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Siderophore-mediated iron uptake in two clades of *Marinobacter* spp. associated with phytoplankton: the role of light

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Abstract Iron is an essential element for oceanic microbial life but its low bioavailability limits microorganisms in large areas of the oceans. To acquire this metal many marine bacteria produce organic chelates that bind and transport iron (siderophores). We have previously shown that algal-associated heterotrophic bacteria belonging to the γ-proteobacterial *Marinobacter* genus release the siderophore vibrioferrin (VF). The iron-VF complex was shown to be both far more photolabile than all previously examined photolabile siderophores and to generate a photoproduct incapable of re-chelating the released iron. Thus, the photogenerated iron was shown to be highly bioavailable both to the producing bacterium and its algal partner. In exchange, we proposed that algal cells produced dissolved organic matter that helped support bacterial growth and ultimately fueled the biosynthesis of VF through a light-dependent "carbon for iron mutualism". While our knowledge of the importance of light to phototrophs is vast, there are almost no studies that examine the effects of light on microbial heterotrophs. Here, we characterize iron uptake mechanisms in "algal-associated" VF-producers. Fe uptake by a VF knock-out mutant mimics the wild-type strain and demonstrates the versatility of iron uptake mechanisms in *Marinobacter* VF-producers. We also show that VF-producers selectively regulate a subset of their siderophore-dependent iron uptake genes in response to light exposure. The regulation of iron uptake and transport genes by light is consistent with the light driven algal–bacterial "carbon for iron mutualism" hypothesis in the marine environment.

Keywords Iron · Transport · *Marinobacter* · Light · Gene expression · Siderophores

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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 (Rue and Bruland 1995; Wu and Luther 1995; Gledhill and van den Berg 1994). Multiple iron fertilization experiments in high-nutrient-low-chlorophyll (HNLC) regions of the oceans have corroborated the importance of iron and its limitation to marine microorganisms (Coale et al. 1996). In response to this limitation, diverse marine bacterial species excrete small organic compounds, called siderophores, which bind iron with exceptional affinity and facilitate its transport into cells (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 (Rue and Bruland 1997; Boye et al. 2005; Vraspir and Butler 2009). One of the emerging structural features that seem to differentiate terrestrial from marine siderophores is the near universal presence in the latter of α-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 (Barbeau et al. 2001). Since the photo-generated 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 potentially continuing to restrict its bioavailability (Barbeau et al. 2001; Küpper et al. 2006; Abergel et al. 2008). 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 belong to the class of γ-proteobacteria and these motile, halophilic or halotolerent bacteria all share the ability to use petroleum hydrocarbons as sole energy and carbon sources. They are a ubiquitous species in the world's oceans, having been isolated from a wide variety of marine environments ranging from hydrothermal vents to Antarctic sea ice (Kaye et al. 2011; Glatz et al. 2006). They have also been identified as members of the bacterial flora associated with other marine organisms (Romanenko et al. 2005). Indeed we and others have observed that among the most notable members of the bacterial communities associated with marine phytoplankton including diatoms (Kaeppel et al. 2011), coccolithophores (Amin et al. 2009a) and dinoflagellates (Alavi et al. 2001; Seibold et al. 2001; Green et al. 2004) were bacteria from several *Marinobacter* clades.

While these algal-associated species were closely related to other *Marinobacter* species (e.g. *M. hydrocarbonoclasticus* or *Marinobacter* sp. DS40M8), most of the tested strains did not produce the types of siderophores commonly produced by free-living members of the genus (Barbeau et al. 2002; Martinez et al. 2000). Rather we have shown that only the extremely photolabile siderophore, vibrioferrin (VF), is produced by the two clades of *Marinobacter* that appear to be algal-associated. We have further shown that the photo-generated iron, Fe(III)' Eqs. 1, 2 was shown to be highly bioavailable both to the producing bacterium and its algal partner (Amin et al. 2009a).

$$\mathrm{Fe(III)} - \mathrm{VF} + h\nu \rightarrow \mathrm{Fe(II)}' + \mathrm{VF}^{*} + \mathrm{CO_2} \quad (1)$$

$$\mathrm{Fe(II)}' + \mathrm{O_2} \rightarrow \mathrm{Fe(III)}' \quad (2)$$

This led to the hypothesis that algal cells produced dissolved organic matter (DOM) that helped support bacterial growth and ultimately fuel the biosynthesis of VF through a light-dependent mutualism. Here, we assess in more detail the iron uptake systems and the underlying effects of light on iron speciation and gene expression in *Marinobacter* clades that produce VF. Understanding the effects of light on the *Marinobacter* clades of interest is essential to understanding their ecological relevance to the growth of algae in natural habitats and can serve as a promising step towards validating a "carbon-for-iron" based mutualism in bacterial–algal interactions in the marine environment.

Materials and methods

Genomic analysis

Hidden Markov models (HMMs) of iron uptake protein families were used to search the genome of *M. algicola* DG893 (NCBI accession no. NZ_ABCP01000000) to

identify siderophore-mediated iron acquisition systems. Genes involved in TonB-dependent outer membrane receptors and ABC-type transporters were manually annotated for final assignments 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 *Vibrio parahaemolyticus* (NCBI accession no. BAC16544.1) vibrioferrin-biosynthetic genes 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%. Sequences were aligned using ClustalW (default settings). Fur binding sites were identified using HMMs profiles built with *V. cholera* O395 and *E.coli* sequences and using regular expression pattern search with RegEx (0.3.1b by gskinner.com). The *M. algicola* DG893 Fur consensus sequence was built with WebLogo 3 (http://weblogo.berkeley.edu/logo.cgi) (Crooks et al. 2004).

Bacterial growth and siderophore isolation

All experiments were conducted using either *Marinobacter* sp. DG879 or *M. algicola* DG893 which are representative members of the two different VF producing algal-associated clades (Amin et al. 2011). Both species gave essentially identical results and were grown, and harvested as previously described (Amin et al. 2009a; Amin et al. 2007). Growth of DG893 wild-type and DG893 *pvsAB*$^{-}$ mutant was monitored by measuring the optical density of cultures at 600 nm. Cultures were grown in acid-washed 30-ml polycarbonate tubes in media previously passed through Chelex-100 resin as described below. Cultures were constantly shaken at 145 rpm at 20°C in the dark. No difference in growth rates was observed between cultures grown in the dark versus ambient light.

^{55}Fe uptake measurements

^{55}Fe-VF was prepared by adding a standard solution of $FeCl_3 \cdot 6H_2O$ (Aldrich; 1 mg/ml) and $^{55}FeCl_3$ (1522 MBq/ml; Perkin–Elmer) to VF (final Fe_T:ligand = 1:3; final $FeCl_3$:^{55}Fe = 1:0.1). The complex was allowed to equilibrate for at least 24 h in the dark prior to use. ^{55}Fe(III)-EDDHA (ethylenediamine-*N*,*N*′-bis(2-hydroxyphenylacetic acid)) and $^{55}FeCl_3$ were prepared in a similar way by using a concentrated stock of EDDHA or the $FeCl_3$ stock. Cells were grown in artificial seawater (ASW) containing per liter: 15 g NaCl, 0.75 g KCl, 12.4 g $MgSO_4 \cdot 7H_2O$, 3 g $CaCl_2 \cdot 2H_2O$, 1 g NH_4Cl, 0.1 g β-glycerophosphoric acid and 2 g Casamino acids with the final pH adjusted to 8.0. The ASW was passed through Chelex-100 resin (BioRad) to remove trace metals and supplemented with 100 nM $FeCl_3$. Cultures were harvested at mid-exponential growth phase by centrifugation at 6000 rpm for 20 min using Sorvall RC5C+ centrifuge followed by washing three times with iron-free ASW. The harvested cells were then diluted with fresh media to an optical density of 0.3 at 600 nm and were shaken at 130 rpm and 20°C for 30 min in the dark to acclimate to 20°C after which the experiment was started by adding ^{55}Fe-VF, ^{55}Fe-EDDHA, or $FeCl_3$ to a final concentration of 1 μM. Triplicate cultures for each experiment (Fe-VF dark, FeVF light, Fe-EDDHA and $FeCl_3$) were incubated in a rotary shaker (130 rpm) at 20°C throughout the experiment. All cultures were kept in the dark with the exception of the Fe-VF light cultures, which were exposed throughout the experiment to a mercury vapor lamp (350 μmol photons m^{-2} s^{-1}). Time-course uptake experiments were performed in a similar manner with 5-ml aliquots taken out throughout the experiment. Concentration dependent ^{55}Fe-VF uptake rates were measured by growing the cells as described above and adding ^{55}Fe-VF to final concentrations of 1, 2, 10 and 50 μM. Cultures for all experiments (except the time course) were then incubated for 1 h and filtered, using a Millipore 1225 sampling vacuum manifold, onto 0.6 μm-pore size polycarbonate (PC) filters (Millipore). Filtered cells were washed with 5 ml ASW followed by 5 ml Ti(III)–citrate–EDTA reagent and a final 5 ml rinse with ASW to reductively remove iron oxides and iron nonspecifically bound to cell surfaces (Hudson and Morel 1989). Uptake of ^{55}Fe was measured using a Beckman–Coulter LS6500 liquid scintillation counter using the tritium channel.

Preparation of VF biosynthesis knockout mutants

Briefly, *pvsAB* PCR amplicons were modified by restriction endonuclease digestion and re-ligation or overlap PCR to delete regions of one or both

biosynthetic genes and this truncated product cloned into the suicide vector pDM4 (Milton et al. 1996). The vector pDM4-*pvsAB1* was then mobilized into DG893 by conjugal transfer from an *Escherichia coli* S17-1 λpir donor. Chromosomal integration of pDM4-*pvsAB1* (single crossover) was selected for by growth of transconjugants on marine agar with 30 μg/ml chloramphenicol (CamR). PCR was used to screen for the truncated copy of *pvsAB*. Allelic exchange was then accomplished by growing DG893*pvsAB1*::CamR on marine agar containing 5% sucrose, where expression of *sacB* is the counter-selection marker for allelic exchange. Sucrose-resistant survivors were confirmed for chloramphenicol sensitivity (CamS). To confirm loss of VF production, CamS colonies were patched on CAS agar plates and colonies that showed no orange halo were deemed VF mutants. CAS liquid assay was used to confirm the siderophore negative phenotype based on assaying cells and cell-free supernatants of putative *pvsAB*$^{-}$ mutants. PCR and DNA sequencing were used to confirm the nature of the mutation.

CAS assay

Cultures of DG893 wild-type and DG893 *pvsAB*$^{-}$ were grown in the dark as described above. Aliquots of each strain were withdrawn immediately before cultures entered stationary phase and were centrifuged at 13,000 rpm for 5 min. The supernatant from each strain was combined with the CAS shuttle solution in a 1:1 volume ratio and allowed to equilibrate in the dark for 10 min (Schwyn and Neilands 1987).

Gene expression

Cells were inoculated into ASW as described above, supplemented with either 50 nM (Fe-limited) or 10 μM (Fe-sufficient) $FeCl_3$ and grown in the dark or exposed to a fluorescent lamp (30 μmol photons m^{-2} s^{-1} measured output) with constant shaking at 150 rpm and 28°C. To ensure acclimation of the cultures to their respective conditions, we used semi-continuous batch culturing techniques to maintain logarithmic growth by passing a small inoculum to fresh media under light or dark conditions every 2 days for at least 3 passes. Cells were harvested at mid-exponential growth phase (ca. 2–3 days) at an optical density of ca. 0.3 at 600 nm. Cells were lysed enzymatically using lysozyme (Fisher) according to RNAprotect® Bacteria Reagent kit protocols (Qiagen). RNA was isolated using RNeasy® Mini kit according to manufacturer's instructions (Qiagen). Genomic DNA co-eluted with RNA was digested using RNase-free DNase (Qiagen). Nucleic acid concentrations were followed throughout the process spectrophotometrically using a Cary 50 UV–Vis spectrophotometer. Subsequently, cDNA synthesis was performed on 2 μg RNA using iScript cDNA synthesis kit (Bio-Rad), which contains oligo(dT) and random hexamer primers. Ten-fold dilutions of the cDNA stocks were prepared to afford a working stock for real-time quantitative PCR.

Primers were designed using either VectorNTI software package (Invitrogen) or Primer-BLAST on NCBI (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). Primers were BLAST searched against the *M. algicola* DG893 genome to check for possible non-specific targets, and amplicons were further checked with standard PCR. The complete list of primers used in this study is provided in Table 1. Primers specific for *gyrA* and *rpoD* were used as housekeeping genes and neither exhibited any variations under conditions used in this article. Real-time PCR (RT-PCR) analyses were performed on three different biological experiments each with four growth conditions, Fe-limited (50 nM) and Fe-replete (10 μM) in the dark and in the light, to quantify relative abundance of iron uptake genes using the iQ SyberGreen Supermix kit (Bio-Rad) and relevant primers (0.2 μM). Reported data represent statistical analyses from all three independent experiments. Temperature profiles for RT-PCR consisted of an initial incubation at 50°C for 10 min, 95°C for 5 min, followed by 45 cycles of 95°C for 10 s and 60°C for 1 min. Six dilutions of DG893 genomic DNA were used to construct a standard curve for sample quantification. Experiments were carried out using iQ5 cycler (Bio-Rad) with efficiencies of the PCR reaction ranging from 92 to 104%.

Results

Genomics

The genome of *Marinobacter algicola* DG893 harbors several genes that are candidates for involvement in siderophore-mediated iron acquisition. The indigenous siderophore produced by *M. algicola* has been

Table 1 List of primers used in qPCR to quantify gene transcripts of important iron uptake genes and in constructing the VF mutant (DG893 *pvsAB*$^{-}$)

Target gene	Primer sequences	Putative function	Accession no.
pvsAB	GARTGYGAYGTITTYAAY	Vibrioferrin biosynthesis	ZP_01892156
	CCRTARAAYTTRTTDATRTC		ZP_01892155
pvsA	TGTGATCAGTTCTGGCAAGG	Vibrioferrin biosynthesis gene	ZP_01892156
	CCAATGGTCTCCAGAGTGTG		
fbpA	TCGCTCACCATGGCGAAGAAGC	Periplasmic iron binding protein	ZP_01892821
	AATGTCGCACTCGCCGGCAT		
pvuA	GGTTCTGGCCGAAGAAGAAGG	VF outer membrane receptor	ZP_01892151
	ACGAAGCGGACATGAGTCG		
fhuA	TGGACCGTCGTGCCATTGCC	Ferric hydroxamate uptake protein	ZP_01893439
	CGGGCTTTCTGCAAGGCGGA		
foxA	CTGAACCTACCGCCCTGTATCC	TonB-dependent outer membrane siderophore receptor	ZP_01893080
	CGATTGGTGTTCTCGATCTCC		
gyrA	GTGCATCGCCGCGTACTGTT	DNA gyrase subunit A	ZP_01895129
	ATAACGCATGGCAGCCGCGT		
rpoD	GGACAGGTGTTTGCGCCGTT	RNA polymerase	ZP_01893908
	TGCGCGGCATCTTGCACTCA		

identified as vibrioferrin (Amin et al. 2007). The vibrioferrin biosynthesis operon (*pvsABCDE*), its TonB-dependent outer membrane receptor (*pvuA*) and ABC-type uptake system *(pvuBCD*) were originally identified in *Vibrio parahaemolyticus* (Tanabe et al. 2003). Homologous genes in *M. algicola* DG893 were identified, with a putative aldolase gene (denoted *pvsX*) positioned immediately upstream of the VF biosynthetic gene *pvsA* (Fig. 1). PvsX shows no homologies with any protein of *V. parahaemolyticus*, but shares 47% identity with the *Pseudomonas syringae* achromobactin biosynthetic gene *acsB* (YP_235665.1) (Berti and Thomas 2009). Since both vibrioferrin and achromobactin are citrate-containing siderophores, they likely share homologies in their biosynthetic pathway, and we propose *pvsX* is likely to form a part of the VF biosynthetic machinery. This is supported by the fact that *pvsX* is likely the first gene of the DG893 VF operon as a putative Fur box has been identified immediately upstream. Besides using its own siderophore for iron acquisition, *M. algicola* DG893 can also use exogenous siderophores produced by other species and has non-siderophore uptake systems as well. These are described in a companion publication (Amin et al. 2011).

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). To characterize genes regulated by iron and/or Fur genes, 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 of *M. algicola* DG893. Those 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. Putative *M. algicola* Fur binding sites were aligned to identify the conserved core region encompassing three repeats of 6 bp NATAAT, with a second repeat in the opposite orientation which forms a 19 bp palindrome (Fig. 2). This indicates that VF (Pvs-Pvu) and FecABCD iron uptake systems are regulated by Fur, but that the

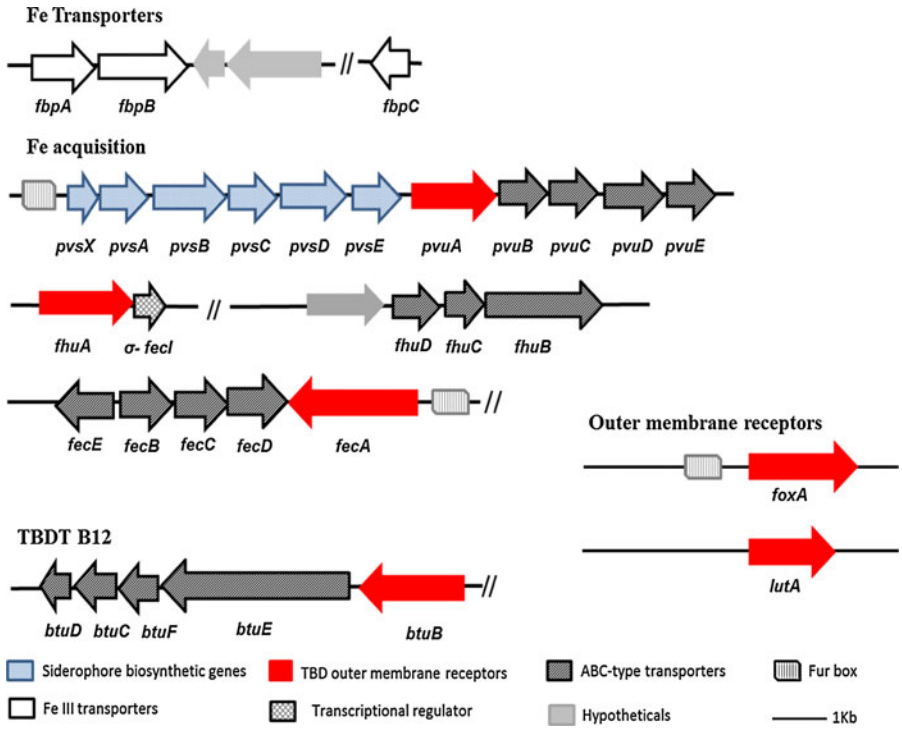

Fig. 1 Iron uptake and siderophore mediated uptake systems of *M. algicola* DG893, grouped into iron transporters, iron acquisition and outer membrane receptors. The TonB-dependent transporter (TBDT) for vitamin B_{12} is grouped with those for siderophores, as they share a similar domain organization and utilize an analogous mechanism of ligand transport. *Arrows* representing each gene indicate direction of transcription. The *double-hashed line* separating independent gene clusters indicates that they are not contiguous in the genome

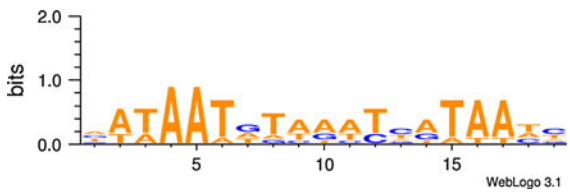

Fig. 2 The *M. algicola* Fur box consensus sequence. Predicted Fur boxes within the promoter regions of known iron acquisition genes in *M. algicola* were used as a training set to derive a consensus sequence and WebLogo 3.1 was used to build a consensus sequence logo, in which the *height of the individual letters* represents the relative frequency of that *letter* at the given position, and the overall *height of the stack* represents the degree of conservation at that position

FhuABCD transporter is dependent on the σ factor FecI (Enz et al. 1995).

Fe uptake: light versus dark

In the dark vibrioferrin mediated uptake of ^{55}Fe in *Marinobacter* sp. DG879 was linear as a function of time for at least 120 min. This was inferred to be an active transport process as determined by its inhibition by carbonyl cyanide 3-chlorophenyhydrazone (CCCP), a decoupler of oxidative phosphorylation. Consistent with our previous results using natural attenuated sunlight, artificial light provided by the mercury vapor lamp (350 µmol photons m^{-2} s^{-1}) enhanced iron uptake rates from ^{55}Fe-VF in DG879 by >30% over that in the dark (Fig. 3a) indicating that the inorganic soluble iron species (referred to hereafter as Fe(III)') formed by photolysis was more bioavailable to the bacteria than iron bound to VF itself. Similar results were seen with *M. algicola* DG893, a representative of another clade of VF producing algal-associated *Marinobacter* (Fig. 4). Rates of uptake from $^{55}FeCl_3$ were five-fold higher than uptake rates from Fe-VF in the dark ($P < 0.002$), demonstrating the high bioavailability of photochemically derived inorganic iron relative to organically complexed forms of iron. That the uptake rate of Fe-VF in the light was between that of Fe-VF in the dark and that of inorganic Fe(III) (i.e. $FeCl_3$) is consistent with photolysis of a significant fraction of the Fe-VF to produce Fe(III)′ during the course of the uptake experiment.

Because it is likely that DG893 continues to produce free VF during the course of the uptake experiment, we prepared a knockout mutant by allelic exchange (Milton et al. 1996) that was unable to produce PvsA and PvsB, and thus lacked the ability to produce VF (DG893 *pvsAB*$^-$) (Fig. 5a). The inability of DG893 *pvsAB*$^-$ to produce VF was phenotypically

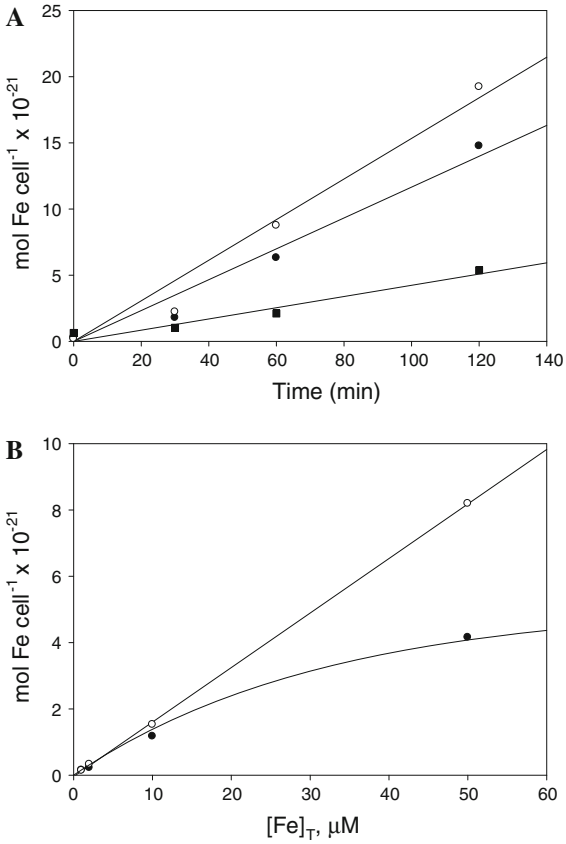

Fig. 3 VF mediated iron uptake in *Marinobacter* sp. DG879 in the light and dark. **a** Iron uptake from photolyzed and intact ^{55}Fe-VF. ^{55}Fe uptake by *Marinobacter* sp. DG879 was performed at 20°C using 1 μM Fe(III) and 3 μM total VF either kept in the dark (*closed circles*) or photolyzed previously (*open circles*) using a mercury vapor lamp (350 μmol photons m^{-2} s^{-1}). The decoupler of oxidative phosphorylation (*closed squares*), CCCP (carbonyl cyanide 3-chlorophenyhydrazone) was used as a metabolic inhibitor; incubations were carried out in the dark. **b** Concentration-dependent VF-mediated iron uptake rates. ^{55}Fe uptake was performed at 20°C using varying concentrations of Fe(III)-VF. Cultures were kept in the dark and either contained intact ^{55}Fe-VF (*closed symbols*) or previously photolyzed ^{55}Fe-VF (*open symbols*) using a mercury vapor lamp

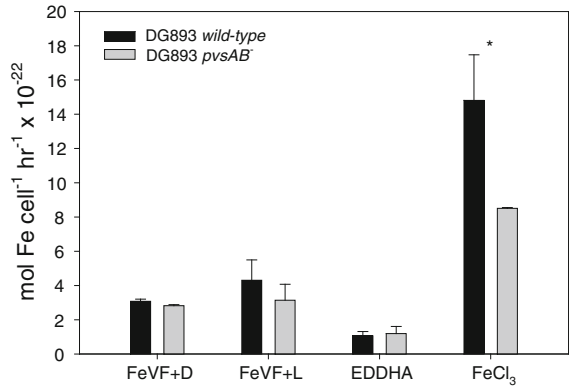

Fig. 4 ^{55}Fe uptake in *M. algicola* DG893 and its VF knockout mutant from various iron sources. ^{55}Fe uptake was performed at 20°C using 1 μM Fe(III) and 3 μM VF incubated either in the dark or exposed to a mercury vapor lamp (350 μmol photons m^{-2} s^{-1}), 3 μM total EDDHA, or no ligand for $FeCl_3$. EDDHA and $FeCl_3$ experiments were kept in the dark. *Error bars* represent s.d. from biological triplicates. $P < 0.01$ is denoted by * above the columns

confirmed by the negative response of the mutant's cells and supernatant to the CAS assay when compared to DG893 wild-type (Fig. 5b) (Schwyn and Neilands 1987). However growth curves indicated that DG893 *pvsAB*$^-$ grew as well as the wild type under both iron deficient and iron replete conditions (Fig. 5c). Iron uptake rates by DG893 *pvsAB*$^-$ from ^{55}Fe-VF in the dark, exposed to a mercury vapor lamp or from ^{55}Fe-EDDHA, were both similar to that of the wild-type (Fig. 4). Interestingly, the uptake rates of $FeCl_3$ were significantly less in DG893 *pvsAB*$^-$ relative to the wild-type ($P < 0.01$). The increased rates of $^{55}FeCl_3$ uptake by the wild-type were attributed to the presence of endogenously produced VF, which augments the uptake of Fe(III)' so that the total observed rate is the sum of Fe(III)' and VF-mediated uptake rates.

The presence of two distinct uptake systems, one for intact Fe-VF and another for soluble inorganic Fe(III) was further supported by an examination of the effects of concentration on the rate of uptake in *Marinobacter* DG879 in the light and in the dark (Fig. 3b). In the dark the rate of uptake (intact Fe-VF) showed saturation kinetics with an apparent Michaelis–Menten constant of 17.6 μM, typical for a receptor mediated siderophore uptake system (Carrano et al. 1996). However, in the light there was no evidence of saturation up to the maximum tested concentration of 50 μM, indicating that light caused a change in the uptake mechanism from a classical low capacity high affinity siderophore based uptake system to one of higher capacity and lower affinity (possibly via facilitated diffusion through porins) for the "free" metal.

Gene expression: light versus dark

The influence of light exposure on the photochemistry of VF and subsequent iron uptake imply that these

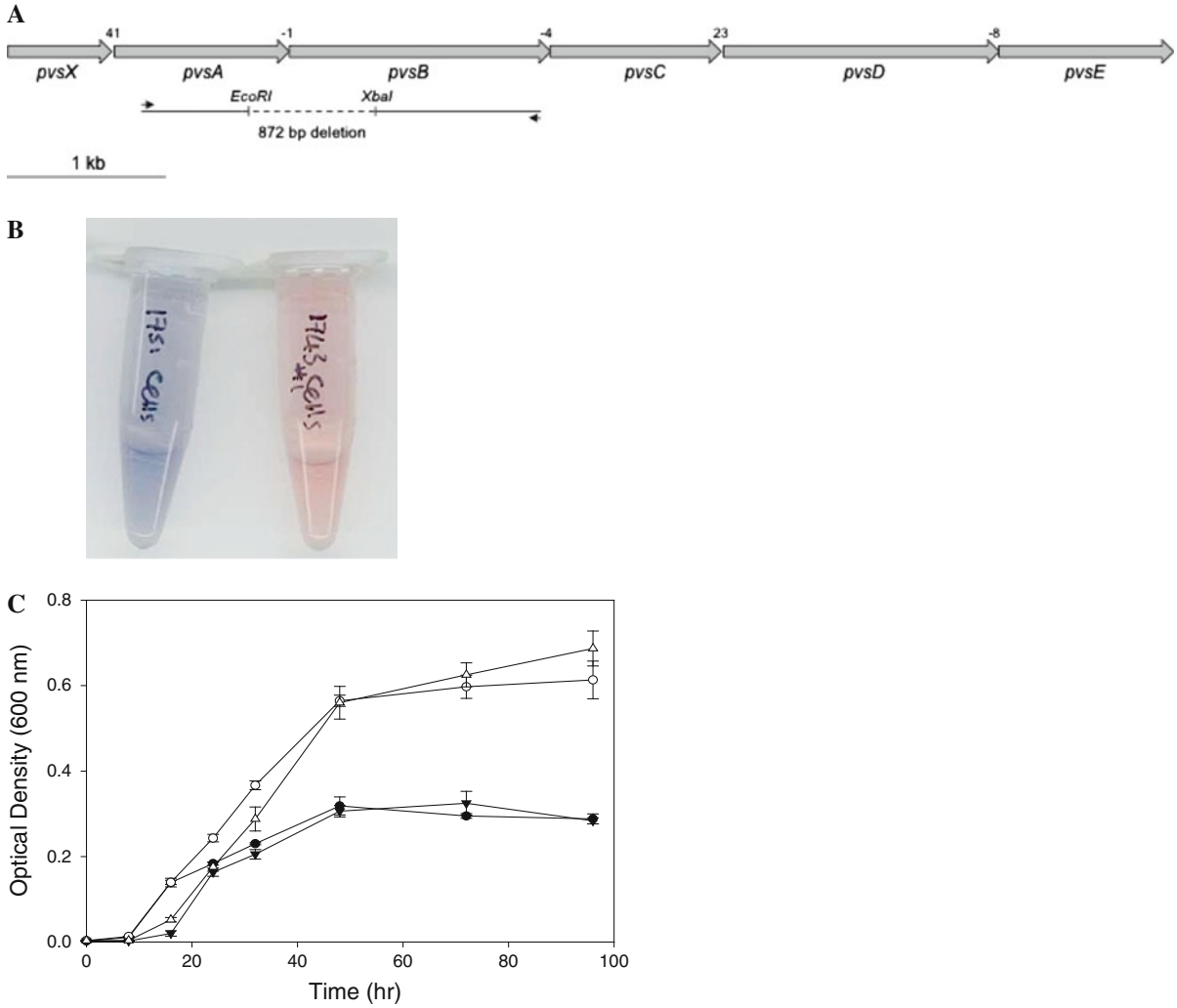

Fig. 5 Construction and characterization of the *M. algicola* DG893 *pvsAB*$^{-}$ deletion mutant. **a** Genetic map of the *M. algicola* DG893 *pvs* region showing the construction of the *M. algicola* DG893 *pvsAB*$^{-}$ deletion mutant. The mutation was constructed by amplifying the *pvsA* and *pvsB* genes using primers PvsAf1 and PvsBr1 denoted by the *arrows* (Table 1). The amplicon was cloned and digested by the indicated endonucleases and religated to produce an 872 bp deletion according to the Methods. **b** Inability to produce the siderophore VF was confirmed by the Chrome Azurol S (CAS) assay. Supernatants of DG893 wild-type or DG893 *pvsAB*$^{-}$ cultures were mixed with the CAS solution in a 1:1 ratio and allowed to equilibrate for 10 min. Siderophore production changes the *blue color* of the CAS solution to *pink* (DG893 wild-type, right) while the supernatant of DG893 *pvsAB*$^{-}$ does not cause a color change (*left*). **c** Growth of DG893 wild-type (*triangles*) and DG893 *pvsAB*$^{-}$ mutant (*circles*) under iron replete (10 μM, *open symbols*) and limited (50 nM, *closed symbols*) conditions. Cultures were grown at 20°C in 30-ml polycarbonate tubes in media passed through chelex-100 resin to remove residual iron. *Error bars* represent s.d. of triplicate cultures. (Color figure online)

organisms might regulate their iron uptake genes to cope with a diurnal shifting in iron speciation. Although, to our knowledge, no similar example has been demonstrated in a microbial heterotroph, adopting such a strategy could benefit the bacteria by selectively producing specialized proteins that assimilate the prevalent Fe species in solution. To test this hypothesis, we used real-time PCR to quantify mRNA transcripts of several potentially important iron uptake genes under light versus dark conditions. A complete list of genes tested and primers used in this study is shown in Table 1. Cells were acclimated by growing under iron limited (50 nM) or iron replete (10 μM) conditions while exposed to a fluorescent lamp

(30 μmol photons m^{-2} s^{-1}) or kept in the dark using semi-continuous batch culturing techniques as described in the "Materials and Methods". This low light intensity allowed for appreciable Fe-VF photolysis rates based on previously measured kinetics (Amin et al. 2009b) while ensuring a minimal effect on the bacteria as compared to potentially strong irradiation emanating from a mercury vapor lamp or attenuated sunlight. Under iron limitation in the dark, transcription of VF biosynthetic and receptor genes, *pvsA* and *pvuA*, respectively, and the periplasmic iron binding *fbpA* were up-regulated by 45-, 65-, and 25-fold, respectively compared to iron sufficient cells. In addition, non-VF related siderophore uptake genes, *fhuA* and *foxA*, were also up-regulated in the dark by 56- and 37-fold, respectively (Fig. 6). These results are consistent with an iron-dependent regulation of siderophore biosynthesis and uptake genes in bacteria. However, cells exposed to the low light intensity of the fluorescent lamp under iron-limited conditions showed that transcripts of *pvuA* and *fbpA*, while still up-regulated relative to high iron conditions, were surprisingly less so (2–3 fold) relative to dark conditions (Fig. 6). While this is a small change it appears to be statistically significant ($P < 0.01$). Other putative siderophore receptor genes, *fhuA* and *foxA*, and the VF biosynthesis gene, *pvsA* seemed to be largely unaffected by light. Under iron sufficient conditions light had no discernible effect on the abundance of any of the transcripts.

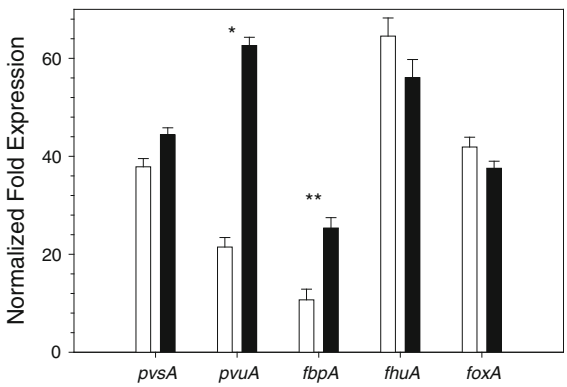

Fig. 6 The effect of light on transcript levels of iron uptake genes. Cultures were grown under iron limited conditions (50 nM) and were exposed to a fluorescent bulb (30 μmol photons m^{-2} s^{-1}, *clear columns*) or kept in the dark (*dark columns*) at 20°C. RNA was extracted and transcript levels quantified according to the "Materials and Methods". Two housekeeping genes (*gyrA* and *rpoD*) were used as controls as they did not vary appreciably under all experimental conditions. Transcripts were normalized relative to transcript quantities under iron-replete conditions (10 μM). *Error bars* represent s.d. of biological triplicates (3 sets of cultures and RNA extractions). $P < 0.001$ is indicated by * and $P < 0.01$ is indicated by ** for *light* versus *dark values*

Discussion

In a previous communication, we proposed the existence of a mutualism between VF-producing *Marinobacter* spp. and members of the dinoflagellate and coccolithophore algal lineages (Amin et al. 2009a). This mutualism is based on these bacteria producing the photolytically active siderophore VF, which provides an enhanced supply of Fe(III)' to the algae, and in return, the bacteria benefit from the release of photosynthate supporting their growth. To our knowledge, this study represents the first report that links light and its influence on iron speciation to the genetic response of a marine microbial heterotroph.

After light exposure and the resulting rapid increase in iron availability (half-life of Fe(III)' generation is <7 min in attenuated sunlight) (Amin et al. 2009a), the bacterial response included down regulation of *fbpA* and *pvuA*, which is involved in shuttling the siderophore-bound iron into the cytoplasm. This behavior suggests that a feedback mechanism exists between receptor biosynthesis and the Fe-VF concentration in solution, which serves to reduce unneeded transcripts of the receptor when the concentration of the Fe-VF complex decreases. This feedback mechanism is consistent with earlier studies on Pseudomonads, where the presence of the siderophore positively regulates the expression of its receptor (Venturi et al. 1995). The down regulation of *pvuA* and *fbpA* in the light further suggests that while light is affecting expression of VF related iron uptake genes, the biosynthesis of VF is not affected so as to establish a steady concentration of Fe(III)'. Interestingly, *fhuA* and *foxA* were not differentially regulated by light, indicating that light affects only VF-mediated iron uptake. The cumulative outcome of the bacterial response then suggests that shifts in extracellular iron speciation can be directly perceived by the bacteria via the described light-dependent regulation of iron uptake genes. A model for the effects of the diurnal cycle on VF-producing *Marinobacter* spp. has been proposed in Fig. 7.

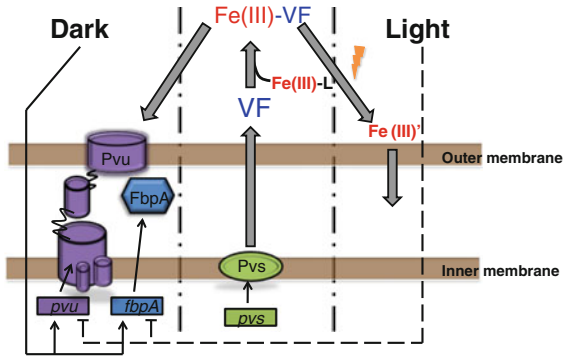

Fig. 7 A model for light mediated iron speciation and regulation of siderophore gene expression in *Marinobacter* species. VF is produced by iron-limited cells via the *pvs* operon and excreted outside the cell where it forms Fe(III)-VF by binding iron from organic ligands that chelate iron in seawater. In the dark, Fe(III)-VF is transported intracellularly by Pvu and Fbp and the *pvs*, *pvu* and *fbp* operons are positively regulated. In the light, Fe(III)-VF quickly degrades producing Fe(III)' and the *pvu* and *fbp* operons are repressed. *Boxes* represent gene operons while other shapes represent proteins. *Pvs*/Pvs = VF biosynthesis operon/enzymes; *pvu*/Pvu = VF receptor operon/proteins; *fbp*/FbpA = periplasmic Fe binding operon/protein. → denotes positive regulation; *dashed line* denotes repression

Our uptake experiments on *Marinobacter* spp. suggest that uptake of Fe(III)' is an important mechanism of iron assimilation especially in a bacterium that excretes a short-lived photolabile siderophore. In fact the ability of DG893 *pvsAB*$^{-}$ mutant, incapable of producing VF, to grow and assimilate inorganic iron as efficiently as the wild type highlights the importance of iron as a micronutrient and the various mechanisms available to bacteria for assimilating it. Surprisingly, our knowledge of Fe(III)' uptake mechanisms in marine microbial heterotrophs is limited. In the *Marinobacter* system presented here, it appears that Fe(III)' is transported across the cell membrane via a high capacity low affinity uptake system, which is different from the receptor mediated-siderophore uptake system based on the uptake kinetics. The ferric binding protein system (FbpABC) has long been hypothesized to shuttle inorganic iron species within the periplasm into the cytoplasm based on several crystal structures showing FbpA bound to inorganic iron substrates (Ferreiros et al. 1999; Shouldice et al. 2003). However, no conclusive evidence has so far been presented regarding the accessibility of FbpA to the outer membrane of bacteria and whether FbpA is the ultimate Fe(III)' binding target. Our results on *fbpA* transcript expression suggest that it responds to Fe-siderophore rather than the Fe(III)' substrate. This is consistent with recent evidence that shows that the FbpABC operon in the pathogenic bacterium *Neisseria gonorrhoeae* is required for the assimilation of iron from exogenous siderophores, suggesting that FbpA is involved in shuttling iron from siderophore transport systems (Strange et al. 2011).

The photochemistry of VF leads to a unique influence of light on the ecophysiology of these heterotrophic marine bacteria, where unlike phototrophs, there is no a priori reason why light should affect heterotrophic bacterial behavior. It has been shown previously that heterotrophic bacterial utilization of the algal organic photosynthate, glycolate (measured as transcript expression of the glycolate oxidase gene *glcD*), varied with diel cycle in response to likely changes in excretion rates from algal cells during day and night (Lau et al. 2007). A similar pattern in the *Marinobacter* system with regards to the VF receptor, *pvuA*, and *fbpA* may be attributed to bacterial sensing of light, but this may also be interpreted as a signaling mechanism between the bacteria and phytoplankton. For example, a reduction in the effective Fe-VF concentration due to photolysis may signal to the bacteria to up-regulate various carbon-utilizing proteins in anticipation of increased algal excretion of these substrates during daylight. Further experiments are being designed to answer these questions.

Further understanding of iron speciation and the effects of light on heterotrophic bacterial siderophore production and their subsequent interactions with phytoplankton is needed especially in view of the anticipated changes in iron bioavailability to phytoplankton in response to the rising CO_2 concentrations and resulting lower seawater pH (Shi et al. 2010; Sunda 2010). The outcome of this study implies that the potential reduction of iron bioavailability to phytoplankton could be alleviated for algal species that associate with bacteria producing photochemically active siderophores such as VF. In turn, the degree of success by which phytoplankton can participate in a mutualism with such bacteria should select for certain algal lineages and consequently may influence the biodiversity of future algal communities.

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