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Amphiphilic Siderophore Production by Oil-associating Microbes

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The Deepwater Horizon oil spill in 2010 released an unprecedented amount of oil into the ocean waters of the Gulf of Mexico. As a consequence, bioremediation by oil-degrading microbes has been a topic of increased focus. One factor limiting the rate of hydrocarbon degradation by microbial communities is the availability of necessary nutrients, including iron. The siderophores produced from two *Vibrio spp.* isolated from the Gulf of Mexico following the Deepwater Horizon oil spill, along with the well-studied oil-degrading microbe, *Alcanivorax borkumensis* SK2, is studied under iron-limiting conditions. Here we report the amphiphilic amphibactin siderophores produced by the oil-associated bacteria, *Vibrio sp.* S1B, *Vibrio sp.* S2A and *Alcanivorax borkumensis* SK2. These findings provide insight into oil-associating microbial iron acquisition.

Introduction

The marine environment is subject to contamination by organic pollutants from a variety of sources, including crude oil.[1,2] In 2010, the Deepwater Horizon blowout at the MC252 Macondo well site released more than 4 million barrels (636 million liters) of oil into the Gulf of Mexico.[3] One strategy for the remediation of the deep underwater plume has been to use the intrinsic hydrocarbon-degrading potential of marine microorganisms to break down the oil.[1,4,5] Crude oil is a complex mixture containing thousands of different hydrocarbon compounds which differ in solubility and volatility and are degraded at different rates.[4] Effective microbial biodegradation is dependent on a variety of environmental factors, including a favourable response by the indigenous microorganisms. Reports profiling the most dominant oil-degraders in plume samples following the spill revealed an abundance of γ-proteobacteria in both deep water, surface water and oil-contaminated sand samples; *Oceanospirillales*, *Colwellia* and *Cycloclasticus* dominated the deep water samples, *Pseudoalteromonas, Vibrio, Acinetobacter* and *Alteromonas* prevailed in the surface samples,[4,5] while the famously ubiquitous oil-degrader *Alcanivorax borkumensis* was the dominant hydrocarbon-degrading bacterial group enriched by oil contamination of Gulf beach sands.[6] These results indicate a dynamic population of hydrocarbon-degraders with different compound preferences existing in the Gulf. The implication is that these bacterial communities could play an important role in bioremediation.

In addition to the microbial population, the rate of microbial biodegradation is influenced by the availability of nutrients; specifically nitrogen, phosphorus, and iron.[7,8] The iron-dependence of efficient hydrocarbon degradation could be related to the iron-containing oxygenases required for initial hydrocarbon breakdown, such as, the alkane monooxygenase, AlkB2, and cytochrome P450 (CYP).[11] AlkB2 is a non-heme diiron alkane monooxygenase that is required for the first step of hydrocarbon degradation, hydroxylating the terminal position of the alkane.[9,10] This pathway is found in the vast majority of medium to long chain alkane-oxidizing aerobic bacteria.[11]

The rate of hydrocarbon degradation has been demonstrated to increase in the presence of iron, however, the mechanisms of iron acquisition by these microbes have not been well characterized.[12,13] Many marine bacteria produce siderophores in response to the extremely low concentration of soluble iron in surface ocean waters. Siderophores are low molecular weight, iron(III) chelators that bacteria secrete to solubilize and transport iron(III) into the cell. A distinct characteristic of marine siderophores is the predominance of suites of amphiphilic siderophores composed of a peptidic head group appended by one of a series of fatty acid tails, which is not commonly seen in terrestrial siderophores (Fig. 1).[14-18] The fatty acid tails differ in length and functionality; however, little is known about the interaction of the amphiphilic siderophores with the bacterial cells.[17] Amphiphilic siderophores with smaller head groups and longer fatty acid tails, as seen with the amphibactin, moanachelins, and loihichelins are quite hydrophobic and predominantly associate with the bacterial membrane.[14,17,19] The aquachelins, however, are quite hydrophilic and are secreted into the extracellular environment.[16] Other marine siderophores, like the marinobactins, range in hydrophilicity among the suite resulting in a mixed environment of membrane-associated and secreted siderophores.[20]

Siderophore producing marine bacteria have been isolated from a wide range of ocean environments, including the Santa Barbara Channel in the vicinity of natural oil seepage sites, and the Gulf of Mexico at the site of the Deepwater Horizon oil spill. Previously, we have identified and characterized siderophore production from two oil-associated microbes. *Pseudoalteromonas* sp. S2B and *Vibrio* sp. S4BW were isolated from the Gulf of Mexico following the Deepwater Horizon oil spill and produce

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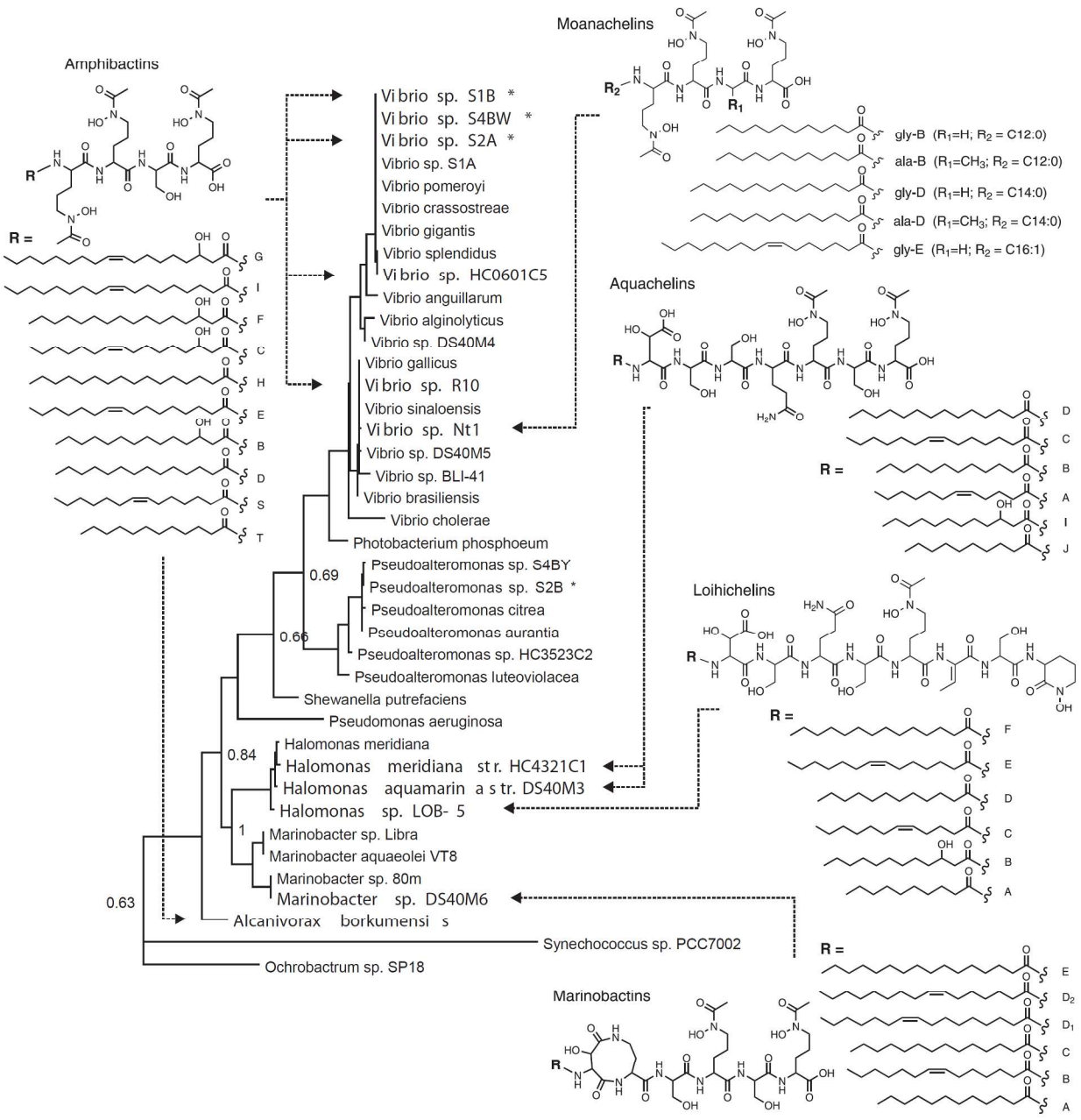

Fig. 1 Phylogenetic tree of selected siderophore-producing bacteria and related species based on maximum likelihood analysis of SSU rDNA sequences.[23-29] Amphiphilic siderophore-producing bacteria are labelled in bold, with their corresponding structures. The asterisked (*) bacteria are oil-associated strains. Sequences of related bacteria and other known siderophore-producing bacteria were obtained from green genes[30] or GenBank.[31] (For moanachelin gly-C (R1 = H; R2 = C14:1) and marinobactin F (C18:1), not shown, the position and E/Z orientation of the double bond has not been determined).[19,20]

the lystabactins and ochrobactins-OH siderophores, respectively.[21,22] We report the siderophore production of two more oil-associated microbes from the Gulf of Mexico following the Deepwater Horizon oil spill, as well as siderophore production by the well-studied hydrocarbon degrading bacterium, *Alcanivorax borkumensis* SK2. All three isolates were determined to produce the amphibactins, a suite of amphiphilic siderophores. This amphiphilic property might aid in bacterial iron(III) solubilization in an oil-contaminated environment.

Experimental methods

Bacteria isolation/16S rDNA sequencing

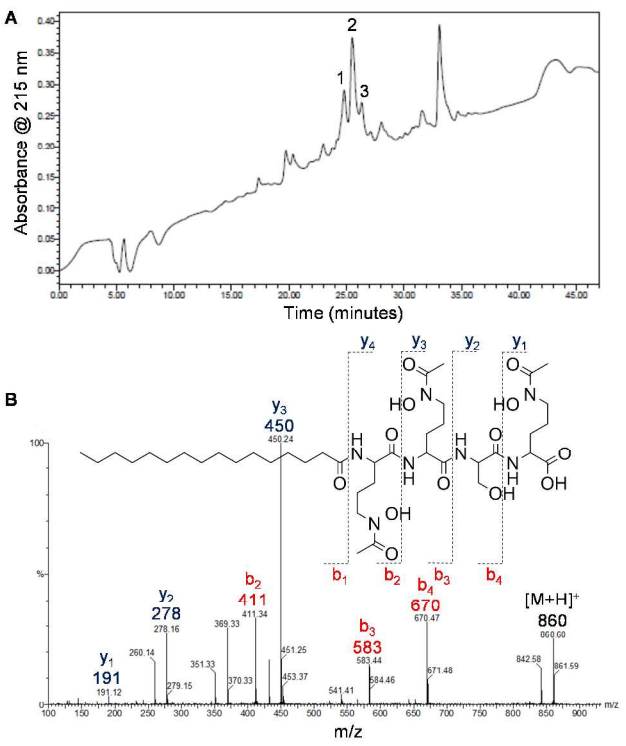

Fig. 2 (A) HPLC trace of siderophores from the ethanol extracted pellet of *Alcanivorax borkumensis* SK2. Peaks 1-3 correspond to amphibactin E, H, and I, respectively. (B) Tandem mass spectrum of amphibactin H.

Bacteria were isolated from surface waters on June 4th and 5th, 2010 following the Deepwater Horizon oil spill in the Gulf of Mexico on April 20th 2010. Samples were taken from areas with visible amounts of oil on the surface and gifted to us by Dr. Molly Redmond and Professor David Valentine in the Department of Earth Sciences at UCSB. The water samples were checked for siderophore production by plating on CAS indicator plates.[32] Siderophore-producing bacteria were identified through 16S rDNA sequencing using the universal bacterial 16S rDNA primers, 27F and 1492R. The samples were sequenced by the UC Berkeley DNA Sequencing facility and the sequences were aligned with ClustalW. The bacterial species were identified by comparing the sequences to published sequences using Blastn. *Alcanivorax borkumensis* SK2 was a gift from Professor D. Valentine.

Bacterial growth and Siderophore Isolation

Alcanivorax borkumensis SK2 was grown in an iron-free modified NSW medium containing 1g NH_4Cl/L, 2 g Casamino acids/L, 0.1 g glycerol phosphate/L, 5 g sodium pyruvate/L, and 3 ml glycerol/L. The bacterium was cultured at 180 rpm on a rotary shaker at 30°C for about 3 days. *Vibrio* sp. S2A and *Vibrio* sp. S1B were grown in an artificial seawater medium (ASG-Fe) containing 10 g Casamino acids/L, 1 g NH_4Cl/L, 1 g glycerol phosphate/L, 12.3 g $MgSO_4 \cdot 7H_2O$/L, 16.5 g NaCl/L, and 0.75 g KCl/L. Prior to induction, filter sterilized solutions of 10 ml of 1.0 M HEPES/L, 2 ml of 1.0 M $NaHCO_3$/L, and 10 ml vitamin stock/L (40 mg of biotin, 4 mg of niacin, 2 mg of thiamin, 4 mg of p-aminobenzoic acid, 2 mg of calcium pantothenic acid, 20 mg of pyridoxine hydrochloride, 2 mg of cyanocobalamin, 4 mg of riboflavin, and 4 mg of folic acid in 200 ml of doubly deionized water) were added to the ASG medium. The cultures were grown for approximately 2 days at 180 rpm on a rotary shaker and ambient temperature.

Table 1 The amphibactin siderophores produced by each oil-associated species.

A. borkumensis SK2			*Vibrio* sp. S2A			*Vibrio* sp. S1B		
m/z	Amphi-bactin	FA[a]	m/z	Amphi-bactin	FA[a]	m/z	Amphi-bactin	FA[a]
858	E	C16:1	832	D	C14:0	830	S	C14:1
860	H	C16:0	858	E	C16:1	832	D	C14:0
886	I	C18:1	860	H	C16:0	858	E	C16:1
			886	I	C18:1	860	H	C16:0

[b]FA = Fatty acid tail length and degree of unsaturation

To isolate siderophores, the cultures were harvested at 6000 RPM for 30 minutes at 4°C using a SLA-3000 rotor. The supernatants were decanted and siderophores were extracted from the cell pellets using 100 ml of 90% ethanol per 2 L of bacterial culture. The resuspended pellets were shaken at 120 RPM for 20-48 hours at ambient temperature. The pellet extract was filtered through a 0.22 µm filter and concentrated using a rotary evaporator. The concentrated extract was diluted 1:5 in water and loaded onto a 1 ml C-18 SepPak cartridge. The cartridge was washed with 50% methanol in water and siderophores were eluted with 80%-100% methanol. The siderophores were further purified using HPLC and a Vydac C4 prep column. The compounds were eluted using a linear gradient of 50% methanol in water + 0.05% trifluoroacetic acid to 90% methanol + 0.05% trifluoroacetic acid over 35 minutes. The siderophores were identified using ESI-MS/MS on a Micromass QTOF2 Quadrupole/Time-of-Flight Tandem mass spectrometer using argon as the collision gas.

Analysis of amphibactin biosynthesis genes from *Alcanivorax borkumensis* SK2

The genome of *Alcanivorax borkumensis* SK2 is publicly available from the National Center for Biotechnology Information (NCBI). To investigate the genes responsible for the biosynthesis of the amphibactins, the genome was screened for genes encoding putative non-ribosomal peptide synthetases. The publicly available NRPS/PKS analysis website was used to predict the amino acid specificity of the NRPS adenylation domains.[33]

Results and Discussion:

Bacteria isolation and 16S rDNA sequencing

Efficient hydrocarbon degradation is limited by various nutrients in the marine environment, including iron(III). To investigate how oil-associated bacteria obtain iron(III) from the environment, bacteria isolated from the Gulf following the Deepwater Horizon oil spill were analyzed for siderophore production. Two bacterial species isolated from surface waters following the Deepwater Horizon oil spill were isolated and predicted to produce siderophores as determined by observing the appearance of

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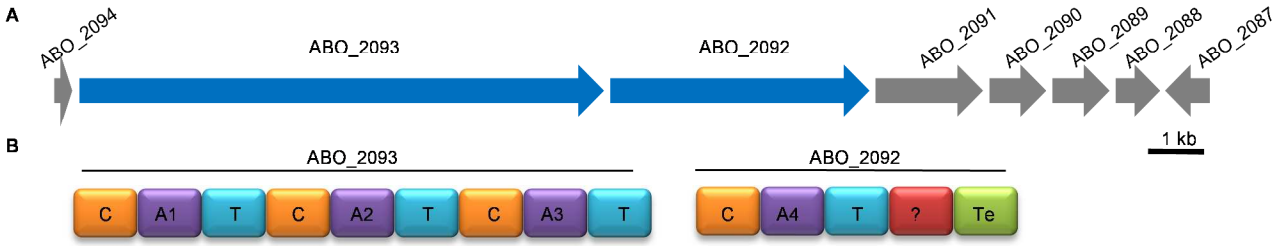

Fig. 3 (A) Gene map of putative amphibactin biosynthetic genes from *Alcanivorax borkumensis* SK2. (B) The predicted modular organization of the NRPS genes

Table 2 Predicted function of the ORF involved with amphibactin biosynthesis in *Alcanivorax borkumensis* SK2

Locus Tag	ORF Predicted function	Gene Annotation	Accession Number
ABO_2087	Phosphopantetheinyl transferase	EntD	YP_693807
ABO_2088	Ferric iron reductase		YP_693808
ABO_2089	L-ornithine N^5-monooxygenase		YP_693809
ABO_2090	Acetyltransferase	IucB	YP_693810
ABO_2091	Ferrioxamine receptor	foxA	YP_693811
ABO_2092	NRPS		YP_693812
ABO_2093	NRPS		YP_693813
ABO_2094	Mbth-like protein		YP_693814

Table 3 Predicted adenylation domain specificity of the amphibactin biosynthesis NRPS using the PKS/NRPS analysis web-site.

Locus Tag	Residues	Predictions
ABO_2093 A1	D G - C T G G V	No hit
ABO_2093 A2	D G - C T G G V	No hit
ABO_2093 A3	D V W H I S L I	Serine (100%)
ABO_2092 A4	D G - C T G G V	No hit

orange halos surrounding bacterial colonies on a CAS plate. The 16S rDNA sequencing data identified the siderophore producing bacteria to be two different *Vibrio* species designated as *Vibrio* sp. S2A (GenBank ID: KJ439559) and *Vibrio* sp. S1B (GenBank ID: KJ439558). *Vibrio* species have been previously found in oil-rich marine environments and a few species are known to degrade hydrocarbons.[34-36] An *Alcanivorax spp.* was not isolated from this water sample, but due to its abundance in previous oil-contaminated samples, we also screened *Alcanivorax borkumensis* SK2 for siderophore production.

Siderophore Isolation and Characterization

Alcanivorax borkumensis SK2 is often the dominant microbe found in oil-polluted waters and has been extensively studied for its ability to degrade hydrocarbons; however, the siderophore-mediated iron acquisition of the bacteria has not been investigated. The HPLC trace of the ethanol extracted pellet resulted in the isolation of three CAS active peaks labelled 1-3 (Fig. 2A). Mass spectrometry of the eluted compounds identified peaks 1-3 as amphibactin E, H and I, respectively, as summarized in Table 1. Tandem mass spectrometry analysis (Fig. 2B, Fig. S1) indicated that all three siderophores have the same 'y' ions with m/z values of 191, 278, and 450, corresponding to a loss of 191, 87 and 172 for a C-terminus N^5-acyl-N^5-hydroxyornithine followed by a serine and another N^5-acyl-N^5-hydroxyornithine. The m/z value for the 'y' ion of the N-terminus N^5-acyl-N^5-hydroxyornithine connected to the fatty acid could not be detected by tandem mass spectrometry.

The siderophores did, however, differ in the masses of their 'b' ions (Table S1) which is indicative of a suite of amphiphilic siderophores that are comprised of the same head group but varied in the nature of the fatty acid tails.[16] The difference in the mass of the 'b' ions can be used to predict the tail lengths of the isolated siderophores.

Both *Vibrio* sp. S2A and *Vibrio* sp. S1B were also determined to produce the amphibactin siderophores. *Vibrio* sp. S2A produced amphibactin D, E, H, and I corresponding to the m/z values of 832, 858, 860, and 886 (Table 1, Fig. S2) and *Vibrio* sp. S1B produced amphibactins S, D, E, and H, respectively corresponding to the m/z values of 830, 832, 858, and 860 (Table 1, Fig. S3). The 'b' ions used to predict tail lengths are provided in Table S1. Each of the species analyzed produces a suite of amphibactin siderophores with C14-C16 tails varying in degree of unsaturation. Interestingly, no amphibactins with hydroxylated fatty acid tails were isolated as has been previously seen with other amphibactin-producing bacteria.[17,37] Small amounts of amphibactins with shorter tail lengths were found in the culture supernatant of both *Vibrio* spp. (data not shown); however, the majority of siderophores were extracted from the pellet.

The production of amphiphilic siderophores by oil-associated microbes is likely to be very beneficial in an oil-rich environment. Many oil-degrading bacteria produce biosurfactants to disperse and emulsify hydrocarbons.[38,39] The amphiphilic nature of the amphibactins allows the siderophore to behave as a biosurfactant, aiding in both the iron uptake process and hydrocarbon degradation by preventing siderophore diffusion and increasing the solubility of oil at the bacterial membrane.

Putative biosynthetic pathway for the amphibactin siderophores in *Alcanivorax borkumensis* SK2

The genes responsible for the biosynthesis of the amphibactin siderophores from *Alcanivorax borkumensis* SK2 were investigated to determine if the predicted structure matched the obtained mass spectrometry data. Due to the peptide based structure of the amphibactins, and the inclusion of non-proteinogenic amino acids, it was predicted that the siderophores would be synthesized by non-ribosomal peptide synthetases (NRPS). NRPS are large, multi-modular synthetases that carry-out the step-wise formation of peptides in an assembly-line manner.[40] The adenylation domain within a NRPS module is responsible for activation and incorporation of the amino acid into the growing peptide chain. The identity of the amino acid to be incorporated can be predicted based on the sequence of a ten amino acid region in the adenylation domain binding pocket.[41,42] The predicted functions of the putative amphibactin biosynthetic genes are summarized in Table 2 and the biosynthetic gene map is organized in Fig. 3A. The organization and amino acid specificity of the NRPS modules responsible for amphibactin biosynthesis were analyzed using the publicly available software, PKS/NRPS predictor.[33] BLAST analysis indicated the presence of two NRPS genes, ABO_2093 and ABO_2092, which are comprised of four classical NRPS modules, three on ABO_2093 and one on ABO_2092. The first NRPS domain begins with an N-terminal condensation domain as has been seen previously with other lipopeptide natural products like surfactin.[43] Since no fatty acyl CoA ligase is present in the biosynthetic gene cluster, this suggests that an external acyl CoA ligase is responsible for acylation of the N-terminal N^5-acyl-N^5-hydroxyornithine to initiate siderophore biosynthesis.

According to the modular organization of the NRPS (Fig. 3B), four adenylation domains are involved in the biosynthesis of the amphibactins consistent with the four amino acids present in the head group. The amino acid specificity of the adenylation domains predicts the third amino acid from the N-terminus to be a serine. The signature sequences of the other three adenylation domains are identical; however, the amino acid code (DG-CTGGV) does not have any significant homology to adenylation domains of known specificity (Table 3). Interestingly, genes ABO_2089 and ABO_2090 are predicted to be involved in the tailoring of ornithine to form N^5-acyl-N^5-hydroxyornithine. Gene ABO_2089 is a putative L-ornithine N^5-monooxygenase responsible for the hydroxylation of ornithine and ABO_2090 is predicted to be an acetyltransferase similar to IucB, the tailoring enzyme responsible for the acetylation of hydroxylysine during aerobactin biosynthesis in *E. coli*.[44] Due to the presence of three N^5-acyl-N^5-hydroxyornithines seen in the structure of the amphibactins at the location of these adenylation domains and the presence of ornithine tailoring enzymes, it can be postulated that these domains encode for N^5-acyl-N^5-hydroxyornithine.

No epimerization domains were predicted suggesting that all four amino acids are in the L-configuration. Gene ABO_2092 is predicted to have an unknown domain preceding the thioesterase domain. BLAST analysis shows the N-terminal part of the unknown domain (about 300 AA) has homology to NRPS condensation domains while the remaining 130 AA of the domain have conserved motifs of the NRPS-para261 superfamily. It is not clear whether this domain is involved with the biosynthesis of the amphibactins or necessary for the termination of the molecule from the NRPS.

Conclusions

While it is known that many alkane-degrading enzymes require iron as a cofactor, the siderophore-mediated iron uptake pathway for many oil-degrading microbes, including *Alcanivorax spp*., had not been investigated. In this work, we have determined the siderophore production by three oil-associated microbes, *Vibrio* sp. S1B, and *Vibrio* sp. S2A, isolated from the Gulf of Mexico following the Deepwater Horizon oil spill and *Alcanivorax borkumensis* SK2. All three species produce the amphibactin siderophores with fatty acid tails ranging from 14-18 carbons as determined by mass spectrometry. An analysis of the putative biosynthetic genes involved in amphibactin biosynthesis further verified the structure of the amphibactin head group with four amino acids containing three N^5-acyl-N^5-hydroxyornithines and one serine. Further characterization of the amphibactin structure and biosynthetic pathway from *Alcanivorax borkumensis* SK2 is under way. The production of amphiphilic siderophores could be beneficial for both the solubilization of oil and iron uptake at the cell surface by acting as a biosurfactant for oil emulsification and associating with the cell membrane to prevent siderophore diffusion.

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Notes and references

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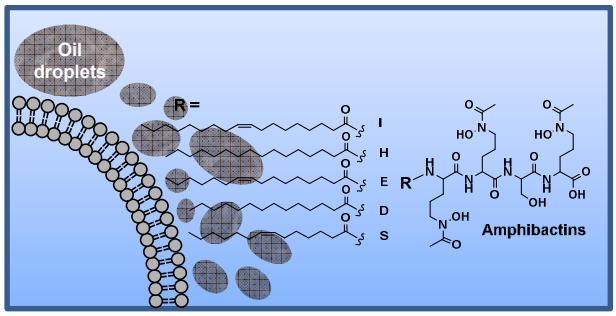

Amphibactin siderophores have been isolated from oil-associated *Vibrio spp.* following the Deepwater Horizon oil spill, and from *Alcanivorax borkumensis* SK2.