

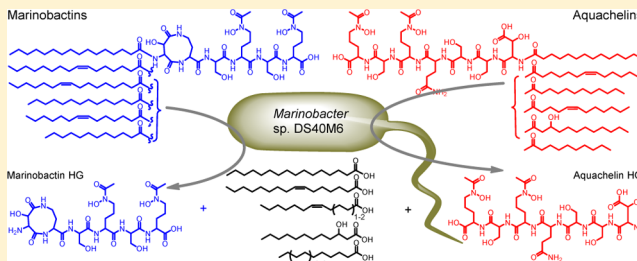
## Microbial Tailoring of Acyl Peptidic Siderophores

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## S Supporting Information

**ABSTRACT:** Marine bacteria produce an abundance of suites of acylated siderophores characterized by a unique, species-dependent headgroup that binds iron(III) and one of a series of fatty acid appendages. *Marinobacter* sp. DS40M6 produces a suite of seven acylated marinobactins, with fatty acids ranging from saturated and unsaturated C12–C18 fatty acids. In the present study, we report that in the late log phase of growth, the fatty acids are hydrolyzed by an amide hydrolase producing the peptidic marinobactin headgroup. *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 composed of a peptidic headgroup 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 the stationary phase. The *Marinobacter* cell-free extract containing the acyl amide hydrolase is active toward 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 headgroup is also produced. The present study demonstrates that coculturing 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 *H. aquamarina* str. DS40M3 were isolated from the same sample of seawater.



Many bacteria secrete siderophores to facilitate iron acquisition when growing in low-iron aerobic conditions.<sup>1</sup> 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.<sup>2–4</sup>

Many marine bacteria produce large suites of acyl peptidic siderophores in which a species-dependent headgroup is appended by a fatty acid that varies in chain length, degree of unsaturation, and hydroxylation (Figure 1A).<sup>5–9</sup> Suites of amphiphilic siderophores are also produced by pathogens<sup>10</sup> and most recently reported from numerous environmental isolates<sup>11–15</sup> (Figure 1B). On the basis of 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*.<sup>16–23</sup>

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,<sup>24</sup> the amphibactins,<sup>24</sup> the mycobactins<sup>25,26</sup>) is greater for longer chain acyl-siderophores

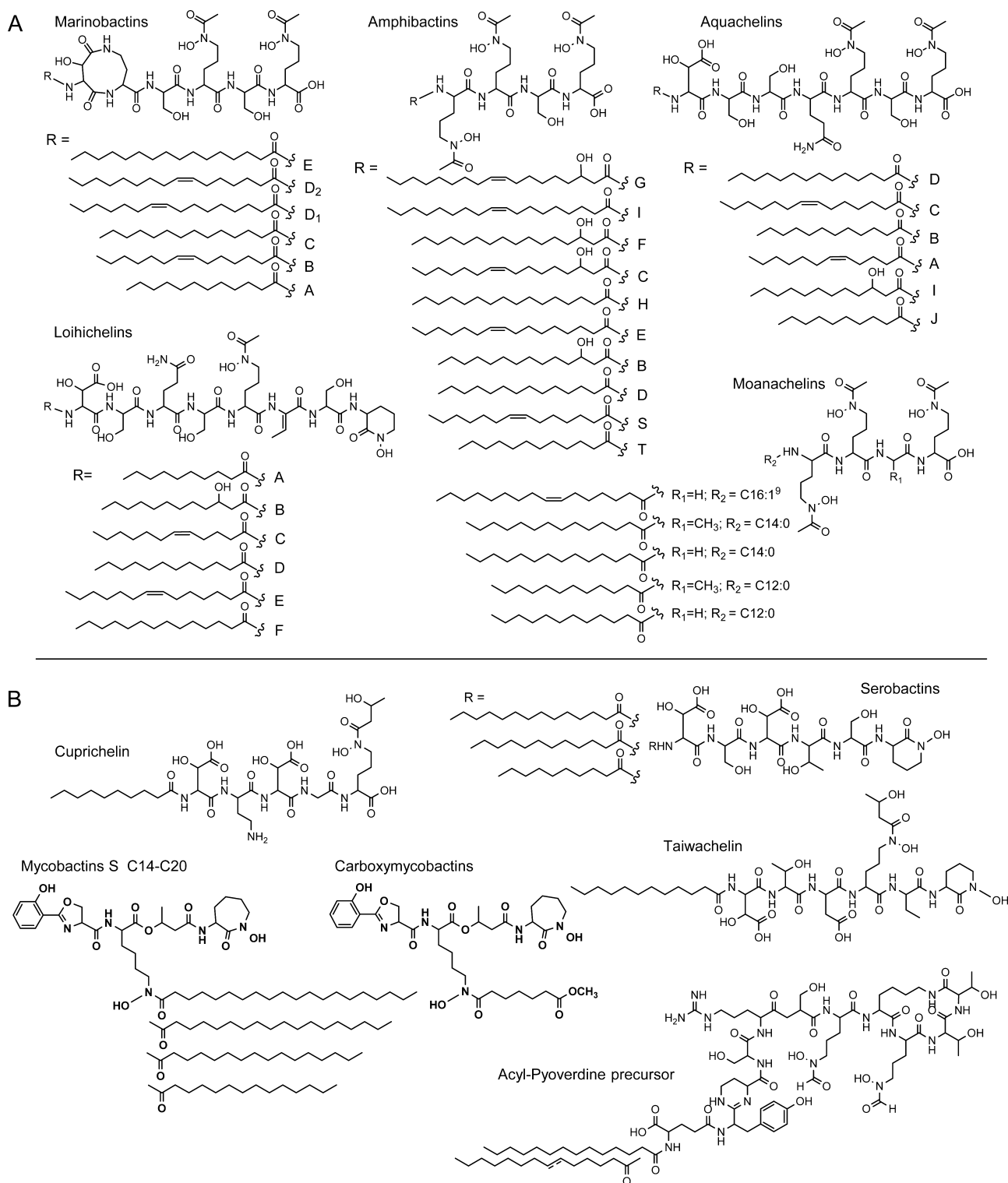
than those with shorter fatty acids or those with unsaturated and hydroxylated fatty acids. For example, the partition coefficients for the suite of marinobactins A–E ( $36\text{--}5818\text{ M}^{-1}$ ) varies widely with the length of the fatty acid, as well as the extent of unsaturation in the fatty acid.<sup>24</sup> 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.<sup>5</sup>

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-hydroxyphenoxazoline-containing peptidic headgroup and a long fatty acid (C16–C21) appendage. Carboxymycobactins are relatively hydrophilic and composed of the same peptidic headgroup, 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.<sup>27,28</sup>

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**Figure 1.** Structures of selected amphiphilic siderophores: (A) Suites of marine siderophores include the marinobactins,<sup>5</sup> aquachelins,<sup>5,8</sup> amphibactins,<sup>6,8</sup> loihichelins,<sup>7</sup> and moanachelins;<sup>9</sup> (B) Siderophores from other environmental isolates include cuprichelin,<sup>12</sup> taiwachelin,<sup>13</sup> and the serobactins;<sup>11</sup> siderophores from pathogens include mycobactins,<sup>26</sup> carboxymycobactins,<sup>26</sup> and the acyl precursor to pyoverdine<sup>17,21</sup> are also shown, along with pyoverdine. (Note: For moanachelin gly-C14:1 (R<sub>1</sub> = H; R<sub>2</sub> = C14:1)<sup>9</sup> and marinobactin F (C18:1),<sup>31</sup> not shown, the position and E/Z orientation of the double bond have not been determined.)

As mentioned above, an amphiphilic siderophore precursor was discovered in the biosynthetic pathway of pyoverdine in *P. aeruginosa*.<sup>17,21</sup> 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.<sup>29</sup> Interestingly, the major quorum sensing molecule of *P. aeruginosa*, N-

dodecanoic homoserine lactone (3-oxo-C12-HSL), is also hydrolyzed by PdvQ, providing a payoff between quorum quenching and pyoverdine biosynthesis.<sup>30</sup>

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 headgroup (marinobactin HG or  $M_{HG}$ ), the structural analysis of this headgroup, 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.

## ■ EXPERIMENTAL PROCEDURES

**Microorganisms.** *Marinobacter* sp. DS40M6 and *H. aquamarina* str. DS40M3 were isolated from open ocean water off the west coast of Africa<sup>5,32</sup> and were grown in liquid M6 media. M6 media contains 31 g of NaCl, 6 g of  $Na_2HPO_4 \cdot 7H_2O$ , 1.5 g of KCl, 2 g of  $NH_4Cl$ , 10 g of  $Na_2$  succinate, 0.4 g of  $MgSO_4 \cdot 7H_2O$ , 0.2 g of  $CaCl_2 \cdot 2H_2O$ , 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 of yeast extract, 5 g of peptone, and 15 g of 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,<sup>33</sup> indicating the presence of apo-siderophores.

For bacterial growth in mixed culture, 4 mL of growth media was 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 1 L or 2 L 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 headgroup 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.<sup>5</sup>  $M_{HG}$  was purified by RP-HPLC with a preparative  $C_{18}$  column (22 mm ID  $\times$  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 Headgroup Formation.** For *Mono-culture Growth*. An aliquot of either *Marinobacter* sp. DS40M6

or *H. aquamarina* str. DS40M3 culture was removed daily and centrifuged to pellet the bacterial cells, and the supernatant was filtered through a sterile 0.22  $\mu m$  filter and frozen at  $-20^\circ C$  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  $\times$  250 mm L, 214TP54, Grace Vydac) using a gradient from 100% solvent A (99.95%  $ddH_2O$ , 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%  $H_2O$ , 0.05% TFA), held for 10 min, to 100% solvent B (50% acetonitrile, 49.95%  $H_2O$ , 0.05% TFA) over 30 min, followed by a 5 min 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 were used to determine the mass and partial amino acid connectivity of the marinobactin and aquachelin head groups. For  $M_{HG}$ ,  $^1H$ ,  $^{13}C$ , and various two-dimensional nuclear magnetic resonance (NMR) techniques, including  $^1H$ – $^1H$  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  $^1H$ – $^{15}N$  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 dimethyl sulfoxide- $d_6$  (DMSO- $d_6$ , 99.9%, Cambridge Isotope, Inc.) ( $d = 2.46$  ppm), and experiments were run at  $23.5^\circ C$  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 4–5 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  $\mu M$   $^{15}N$ -marinobactin E (final concentration) was added to a culture of *Marinobacter* sp. DS40M6, which had been grown for 4–5 days. The cells were pelleted 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 min and at 4 days after addition of  $^{15}\text{N}$ -marinobactin E and analyzed for the presence of marinobactins and  $M_{\text{HG}}$ . 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  $^{15}\text{N}$ -marinobactins.

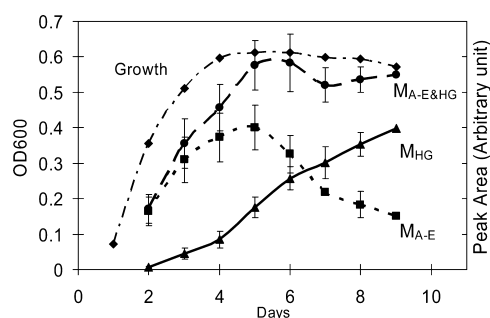
**Preparation of Cell-Free Extract.** *Marinobacter* sp. DS40M6 was grown in M6 medium as previously described and typically required 4–5 days to reach stationary phase. The optical density of the culture at 600 nm was allowed to reach approximately 0.4–0.6 to maximize the cell mass harvested from liquid culture. The cells were pelleted by centrifugation and resuspended 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 ultrasound sonicator (Sonifier 250, Branson). The sonicated homogenate was centrifuged at 7800g for 10 min to remove large cell debris. The supernatant was ultracentrifuged without detergent at 108,000g for 90 min at 4 °C (Discovery M120SE, Sorvall, Hitachi) to obtain a pellet containing the membrane fraction. This pellet was resuspended in buffer-A with 2% Triton X-100 by gentle pipetting and allowed to equilibrate for 30 min in an ice bath. The final ultracentrifugation of the suspension at 108,000g for 60 min at 4 °C resulted in enzymatic activity in the supernatant. The enzyme-detergent extract was concentrated by ultrafiltration 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 to 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<sub>2</sub>O with 0.05% TFA for 3 min, 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.

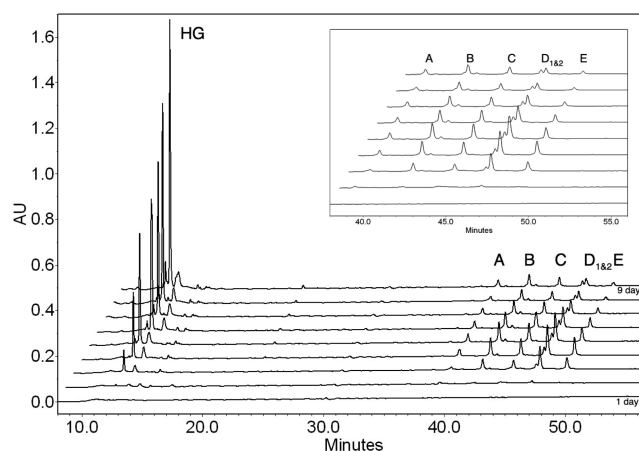
## RESULTS

### 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).<sup>5,31</sup> Beginning in the late log phase of growth and continuing into the stationary phase, a new hydrophilic siderophore is produced, concomitant with a decrease in the amount of marinobactins A–E in the culture supernatant (Figures 2 and 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 ( $M + H$ )<sup>+</sup>, a doubly charged ion peak at  $m/z$  375.6 ( $M + 2H$ )<sup>2+</sup>, and a minor ion peak at  $m/z$  803, which is consistent with Fe(III) coordination ( $[M - 3H + \text{Fe(III)}] + H$ )<sup>+</sup> (Supporting Information, Figure S1). The mass at  $m/z$  750 matches the mass of the marinobactin peptide headgroup. The exact mass of  $M_{\text{HG}}$ ,  $m/z$  750.3240 for ( $M + H$ )<sup>+</sup>, is consistent with



**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:  $M_{\text{HG}}$  production; dotted line with square: the sum of the marinobactin A–E siderophores; dashed-line with circle: the sum of all marinobactins (A–E and HG). The left y-axis denotes the optical density at 600 nm and the right denotes HPLC peak areas in arbitrary units from Figure 3.

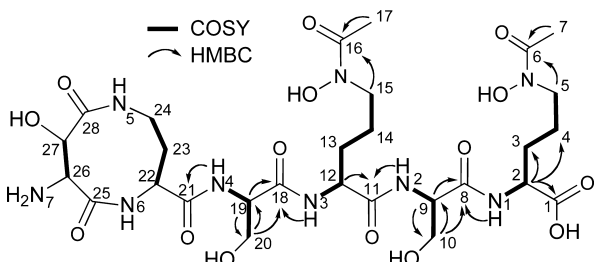


**Figure 3.** Marinobactins A–E and  $M_{\text{HG}}$  production over time: RP-HPLC chromatograms of *Marinobacter* sp. DS40M6 culture supernatant from day 1 (bottom) to day 9 (top). The peak growing in with a retention time of ~12 min is  $M_{\text{HG}}$ ; the retention times of marinobactins A–E are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

$\text{C}_{28}\text{H}_{48}\text{N}_9\text{O}_{15}$ . The shorter retention time of  $M_{\text{HG}}$ , compared with the amphiphilic marinobactins A–E, is consistent with a more hydrophilic compound, such as one lacking a fatty acid appendage (Figure 3).  $M_{\text{HG}}$  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.  $M_{\text{HG}}$  retains the ability to coordinate Fe(III) similar to the acyl-marinobactins.

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  $M_{\text{HG}}$  production increases steadily during growth, becoming the predominant siderophore in the culture medium in stationary phase.

**Marinobactin HG Structure Determination.** Fragmentation analysis of the new hydrophilic siderophore,  $M_{\text{HG}}$ , by tandem mass spectrometry establishes the partial connectivity of the amino acids (Figure S2, Supporting Information), consistent with marinobactins A–E.

Table 1. NMR Assignments:  $^{13}\text{C}$  and  $^1\text{H}$  NMR Chemical Shifts ( $\delta$ , ppm) of  $M_{\text{HG}}$  in  $\text{DMSO-}d_6^a$ 


		$\delta_{\text{C}}$	$\delta_{\text{H}}$	type	COSY	HMBC
(N-Ac, N-OH)-Orn	C1	174.0				2
	C2	52.2	4.14	C	3, NH1	
	C3	29.0	1.65, 1.50	$\text{CH}_2$	2, 4	2
	C4	23.4	1.5	$\text{CH}_2$	3, 5	
	C5	47.0	3.42	$\text{CH}_2$	4	
	C6	170.8		C		5, 7
	C7	20.9	1.93	$\text{CH}_3$		
Ser	N1		8.02	NH	2	
	C8	170.2		C		NH1, 9, 10
	C9	55.4	4.30	CH	10, NH2	10
	C10	62.3	3.51	$\text{CH}_2$	9	9
(N-Ac, N-OH)-Orn	N2		7.85	NH	9	
	C11	171.9		C		NH2, 12
	C12	52.8	4.30	CH	13, NH3	
	C13	29.8	1.42, 1.63	$\text{CH}_2$	12, 14	
	C14	23.4	1.5	$\text{CH}_2$	13, 15	
	C15	47.2	3.42	$\text{CH}_2$	14	
	C16	170.9		C		15, 17
Ser	N3		8.19	NH	12	
	C18	170.1		C		NH3, 19, 20
	C19	55.9	4.35	CH	20, NH4	20
	C20	62.4	3.54	$\text{CH}_2$	19	19
DHBA	N4		8.35	NH	19	
	C21	169.7		C		NH4
	C22	51.3	4.32	CH	23	
	C23	22.0	1.91	$\text{CH}_2$	22	
	C24	36.9	3.27, 3.35	$\text{CH}_2$	23	
	N5		9.68 br	NH	24	
$(\alpha\text{-OH})\text{-Asp}$	N6		9.68 br	NH		
	C25	172.7		C		
	C26	54.9	4.06	CH	27	
	C27	71.7	4.37	CH	26	
	C28	172.7		C		
	N7		nd	$\text{NH}_2$		

<sup>a</sup>See NMR spectra in Figures S5–S7; br, broad; nd, not detected. The orientation of  $\beta$ -hydroxyaspartic acid and diamminobutyric acid in the nine-membered ring was established by HMBC on marinobactins C and E.<sup>34</sup> The  $\text{C}_\alpha$  of  $\beta$ -hydroxyaspartate, C26, couples to the  $\text{N}_6$   $^1\text{H}$  at 9.8 ppm and not with the  $\text{N}_5$   $^1\text{H}$  located further away at 9.72 ppm, consistent with the orientation of the nine-membered ring that is shown here.<sup>34</sup> The structure above shows the  $^1\text{H}$ – $^1\text{H}$  correlation (bold lines) and key HMBC ( $\text{H} \rightarrow \text{C}$ ) correlations.

NMR characterization of  $M_{\text{HG}}$  confirms that it is the peptidic headgroup moiety of the acyl marinobactin siderophores. The  $^{13}\text{C}$  and  $^1\text{H}$  NMR spectral values of  $M_{\text{HG}}$  in  $\text{DMSO-}d_6$  are summarized in Table 1 (Figures S3 and S4, Supporting Information).  $M_{\text{HG}}$  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,  $^1\text{H}$  resonances at  $\delta_{\text{H}}$  0.85 and 1.23–1.26 ( $-\text{CH}_3$  and  $-\text{CH}_2$ , respectively) observed for marinobactin E are not observed in the  $^1\text{H}$  NMR spectrum of  $M_{\text{HG}}$  (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.  $\delta_{\text{H}}$  8 ppm and two in the nine-membered ring at ca.  $\delta_{\text{H}}$  9.85 ppm) in marinobactin E to six in  $M_{\text{HG}}$ , consistent with the conversion of the fatty acid amide of marinobactin E to an amine in  $M_{\text{HG}}$ . The two amide proton signals in the nine-membered ring of  $M_{\text{HG}}$  were broad and overlapped at  $\delta_{\text{H}}$  9.68 ppm. The two amide proton signals of marinobactins C and E were also broad but clearly separated.<sup>34</sup> This change shows the increase in symmetry of the nine-membered ring of  $M_{\text{HG}}$  on fatty acid hydrolysis. Additionally, the proton signal at C26 in

marinobactin E at  $\delta_H$  5.05 is shifted to a higher magnetic field for  $M_{HG}$  at  $\delta_H$  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.

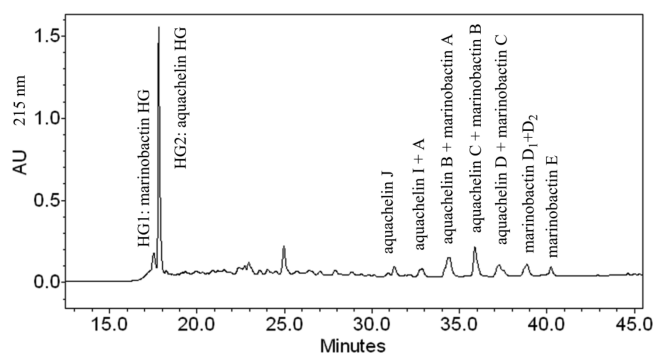
**Microbial Hydrolysis of the Fatty Acid Amide of Marinobactin E to  $M_{HG}$ .**  $M_{HG}$  production was further investigated by adding  $^{15}N$ -labeled marinobactin E back to a culture of *Marinobacter* at day 4 of growth, which is in the range of the late log phase or the beginning of the stationary 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, Supporting Information), 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 nonisotopically 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 nonisotopically 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 headgroup 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 that occurring 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 Coculture.** Growth of *H. aquamarina* str. DS40M3 in pure culture resulted in identification of aquachelins A–D, I, and J (Figure 1).<sup>5,8</sup> However, no aquachelin headgroup formation was detected through the stationary phase of growth and even through cell death (Figure S9, Supporting Information), despite culturing *H. aquamarina* under conditions analogous 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 headgroup increases between days 9 and 14 (data not shown). The appearance of two headgroup peaks indicates that the *Marinobacter* bacterium is modifying not only its own acyl marinobactin siderophores but also the acyl aquachelin siderophores in vivo.

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 headgroup peaks (Figure 4). Extraction of the mixed culture of *Halomonas* and *Marinobacter* using



**Figure 4.** RP-HPLC chromatogram of a 100  $\mu$ L *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. On the basis of mass spectrometric data, the peaks at 17 min are HG1:750  $m/z$ ,  $M_{HG}$ ; HG2:883  $m/z$ , aquachelin headgroup,  $A_{HG}$ . The siderophore peaks between 30 and 42 min 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.

XAD-2 resin resulted in results similar to the aliquot samples and sufficient quantities for identification (Figure 4).

The first smaller peak was identified as the marinobactin headgroup,  $M_{HG}$  (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 headgroup (883  $m/z$ ),  $A_{HG}$ , and tandem mass spectrometry (Figure S10, Supporting Information) 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.<sup>35</sup>) Interestingly, the  $A_{HG}$  peak is the larger of the two headgroup peaks (Figure 4 at 17–18 min). 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 toward marinobactins A–E, converting the isolated suite of marinobactins to the  $M_{HG}$  siderophore. Intriguingly, it also showed activity toward 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 coculture) and loihichelin C (Figure 1). In both cases, the headgroup 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 (Figure 1) and its quorum sensing molecule, 3-oxo-dodecanoic HSL.<sup>29</sup>

## 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 headgroup, M<sub>HG</sub>, 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 headgroup 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.<sup>24</sup>

The NTN-hydrolase, PvdQ, in *P. aeruginosa* catalyzes hydrolysis of the fatty acid from both the acyl-pyoverdine precursor and the endogenous quorum sensing molecule, *N*-(3-oxo-C12)-HSL.<sup>16,17,30</sup> 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<sub>HG</sub> during growth, and another which may be involved in quorum sensing regulation. In this latter 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.<sup>24</sup> 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<sub>HG</sub> 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<sub>HG</sub> 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 coculturing *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 headgroup and overall shorter fatty acids compared to the marinobactins. Production of the aquachelin headgroup, A<sub>HG</sub>, 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, coculturing 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.

## ASSOCIATED CONTENT

### Supporting Information

ESI-MS of M<sub>HG</sub>; MS-MS of M<sub>HG</sub> and <sup>15</sup>N-M<sub>HG</sub>; <sup>1</sup>H, <sup>13</sup>C, <sup>1</sup>H–<sup>1</sup>H COSY, <sup>1</sup>H–<sup>13</sup>C HMBC, and <sup>1</sup>H–<sup>13</sup>C HMQC of M<sub>HG</sub>; HPLC of aquachelin production versus time and HPLC of cocultures of *Marinobacter* sp. DS40M6 and *H. aquamarina* str. DS40M3, MS-MS of A<sub>HG</sub>. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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## Notes

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## REFERENCES

- (1) Sandy, M., and Butler, A. (2009) Microbial Iron Acquisition: Marine and Terrestrial Siderophores. *Chem. Rev.* 109, 4580–4595.
- (2) Luo, M., Fadeev, E. A., and Groves, J. T. (2005) Membrane dynamics of the amphiphilic siderophore, acinetoferrin. *J. Am. Chem. Soc.* 127, 1726–1736.
- (3) Martin, J. D., Ito, Y., Homann, V. V., Haygood, M. G., and Butler, A. (2006) Structure and membrane affinity of new amphiphilic siderophores produced by *Ochrobactrum* sp SP18. *J. Biol. Inorg. Chem.* 11, 633–641.
- (4) Gauglitz, J. M., Zhou, H. J., and Butler, A. (2012) A suite of citrate-derived siderophores from a marine *Vibrio* species isolated following the Deepwater Horizon oil spill. *J. Biol. Inorg. Chem.* 107, 90–95.
- (5) Martinez, J. S., Zhang, G. P., Holt, P. D., Jung, H. T., Carrano, C. J., Haygood, M. G., and Butler, A. (2000) Self-assembling amphiphilic siderophores from marine bacteria. *Science* 287, 1245–1247.
- (6) Martinez, J. S., Carter-Franklin, J. N., Mann, E. L., Martin, J. D., Haygood, M. G., and Butler, A. (2003) Structure and membrane affinity of a suite of amphiphilic siderophores produced by a marine bacterium. *Proc. Natl. Acad. Sci. U. S. A.* 100, 3754–3759.
- (7) Homann, V. V., Sandy, M., Tincu, J. A., Templeton, A. S., Tebo, B. M., and Butler, A. (2009) Loihichelins A-F, a Suite of Amphiphilic Siderophores Produced by the Marine Bacterium *Halomonas* LOB-5. *J. Natl. Prod.* 72, 884–888.
- (8) Vraspir, J. M., Holt, P. D., and Butler, A. (2011) Identification of new members within suites of amphiphilic marine siderophores. *Biometals* 24, 85–92.
- (9) Gauglitz, J. M., and Butler, A. (2013) Amino acid variability in the peptide composition of a suite of amphiphilic peptide siderophores from an open ocean *Vibrio* species. *J. Biol. Inorg. Chem.* 18, 489–497.
- (10) Ratledge, C., and Dover, L. G. (2000) *Annu. Rev. Microbiol.* 54, 881–941.
- (11) Rosconi, F., Davyt, D., Martinez, V., Martinez, M., Abin-Carriquiry, J. A., Zane, H., Butler, A., de Souza, E. M., and Fabiano, E. (2013) Identification and structural characterization of serobactins, a suite of lipopeptide siderophores produced by the grass endophyte *Herbaspirillum seropedicae*. *Environ. Microbiol.* 15, 916–927.
- (12) Kreutzer, M. F., Kage, H., and Nett, M. (2012) Structure and Biosynthetic Assembly of Cupriachelin, a Photoreactive Siderophore from the Bioplastic Producer *Cupriavidus necator* H16. *J. Am. Chem. Soc.* 134, 5415–5422.
- (13) Kreutzer, M. F., and Nett, M. (2012) Genomics-driven discovery of taiwachelin, a lipopeptide siderophore from *Cupriavidus taiwanensis*. *Org. Biomol. Chem.* 10, 9338–9343.
- (14) Meyer, J. M., Van, V. T., Stintzi, A., Berge, O., and Winkelmann, G. (1995) Ornibactin Production and Transport-Properties in Strains of *Burkholderia-Vietnamiensis* and *Burkholderia-Cepacia* (Formerly *Pseudomonas-Cepacia*). *Biometals* 8, 309–317.
- (15) Stephan, H., Freund, S., Beck, W., Jung, G., Meyer, J. M., and Winkelmann, G. (1993) Ornibactins - a New Family of Siderophores from *Pseudomonas*. *Biometals* 6, 93–100.
- (16) Guillon, L., El Mecherki, M., Altenburger, S., Graumann, P. L., and Schalk, I. J. (2012) High cellular organization of pyoverdine biosynthesis in *Pseudomonas aeruginosa*: clustering of PvdA at the old cell pole. *Environ. Microbiol.* 14, 1982–1994.
- (17) Hannauer, M., Schafer, M., Hoegy, F., Gizzi, P., Wehrung, P., Mislin, G. L. A., Budzikiewicz, H., and Schalk, I. J. (2012) Biosynthesis of the pyoverdine siderophore of *Pseudomonas aeruginosa* involves precursors with a myristic or a myristoleic acid chain. *FEBS Lett.* 586, 96–101.
- (18) Hannauer, M., Yeterian, E., Martin, L. W., Lamont, I. L., and Schalk, I. J. (2010) An efflux pump is involved in secretion of newly synthesized siderophore by *Pseudomonas aeruginosa*. *FEBS Lett.* 584, 4751–4755.
- (19) Braud, A., Geoffroy, V., Hoegy, F., Mislin, G. L. A., and Schalk, I. J. (2010) Presence of the siderophores pyoverdine and pyochelin in the extracellular medium reduces toxic metal accumulation in *Pseudomonas aeruginosa* and increases bacterial metal tolerance. *Environ. Microbiol. Rep.* 2, 419–425.
- (20) Yeterian, E., Martin, L. W., Guillon, L., Journet, L., Lamont, I. L., and Schalk, I. J. (2010) Synthesis of the siderophore pyoverdine in *Pseudomonas aeruginosa* involves a periplasmic maturation. *Amino Acids* 38, 1447–1459.
- (21) Drake, E. J., and Gulick, A. M. (2011) Structural Characterization and High-Throughput Screening of Inhibitors of PvdQ, an NTN Hydrolase Involved in Pyoverdine Synthesis. *ACS Chem. Biol.* 6, 1277–1286.
- (22) Drake, E. J., and Gulick, A. M. (2008) Three-dimensional Structures of *Pseudomonas aeruginosa* PvcA and PvcB, Two Proteins Involved in the Synthesis of 2-Isocyano-6,7-dihydroxycoumarin. *J. Mol. Biol.* 384, 193–205.
- (23) Drake, E. J., Cao, J., Qu, J., Shah, M. B., Straubinger, R. M., and Gulick, A. M. (2007) The 1.8 Å crystal structure of PA2412, an MbtH-like protein from the pyoverdine cluster of *Pseudomonas aeruginosa*. *J. Biol. Chem.* 282, 20425–20434.
- (24) Xu, G. F., Martinez, J. S., Groves, J. T., and Butler, A. (2002) Membrane affinity of the amphiphilic marinobactin siderophores. *J. Am. Chem. Soc.* 124, 13408–13415.
- (25) Luo, M. K., Fadeev, E. A., and Groves, J. T. (2005) Mycobactin-mediated iron acquisition within macrophages. *Nat. Chem. Biol.* 1, 149–153.
- (26) Ratledge, C. (2004) Iron, mycobacteria and tuberculosis. *Tuberculosis* 84, 110–130.
- (27) Gobin, J., and Horwitz, M. A. (1996) Exochelins of *Mycobacterium tuberculosis* remove iron from human iron-binding proteins and donate iron to mycobactins in the M-tuberculosis cell wall. *J. Exp. Med.* 183, 1527–1532.
- (28) Rodriguez, G. M., and Smith, I. (2006) Identification of an ABC transporter required for iron acquisition and virulence in *Mycobacterium tuberculosis*. *J. Bacteriol.* 188, 424–430.
- (29) Schalk, I. J., and Guillon, L. (2013) Pyoverdine biosynthesis and secretion in *Pseudomonas aeruginosa*: implications for metal homeostasis. *Environ. Microbiol.* 15, 1661–1673.
- (30) Bokhove, M., Jimenez, P. N., Quax, W. J., and Dijkstra, B. W. (2010) The quorum-quenching N-acyl homoserine lactone acylase PvdQ is an Ntn-hydrolase with an unusual substrate-binding pocket. *Proc. Natl. Acad. Sci. U. S. A.* 107, 686–691.
- (31) Martinez, J. S., and Butler, A. (2007) Marine amphiphilic siderophores: Marinobactin structure, uptake, and microbial partitioning. *J. Inorg. Biochem.* 101, 1692–1698.
- (32) Haygood, M. G., Holt, P. D., and Butler, A. (1993) Aerobactin production by a planktonic marine *Vibrio* sp. *Limnol. Oceanogr.* 38, 1091–1097.
- (33) Schwyn, B., and Neilands, J. (1987) Universal chemical assay for the detection and determination of siderophores. *Anal. Biochem.* 160, 47–56.
- (34) Zhang, G. (1999) Ph.D. Dissertation, *University of California Santa Barbara*, p 331.
- (35) Roepstorff, P., and Fohlman, J. (1984) Proposal for a common nomenclature for sequence ions in mass spectra of peptides. *Biomed. Mass Spectrom.* 11, 601.