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Iron transport in the genus *Marinobacter*

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Abstract *Marinobacter* belong to the class of Gammaproteobacteria and these motile, halophilic or halotolerent bacteria are widely distributed throughout the world's oceans having been isolated from a wide variety of marine environments. They have also been identified as members of the bacterial flora associated with other marine organisms. Here, using a combination of natural products chemistry and genomic analysis, we assess the nature of the siderophores produced by this genus and their potential relationship to phylogeny and lifestyle/ecological niche of this diverse group of organisms. Our analysis shows a wide level of diversity in siderophore based iron uptake systems among this genus with three general strategies: (1) production and utilization of native siderophores in addition to utilization of a variety of exogenous ones, (2) production and utilization of native siderophores only, (3) lack of siderophore production but utilization of exogenous ones. They all share the presence of at least one siderophore-independent iron uptake ABC transport systems of the FbpABC iron metal type and lack the ability for direct transport of ferrous iron. Siderophore production and utilization can be correlated with phylogeny and thus it forms a type of chemotaxonomic marker for this genus.

Keywords Iron · Transport · Siderophores · Marinobacter

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Introduction

Iron, although one of the most important micronutrients in the marine environment is largely bio-unavailable due to its poor solubility and tendency to form colloidal and oxopolymeric species (Bruland et al. 1991; Tortell et al. 1999). The presence of organic ligands of as yet unknown structures that tightly complex iron and increase its solubility, yet reduce the concentration of biologically available inorganic ferric species, further complicate iron speciation and its availability to microorganisms (Gledhill and van den Berg 1994; Rue and Bruland 1995; Wu and Luther 1995). Multiple iron fertilization experiments in high-nutrient-low-chlorophyll (HNLC) regions of the oceans have corroborated the importance of iron to phytoplankton and its limitation to

marine microorganisms (Coale et al. 1996). To alleviate this limitation, diverse marine bacterial species excrete small organic compounds, called siderophores, which bind iron with exceptional affinity in response to iron limitation (Vraspir and Butler 2009). It has been hypothesized that the global production of siderophores by heterotrophic bacteria and some cyanobacteria constitutes the bulk of organic ligands binding iron in the ocean because stability constants of siderophores and these organic ligands are similar, and because ligand concentrations rise sharply in response to iron fertilization events (Boye et al. 2005; Rue and Bruland 1997; Vraspir and Butler 2009). One of the emerging structural features that differentiate terrestrial from marine siderophores is the near universal presence in the latter of α or β-hydroxy acid moieties (Barbeau et al. 2002; Sandy and Butler 2009). These chelating groups make the resulting iron complexes photolabile so that the bound Fe(III) is reduced to Fe(II) with the concomitant oxidation and loss of CO_2 from the ligand via an irreversible internal redox reaction. Since the photogenerated Fe(II) rapidly oxidizes under aerobic oceanic conditions to yield soluble inorganic iron, designated Fe(III)', it was anticipated that most of this iron would become bioavailable to microorganisms. However, thermodynamic measurements unexpectedly indicated that the oxidized siderophore photoproducts maintained an exceptional affinity for Fe(III), recomplexing it and thus possibly continuing to restrict its bioavailability (Abergel et al. 2008; Barbeau et al. 2001; Küpper et al. 2006). Indeed in two of the three examples examined the photoproducts bound Fe(III) with even higher affinity than the parent siderophore (Abergel et al. 2008; Küpper et al. 2006).

Marinobacter (Fig. 1) belong to the class of Gammaproteobacteria and these motile, halophilic or halotolerent bacteria all share the ability to use petroleum hydrocarbons as sole energy and carbon sources (Duran 2010). They are widely distributed throughout the world's oceans as evidenced by their isolation from a wide variety of marine environments ranging from hydrothermal vents (Kaye et al. 2011) to Antarctic sea ice (Glatz et al. 2006). They have also been identified as members of the bacterial flora associated with other marine organisms. Indeed we and others have observed that among the most notable members of the bacterial communities associated with marine phytoplankton including diatoms, coccolithophores and dinoflagellates were bacteria from several *Marinobacter* clades (Green et al. 2004; Alavi et al. 2001; Seibold et al. 2001; Kaeppel et al. 2011). In fact, siderophore production by these clades has been shown to enhance iron availability to associated phytoplankton (Amin et al. 2009). Here, we assess the nature of the siderophores produced by this genus and their potential relationship to phylogeny and lifestyle/ecological niche of this diverse group of organisms.

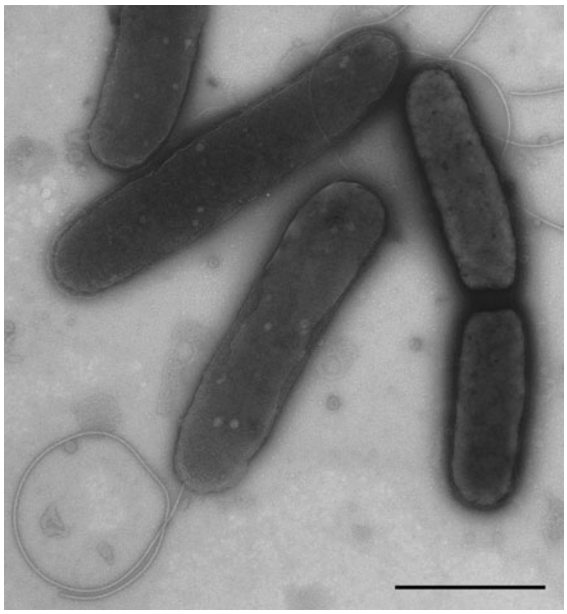


Fig. 1 Transmission electron micrograph of the VF producing *Marinobacter* sp. DG879. Cells were negatively stained with 2% ammonium molybdate. *Scale bar*, 1 μm

Materials and methods

Bacterial growth and siderophore isolation

Marinobacter spp. were grown and harvested as previously described (Amin et al. 2007, 2009).

Siderophore isolation and identification

The supernatant was isolated from bacterial cells by centrifugation at 5000 rpm for 25 min at 4°C using Sorvall RC5C + centrifuge and acidified to pH 2.5. Amberlite XAD-2 resin, (ca. 100 g/l, SUPELCO), was added to the supernatant and the suspension

shaken for 12 h. Subsequently, the resin was poured into a column, washed with several bed volumes of Mille-Q water and finally eluted with methanol. Concentration of the methanol eluent by rotary evaporation yielded crude culture extracts. Siderophores were isolated from the extracts by purification on a size exclusion column (Biogel P-2, BioRad). Siderophore-containing fractions were identified via the Chromeazurol S, CAS, assay (Schwyn and Neilands 1987) combined and repurified using semi-preparative reverse-phase HPLC. A Phenomenex Synergi-Hydro C18 column was used with the following gradient: (A = 0.1% TFA in water, B = 0.1% TFA in Acetonitrile) 0–15% B in 30 min, 15–95% B in 15 min, 95% B for 5 min, and 95–0% B in 10 min at a flow rate of 6 ml/min and monitoring of the eluent at 220 nm. The siderophore containing fractions were further purified on a Phenomenex Luna C18 column with the following gradient: 0–3% B in 5 min, 3–12% B in 5 min, 12–15% B in 5 min, 15% B for 2 min, and 15–0% B in 5 min at a flow rate of 5 ml/min. The appropriate fractions were then pooled and lyophilized. The identity of isolated siderophores was confirmed by high resolution MS, ^{1}H, ^{13}C, gCOSY TOCSY, HMQC, HMBC, DEPT, NOESY NMR. All 1 and 2-D NMR experiments were carried out on a Varian 500 MHz instrument using standard pulse sequences available on the instrument while routine ESI-MS and MS^{n} spectra were obtained on a Finnigan LCQ ion-trap mass spectrometer equipped with an ESI source (Finnigan MAT, San Jose, CA). MS/MS spectra were obtained utilizing a collision voltage between 20 and 50 V and argon as the collision gas. Isotope distribution patterns were simulated using the program IsoPro 3.0. High-resolution mass spectra were obtained on a Thermo Finnegan MAT900XL instrument located in the UCSD mass spectroscopy facility.

Siderophore growth bioassay

Bacteria were grown on separate agar plates with supplemented seawater that was rendered iron-deficient by the addition of 150 μM EDDHA and 150 μM 2,2′-bipyridyl to the agar broth prior to autoclaving. 200 μl aliquots of bacterial solution broth cultures were added to the plates prior to agar solidification. Sterile filter disks were then impregnated with 20 μl of 250 μM solutions of VF, water (as negative control) and iron citrate (as positive control) and various known siderophores (e.g., aerobactin, petrobactin and desferrioxamine E) and air dried. Filter disks were then placed on the agar plates, which were then incubated at 28°C overnight. A positive response (siderophore utilization) was indicated by a halo of growth around the disk.

Marinobacter phylogeny

Genomic DNA was extracted and the 16S rRNA gene amplified by PCR according to Green et al. 2004. Phylogenetic affiliation was established following automatic alignment by the NAST aligner (http://greengenes.lbl.gov) and importation of the aligned sequences into ARB software using the ARB parsimony tool (Ludwig et al. 2004). The alignment was refined and ambiguous positions were masked from the analysis. Phylogenetic inference of the masked alignment was based on maximum likelihood (PHYML) using the GTR model of nucleotide substitutions, as implemented in ARB. Gammaproteobacteria of the genus *Salicola* were used as the out-group.

Determination of the presence of vibrioferrin biosynthetic genes

Degenerate PCR primers were designed to amplify a region between the N-terminus of PvsA (PvsAf1: GARTGYGAYGTNTTYAAYCC) and C-terminus of the PvsB (PvsBr1: CCRTARAAYTTRTTDATRTC), two of the enzymes involved in vibrioferrin biosynthesis. PCR amplification used standard PCR buffer conditions with 1 μM of each primer and 2 mM Mg^{2+}. Cycling conditions used an initial denaturation step of 94°C for 5 min, followed by 10 cycle step-down annealing profile starting at 58°C, extension at 72°C for 3 min and denaturation at 94°C for 10 s, then a further 30 cycles of annealing at 48°C (30 s), 72°C for 3 min and 94°C for 10 s, and a final 72°C for 10 min extension. The expected PCR product was ca. 3 kbp.

Genomic analysis

Genes involved in ferrous and ferric iron uptake were taken from the Swissprot and RefSeq database to search the genomes of *M. aquaeolei* VT8 (NC_008740), *Marinobacter* sp. ELB17 (NZ_AAXY00000000), and

M. adhaerens HP15 (CP001978) to identify siderophore-independent uptake systems. Hidden Markov models (HMMs) of siderophore mediated iron uptake proteins were built by using a training set of functionally characterized proteins, which were aligned by ClustalW, to identify TonB-dependent outer membrane receptors and ABC-type transporters involved in siderophore mediated iron acquisition. Manual annotation for final assignments was based on maximum BLAST e-values and the description of the top hits in the general protein database. Cluster of orthologous groups (COG) assignments were performed as needed (National Center for Biotechnology Information GenBank and BLAST). The protein sequences of *M. algicola* DG893 were used as a query in BLAST searches using the BLASTP algorithm 2.2.16 with a cutoff e-value of 1 e-5 or an amino acid similarity of >30% to compare identified genes within the four genomes. Fur binding sites were identified using HMMs profiles built with *M. algicola* DG893 sequences and using regular expression pattern search with RegExr (0.3.1b by gskinner.com).

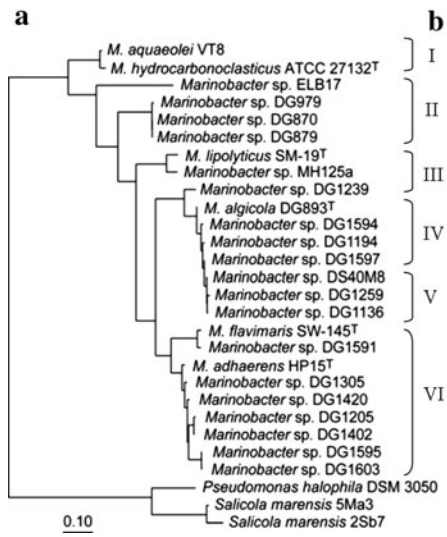
Results

Siderophore production

Production of siderophores was determined by a combination of bioinformatics/molecular biology and isolation and chemical characterization. In this way we have identified at least four distinct groups of siderophores produced by members of the *Marinobacter* genus. These include the petrobactins, marinobactins, vibrioferrin and desferrioxamine E (along with desferrioxamine G, its linear hydrolysis product). Three of these are mixed ligand siderophores, which utilize catechols (petrobactins), hydroxamates (marinobactins) and carboxylates (vibrioferrin) around a citrate-based core. The presence of the citrate-based core renders all of these siderophores photolabile. The last group of siderophores isolated from the *Marinobacter* genus (desferrioxamine E and G) are hydroxamate-containing siderophores with no citrate moiety. Although they were isolated originally from *Streptomyces* spp., we now know that desferrioxamines are produced by at least one marine bacterium in culture (Martinez et al. 2001) and they have recently been reported to be widespread in the Atlantic Ocean (Mawji et al. 2011). So far, desferrioxamines are the only group of siderophores produced by marine bacteria that are not photolabile.

A remarkable correlation emerges between siderophore production by several clades of *Marinobacter* spp. and their phylogeny based on 16S rRNA sequences (Fig. 2). The petrobactins, which are a small family of siderophores that include the parent molecule and its sulfonated derivatives (Fig. 3a), are produced by the *Marinobacter* spp. that constitute group I in Fig. 2. *Marinobacter* sp. ELB17, isolated from an Antarctic lake, and *Marinobacter* spp. DG870, DG879 and DG979, isolated from dinoflagellate cultures, produce the siderophore vibrioferrin (Fig. 3b) and constitute group II. This siderophore is also produced by *Marinobacter* spp. lying in group IV, which are again isolated from dinoflagellate and coccolithophore cultures. The ferric vibrioferrin complex has been shown to be more photolabile than previously examined photolabile siderophores and this characteristic along with its commonality among algal-associated *Marinobacter* spp. suggest a mutualistic interaction between these bacteria and some phytoplankton species. Interestingly, species in group III (*M. lipolyticus* SM-19, *Marinobacter* spp. DG1239 and MH125a) are the only members of the *Marinobacter* genus that do not produce a photolabile siderophore, instead they all produce desferrioxamines (Fig. 4c, d). While all of the aforementioned siderophores appear to be hydrophilic, the marinobactins consist of a suite of siderophores with fatty acid tails of varying lengths rendering them more or less hydrophobic (Fig. 3e) (Martin and Butler 2007). We isolated only a single class of marinobactin from late stationary phase cultures of DS40M8 and DG1136 (Fig. 2, group V) and found it to consist of the head-group common to this class of siderophore sans any fatty acid tail. Such cleavage of the fatty acid tails in late growth phase cultures of the marinobactins produced by DS40M8 via enzymatic deacylation has previously been reported (Butler, personal communication).

While we have identified the siderophores produced by almost all of the *Marinobacter* clades, those produced by one large group remains undetermined (Fig. 2, group VI). While *Marinobacter* spp. belonging to this group display weak, "off color" halos when growing on CAS plates possibly indicative of siderophore production, we have been unable to



b

Strain Origin	Siderophore Production	Siderophore Utilization VF	PB	AB	Cit	FC
SW	$PB/PBSO_3$		+	-	-	+
SW	PB	-	+	+	+	
SW	VF	+	-	-	-	-
Dn	VF	+	-		+	
Dn	VF	+	-	+	-	
Dn	VF	+	+		+	
SW	DFE	-	+		+	
Dt	DFE	+	+	+	+	
Dn	DFE	+	-			
Dn	VF	+	-	+	+	+
Dn	VF	+				
Co	VF	+	-	-	-	
Co	VF	+	-	-	-	
SW	MB					
Dn	MB	+	+/-			
Dn	MB	+	+/-			
SW	?	-	-	-	+	
Co	?	-	-	+	-	
Dt	?	-	-	+	-	+
Dn	?	-	-	+	-	
Dn	?	-	-	+	-	
Co	?	-	-	+	+/-	
Co	?	-	-	+	+	
Co	?	-	-	+	-	
Dn	?	-	-	-	+	

Fig. 2 16S rRNA gene phylogeny of the *Marinobacter* clade and siderophore production and utilization profile. **a** Maximum likelihood neighbor-joining tree of *Marinobacter* 16S rRNA genes. **b** Siderophore production and utilization by each strain of *Marinobacter* listed, as determined by LC-MS, NMR, and PCR screening of VF biosynthetic genes and siderophore growth promotion assays. Seawater (*SW*), dinoflagellate (*Dn*), coccolithophore (*Co*), diatoms (*Dt*), vibrioferrin (*VF*), petrobactin (*PB*), desferrioxamine E (*DFE*), marinobactin (*MB*), unknown class or no siderophore (?), aerobactin (*AB*), ferric citrate (*Cit*) and ferrichrome (*FC*). *Bar* denotes nucleotide substitutions per site

isolate any such siderophores from liquid-grown culture, despite changes to the carbon source (CAS amino acids, glucose or succinate) or iron concentration in the media. The small diameter of the halos suggested that perhaps these organisms where producing membrane bound rather than soluble siderophores but extraction with solvents previously shown to reveal the presence of such siderophores (Martinez et al. 2003) was also negative. Since it was previously reported that siderophores produced by one species of marine bacteria can induce production of siderophores from a previously nonproducing and unrelated bacterium (Guan et al. 2001) or promote the growth of previously uncultivable bacteria (D'Onofrio et al. 2010) it is possible that that siderophore production by this clade may depend on the presence of other organisms. Alternatively, because the CAS dye also undergoes color changes at low pH that mirror those caused by iron binding, it is possible that these bacteria do not produce siderophores at all but rather simply acidify the surrounding media as reported for some other organisms (Winkelmann, personal communication). However work continues to try and find conditions that would allow for the isolation and characterization of any putative siderophore(s) produced by this group.

Siderophore utilization

Like many terrestrial bacteria, members of the *Marinobacter* genus can utilize exogenous siderophores in addition to those that they produce. Here we have used both bioinformatics (vide infra) PCR and growth promotion assays (Fig. 4) to identify some of their capabilities to utilize these exogenous siderophores. These siderophores included vibrioferrin, petrobactins, aerobactin, citrate and ferrichrome. Unlike production, siderophore utilization did not seem to follow the same pattern *vis-a-vis* 16S rRNA phylogeny with one exception. Group VI isolates generally all share the ability to assimilate iron from aerobactin but not from other siderophores tested. Although citrate is usually considered a "bioavailable" source of iron, our growth bioassay suggests otherwise since nearly half of the tested isolates were not able to grow on Fe-citrate.

Genomics

We have previously reported a detailed analysis of the iron uptake systems in the genome of *Marinobacter algicola* DG893 (Amin et al. 2011). Here we compare the *M. algicola* DG893 results with data

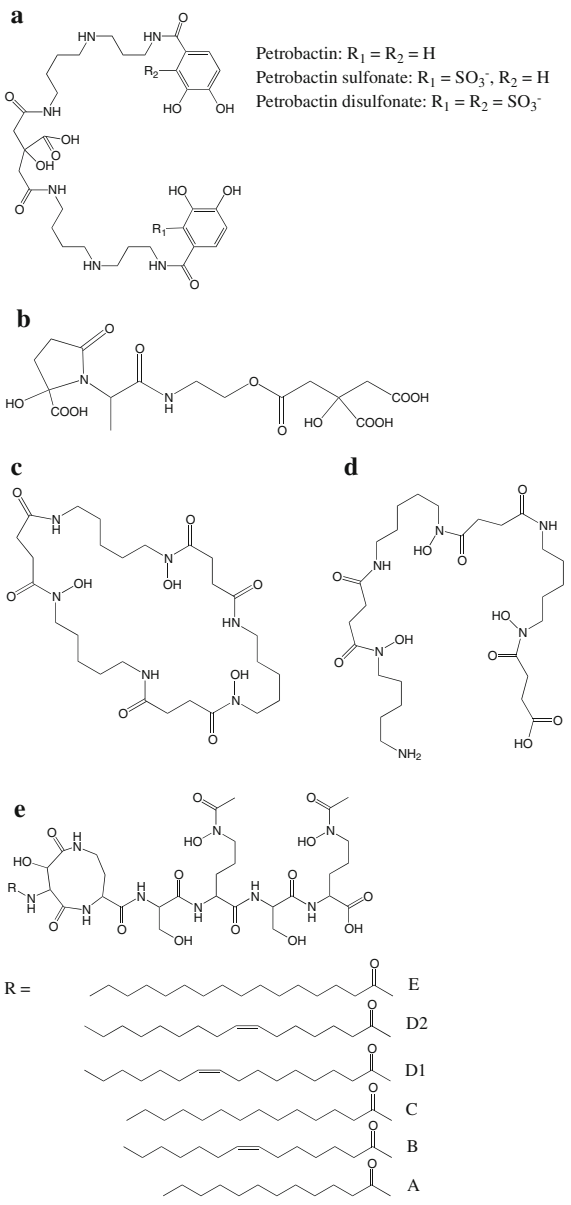


Fig. 3 Structures of the siderophores produced by the *Marinobacter* genus. **a** The petrobactins, **b** vibrioferrin, **c** desferrioxamine E, **d** desferrioxamine G, **e** the marinobactins

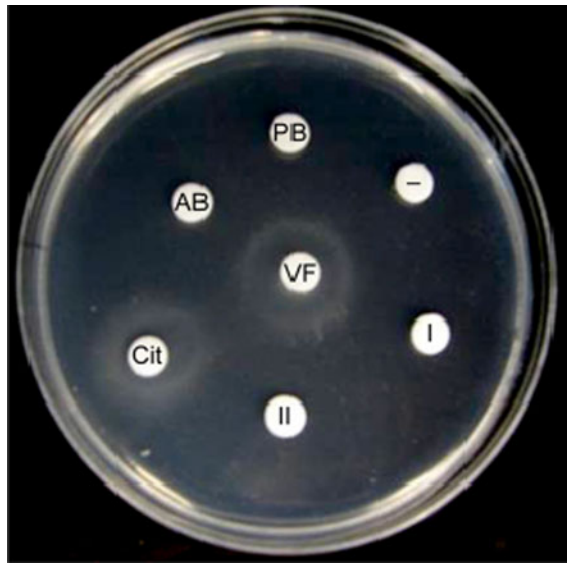


Fig. 4 Growth promotion assay for *Marinobacter* sp. DG870. *AB* aerobactin, *PB* petrobactin, *cit* Fe-citrate, *VF* vibrioferrin. [siderophore] = 40 μM, [Fe-citrate] = 150 μM. Bacteria and siderophores were inoculated as described in the "Materials and methods" section

mined from the publically available genomes of three other *Marinobacter* species. As expected the genomes of *M. aquaeolei* VT8 and *Marinobacter sp.* ELB17 harbor several genes that are candidates for involvement in siderophore-mediated iron acquisition. The indigenous siderophore produced by *M. algicola* DG893 has been identified as vibrioferrin (Amin et al. 2007). The vibrioferrin biosynthesis operon (*pvsXABCDE*), its TonB-dependent outer membrane receptor (*pvuA*) and ABC-type uptake system *(pvuBCD*) could also be identified in *Marinobacter sp.* ELB17 and vibrioferrin production and utilization have been confirmed experimentally. Thus vibrioferrin appears to be the indigenous and only siderophore produced by ELB17. We and others (Homann et al. 2009) find experimentally that the only siderophore produced by *M. aquaeolei* VT8 is petrobactin (and its sulfonated derivatives). This mixed catecholate-citrate siderophore is also produced by the type strain, *M. hydrocarbonoclasticus* (Barbeau et al. 2002), and terrestrial bacteria of the genus *Bacillus*, notably *B. anthracis* (Wilson et al. 2006). The biosynthetic pathway for petrobactins has been reported (Lee et al. 2007) and we find genes homologous to *asbA*, *asbB*, *asbC*, *asbD*, *asbE* and *asbF* comprising an iron dependent operon structure with an upstream fur box in *M. aquaeolei* VT8. Unfortunately we have not been able to isolate any native siderophore from *M. adhaerens* HP15 nor have we identified any likely candidates from our analysis of its genome.

Besides using their own siderophore for iron acquisition, members of the genus *Marinobacter* can also use exogenous siderophores produced by

other species as mentioned above. Thus, a hydroxamate siderophore uptake system has been identified in *M. algicola* DG893, *M. adhaerens* HP15 and *M. aquaeolei* VT8, corresponding to FhuABCD, the *E. coli* ferrichrome siderophore uptake system (Köster 2001). This system appears to be absent from ELB17. Additionally, two other TonB dependent outer membrane receptors, LutA (aerobactin) and FoxA (ferrioxamine), were also identified and could allow the uptake of additional hydroxamate-containing siderophores. Iron uptake through the ferric citrate system, FecABCD, of the *E. coli* ABC transport system which includes periplasmic binding protein, inner membrane permease, ATPase component which powers substrate transport and the TonB dependent outer membrane receptor protein were identified only in *M. algicola* DG893. Catechol type siderophore uptake systems related to *E. coli* FepABCDEG genes could not be identified in any of the *Marinobacter* genomes.

Siderophore-independent iron uptake systems were also identified in all the *Marinobacter* genomes (Table 1). These iron metal type ABC transporters included the FbpABC system, classified as a periplasmic ferric iron type ABC transporter (Köster 2001). Significantly, none of the *Marinobacter* genomes carry genes for the direct transport of ferrous iron, such as FeoAB from *V. cholera* and *E. coli* (Kammler et al. 1993, Wyckoff et al. 2006).

It has been known for many years that siderophores and other iron uptake systems are repressed at high levels of iron. This control is typically mediated via the global iron-response transcriptional regulator Fur (ferric uptake regulator). In the presence of high quantities of iron Fur binds Fe^{2+} and the resulting Fe-fur complex then recognizes and specifically binds to a 19 bp DNA sequence known as the Fur box (Escolar et al. 1999) and blocks transcription of the downstream gene(s). To characterize genes regulated by iron and/or Fur, a genome-wide search for conserved Fur boxes was carried out, with emphasis on the mentioned iron acquisition and transport systems. A Fur recognition weight matrix was derived from a pool of recognized Fur-binding sites of several bacteria. This matrix was used to locate potential Fur-binding sites by computing the information content of each 19 bp sequence of a sliding window passed over the complete genome. The potential Fur sequences were used to construct a HMM profile for further screening of a set of more stringent search criteria in order to locate potential species-specific Fur binding sites and to reduce the rate of false positives. Using this strategy, several putative Fur boxes were identified and indicate that the VF (Pvs-Pvu), petrobactin (AsbABCDF) and citrate (FecABCD) iron uptake systems are regulated by Fur, but that the FhuABCD transporter is dependent on the σ factor FecI (Enz et al. 1995). Interestingly, no recognizable Fur box could be identified for the ferric iron uptake system FbpABC, although Fbp transporters have been previously reported to be regulated by Fur (Desai et al. 1996, Mey et al. 2005).

Discussion

Through the last decade a relatively comprehensive catalogue of the microbial diversity of coastal and open-ocean regions has been achieved (Giovannoni and Rappé 2000). Yet, how this biodiversity and its interactions therein, structure the marine ecosystem is now a question of prime importance (Azam and Worden 2004). The consequences of interactions between heterotrophic bacterial activity and phytoplankton within the backdrop of global climate change may have profound and unforeseen effects on global primary productivity and economics as well as ecosystem modification. Bell and Mitchell first introduced the concept of the "phycosphere" to describe the zone around phytoplankton, under which influence, microbial activity is altered as compared to that of the surrounding seawater (Bell and Mitchell 1972). Phytoplankton enrich this zone through active excretion of photosynthates (photosynthetically fixed carbon compounds) which in turn attract and maintain a specific microflora (Bell et al. 1974). There is a growing body of evidence to suggest that there are direct interactions at the microscale between phytoplankton and heterotrophic bacteria, and that these interactions are likely to be quantitatively significant in terms of bacterial coupling to primary production (Azam and Malfatti 2007). The relationship between phytoplankton and bacteria in the phycosphere is likely to be complex and variable over short timeframes. Clustered bacteria could benefit the phytoplankton cell by employing hydrolytic enzymes to degrade dissolved organic matter (DOM), thus

Table 1 Identification and comparison of genes associated with iron acquisition and siderophore mediated iron acquisition in the genus *Marinobacter* (identities refer to DG893)

Gene	Function	*Marinobacter algicola* DG893 Protein ID	*Marinobacter adhaerens* HP15			*Marinobacter sp.* ELB17			*Marinobacter aquaeolei* VT8		
			Protein ID	Identities (%)	e-Value	Protein ID	Identities (%)	e-Value	Protein ID	Identities (%)	e-Value
Iron acquisition											
fbpA	iron(III) ABC transporter, periplasmic iron-compound-binding protein	ZP_01892821.1	ADP97220.1	99	0.0E+00	ZP_01739870.1	83	2.0E−148	YP_958371.1	84	0
fbpB	Iron(III) ABC transporter, permease protein	ZP_01892822.1	ADP97219.1	89	0.0E+00	ZP_01739869.1	84	0.0E+00	YP_958370.1	84	0
fbpC	Iron(III) ABC transporter ATP-binding subunit	ZP_01892642.1	ADP99826.1	45	1.0E−105	ZP_01736295.1	43	4.0E−83	YP_957633.1	84	1.00E−82
fbpu	Iron(III) ABC transporter, periplasmic iron-compound-binding protein	–	ADP99607.1	69*	2e−173*	–			–		
fbpv	Iron(III) ABC transporter, permease protein	–	ADP99606.1	66*	3e−165*	–			–		
fbpw	Iron(III) ABC transporter ATP-binding subunit	–	ADP99605.1	73*	0.0*	–			–		
feoABC	Iron(II) iron transport	–	–			–			–		
TonB-dependend siderophore outer membrane receptors											
fhuA	TonB-dependend ferrichrome siderophore receptor	ZP_01893080.1	ADP99437.1	53	2.0E−127	–			YP_959458.1	67	7.00E−150
fecA	TonB-dependend ferric citrate siderophore outer membrane receptor	ZP_01895081.1	–			–			–		
pvuA	TonB-dependend vibrioferrin siderophore outer membrane receptor	ZP_01892151.1	–			ZP_01739345.1	80	0.0E+00	–		
fatB	TonB-dependend petrobactin siderophore protein	–	–			–			YP_960789.1	24*	2E−44*
lutA	TonB-dependend ferric aerobactin siderophore receptor	ZP_01895310.1	ADP98706.1	37	6.0E−11	–			–		
foxA	TonB-dependend receptor Fe(III) ferrioxamine receptor	ZP_01893439.1	ADP97174.1	32	4.0E−99	–			–		
vciA	TonB-dependend iron(II) outer membrane receptor	–	–			–			–		
Siderophore mediated iron acquisition ABC transporters											
Hydroxamates											
fhuB	ABC-type Fe^{3+}-siderophore transport system permease component	ZP_01892432.1	ADP96601.1	82	0.0E+00	–			YP_959456.1	46	2.00E−285

Table 1 continued

Gene	Function	*Marinobacter algicola* DG893 Protein ID	*Marinobacter adhaerens* HP15			*Marinobacter sp.* ELB17			*Marinobacter aquaeolei* VT8		
			Protein ID	Identities (%)	e-Value	Protein ID	Identities (%)	e-Value	Protein ID	Identities (%)	e-Value
fhuC	ABC-type Fe^{3+}-siderophores ATPase component	ZP_01892431.1	ADP96603.1	85	1.0E−164	–			YP_959454.1	80	0
fhuD	ABC transport of Fe^{3+} siderophores periplasmic binding domain	ZP_01892430.1	ADP96602.1	78	2.0E−178	–			YP_959455.1	79	5.00E−154
fhux	ABC transport of Fe^{3+} siderophores transmembrane region	ZP_01892429.1	ADP96604.1	90	0.0E+00	–			YP_959453.1	90	0
exbD	ferric siderophore transport system, innermembrane protein E	ZP_01895590.1	ADP99652.1	58	1.0E−53	ZP_01740146.1	46	6.0E−43	YP_957479.1	47	3.00E−32
fepABCDG	ferric siderophore enterobactin uptake	–	–			–			–		
Hydroxycarboxylates											
fecB	ABC-type ferric citrate siderophore periplamic binding domain	ZP_01895078.1	–			–			–		
fecC	ABC-type ferric citrate siderophore permease component	ZP_01895079.1	–			–			–		
fecD	ABC-type ferric citrate siderophore permease component	ZP_01895080.1	–			–			–		
fecE	ABC-type ferric citrate siderophore ATPase component	ZP_01895077.1	–			–			–		
Siderophore mediated iron acquisition ABC transporters											
Citrate based Siderophores											
pvuB	ABC-type vibrioferrin transport system permease protein	ZP_01892150.1	–			ZP_01739344.1	24	5.0E−16	–		
pvuC	ABC-type vibrioferrin transport system permease protein	ZP_01892149.1	–			ZP_01739343.1	40	3.0E−44	–		
pvuD	ABC-type vibrioferrin transporter ATP-binding subunit	ZP_01892148.1	–			ZP_01739342.1	73	7.0E−152	–		

Table 1 continued

Gene	Function	*Marinobacter algicola* DG893 Protein ID	*Marinobacter adhaerens* HP15			*Marinobacter sp.* ELB17			*Marinobacter aquaeolei* VT8		
			Protein ID	Identities (%)	e-Value	Protein ID	Identities (%)	e-Value	Protein ID	Identities (%)	e-Value
pvuE	ABC-type vibrioferrin transporter ATP-binding protein	ZP_01892147.1	–			ZP_01739341.1	67	2.0E−118	–		
fpuA	ABC-type petrobactin transport system permease protein	–	–			–			YP_960788.1	48*	5.0E−16
fpuB	ABC-type petrobactin transport system permease protein	–	–			–			YP_960787.1	44*	2E−328
fbuC	ABC-type petrobactin transporter ATP-binding subunit	–	–			–			YP_960786.1	50*	9.0E−237
fbuD	ABC-type petrobactin transporter ATP-binding protein	–	–			–			YP_960785.1	44*	3.0E−49
Siderophores biosynthesis											
pvsX	2,4-dihydroxyhept-2-ene-1,7-dioic acid aldolase	ZP_01892157.1	–			ZP_01739400.1	74	9.0E−105	–		
pvsA	vibrioferrin biosynthesis protein Carbamoylphosphate synthase subunit	ZP_01892156.1	–			ZP_01739399.1	59	0.0E+00	–		
pvsB	vibrioferrin biosynthesis protein	ZP_01892155.1	–			ZP_01739398.1	59	0.0E+00	–		
pvsC	vibrioferrin permease component	ZP_01892154.1	–			ZP_01739397.1	56	1.0E−133	–		
pvsD	vibrioferrin biosynthesis protein	ZP_01892153.1	–			ZP_01739396.1	62	6.0E−160	–		
pvsE	vibrioferrin diaminopimelate decarboxylase	ZP_01892152.1	–			ZP_01739341.1	67	2.0E−118	–		
asbA	petrobactin biosynthesis	–	–			–			YP_960779.1	48*	6.0E−69
asbB	petrobactin biosynthesis	–	–			–			YP_960780.1	40*	5.0E−68
asbC	petrobactin biosynthesis, acyl-CoA synthetase	–	–			–			YP_960781.1	38*	1.0E−33
asbD	petrobactin biosynthesis, acyl carrier protein	–	–			–			YP_960782.1	44	2.0E−18
asbE	petrobactin biosynthesis, Ferrochelatase	–	–			–			YP_960783.1	44*	2.0E−170
asbF	petrobactin biosynthesis, 3-DHS dehydratase	–	–			–			YP_960784.1	49*	
Transcriptional regulators											
Fur	ferric uptake regulator (Fur)	ZP_01893541.1	ADP98849.1	94	1.0E−95	ZP_01735856.1	83	2.0E−84	YP_960623.1	95	1.0E−195
σ-FecI	sigma-24 (FecI-like) protein	ZP_01893081.1	ADP96714.1	60	6.0E−88	–			–		

*Identities referred to RefSeq

enhancing nutrient regeneration near the phytoplankton cell surface at the expense of dissolved organic nutrients. The inorganic nutrient "hot-spot" surrounding the phytoplankton cell could make its microenvironment eutrophic in what might otherwise be oligotrophic seawater, thus enhancing algal growth by increasing nutrient bioavailability. Thus, bacterial-algal interactions in the phycosphere are likely to be strongly influenced by the supply of available nutrients. While nitrogen and phosphorous have most often been considered in this context, a broad hypothesis that links these bacterial 'symbionts' to the growth of dinoflagellates and coccolithophores is in their possible control of the supply of iron. In a previous communication, we proposed the existence of a mutualism between certain algal-associated *Marinobacter* spp. and members of the dinoflagellate and coccolithophore algal lineages (Amin et al. 2009). This mutualism was proposed to be based on these bacteria providing an enhanced supply of Fe(III) to the algae, and in return, the bacteria benefit from the release of photosynthate supporting their growth.

While iron transport systems in *Marinobacter* have been briefly considered in the context of various genome studies (Singer et al. 2011; Amin et al. 2011), here we have combined a detailed genomic analysis with the experimental verification of siderophore production and utilization. Like other secondary metabolites, siderophore biosynthesis genes undergo extensive horizontal gene transfer that prevents our ability to assign specific siderophores to bacterial clades. However, siderophore production in the *Marinobacter* genus provide a type of chemotaxonomic marker which may provide clues into the ecological niches occupied by various members of this diverse group of bacteria. Thus most of the *Marinobacter* that we view as algal associated belong to two clades both of which are characterized by the production of the siderophore vibrioferrin (groups II and IV in Fig. 2). It is interesting to note that vibrioferrin possesses the weakest binding affinity to iron and its iron complex has the fastest photodegradation rate under seawater conditions among all the siderophores discussed here (Amin et al. 2009). These characteristics suggest a metabolic cost, mainly less efficient iron acquisition, for the producing species. However this may be compensated for by ready acquisition of dissolved organic carbon (DOC) obtained by clustering around algal cells which also may provide a means for avoiding competition with other bacterial "degraders." *Marinobacter* sp. ELB17 is an exception in group II in that it clusters phylogenetically at an intermediate position between the algal-associated *Marinobacter* spp. DG979, DG870 and DG879 and the oil-degrading specialist *M. aquaeolei* VT8 (Fig. 2). While it produces vibrioferrin it has not been reported to be algal associated. In addition it lacks alternative iron uptake capabilities using any exogenous siderophores we tested.

The three other siderophores (petrobactins, ferrioxamines E & G and the marinobactins) that we find produced by members of the genus represent small but unique clusters that are not obviously algal-associated. Since the ferrioxamine iron has generally been found not to be available to phytoplankton (Hutchins et al. 1999) it is unlikely that that group would be algal-associated at least from a mutualistic view. The lack of any photochemistry of the ferrioxamine family and their high affinity for iron also strengthen this point. While the petrobactins and marinobactins are both photoactive and are related to the aquachelins, which have been shown to be more effective at increasing the bioavailability of iron and facilitated its uptake in a natural assemblage of marine planktonic organisms after photolysis (Barbeau et al. 2001), it is unclear which groups of organisms (bacteria or phytoplankton) were benefiting. Given that these siderophores retain significant Fe(III)-binding capacity even after photolysis they seem unlikely to be able to provide iron to phytoplankton.

Marinobacter adhaerens HP15 (Gärdes et al. 2010) is a representative of the *Marinobacter* clade where we have been unable to detect siderophore production or find siderophore biosynthesis genes at all. Instead we find that while it is capable of using a limited set of exogenous siderophores, most notably aerobactin, it has two distinct non-siderophore ABC transport systems of the FbpABC iron metal type rather than the one seen in the other genomes. In addition we find that there are two bacterioferritins present in HP15 compared to the single one seen with DG893, ELB17 and VT8. Together these observations suggest that perhaps HP15 and the other members of this clade have a lower iron requirement due in part to more efficient iron uptake and storage systems. Thus it and other algal-associated strains of *Marinobacter* in this clade may not provide algae

with iron but rather with vitamins such as B_{12} for which most algae are auxotrophic (Tang et al. 2010). This remains an area of active research.

While there is a wide level of diversity in siderophore-based iron uptake systems among the *Marinobacter* genus (some produce both native siderophores and utilize a variety of exogenous ones, others produce and utilize native siderophores only, and still others that appear not to produce a native siderophore but are capable of using exogenous ones) they all share the presence of siderophore-independent iron uptake ABC transport systems of the FbpABC iron metal type and lack the ability for direct transport of ferrous iron. This suggests that the iron is primarily taken up as Fe(III) rather than as Fe(II) (as demonstrated experimentally for DG 870, (Amin et al. 2009). A better characterization of siderophores from this genus and the discovery of new isolates that come from a variety of sources are needed to fully understand iron acquisition in these organisms and in their associated phototrophs.

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