-M$_{HG}$ did occur, although it was much less than occurred with the resuspended cell pellet, suggesting that some amide hydrolase is released during cell harvesting and that the enzyme may be peripherally membrane associated.

Time-dependent siderophore production by *Marinobacter* sp. DS40M6 and *H. aquamarina* str. DS40M3 in co-culture

Growth of *Halomonas aquamarina* str. DS40M3 in pure culture resulted in identification of aquachelins A-D, I and J (Figure 1) (*5, 8*). However, no aquachelin head group formation was

detected through stationary phase of growth and even through cell death (Figure S9), despite culturing *H. aquamarina* under analogous conditions to *Marinobacter* sp. DS40M6.

Siderophore production in a mixed culture of *Marinobacter* sp. DS40M6 and *H. aquamarina* str. DS40M3 was followed for 30 days and resulted in the concomitant production of the suites of marinobactins and the aquachelins. The amide hydrolase enzymatic activity of *Marinobacter* is evident and the amount of head group increases between days 9 and 14 (data not shown). The appearance of two head group peaks indicates that the *Marinobacter* bacterium is modifying not only its own acyl marinobactin siderophores, but also the acyl aquachelin siderophores *in vivo*.
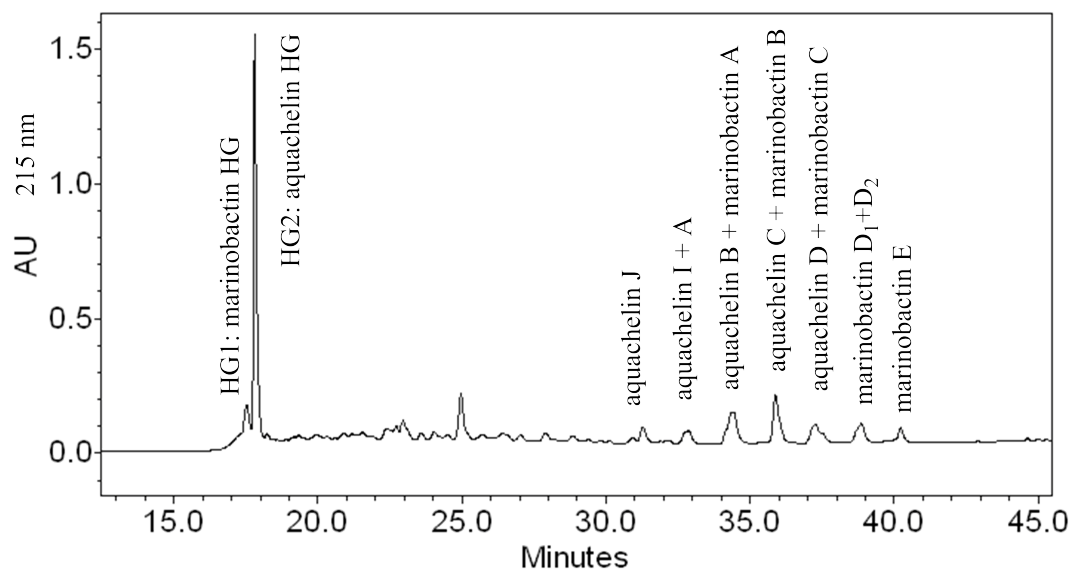

Figure 4 RP-HPLC chromatogram of a 100 µL *Marinobacter* sp. DS40M6 and *Halomonas aquamarina* str. DS40M3 mixed culture sample from day 10 of growth, injected onto an analytical C_4 column (Vydac) (aliquot from the XAD extract). The sample was monitored at 215 nm. Based on mass spectrometric data, the peaks at 17 minutes are HG1: 750 m/z, M_{HG}; HG2: 883 m/z, aquachelin head group, A_{HG}. The siderophore peaks between 30 and 42 minutes are aquachelin J (1037 m/z); aquachelin I (1081 m/z); aquachelin A (1063 m/z); aquachelin B (1066 m/z) and marinobactin A (932 m/z); aquachelin C (1091 m/z) and marinobactin B (958 m/z); aquachelin D (1093 m/z) and marinobactin C (960 m/z); marinobactin D (986 m/z); marinobactin E (988 m/z). The peaks between 20 and 30 min in the XAD extract sample are not siderophores or siderophore fragments.

At day 10 of growth in the mixed culture, the presence of the suites of marinobactin and aquachelin siderophores are clearly present, as well as two head group peaks (Figure 4). Extraction of the mixed culture of *Halomonas* and *Marinobacter* using XAD-2 resin resulted in similar results to the aliquot samples and sufficient quantities for identification (Figure 4).

The first smaller peak was identified as the marinobactin head group, M_{HG}, (750 m/z). The tandem mass spectrum of M_{HG} correlates well with the expected fragmentation pattern. The second larger peak was identified as the aquachelin head group (883 m/z), A_{HG}, and tandem mass

spectrometry (Figure S10) reveals the majority of the $y+2H^+$ and b fragments expected for fragmentation of A_{HG}. (The 'y' and 'b' nomenclature refers to the charge when retained by the COOH-terminal fragment or the NH_2-terminal fragment of the peptide, respectively (*35*). Interestingly, the A_{HG} peak is the larger of the two head group peaks (Figure 4 at 17-18 minutes). The amount of A_{HG} compared to M_{HG} may be due to the concentration difference between the two suites of siderophores. The *Halomonas* culture has a faster growth rate, reaching a higher cell density in culture (data not shown) than the *Marinobacter* culture. Thus *H. aquamarina* DS40M3 produces the acyl aquachelin siderophores earlier, as well as perhaps in higher concentration than occurs for the acyl marinobactin siderophores. Therefore more A_{HG} is present than M_{HG}. The *Marinobacter* acyl amide hydrolase enzymatic activity is predominantly constrained to its cellular membrane, thus hydrolysis of the acyl aquachelins results primarily from partitioning of the acyl aquachelins within the *Marinobacter* membrane.

Acyl amidase activity in *Marinobacter* sp. DS40M6 cell-free extract

The acyl amidase activity in a cell-free extract prepared from *Marinobacter* sp. DS40M6 was confirmed to be active towards marinobactins A-E, converting the isolated suite of marinobactins to the M_{HG} siderophore. Intriguingly, it also showed activity towards other non-native acyl peptidic siderophores, as well as N-octanoyl homoserine lactone (C8-HSL). The enzyme hydrolyzed the fatty acid amide bond of two distinct acyl peptidic siderophores, aquachelin C (also described above in co-culture) and loihichelin C (**Error! Reference source not found.**). In both cases the head group siderophore was identified based on mass spectrometric data (not shown). The partially purified enzyme extract also catalyzed hydrolysis of the shorter fatty acid amide of the quorum sensing molecule, C8-HSL as analyzed by HPLC (data not shown). Attempts to purify the amidase to homogeneity resulted in progressive loss of activity. Acyl amide hydrolysis activity was not observed using a boiled sample of the cell-free extract, as investigated using the marinobactins A-E. Current investigations are directed toward cloning and expression of the recombinant acyl amide hydrolase, which is likely related to the NTN hydrolase in *P. aeruginosa* that deacylates the acyl pyoverdine precursor (**Error! Reference source not found.**) and its quorum sensing molecule, 3-oxo-dodecanoic HSL (*29*).

Discussion

We have shown that the fatty acid appendages of the marinobactins A-E produced by *Marinobacter* sp. DS40M6 are hydrolyzed during bacterial growth, producing the marinobactin head group, M_{HG}, as established by NMR and mass spectrometry. Fatty acid hydrolysis is carried out by an amide hydrolase present in cell-free extracts of *Marinobacter* sp. DS40M6. The acyl amidase also catalyzes the hydrolysis of fatty acids from other structurally distinct acylated peptidic siderophores, including aquachelin C and loihichelin C, producing the respective head group peptides as identified by mass spectrometry. In addition to acyl-siderophore hydrolysis, the *Marinobacter* acyl amidase hydrolyzes N-octanoyl-HSL, which was chosen as a test substrate and one that also serves as a quorum sensing compound in some bacteria. Figure 3

shows the faster disappearance of marinobactin E compared to marinobactins with shorter fatty acid appendages (e.g., marinobactins A and B). This rate difference is consistent with the location of the acyl amide hydrolase in the bacterial membrane since the partitioning of the marinobactins within bilayer membranes is greater for the longest saturated fatty acids and decreases with unsaturation or shorter chain length (*24*).

The NTN-hydrolase, PvdQ, in *P. aeruginosa* catalyzes hydrolysis of the fatty acid from both the acyl-pyoverdine precursor the endogenous quorum sensing molecule, N-(3-oxo-C12)-HSL (*16, 17, 30*). Thus functional similarities exist between the *Marinobacter* acyl amide hydrolase and the *P. aeruginosa* NTN hydrolase. In the case of *P. aeruginosa*, the acylated pyoverdine precursor is first hydrolyzed in the periplasm and subsequent biosynthetic steps are required to form pyoverdine prior to release from the bacterium, which differs from that of acyl marinobactin biosynthesis. Further work is in progress to clone and express a NTN-hydrolase from *Marinobacter* sp. DS40M6 to investigate the enzyme reactivity and the relationship between acyl siderophore hydrolysis of native and exogenous siderophores, as well as a possible role in quorum sensing regulation.

While the quorum sensing compounds of *Marinobacter* sp. DS40M6 are not known, the fatty acid amide hydrolase activity on acyl-HSLs raises a possible role in quorum regulation, and one that is under investigation for *P. aeruginosa*. It is possible that the *Marinobacter* acyl amidase has multiple roles for the bacterium, one of which is formation and release of M_{HG} during growth, and another which may be involved in quorum sensing regulator. In this later regard, experiments are in progress to—identify and isolate the quorum sensing compounds of *Marinobacter* sp. DS40M6.

In contrast to hydrolysis of the acyl pyoverdine precursor by the periplasmic NTN-hydrolase, the marinobactins are not hydrolyzed in the periplasm and in fact are released as the suite of acylated marinobactins, likely partitioning to varying degrees within the outer membrane (*24*). Hydrolysis appears to occur predominantly in the outer membrane and to a much lesser extent extracellularly by release of a small amount of the hydrolase. The M_{HG} retains the ability to coordinate iron(III), as all three of the iron-coordinating moieties are still present and not impacted by deacylation. As both the acyl marinobactins and M_{HG} are capable of coordinating iron(III) and both are present in the external milieu, either form of the molecule could be active in iron uptake, and they may perhaps even play different roles during iron uptake for *Marinobacter* sp. DS40M6. It is interesting to speculate in cases where a bacterium modifies its own siderophore, which form of the molecule is the true intended siderophore.

A surprising result in this investigation is that co-culturing *Marinobacter* sp. DS40M6 and *H. aquamarina* str. DS40M3 results in production of both the marinobactin and aquachelin head groups. The ability of one bacterium to structurally alter the siderophores of another bacterium is intriguing and the benefits in the natural environment are not clear. The aquachelin siderophores

are more hydrophilic than the marinobactins as a result of their longer peptidic head group and overall shorter fatty acids compared to the marinobactins. Production of the aquachelin head group, A_{HG}, would indicate that a portion of aquachelin siderophores dissociate from the *H. aquamarina* cells and either associate with the *Marinobacter* cells by partitioning in the outer membrane, or encountering the peripherally membrane-bound amidase enzyme in *Marinobacter* sp. DS40M6. Thus the aquachelin siderophores are encountering the *Marinobacter* enzyme at its cell surface, although it cannot be ruled out that growth of *H. aquamarina* str. DS40M3 with the *Marinobacter* cells induces expression of a native *H. aquamarina* acyl amide hydrolase enzyme. In sum, co-culturing bacteria can lead to metabolically altered and tailored metabolites compared to a single pure culture, which is especially interesting given the importance of siderophore-mediated iron acquisition for bacterial growth.

Acknowledgment

Supporting Information Available: ESI-MS of M_{HG}; MS-MS of M_{HG} and ^{15}N-M_{HG}; ^1H, ^{13}C, ^1H-^1H COSY, ^1H-^{13}C HMBC, and ^1H-^{13}C HMQC of M_{HG}; HPLC of aquachelin production *vs* time and HPLC of co-cultures of Marinobacter sp. DS40M6 and *H. aquamarina* str DS40M3, MS-MS of A_{HG} This information is available via the Internet http://pubs.acs.org.

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