Title: Transcriptome response of high- and low-light adapted *Prochlorococcus* strains to changing iron availability

Running title: *Prochlorococcus* response to iron stress

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Abstract

Prochlorococcus contributes significantly to ocean primary productivity. The link between primary productivity and iron in specific ocean regions is well established and iron-limitation of *Prochlorococcus* cell division rates in these regions has been demonstrated. However, the extent of ecotypic variation in iron metabolism among *Prochlorococcus* and the molecular basis for differences is not understood. Here, we examine the growth and transcriptional response of *Prochlorococcus* strains, MED4 and MIT9313, to changing iron concentrations. During steady-state, MIT9313 sustains growth at an order-of-magnitude lower iron concentration than MED4. To explore this difference, we measured the whole-genome transcriptional response of each strain to abrupt iron starvation and rescue. Only four of the 1159 orthologs of MED4 and MIT9313 were differentially-expressed in response to iron in both strains. However, in each strain, the expression of over a hundred additional genes changed, many of which are in labile genomic regions, suggesting a role for lateral gene transfer in establishing diversity of iron metabolism among *Prochlorococcus*. Furthermore, we found that MED4 lacks three genes near the iron-deficiency induced gene (*idiA*) that are present and induced by iron stress in MIT9313. These genes are interesting targets for studying the adaptation of natural *Prochlorococcus* assemblages to local iron conditions as they show more diversity than other genomic regions in environmental metagenomic databases.

Keywords: cyanobacteria/ iron/ transcriptome

Introduction

The marine cyanobacterium *Prochlorococcus* is the most abundant photosynthetic cell in the ocean and an important biogeochemical agent (Partensky *et al,* 1999). Specific environmental parameters, such as light, temperature, phosphorus, nitrogen, cobalt, and copper, contribute to the diversity of *Prochlorococcus*, shaping its distribution and contribution to marine primary productivity. Iron is required for photosynthesis and limits primary productivity in specific ocean regions (Boyd *et al*, 2007; Moore *et al*, 2004), so may be another important factor in *Prochlorococcus* ecology. Evidence suggesting this is the limitation of *Prochlorococcus* cell division rates by iron in the Equatorial Pacific (Mann & Chisholm, 2000) and variations in the abundance of *Prochlorococcus* iron-related genes between oceans (Rusch *et al*, 2010).

Induction of iron stress is a useful approach towards understanding iron metabolism. At the physiological level, loss of chlorophyll – chlorosis (Öquist 1971, 1974), reductions in iron quota, and diminished cell volume are common iron-stress responses in cyanobacteria and other phototrophs (Berman-Frank *et al*, 2001; Sunda & Huntsman, 1995, 1997). At the molecular level, diverse phototrophs up-regulate the iron-free electron transfer gene, flavodoxin (*isiB*), and down-regulate the iron-requiring electron transfer gene, ferredoxin (*petF*), possibly reducing iron quota or redirecting iron to other cellular processes (McKay *et al*, 1999; Erdner & Anderson, 1999). This expression pattern was demonstrated in *Prochlorococcus* MED4, SS120, and MIT9313 (Bibby *et al,* 2003). The same study also revealed that when iron-starved, MIT9313's PSI complex associates with the chlorophyll-binding accessory protein PcbB. In contrast, a similar protein associates with SS120's PSI complex under iron-replete and iron-starved conditions, while neither condition induces such a

change in MED4. These results suggest variability in the iron-stress response of *Prochlorococcus* strains and invite further study.

Comparative genomics provides additional insights into iron metabolism and ecotypic diversity regarding iron. The *Prochlorococcus* core genome (genes shared by twelve *Prochlorococcus* isolates - Kettler *et al.* (2007) and MIT9202, this study), contains numerous iron-related genes. In addition to the aforementioned *isiB* and *petF*, the core genome contains several Fe-S proteins and ferredoxins, the iron storage molecule ferritin (*ftn*), and two ferric uptake regulator (*fur*) genes. Individual genomes vary in the numbers of each iron-related core gene they contain, possibly leading to distinct physiologies.

For iron transport, *idiA* (*futA/afuA), futB,* and *futC*, homologous to components of a periplasmic binding protein-dependent Fe^{3+} ABC transporter (Webb *et al,* 2001), are in the core genome. Various components of other iron transport systems (and other iron-related genes) exist in the *Prochlorococcus* flexible genomes (genes not shared by all *Prochlorococcus* genomes). Yet, a complete iron transport system has not yet been recognized in *Prochlorococcus*, or any marine picocyanobacteria (Kettler *et al*, 2007; Hopkinson & Morel 2009; Webb *et al,* 2001). Thus, it remains unclear which iron species (i.e. free or ligand-bound) are available to *Prochlorococcus*, how they acquire it, and if differences exist among ecotypes.

Here, we examine the response of *Prochlorococcus* to iron with the goal of beginning to understand how iron influences *Prochlorococcus* ecology. To this end, we investigated *Prochlorococcus* ecotypes MED4 and MIT9313 (Rocap *et al,* 2003), to tease out iron responses that are general to, or variable among, *Prochlorococcus* ecotypes. These ecotypes differ in several ways possibly relevant to their iron

metabolisms including cell size, light physiology, N metabolism, origin of isolation, and gene content (Table 1). We first tested the strains' steady-state growth rates over ranges of precisely-controlled iron concentrations to see if they differed in the iron required for growth. Then, we used short-term microarray experiments to identify iron-stress and recovery-responsive transcripts and ask how similar these ecotypes are in their transcriptional response to iron. Finally, we explored a set of particularly interesting iron-stress induced genes in environmental metagenomic databases to understand their distribution among wild *Prochlorococcus* populations and potential role in adaptation to local iron regimes.

Methods

Culture conditions

Prochlorococcus MED4ax and MIT9313ax were grown in PRO99 (Moore *et al*, 2007) modified for trace-metal clean work through microwave sterilization of seawater, increased EDTA (11.7μM), Chelex-100 treatment of major nutrients (Biorad, Hercules, CA), and soaking polycarbonate culture vessels in 0.1% Citranox (Alconox, White Plains, NY), 10% Baker Instra-analyzed HCl (Mallinckrodt Baker, Phillpsburg, NJ), and pH2 H_2O for 24 hours each (Keller *et al*, 1988; Price *et al*, 1988; Saito *et al*, 2002). EDTA-buffered media provided chemostat-like conditions by buffering >99% of the iron and maintaining a small defined Fe' (dissolved inorganic iron species) concentration throughout the experiment (Sunda and Huntsman, 2005). These cultures are chemostat-like because dissociation of the FeEDTA complex continually replenishes Fe' throughout the growth curve. Thus, Fe' is not depleted from the media as is typical for batch growth limited by nutrients like N or P. The

ratio of Fe' to Fe_{total} used was 0.039, using the empirical estimates by Sunda and Huntsman (2003) at 10μM EDTA, consistent with the approximately order-of-magnitude lower ratio found at 100μM EDTA as used for eukaryotic phytoplankton iron studies (Sunda and Huntsman, 1995).

For steady-state growth experiments, we chose an irradiance of 20μ$Em^{-2}s^{-1}$ (continuous, 25ºC), representing 16% and 40% of the light required for MED4 and MIT9313 maximum growth rates, respectively (Moore *et al,* 1999). This irradiance was useful for three reasons: First, because photochemical reactions strongly influence Fe' (Sunda and Huntsman, 2003), using a single irradiance allowed a consistent range of Fe' concentrations in the media, enabling strain-to-strain comparisons. Second, at 20μ$Em^{-2}s^{-1}$, MED4 and MIT9313 grew at similar rates when nutrient-replete. This was important as specific growth rate, steady-state iron uptake rates, and iron quota are interrelated (Sunda and Huntsman, 1997) and we aimed to isolate the effects of iron uptake and quota differences on growth rate. Thirdly, from a practical perspective, growth at sub-maximal irradiances facilitates experimental work due to higher pigment-per-cell and sensitivity for bulk fluorescence and flow cytometry. To initiate steady-state experiments, mid-log phase iron-replete cultures were centrifuged (8500rpm, 10 minutes), rinsed twice with sterile seawater, and inoculated in duplicate at added Fe_{total} concentrations of 0, 0.00003, 0.0001, 0.000567, 0.001, 0.003, 0.01, 0.1, and 1μM, resulting in added Fe' concentrations of 0, 0.0012, 0.0039, 0.0221. 0.039, 0.12, 0.39, 3.9 and 39nM, respectively, based on the chemical equilibrium with EDTA described above. Cultures were transferred at mid-log phase until growth rates reached steady-state.

For microarray experiments, where we aimed to measure gene expression in response to abrupt changes in iron, rather than compare cells in steady-state growth at precise iron concentrations, we grew MED4 at 27μE$m^{-2}s^{-1}$ (continuous, 21ºC) (chosen to match other experiments - Martiny *et al*, 2006; Steglich *et al*, 2006) and MIT9313 at 20μE $m^{-2}s^{-1}$ (continuous, 25ºC), in iron-replete (1μM) media prepared as above. To induce iron stress, triplicate cultures were centrifuged, washed (as above), divided, and re-suspended in either iron-replete (1μM Fe) or no added-iron media. RNA and cell number samples were collected (as in Lindell *et al,* 2007) at 0, 12, 24, 48, and 70 hours (MED4) and 0, 16, 28, 53, and 72 hours (MIT9313) with iron addition to no added-iron cultures to replete levels at 49 hours (MED4) and 54 hours (MIT9313). Additional cell number samples were collected at selected time-points.

We used an Influx flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA) to measure cell numbers for growth-rate calculations and determination of relative cell size and chlorophyll-per-cell (Olson *et al,* 1990a, 1990b), measuring a minimum of 10000 cells per sample. Relative cell size and chlorophyll per cell were approximated by normalizing forward-angle light scatter (FALS) and red fluorescence per cell, respectively, to 2μm-diameter Fluoresbrite beads (Polysciences, Inc., Warrington, PA).

RNA preparation

MED4 and MIT9313 RNA was extracted using the mirVana miRNA kit (Ambion, Austin, TX, USA) as in Lindell *et al.* (2007) with lysozyme added to MIT9313 samples (Tolonen *et al*, 2006) for better lysis. DNA was removed using Turbo DNase (Ambion) as in Lindell *et al*. (2007). Due to low yield, DNase-treated

MIT9313 RNA was concentrated using Micron Y-30 columns (Millipore, Billerica, MA) then amplified using the Message Amp TM II-Bacteria Prokaryotic RNA Amplification Kit (Ambion) following the manufacturer's protocol.

Array Normalization and Analysis

Custom Affymetrix (Santa Clara, CA) cDNA arrays, MD4-9313, were used to measure whole-genome expression with processing and design as in Lindell *et al.* (2007) for duplicate (MED4 samples), or triplicate (most MIT9313 samples, otherwise duplicate). Normalization methods, implemented in Matlab (The Mathworks, Inc., Natick, MA), followed Choe *et al.* (2005) with robust multi-chip average (RMA) to normalize background signal between arrays and lowess normalization at the probe-set level to correct influences of expression-signal intensity on fold change.

We made five comparisons to assess the iron stress and rescue response for each strain. We compared no added-iron (-Fe) to iron-replete (+Fe) expression at each of the four iron-stress time-points (see above) and report fold change for these as $\log_2$(-Fe/+Fe). Due to poor hybridization for a MED4 48-hour iron-replete sample, 48-hour no added-iron samples were compared to 24-hour iron-replete samples. A fifth comparison (R) was made between -Fe cultures before (48-hours MED4, 53-hours MIT9313) and after (70-hours MED4, 72-hours MIT9313) iron rescue and for this we report fold change as $\log_2$(after rescue/before rescue). Bayesian statistical analyses were performed in Cyber-T (Baldi and Long, 2001) and Q-VALUE (Storey and Tibshirani, 2003) was used to calculate false discovery rates (FDR) (q-value) as in Martiny *et al*. (2006). Following recommendations of Choe *et al.* (2005), we chose

stringent thresholds and define differentially-expressed genes as those with q-value less than 0.01 and $\log_2$(fold change) greater than 1 or less than -1 in one or more comparisons (Table S1 and S2). Raw data files and normalized expression levels are available in NCBI's Gene Expression Omnibus (Edgar *et al*, 2002) and are accessible through GEO Series accession number GSE26533 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSExxx

Hierarchical clustering (Cluster – Eisen *et al*, 1998) was performed with $\log_2$(fold change) for every gene in all comparisons using Complete Linkage Clustering and Correlation (centered) as the similarity metric. TreeView (http://rana.lbl.gov/EisenSoftware.htm) was used to visualize and analyze cluster results. Cluster membership is presented in Table S1 and S2 for differentially-expressed genes.

Recruitment of Prochlorococcus*-like *Global Ocean Survey (GOS) reads to sequenced genomes

We established a collection of *Prochlorococcus*-like reads from the global ocean survey (GOS) database as in Rusch *et al*. (2007) using thirteen *Prochlorococcus* genomes (Kettler *et al*. 2007 and MIT9202, this study). We required sequence alignments to *Prochlorococcus* genomes of at least 50% of the read's length (after trimming the vectors). As a result, we report 402771 *Prochlorococcus*-like GOS reads out of the total 9893120 GOS reads. AS9601 best represents *Prochlorococcus* in the GOS database, thus, this genome was used to assess quantity and diversity of natural *Prochlorococcus* genes in the *idiA* region. Similar analyses using MIT9312 and MED4 appear in the Supplementary Materials.

RESULTS and DISCUSSION

Steady-state growth over a range of iron concentrations

MED4 and MIT9313 displayed different relationships between steady-state growth rate and iron concentration under the chemostat-like (i.e. constant Fe' concentration – See Methods) conditions of metal-ion buffered media in batch cultures (Sunda *et al*, 2005). Under these light and temperature conditions, MIT9313 grew at the lower Fe' concentrations of 0.022 and 0.004nM whereas MED4 could not (Figure 1), revealing a large difference in the fitness of these ecotypes at low iron.

These results were contrary to expectations based on the smaller size of MED4 relative to MIT9313 (Table 1), and the advantage in nutrient acquisition higher surface-area-to-volume ratios are expected to confer. Similarly, from the perspective of light physiology, we imagined that low-light adapted cells (MIT9313) would contain more iron-rich photosystems (Jordan *et al,* 2001), thus requiring more iron than high-light adapted cells (MED4). However, another important difference between the strains, possibly related to MIT9313's better tolerance of lower iron concentrations than MED4, is the habitat from which they were isolated – MIT9313 from the North-Western Atlantic and MED4 from the Mediterranean Sea (Rocap *et al,* 2003). Modeling studies (Jickells *et al,* 2005), and direct Fe_{total} measurements of unfiltered samples indicate that concentrations in the Mediterranean Sea (approximately 20-40nM) are dramatically higher than in the North-Western Atlantic (1.5-3.2nM), where MIT9313 was isolated (Sherrell and Boyle, 1998; Wu and Luther, 1995). Thus, the difference in response of the ecotypes to low iron in culture may originate from the selective pressures imposed by iron availability in the waters where

they were isolated – as observed for phosphorus acquisition in *Prochlorococcus* (Martiny *et al,* 2006; Coleman and Chisholm, 2010) – through enhanced mechanisms for coping with secondary consequences of iron stress, lower iron quotas, or alternative iron acquisition systems. Indeed, Rusch *et al*. (2010) suggested that low-iron waters select for cells with genomes encoding fewer iron-requiring proteins. Also consistent with our hypothesis is MIT9313's expression of a PSI-associated chlorophyll-binding accessory protein during iron stress, while a similar gene and PSI-associated protein are absent in MED4 (Bibby *et al,* 2003). Finally, it is perhaps noteworthy that MIT9313 is more sensitive to copper than MED4 (Mann *et al,* 2002), which could have its origins in high-affinity metal transporters that allow survival at low metal concentrations.

Physiological response to sudden iron stress and rescue

To understand the molecular basis for the difference between MED4 and MIT9313 in steady-state at low iron concentrations and to identify iron-related genes in each genome, we measured whole-genome expression in response to abrupt iron deprivation and rescue in experiments designed to induce stress without large changes in growth rate (as large changes in growth rate could yield gene expression changes not specific to iron). After initiation of short-term iron stress, growth rates of both MED4 and MIT9313 experimental (-Fe) cultures matched the controls (+Fe) for 48 hours (53 hours - MIT9313) at 0.58 and 0.61 day^{-1}, respectively (Figure 2A-B). However, relative red fluorescence (proxy for chl*a*) per cell and relative FALS (proxy for cell size) per cell decreased in experimental cultures after 20 hours, evidence that iron stress had set in by this time (Figure 2C-F). The decrease in chl*a* is consistent

with symptoms of chlorosis (Wilhelm 1995), and the decrease in size is consistent with observations of iron-stressed *Prochlorococcus* in the field and cultured iron-stressed diatoms and dinoflagellates (Cavender-Bares *et al*, 1999; Mann & Chisholm, 2000; Sunda and Huntsman, 1995). Upon iron rescue, we observed no significant increase in chla or size in either strain (Figure 2C-F). However, the growth rate of the MED4 experimental culture declined to 0.24 $days^{-1}$, while the MIT9313 experimental culture continued to match the control (Figure 2A-B), possibly indicating more severe iron stress in MED4. Thus, we expect some of the MED4 gene expression changes after rescue (R) to be related to growth rate changes, rather than related directly to iron availability.

Global features of the transcriptional response to sudden iron stress and rescue

Transcription of 6.2% of MED4 genes and 3.8% of MIT9313 genes responded to iron stress and rescue (Figure 3A-B). Differential expression was first evident at 12 hours (Figure 3), when changes in physiological indicators of iron stress were not yet evident (Figure 2C-F). In addition, gene expression responded dramatically to iron rescue in both strains though size and chl*a* did not, revealing a temporal divide between recovery from iron stress at the molecular and physiological levels.

Hierarchical clustering revealed several clusters containing differentially-expressed genes with predicted functions (Figure 3C-D) and some clusters containing only genes of unknown function (Table S1 and S2). In fact, over 60% of differentially-expressed genes in each strain were of unknown function.

Over 80% of differentially-expressed genes were from the flexible genomes (genes not shared by all *Prochlorococcus*) of MED4 and MIT9313 (Table S3 – third

column), a disproportionally-high fraction relative to the proportion of flexible genes in each genome (39.5% - MED4, 57.9% - MIT9313). Additionally, a disproportionate fraction (67.9% compared to 22.1% of MED4's whole genome – $p<0.001$, and 35.3% compared to 21.2% of MIT9313's whole genome – $p<0.01$, Fisher's exact test) of the differentially-expressed genes reside in genomic islands and/or hypervariable regions (Coleman *et al*, 2006; Kettler *et al*, 2007) (Figure 4), and they are almost exclusively flexible genes. It is hypothesized that genomic islands and hypervariable regions are evidence of horizontal gene transfer events in *Prochlorococcus* and contain genes related to relatively recent adaptations to local environmental conditions (Coleman *et al,* 2006; Kettler *et al*, 2007), which is consistent with our hypothesis that iron-related gene content of these strains is due to the iron regime at their origin of isolation.

MED4 genomic island 5 (ISL5), in particular, was a 'hotspot' for differentially-expressed genes in our experiments (Figure 4) – including high-light inducible (*hli)* genes, and numerous genes of unknown function. Some ISL5 genes respond to P-stress (Martiny *et al,* 2006), but these are not the same genes – supporting the hypothesis that island genes are involved in specific metabolic pathways rather than a general stress response (Coleman *et al*, 2006), and that genes in particular genomic islands have diverse functions.

Features of specific differentially-expressed genes

<u>*MED4-MIT9313 shared genes that respond to iron in both strains:*</u> Of the 1159 MED4-MIT9313 shared genes (MED4-MIT9313 bi-directional orthologs - Table S3), only four were differentially-expressed in both strains under our conditions of iron

stress and recovery (Figure 5, bold cyan). Two of these, *petF* (ferredoxin) and *isiB* (flavodoxin), are also *Prochlorococcus* core genes and belong to well-characterized iron-response systems in cyanobacteria and other photosynthetic organisms – See Introduction (McKay *et al*, 1999; Erdner & Anderson, 1999). As previously shown (Bibby *et al*, 2003), ferredoxin was down-regulated and flavodoxin was up-regulated during iron stress in both MED4 and MIT9313, suggesting flexibility in the iron requirements of *Prochlorococcus* depending on iron availability.

Another MED4-MIT9313 shared gene (though not a *Prochlorococcus* core gene) differentially-expressed in both strains is the high-light inducible (*hli*) gene *hli05/hli08* (PMM1404/PMT1154), which is up-regulated in MED4 during iron stress and down-regulated in both strains upon rescue (Table S1 and S2). Although a large number of *hli* genes were differentially expressed in our experiments (Tables S1 and S2), this is the only one that is both shared by, and differentially expressed in, both strains. Hli proteins may have a role in protecting photosystems from oxidative damage, which has been shown to be a consequence of iron stress in cyanobacteria (Latifi *et al*, 2005) and may be a common feature of the response to changing iron availability in MED4 and MIT9313.

A fourth MED4-MIT9313 shared gene (also a *Prochlorococcus* core gene) differentially-expressed in both strains is the iron-deficiency induced gene (*idiA*), homologous to *afuA* in *Synechococcus* PCC7942 and *idiA* in *Synechococcus* PCC6301 (Webb *et al,* 2001; Michel, 1999, 2001), was up-regulated during iron stress, as previously observed (Katoh *et al*, 2001; Singh *et al*, 2003; Webb *et al*, 2001) and down-regulated following rescue. IdiA is hypothesized to be a periplasmic iron-binding protein component of an iron ABC-transporter system including an ATP-

binding protein (*futC*), and permease (*futB*) (Webb *et al*, 2001), which were not differentially-expressed in either *Prochlorococcus* strain, consistent with their constitutive expression in *Synechocystis* PCC6083 (Katoh *et al*, 2001).

Different genes, similar functions: It was surprising that so few MED4-MIT9313 shared genes were commonly differentially-expressed by both strains (Figure 5, Tables S1 and S2). However, looking more closely, we found groups of genes (though different in each strain, and including both MED4-MIT9313 shared and non-shared genes) with common functions. Specifically, though PSII (2-3 atoms Fe) genes were not down-regulated during iron stress in either strain, genes encoding the iron-rich (12 atoms Fe) PSI complex were down-regulated in response to iron stress in both strains. Notably, these PSI components – *psaM* in MIT9313, *psaI* and *psaL* in MED4 – are transmembrane-α helices that stabilize cofactors of the PSI core antennae system (Jordan *et al*, 2001). Possibly, during iron stress, the down-regulation of these stabilizing factors makes room for other proteins that maintain PSI function, releases iron for other uses, and/or precedes down-regulation of the major PSI components (such as *psaA*). Similarly, cytochrome b6/f complex components were down-regulated in both strains, including heme-binding *petA* (cytochrome f) and the Rieske iron-sulfur subunit, *petC*, in MED4, and the cytochrome b6/f complex subunit VII in MIT9313. Thus, both MED4 and MIT9313 may be able to conserve iron by down-regulating these iron-requiring proteins. We speculate that differential expression of some, rather than all, components of these complexes under our conditions may be due to different functions, mRNA half-lives, and/or protein turnover rates for each (Steglich *et al,* 2010).

Though essential for homeostasis, iron can trigger production of damaging reactive oxygen species (Latifi *et al,* 2005). Thus, we expect to find a sophisticated regulatory system for iron uptake, storage, and use in *Prochlorococcus*. Surprisingly, *fur* –a negative repressor of iron transport and storage genes (Hantke, 2001; Gaballa *et al*, 2008) – was not differentially expressed in MED4 or MIT9313. In addition to Fur, regulatory RNAs are known to be important in cyanobacterial iron metabolism. In iron-stressed *Synechocystis* and *Anabaena, cis*-encoded regulatory RNAs, IsiR and *a-furA,* regulate aspects of photosynthesis and diverse metabolic processes (Massé *et al*, 2007). *In* Prochlorococcus, numerous small non-coding RNAs (sRNAs) responded to nitrogen, phage, and light stress conditions (Steglich et al, 2008) and one particular sRNA, Yfr1, has been shown to target the transcription of two specific outer membrane proteins (Richter et al, 2010). We observed differential expression of several, albeit different, sRNAs in MED4 and MIT9313 (Supplementary Table 1 and 2),suggesting that sRNAs are also important regulatory agents for *Prochlorococcus* iron metabolism. This observation is also consistent with metatranscriptomic data from the Pacific revealing sRNAs flanked by *Prochlorococcus* iron transport genes (Shi *et al,* 2009). The differentially-expressed sRNAs fall into distinct hierarchical clusters (Supplementary Table 1 and 2), and some sRNAs, for example Yfr11 and Yfr19, are also controlled by light and phage infection, suggesting a range of target genes and functional roles for sRNAs in *Prochlorococcus* iron metabolism (Steglich *et al*, 2008, Richter et al, 2010).

Finally, both strains differentially expressed nitrogen transporters, though the genes are not MED4-MIT9313 shared genes, demonstrating another similarity in the abrupt response to iron of MED4 and MIT9313. In MED4, PMM0370 – a putative

cyanate transporter, was down-regulated following rescue and could signal a transition from the use of a different N species during iron stress (possibly cyanate) that may require less iron for assimilation. In MIT9313, PMT2240 (formate/nitrite transporter) was down-regulated with iron stress. Cyanobacterial nitrite reductases require iron (Luque *et al*, 1993), and though the MIT9313 nitrite reductase was not differentially-expressed, down-regulation of the nitrite transporter during iron stress may trigger events that would ultimately lead to this, possibly reducing iron requirement.

Different genes, different functions: In addition to genes differentially expressed by MED4 and MIT9313 that have similar functions despite being different genes, many other genes (both shared and non-shared) were differentially-expressed under our conditions of iron stress and recovery (Figure 5; Tables S1-2). This set includes MED4-MIT9313 shared genes that were differentially-expressed in only one strain and genes not shared by the two strains. Possibly, these genes are important in establishing the different tolerances of MED4 and MIT9313 to low iron (Figure 1).

Numerous MED4-MIT9313 shared genes were differentially-expressed only in MIT9313 under our conditions of iron stress. One of the two ferritin (iron storage) genes in MIT9313 (PMT0499), for example, was up-regulated 16 hours after iron deprivation, while the single ferritin gene in MED4 was not differentially expressed. PMT0862, another MED4-MIT9313 shared gene predicted to encode a Fe-S oxidoreductase, was also strongly up-regulated in MIT9313 during iron stress but was unchanged in MED4. The pentapeptide repeat gene, PMT1554, is another interesting case as it was strongly induced in MIT9313 during iron stress, yet not differentially

expressed in MED4. Tandemly-repeated series of amino acids characterize the pentapeptide repeat protein family, which is well represented in both prokaryotic and eukaryotic genomes though few are of known function (Vetting *et al,* 2006). Lastly, the shared gene *rimI* (PMT0510) encodes a GCN5-related N-acetyltransferase protein and was down-regulated in MIT9313 during iron stress, but not differentially-expressed in MED4.

Conversely, several MED4-MIT9313 shared genes were differentially-expressed in MED4 but not MIT9313 under these experimental conditions (Figure 5). Notably, the transcription of *hemA* (glutamyl-tRNA reductase), of the chlorophyll synthesis pathway, was strongly down-regulated in MED4 with iron stress, which is consistent with chlorosis in the iron-stressed cells (Figure 3E and F). It is unclear why *hemA* was not differentially-expressed in MIT9313 where chlorosis was also evident. Another MED4-MIT9313 shared gene, PMM1283, was up-regulated during iron stress in MED4, but was unchanged in MIT9313, though it was up-regulated under more severe (120 hours) iron stress in MIT9313 in another study (Gómez-Baena *et al,* 2009). PMM1283 is annotated as an integral-membrane protein interacting with the metalloprotease FtsH (a protein that possibly degrades the D1 protein of PSII during photoinhibition), thus by association, PMM1283 may be involved in facilitating similar photosystem rearrangments required during iron stress as during photoinhibition (Bieniossek *et al,* 2006; Silva *et al,* 2003). Lastly, PMM0345 (a peroxiredoxin and possible bacterioferritin comigratory protein) is induced in MED4 during iron stress but not in MIT9313. Both genomes contain two such genes but only this one is differentially expressed. As bacterioferritins are involved in iron storage, the induction of this gene under iron limitation may indicate changes in iron

allocations and storage within MED4 that are distinct from changes facilitated by ferritin, which was up-regulated in MIT9313 during iron stress.

PMM0805 and PMT0498 are an intriguing pair of differentially-expressed genes as their genomic context is preserved in MED4 and MIT9313, but the genes themselves are not bi-directional orthologs in these genomes (Kettler *et al,* 2007). These genes are located between a CRP-family bacterial regulatory protein (a shared gene iron-induced only in MED4) and ferritin (a shared gene only iron-induced in MIT9313), and are close to other iron-related (but not differentially-expressed) genes such as *futC* (ATP-binding component of an iron ABC transporter – present in both genomes), iron transport gene *fepC* in MED4, and a second copy of ferritin in MIT9313 (PMT0495). Strangely, the iron-stress response of the two genes was different: PMM0805 was up-regulated while PMT0498 was down-regulated (Table S1 and S2). The origins of this difference are unclear, but could result from the slightly different light and temperature conditions (See Methods) or different degrees of iron stress experienced by MED4 and MIT9313 as a result of their individual iron requirements and different growth conditions. Alternatively, differences in the regulation of genes in this region or functional differences between this pair of genes could explain the different transcriptional responses. Clearly, this genomic region is of great interest regarding *Prochlorococcus* iron metabolism.

In contrast, several of MED4's differentially-expressed genes are absent in MIT9313 (belonging to the non-shared genes). PMM1400 (a redox-related protein with homology to the viral protein hemagglutinin neuraminidase) draws our attention as its expression is highly induced in MED4 during iron stress. PMM1400 is also present in all other HL-adapted and NATL *Prochlorococcus* genomes and several

phage genomes (Kettler *et al*, 2007; Sullivan *et al*, 2005). Interestingly, *Prochlorococcus*-like DNA fragments similar to PMM1400 are sparse in predicted iron-limited regions, compared to other ocean regions (Rusch *et al*, 2010). Additionally, though PMM1400 is adjacent to *hli* genes, it is down-regulated during high-light stress while the *hli*s are up-regulated (Steglich *et al*, 2006). Another MED4 gene of particular interest is a possible Mn ABC transporter (*iraI*) that was up-regulated during iron stress, clustered with *idiA* and *isiB*, and is absent in MIT9313. It is located close to one *fur* gene and other putative Mn transporters supporting a role for this gene in trace-metal metabolism. Manganese is an important element in protecting against reactive oxygen species in other bacteria (Daly *et al,* 2004; Posey *et al,* 2000), thus, it may have a similar role in reacting to oxidative stress in MED4 as a consequence of iron stress.

The MIT9313 genome contains several differentially-expressed genes that are not present in MED4 (belonging to the non-shared genes). One notable member of this group is *pcbB*. The association of PcbB with PSI under iron stress has been demonstrated in MIT9313 (Bibby *et al,* 2003) and we observed similar differential expression (Table S2). So, it is tempting to speculate that this protein contributes to MIT9313's increased fitness at low iron concentrations relative to MED4, as it is absent in the latter (Bibby *et al,* 2003). Possibly, PcbB stabilizes PSI during iron stress allowing continuation of light harvesting, protects PSI from oxidative damage, or allows redistribution of iron from PSI during iron stress. Interestingly, *pcbB* is very similar to the constitutively-expressed *pcbA* genes of MED4 and MIT9313 (Bibby *et al,* 2003), confirming a diversity of function among this group of similar genes.

Finally, during iron stress MIT9313 up-regulated three genes in the *idiA* region that are missing in MED4. These genes are particularly fascinating in light of MIT9313's survival at lower iron concentrations than MED4 and are discussed in the next section.

The idiA region: Intriguingly, MED4 is missing three genes between *idiA* and *glyQ* (glycyl-tRNA synthetase alpha subunit, iron-stress induced in MIT9313 only) that are present in MIT9313. Importantly, these three MIT9313 genes between *idiA* and *glyQ* are iron-stress induced and so are intriguing in the context of the different abilities of the two strains to grow at low iron (Figure 1). The genes include PMT0284 (a possible porin), PMT0285 (a possible peptidase of family M20/M25/M40), and PMT0286 (a putative hydroxylase with homology to the pneumococcal iron uptake gene, *piuC*).

Excepting MED4, all sequenced *Prochlorococcus* genomes contain orthologs to the genes between *idiA* and *glyQ* in MIT9313 (Figure 6). These genes have also been observed in *Prochlorococcus* DNA fragments from the Pacific Ocean, and were hypothesized to be involved in iron acquisition based on their proximity to *idiA* and homology to iron transport genes (Coleman and Chisholm, 2007). PMT0286 (*piuC*) is homologous to a component of an iron transport system in *Streptococcus pneumoniae* (Brown *et al*, 2001; Tai *et al*, 2003; Ulijasz *et al*, 2004) and porins (PMT0284) are important molecules in cyanobacteria for small solute uptake through diffusion (Hoiczyk and Hansel, 2000). The up-regulation of these genes under iron stress supports the hypothesis that these genes are involved in *Prochlorococcus* iron metabolism, specifically iron uptake. Possibly, the genes have a functional role in

determining the advantage of MIT9313 over MED4 at lower iron concentrations by allowing more efficient iron transport or providing access to an alternate (i.e. not Fe') species of iron not available to MED4 (Figure 1).

In order to assess how well-represented these genes are among wild *Prochlorococcus*, we explored their prevalence in the GOS database, by quantifying reads recruiting to the *idiA* region of *Prochlorococcus* AS9601 (a well-represented genome in the GOS database). The abundances of reads recruiting to genes of interest (Figure 7A, Figure S1A-S2A for other reference genomes) were similar to those recruiting to nearby genes such as core gene *psbD* (photosystem II D2 protein) that we expect to occur in single copy in *Prochlorococcus* cells, with the notable exception of small island regions where few reads recruited. This suggests that the suite of genes in the *idiA* region is present in most wild *Prochlorococcus* cells in the GOS database and that cells like MED4, missing the three genes, are a minority in the waters sampled in GOS – consistent with our understanding of the longitudinal distribution of the MED4 ecotype (Johnson *et al,* 2006).

However, examination of the similarity of the recruited reads to AS9601 (Figures 7B, Figure S1B-S2B for the other reference genomes) revealed an interesting feature: The reads aligning to the genes between *glyQ* and *idiA* have low sequence similarity (70.36% +/- 7.40%) to the AS9601 genome compared to the average (86.49% +/- 12.27%). Analysis of the same region among the genomes of cultured *Prochlorococcus* isolates revealed similar sequence divergence in this region relative to AS9601 (Figure S3). The relatively high sequence diversity in this set of genes, and their location adjacent to a tRNA, suggest that they could be prone to horizontal gene transfer and loss events. The region (Figure 4, orange shading) is also identified

as a small genomic island, in MED4 and MIT9312 (Coleman *et al,* 2006) and is marked by a spike in the number of gene gain events (Kettler *et al*, 2007) (Figure 4B, gray line) – again, consistent with this being a labile region of the genome, and possibly involved in the adaptation of *Prochlorococcus* to different iron regimes.

CONCLUSIONS

This study used precisely-controlled iron concentrations to test the tolerance of two *Prochlorococcus* ecotypes to low iron availability and whole-genome microarrays to identify genes that respond to abrupt iron stress and rescue. We found that the two closely-related ecotypes, MED4 and MIT9313, have dramatically different lower limits with regard to iron concentrations that sustain steady-state growth. This suggests a role for iron in the physiological and genomic differentiation among *Prochlorococcus.*

Underlying these physiological differences are a number of characteristics at the transcriptome level discovered through imposing abrupt iron stress and recovery: (1) Most genes that are differentially expressed in response to iron are in the flexible genomes of MED4 and MIT9313. In fact, only 4 of the hundreds of genes shared by MED4 and MIT9313 (three of which are also *Prochlorococcus* core genes) are differentially expressed in both strains; (2) Differentially-expressed genes are concentrated in labile regions (genomic islands and hypervariable regions) of the genomes; and (3), Sequence similarity patterns of *idiA*-region genes of sequenced genomes and *Prochlorococcus* DNA fragments from the GOS database, may reflect horizontal gene transfer events of genes possibly related to iron transport. Together, these observations suggest that adaptation to local iron conditions is a contributor to

the diversity among *Prochlorococcus* and possibly to the success of this group in low iron regions of ocean.

We speculate that underlying MIT9313's ability to grow at lower iron than MED4 are three key features: (1) A more efficient iron transport system adapted to low iron concentrations; (2) A capability to better protect itself from deleterious secondary consequences of iron stress, such as oxidative stress; and (3) a lower cellular iron requirement than MED4. In support of (1) is the iron-induced expression of MIT9313 genes adjacent to *idiA* (*piuC*, a possible peptidase, and a possible porin) that are absent in MED4. These genes may enable more efficient iron transport, or provide access to an iron species not accessible to MED4 – e.g. iron bound to ligands. Attribute (2) is supported by the association of a chlorophyll-binding antenna protein (PcbB) – the gene for which is absent in MED4 – with PSI during iron stress in MIT9313 (Bibby *et al*, 2003). We do not have evidence supporting (3), but a recent metagenomic study (Rusch *et al*, 2010) revealed that *Prochlorococcus* from lower iron waters contain fewer iron-binding proteins than from higher iron waters, suggesting a possible mechanism through which iron requirements could vary among *Prochlorococcus,* as we suspect they do between MED4 and MIT9313.

Clearly, the mechanisms that underlie the differential fitness of these two strains at low iron are complex as are the fundamental aspects of iron transport and use in *Prochlorococcus* and marine picocyanobacteria. Furthermore, as cultures have been maintained in high-iron culture media for close to two decades, it will be important compare our results from cultured strains to the iron requirements and gene expression responses of naturally-occurring *Prochlorococcus*. The results reported here offer more questions than answers, but provide an essential baseline for future

studies. In particular, defining the relationship between light availability and iron requirement in *Prochlorococcus*, identifying a complete iron uptake system, and unveiling the function of uncharacterized iron-related genes will be critical for expanding our understanding to a level where a more complete picture could emerge.

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Author contributions

Designed experiments: AWT, SWC, MAS. Conducted experiments: AWT. Analyzed data: AWT and KH. Provided computational tools and environmental metagenomic analysis: KH. Wrote the paper: AWT, SWC, and MAS.

Supplementary Materials

Supplementary information is available at the ISME Journal's website:

http://www.nature.com/ismej/index.html.

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Figure 1

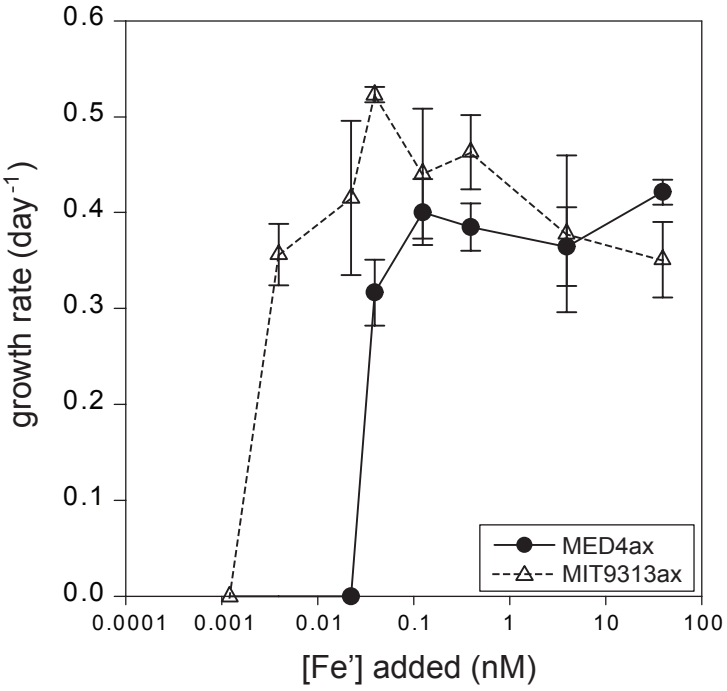

0.6
0.5
0.4
0.3
0.2
0.1
0.0
growth rate (day-1)
0.0001
0.001
0.01
0.1
1
10
100
[Fe'] added (nM)
MED4ax
MIT9313ax

Figure 2

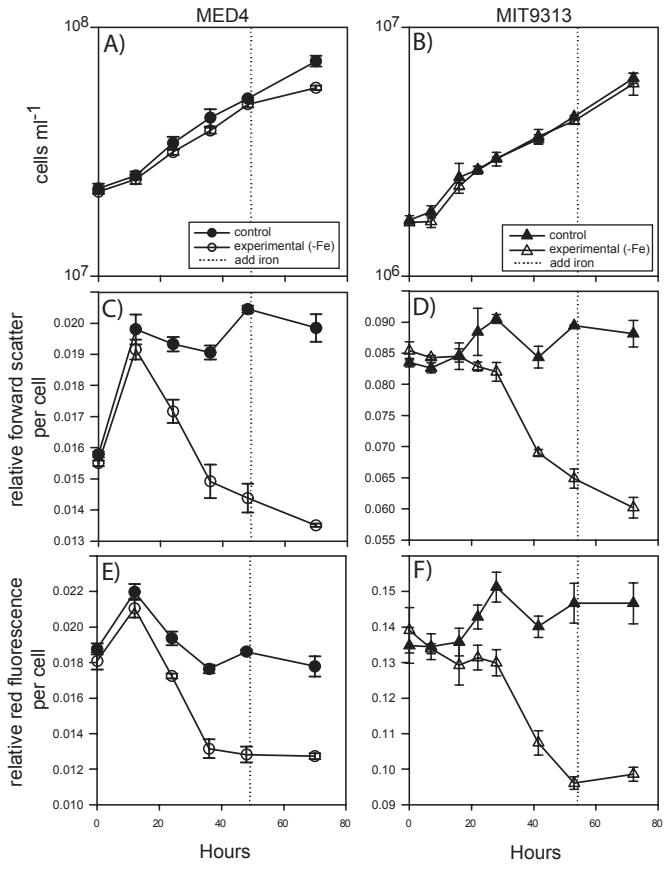

MED4
MIT9313
A)
B)
C)
D)
E)
F)
cells ml^{-1}
relative forward scatter per cell
relative red fluorescence per cell
control
experimental (-Fe)
add iron
Hours
Hours

Figure 3

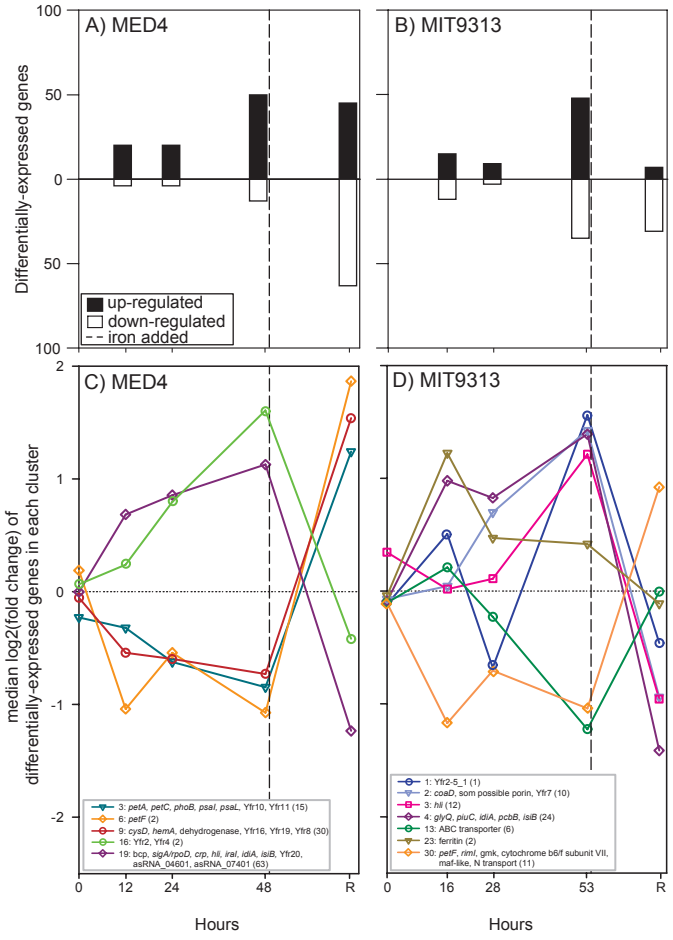

A) MED4
B) MIT9313
C) MED4
D) MIT9313
Differentially-expressed genes
up-regulated
down-regulated
iron added
median log2(fold change) of differentially-expressed genes in each cluster
3: petA, petC, phoB, psaI, psaL, Ytr10, Ytr11 (15)
6: petF (2)
9: cysD, hemA, dehydrogenase, Ytr16, Ytr19, Ytr8 (30)
16: Ytr2, Ytr4 (2)
19: bcp, sigA/rpoD, crp, hli, iraI, idiA, isiB, Ytr20, asRNA_04601, asRNA_07401 (63)
1: Ytr2-5_1 (1)
2: coaD, som possible porin, Ytr7 (10)
3: hli (12)
4: glyQ, pluC, idiA, pcbB, isiB (24)
13: ABC transporter (6)
23: ferritin (2)
30: petF, riml, gmk, cytochrome b6/f subunit VII, maf-like, N transport (11)
Hours
Hours

Figure 4

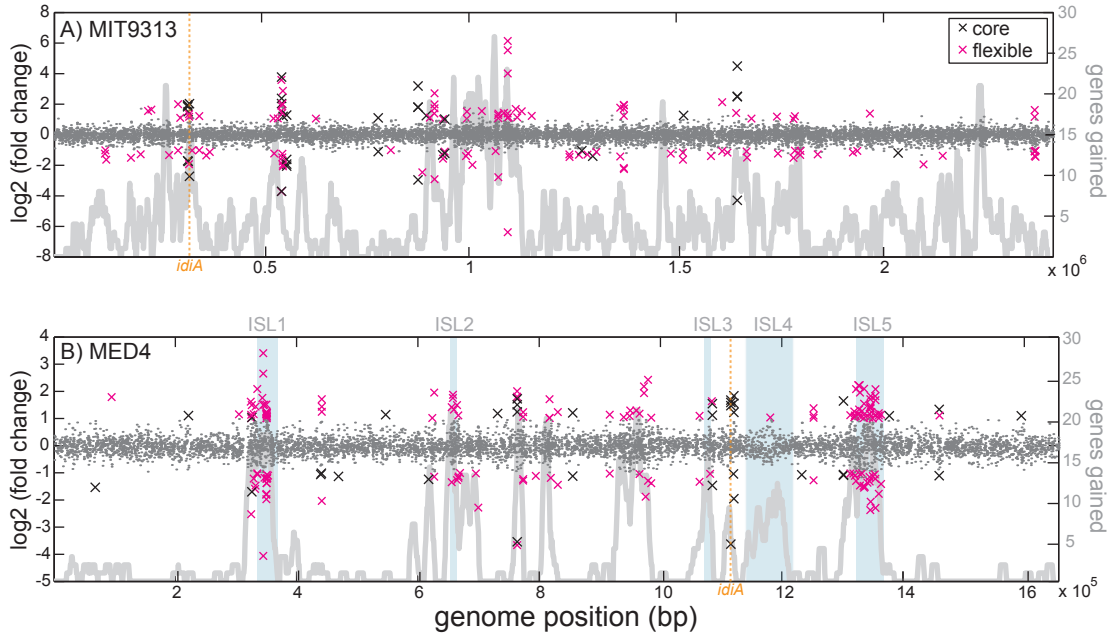

A) MIT9313
core
flexible
log2 (fold change)
genes gained
idiA
x 10^6
B) MED4
ISL1
ISL2
ISL3
ISL4
ISL5
idiA
x 10^5
genome position (bp)

Figure 5

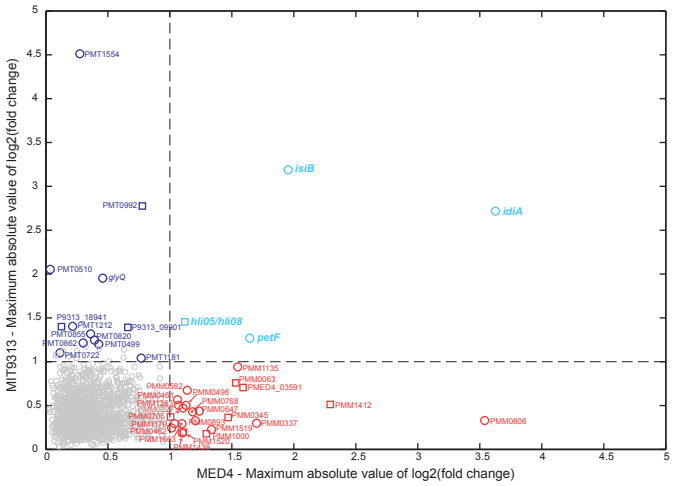

MIT9313 - Maximum absolute value of log2(fold change)
MED4 - Maximum absolute value of log2(fold change)
0
0.5
1
1.5
2
2.5
3
3.5
4
4.5
5
PMT1554
isiB
idiA
PMT0992
PMT0510
glyQ
hli05/hli08
petF
P9313_18941
PMT1212
P9313_09901
PMT0855
PMT0820
PMT0862
PMT0499
PMT0722
PMT1161
PMM1135
PMM0063
PMED4_03591
PMM0582
PMM0496
PMM0768
PMM0647
PMM1412
PMM0345
PMM0337
PMM0806
PMM1519
PMM1000

Figure 6

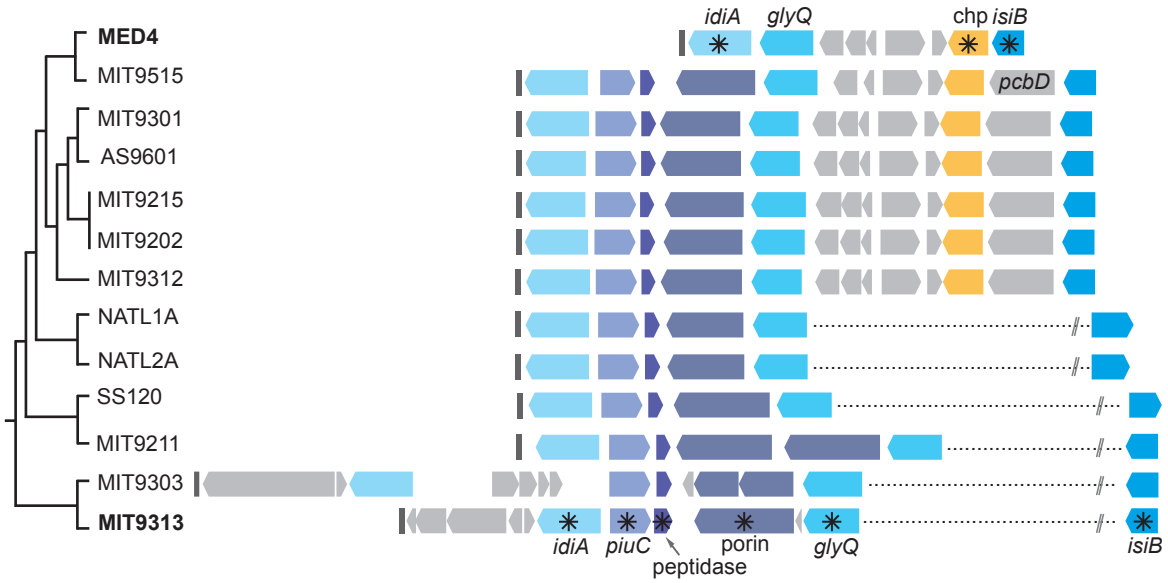

idiA
glyQ
chp
isiB
MED4
MIT9515
pcbD
MIT9301
AS9601
MIT9215
MIT9202
MIT9312
NATL1A
NATL2A
SS120
MIT9211
MIT9303
MIT9313
idiA
piuC
peptidase
porin
glyQ
isiB

Figure 7

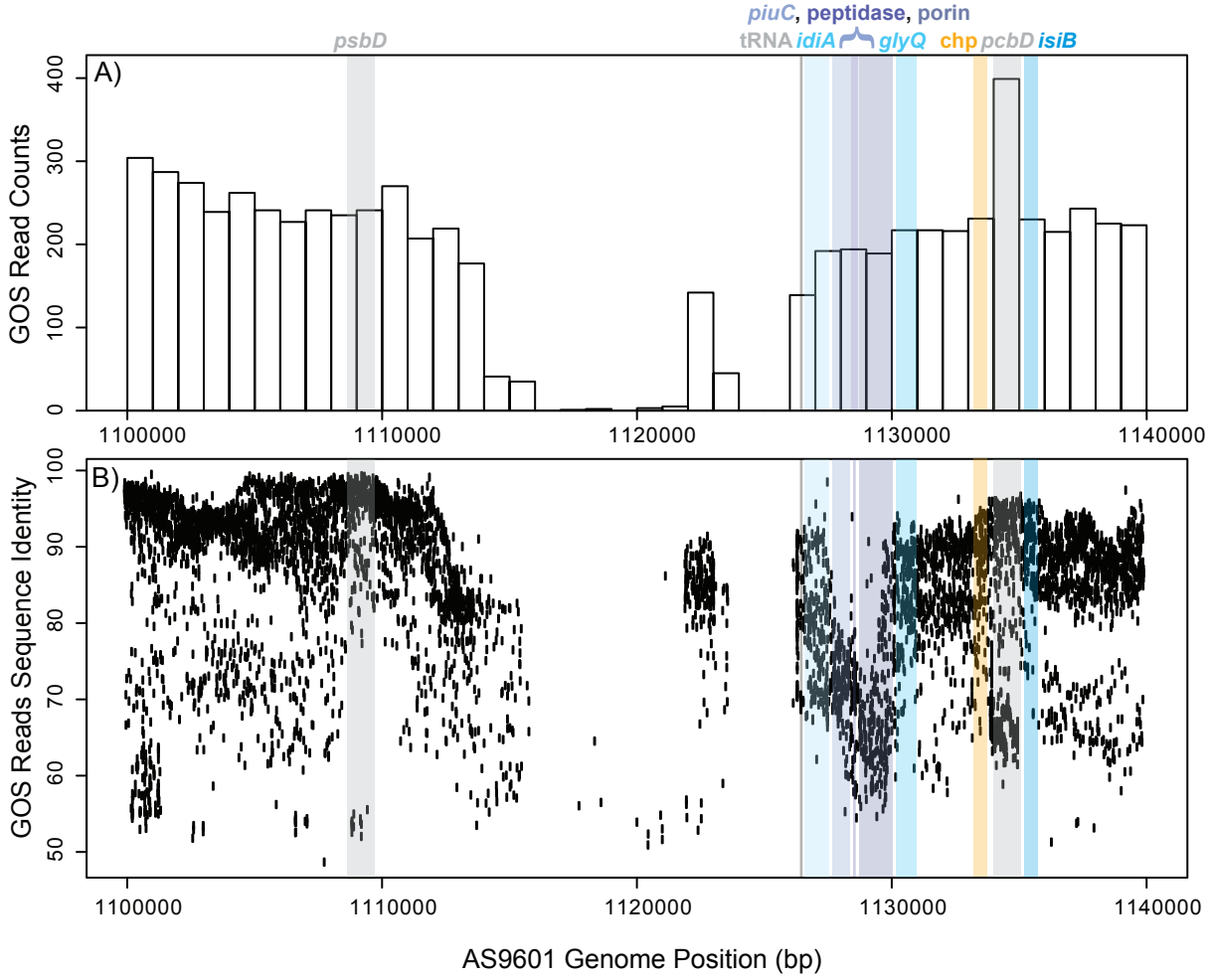

piuC, peptidase, porin
psbD
tRNA idiA glyQ chp pcbD isiB
A)
GOS Read Counts
0
100
200
300
400
1100000
1110000
1120000
1130000
1140000
B)
GOS Reads Sequence Identity
50
60
70
80
90
100
1100000
1110000
1120000
1130000
1140000
AS9601 Genome Position (bp)

Table 1: Properties of MED4 and MIT9313 with potential relevance to iron metabolism. For each iron-related gene listed, the number of orthologs present in each genome is shown, as well as the number of orthologs present in the *Prochlorococcus* (*Pro.*) core genome (i.e. genes shared by all 13 sequenced *Prochlorococcus*).

Origin of isolation [1]	**MED4**	**MIT9313**	***Pro.* core**
Isolation Site	Mediterranean	Gulf Stream	-
Isolation Depth	Surface (5m)	Deep (135 m)	-
Selected iron-related genes [1, 2, 3]			
Ferritin – iron storage	1	2	1
Flavodoxin (*isiB*) – iron-free electron transfer	1	1	1
Ferredoxin (*petF* and others) – iron-requiring electron transfer	6	7	3
idiA/afuA – putative iron ABC transporter, substrate binding protein	1	1	1
futB – putative iron ABC transporter, permease component	1	1	1
futC/sfuC - ABC transporter, ATP binding component, possibly iron transporter	1	1	1
fur – ferric uptake regulator	2	3	2
Cell physiology			
Cell Size (diameter)	0.5 - 0.7µm [4, 5]	0.8 - 1.2µm [5]	-
Light adaptation [6]	High-light	Low-light	-
Nitrogen sources utilized [7]	NH_4, urea	NH_4, urea, nitrite	-
Copper tolerance [8]	Higher	Lower	-

[1] Rocap *et al*. (2003); [2] MicrobesOnline (www.microbesonline.org) (Dehal *et al*, 2010); [3] ProPortal (http://proportal.mit.edu/); [4] Morel *et al*, 1993; [5] Ting *et al*, 2007; [6] Moore *et al*, 1999; [7] Moore *et al*, 2002; [8] Mann *et al*, 2002.

Titles and legends to figures

Figure 1: Steady-state growth rates over a range of dissolved iron (Fe') concentrations for MED4ax and MIT9313ax grown at 20µE $m^{-2}s^{-1}$.

Figure 2: Growth and cell properties of *Prochlorococcus* MED4 and MIT9313 over a time course of iron deprivation and recovery. The time-point at 0 hours was taken immediately after the cells were centrifuged and re-suspended in either +Fe (control) or –Fe (experimental) media. **(A, B)** Changes in cell concentration; **(C, D)** mean forward angle light scatter (FALS) relative to standard beads; **(E, F)** mean red fluorescence relative to standard beads. All points represent the mean of duplicate or triplicate cultures. Dotted line indicates iron addition to the -Fe treatment.

Figure 3: General features of the whole-genome transcription response of MED4 and MIT9313 to iron starvation and rescue. The number of genes that were differentially expressed at each time-point for **(A)** MED4, and **(B)** MIT9313**.** Median expression profiles of differentially-expressed genes in each hierarchical cluster during iron deprivation and rescue (R) for MED4 **(C)** and MIT9313 **(D)**. Clusters are listed in the legend and are identified by the cluster number at the beginning of each line. The differentially-expressed genes with predicted functions in each cluster are listed with the total number of differentially-expressed genes in the cluster given in parentheses at the end of each line. Some clusters contain only differentially-expressed genes of unknown function and these are omitted from the figure (for clarity) but presented in Supplementary Tables 1 and 2.

Figure 4: Genome position and fold change of differentially-expressed genes for **(A)** MIT9313, and **(B)** MED4. Each comparison (See Methods) is represented. Black crosses represent differentially-expressed genes that belong to the *Prochlorococcus* core genome (genes shared by all *Prochlorococcus*) and magenta crosses represent genes from the flexible genome (genes not shared by all *Prochlorococcus*). Gray points represent genes that were not significantly differentially expressed. Numbers of genes gained along the genomes (Kettler *et al*, 2007) are shown in thick gray lines (peaks indicate hypervariable regions) for MED4 and MIT9313. In addition, genomic islands (defined in Coleman *et al*, 2006) are displayed for MED4 as light blue boxes. Genomic islands have not been identified in MIT9313. For both MED4 and MIT9313, the proportion of differentially-expressed genes present in labile regions (genomic islands and/or hypervariable regions) is greater than the proportion of genes from the whole genome that are present in these labile regions ($p<0.001$ MED4, $p<0.01$ MIT9313, Fisher's exact test). Orange lines mark the location of the *idiA* region discussed in the text and displayed in Figures 7, S1 and S2.

Figure 5: The maximum absolute value of fold change expression of differentially-expressed MED4-MIT9313 shared genes (bi-directional orthologs between MED4 and MIT9313) from all comparisons (see Methods). Directionality of fold change for each gene can be found in Supplemental Tables 1and 2. Circles indicate genes that are present in the *Prochlorococcus* core genome and squares indicate genes that are non-core (i.e. flexible) but are MED4-MIT9313 shared genes (bi-directional orthologs between MED4 and MIT9313). Dashed lines mark an absolute value log2(fold change) of 1 (the thresholds for determining significance along with q-value - see Methods). Gray symbols: genes that are not differentially expressed. Cyan symbols:

genes differentially expressed in both MED4 and MIT9313. Red symbols: genes differentially expressed only in MED4. Blue symbols: genes differentially expressed only in MIT9313. *petF* encodes ferredoxin, an iron-requiring electron transfer protein. *isiB* encodes flavodoxin, an iron-free electron transfer protein known to substitute for *petF* during iron stress. *idiA* encodes a possible periplasmic iron-binding protein involved in iron transport. *hli05* and *hli08* are the names for this high light inducible (*hli*) gene in MED4 and MIT9313, respectively.

Figure 6: The *idiA* region of sequenced *Prochlorococcus* genomes. Genomes are organized vertically by phylogeny (Kettler *et al*, 2007). Asterisks indicate iron-induced transcription. The purple-tinted symbols represent genes not present in MED4 and include *piuC* (an uncharacterized iron-regulated protein – PMT0286), a possible peptidase (of the M20/M25/M40 family – PMT0285), and *som* (a possible porin – PMT0284). Blue-tinted symbols represent genes that are present in both MED4 and MIT9313. The yellow symbols represent an iron-induced conserved hypothetical gene (chp) not present in MIT9313. Black boxes represent tRNAs and dotted lines with slashes indicate extensive distances between genes along the genome.

Figure 7: Abundance and diversity of *Prochlorococcus*-like reads from the global ocean survey (GOS) database in the *idiA* region. The AS9601 genome best represents *Prochlorococcus* in the GOS database, thus is used as a reference genome here. Similar plots with MIT9312 and MED4 are provided in the Supplementary Materials (Figure S1 and S2). **A)** Abundance of recruited *Prochlorococcus*-like GOS reads aligning to AS9601, **B)** Percent similarity of recruited *Prochlorococcus*-like GOS

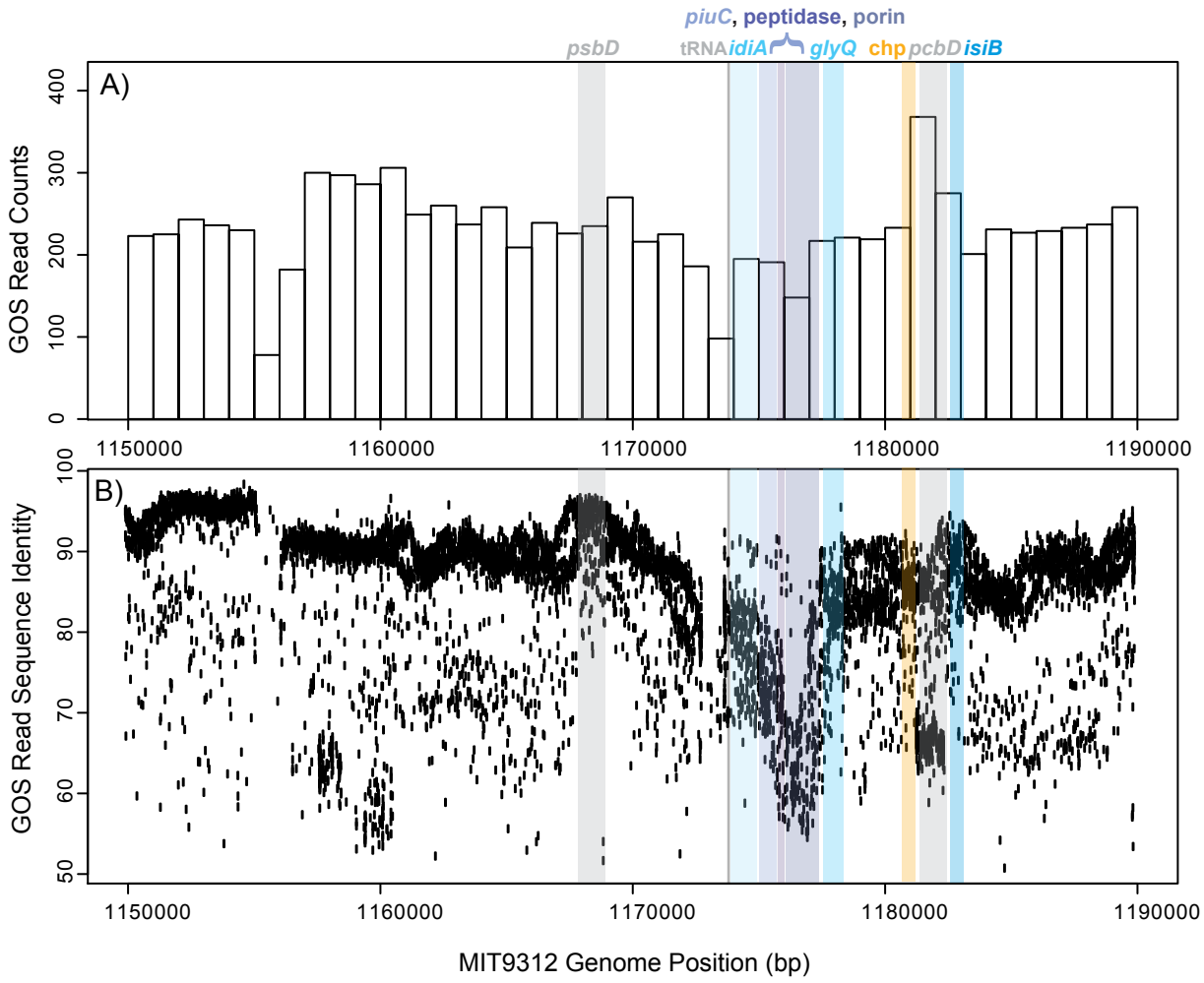
reads to the AS9601 genome. From left to right, genes of interest are *psbD* (a reference core gene encoding the PSII D2 protein), tRNA (black), *idiA* (cyan), *piuC* (purple, AS9601_13531), possible peptidase (purple, AS9601_13541), possible porin (purple, AS9601_13551), *glyQ* (cyan), conserved hypothetical gene (chp – yellow), *pcbD* (gray) – present in multiple copies in some *Prochlorococcus* genomes, and *isiB* (cyan).

Supplementary Figures 1 and 2 (S1, S2): Abundance and diversity of *Prochlorococcus*-like reads from the global ocean survey (GOS) database in the region of *idiA*. MIT9312 used as a reference genome (Figure S2) or MED4 as a reference genome (Figure S3). **A)** Abundance of *Prochlorococcus*-like GOS reads recruiting to MIT9312 or MED4. **B)** Percent similarity of *Prochlorococcus*-like recruited GOS reads to the sequenced genome. From left to right in MIT9312, they are *psbD* (a reference core gene encoding the PSII D2 protein), tRNA (black), *idiA* (cyan), *piuC* (blue – PMT1262), possible peptidase (blue - PMT1263), possible porin (blue – PMT1264), *glyQ* (cyan), conserved hypothetical gene (chp - yellow), *pcbD* (gray) – present in multiple copies in some *Prochlorococcus* genomes, and *isiB* (cyan). From left to right in MED4, they are *psbD* (a reference core gene encoding the PSII D2 protein), tRNA (black), *idiA* (cyan), *glyQ* (cyan), conserved hypothetical gene (chp - yellow), *isiB* (cyan). The genomic context of this region in MED4 and MIT9313 is marked by orange lines in Figure 4.

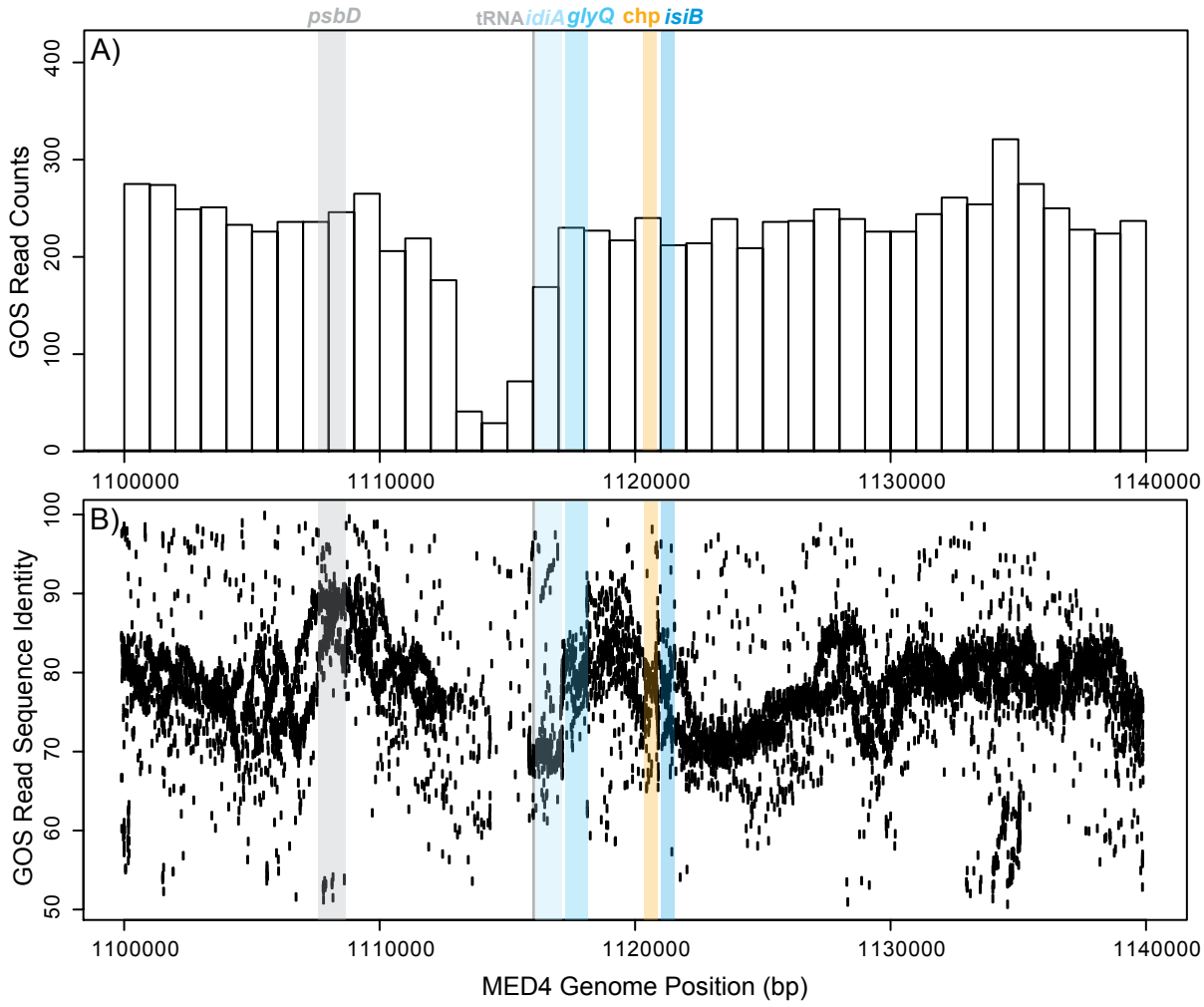
Supplementary Figure 3 (S3): Percent similarity of the *idiA* region genes of the remaining twelve sequenced *Prochlorococcus* isolates to the same genes in AS9601. Similarity was determined as in Rusch *et al*. (2007) and only genes aligning to at least

30% of the AS9601 query sequence were included in the box plot analysis. Horizontal lines in the middle of each box indicate the median percent similarity to AS9601 for each gene group and box top and bottom indicate the 75^{th} and 25^{th} percentile, respectively. See text and Figure 7 legend for gene descriptions.

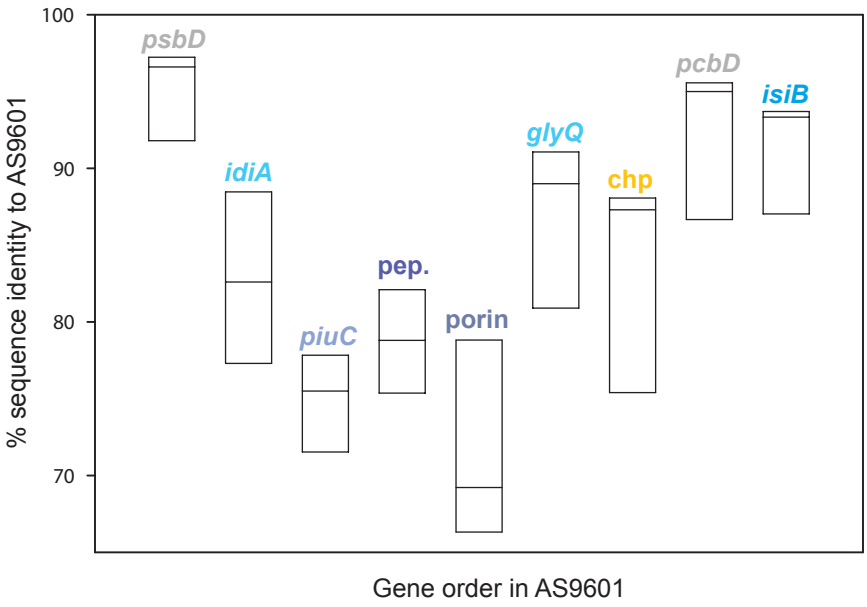
Supplementary Figure 1



Supplementary Figure 2



Supplementary Figure 3



Supplementary Table 1: Fold change and cluster membership of MED4 genes that are differentially expressed in response to changing iron availability.

Gene	Description	Cluster°	0	12	24	48	R*
PMED4_00651 (PMM0063)	conserved hypothetical protein	19	-0.08	**0.62**	**0.81**	**0.91**	**-1.53**
PMED4_00941 (PMM1805)	conserved hypothetical protein	9	-0.09	**-0.69**	**-0.69**	**-0.77**	**1.79**
PMED4_02321 (PMM0227)	ATP-sulfurylase (*cysD)*	9	-0.16	-0.33	**-0.74**	**-0.81**	**1.11**
PMED4_03261 (PMM1808)	conserved hypothetical protein	13	-0.20	0.98	**1.15**	0.06	-0.32
PMED4_03441 (PMM1811)	conserved hypothetical protein	9	-0.31	**-0.74**	**-0.73**	**-0.92**	**1.62**
PMED4_03461 (PMM0336)	conserved hypothetical (with homology to plastoquinol terminal oxidase)	19	-0.05	**1.01**	**0.97**	**1.14**	**-2.52**
PMED4_03462		9	0.43	-0.51	-0.07	-0.34	**1.49**
PMED4_03481 (PMM0337)‡†	conserved hypothetical protein	19	-0.05	**0.66**	**1.04**	**0.79**	**-1.70**
PMED4_03531 (PMM1815)	conserved hypotheical protein	19	0.34	**0.85**	**0.83**	**1.16**	**-1.21**
PMED4_03591 (PMM1873)	protein family PM-16	7	-0.07	-0.01	**-1.59**	-0.61	0.75
PMED4_03621 (PMM0345)‡	putative bacterioferritin comigratory protein	19	-0.10	0.31	**0.82**	**1.44**	**-1.47**
PMED4_03691 (PMM1820)	conserved hypothetical protein	6	0.12	**-1.02**	-0.35	**-1.05**	**2.09**
PMED4_03841 (PMM1826)	conserved hypothetical protein	9	0.31	**-0.76**	**-0.79**	**-0.92**	**1.76**
PMED4_03871 (PMM0362)	hypothetical	19	-0.04	**1.49**	**2.65**	**3.40**	**-4.06**
PMED4_03901 (PMM1828)	conserved hypothetical protein	19	-0.10	0.46	**0.93**	**1.51**	**-1.18**
PMED4_03961 (PMM1829)	conserved hypothetical protein	19	-0.23	**0.64**	**0.71**	**0.87**	**-1.81**
PMED4_03962		19	-0.32	**0.86**	**0.86**	**1.16**	**-1.22**
PMED4_03963		19	-0.18	**1.02**	**1.24**	**1.51**	**-1.97**
PMED4_03964		19	-0.24	0.76	**0.92**	**1.18**	**-1.78**
PMED4_03965		19	-0.03	**0.64**	**1.15**	**1.19**	**-1.37**
PMED4_03971 (PMM1830)	conserved hypothetical protein	13	0.18	**1.22**	0.66	**0.71**	**-1.38**
PMED4_03981 (PMM1831)	conserved hypothetical protein	19	0.18	**1.01**	**0.79**	**1.10**	**-1.07**
PMED4_03991 (PMM1832)	conserved hypothetical protein	19	0.39	**1.07**	**0.89**	**1.21**	**-1.22**
PMED4_04001 (PMM1833)	conserved hypothetical protein	19	-0.05	0.58	**0.65**	**0.69**	**-1.19**
PMED4_04031 (PMM0370)	putative cyanate ABC transporter, substrate binding protein	19	0.00	0.29	**0.56**	**0.75**	**-1.10**

PMED4_05091 (PMM0461)‡†	Cytochrome f (*petA)*	3	0.06	-0.10	**-0.69**	**-1.06**	**0.86**
PMED4_05101 (PMM0462)‡†	Rieske iron-sulfur protein (*petC*)	3	-0.20	-0.35	**-0.71**	**-1.01**	**0.88**
PMED4_05111 (PMM0463)	conserved hypothetical protein	19	0.11	**1.25**	**1.44**	**1.71**	**-2.04**
PMED4_05451 (PMM0496)	Putative principal RNA polymerase sigma factor (*sigA*, *rpoD*)	19	-0.51	0.02	0.37	0.62	**-1.13**
PMED4_06311 (PMM0582)‡†	Nucleoside-diphosphate-sugar epimerases	3	-0.23	-0.31	-0.52	**-0.51**	**1.14**
PMED4_06971 (PMM0647)	conserved hypothetical protein	19	-0.16	0.40	0.45	**0.66**	**-1.24**
PMED4_07081 (PMM1854)	conserved hypothetical protein	19	0.07	**0.56**	0.56	**1.02**	**-0.87**
PMED4_07131 (PMM1856)	conserved hypothetical protein	9	-0.52	**-0.95**	**-0.89**	**-1.14**	**1.95**
PMED4_07481 (PMM1864)	conserved hypothetical protein	19	0.00	**0.80**	**0.82**	**1.42**	**-1.34**
PMED4_07522		9	-0.22	-0.63	-0.26	**-0.58**	**1.75**
PMED4_07531 (PMM1868)	conserved hypothetical protein	9	-0.15	-0.09	0.07	-0.27	**1.87**
PMED4_07571 (PMM1870)	conserved hypothetical protein	16	0.10	0.28	0.46	**1.33**	**-0.56**
PMED4_07691 (PMM1874)	conserved hypothetical protein	19	0.45	0.81	**0.90**	**1.50**	**-1.27**
PMED4_07741 (PMM0702)	possible DUP family	19	-0.14	**0.75**	**0.68**	**1.12**	**-1.19**
PMED4_07751 (PMM1876)	conserved hypothetical protein	19	-0.24	0.44	**0.53**	**0.98**	**-1.14**
PMED4_07791 (PMM0705)‡	two-component response regulator, phosphate (*phoB*)	3	-0.06	-0.32	-0.48	**-1.01**	0.43
PMED4_08081 (PMM0731)	possible COMC family	13	0.06	0.54	0.19	0.35	**-1.02**
PMED4_08161 (PMM1885)	conserved hypothetical protein	22	0.74	**-2.28**	-0.14	0.51	-0.12
PMED4_08531 (PMM0768)‡†	glutamyl-tRNA reductase (*hemA*)	9	0.00	-0.41	**-0.72**	**-0.82**	**1.18**
PMED4_08911 (PMM0805)	Hypothetical (with homology to a Gram-negative pili assembly chaperone)	19	0.09	**1.74**	**1.80**	**2.01**	**-3.66**
PMED4_08921 (PMM0806)‡†	Bacterial regulatory proteins, Crp family	19	0.22	**1.24**	**1.51**	**1.73**	**-3.54**
PMED4_09091 (PMM0817)	possible high light inducible protein (*hli7*)	19	0.31	0.71	**0.95**	**1.23**	**-1.29**
PMED4_09101 (PMM0818)	possible high light inducible protein (*hli6*)	19	0.16	0.68	**0.91**	**1.06**	**-1.23**
PMED4_09321 (PMM0838)	possible Nucleoside diphosphate kinase	19	0.12	0.08	0.49	**0.69**	**-1.11**
PMED4_09651 (PMM1910)	conserved hypothetical protein	16	-0.10	**1.02**	**1.02**	**1.71**	**-0.79**
PMED4_09701 (PMM0861)	possible Virion host shutoff protein	19	0.05	**0.82**	**0.72**	**0.93**	**-1.19**
PMED4_09841 (PMM1915)	conserved hypothetical protein	19	-0.18	**0.81**	0.71	**1.24**	**-1.44**

PMED4_10041 (PMM0893)‡†	possible GTP cyclohydrolase II / 3,4-dihydroxy-2-butanone 4-phosphate synthase (*ribB*)	19	-0.02	**0.55**	**0.64**	**1.21**	**-1.12**
PMED4_10701 (PMM0958)	conserved hypothetical	19	-0.02	0.26	**0.88**	**1.14**	**-1.03**
PMED4_11021 (PMM1925)	conserved hypothetical protein	9	0.06	**-0.55**	-0.39	-0.44	**1.05**
PMED4_11221 (PMM1931)	conserved hypothetical protein	9	-0.27	**-0.59**	-0.28	-0.43	**1.09**
PMED4_11251 (PMM1000)	conserved hypothetical protein	3	-0.07	-0.30	-0.53	**-0.92**	**1.30**
PMED4_11391 (PMM1935)	conserved hypothetical protein	9	0.13	-0.20	-0.36	-0.59	**1.30**
PMED4_11531 (PMM1940)	conserved hypothetical protein	19	-0.01	0.52	**0.79**	**1.18**	**-1.04**
PMED4_11691 (PMM1949)	conserved hypothetical protein	3	-0.47	-0.52	-0.63	**-0.85**	**2.18**
PMED4_11701 (PMM1028)	conserved hypothetical (with homology to a Carboxylesterase)	9	0.20	-0.42	-0.32	**-0.61**	**1.94**
PMED4_11751 (PMM1032)	ABC transporter, substrate binding protein, possibly Mn (*iraI*)	19	-0.16	**0.69**	**0.95**	**0.86**	**-1.88**
PMED4_11781 (PMM1951)	conserved hypothetical protein	3	-0.51	**-0.81**	**-0.69**	**-1.29**	**2.43**
PMED4_11861 (PMM1041)	conserved hypothetical protein (with homology to a Hemagglutinin neuraminidase)	19	0.01	0.60	0.44	**1.03**	**-1.38**
PMED4_12641 (PMM1118)	possible high light inducible protein (*hli4*)	19	0.38	**0.92**	**0.96**	**1.10**	**-1.33**
PMED4_12832		19	-0.36	0.09	0.47	**0.56**	**-1.04**
PMED4_12871 (PMM1961)	conserved hypothetical protein	9	-0.13	**-0.99**	-0.39	**-0.77**	**1.64**
PMED4_12891 (PMM1135)‡†	possible high light inducible protein (*hli14*)	19	0.45	**0.94**	**1.12**	**1.55**	**-1.46**
PMED4_13281 (PMM1164)‡†	putative iron ABC transporter, substrate binding protein (*afuA*)	19	0.00	**1.47**	**1.59**	**1.68**	**-3.63**
PMED4_13351 (PMM1170)‡†	conserved hypothetical protein	19	-0.14	0.61	**0.82**	**0.87**	**-1.04**
PMED4_13361 (PMM1171)‡†	flavodoxin (*isiB*)	19	0.00	**1.23**	**1.56**	**1.84**	**-1.96**
PMED4_13941 (PMM1229)	dehydrogenase, E1 component	9	0.09	-0.17	-0.41	**-0.43**	**1.04**
PMED4_14491 (PMM1283)‡†	Integral membrane protein, interacts with FtsH	19	0.13	**0.55**	**0.85**	**0.84**	**-1.07**
PMED4_14671 (PMM1973)	conserved hypothetical protein	19	0.39	**1.38**	**1.03**	**1.11**	**-1.28**

PMED4_15201 (PMM1352)‡†	ferredoxin (*petF*)	6	0.26	**-1.06**	**-0.73**	**-1.09**	**1.64**
PMED4_15351 (PMM1365)	possible MATH domain	13	0.41	**1.12**	**0.59**	**0.80**	**-1.02**
PMED4_15431 (PMM1979)	conserved hypothetical protein	13	0.07	0.30	0.34	0.19	**-1.03**
PMED4_15442		9	-0.24	**-0.77**	**-0.60**	**-0.77**	**1.26**
PMED4_15451 (PMM1980)	conserved hypothetical protein (with homology to predicted protein family PM-23)	19	0.13	**0.73**	**0.63**	**1.04**	**-0.99**
PMED4_15481 (PMM1982)	conserved hypothetical protein	19	0.32	0.56	**0.97**	**1.11**	**-1.11**
PMED4_15491 (PMM1983)	conserved hypothetical protein	19	0.36	**0.74**	0.58	**1.08**	**-0.85**
PMED4_15531 (PMM1986)	conserved hypothetical protein	19	0.14	**1.10**	1.03	1.07	-0.42
PMED4_15541 (PMM1374)	possible Type I restriction modification DNA s	9	-0.09	-0.84	**-0.67**	**-1.30**	**2.08**
PMED4_15611 (PMM1989)	conserved hypothetical protein	9	-0.14	-0.53	**-0.69**	-0.48	**1.17**
PMED4_15621 (PMM1990)	conserved hypothetical protein	9	-0.30	-0.59	**-0.77**	**-0.87**	**2.22**
PMED4_15631 (PMM1991)	conserved hypothetical protein	9	0.12	-0.59	-0.59	**-0.96**	**2.20**
PMED4_15641 (PMM1379)	putative dape gene and orf2	9	0.03	-0.29	-0.38	-0.49	**1.33**
PMED4_15651 (PMM1992)	conserved hypothetical protein	3	-0.36	-0.39	**-0.71**	**-0.68**	**1.09**
PMED4_15652		9	0.06	**-0.82**	**-1.06**	**-1.47**	**1.96**
PMED4_15701 (PMM1994)	conserved hypothetical protein	9	0.30	-0.26	**-0.87**	**-1.02**	**1.28**
PMED4_15751 (PMM1996)	conserved hypothetical protein	19	-0.25	-0.03	0.75	0.98	**-1.48**
PMED4_15762		9	-0.20	-0.45	-0.17	-0.04	**1.14**
PMED4_15771 (PMM1998)	conserved hypothetical protein	9	-0.57	-0.48	-0.04	-0.08	**1.02**
PMED4_15781 (PMM1999)	conserved hypothetical protein	19	-0.31	0.24	**1.54**	**2.09**	**-1.55**
PMED4_15801 (PMM2001)	conserved hypothetical protein	1	0.02	-0.68	**-1.52**	-1.16	0.22
PMED4_15871 (PMM1391)	possible Helix-turn-helix protein, copG family	13	-0.01	0.49	0.20	0.32	**-1.36**
PMED4_15921 (PMM2004)	conserved hypothetical protein (with homology to protein family PM-15)	19	0.51	0.33	0.54	**1.03**	**-0.92**
PMED4_15931 (PMM1396)	possible high light inducible protein (*hli9*)	19	0.21	0.56	**0.85**	**0.98**	**-1.16**
PMED4_15941 (PMM1397)	possible high light inducible protein (*hli8*)	19	0.28	0.68	**0.93**	**1.17**	**-1.32**

PMED4_15971 (PMM1400)	possible Hemagglutinin-neuraminidase	19	0.23	**1.42**	**1.40**	**1.71**	**-2.37**
PMED4_15981 (PMM2005)	conserved hypothetical protein	19	0.51	**1.37**	**1.47**	**1.83**	**-2.07**
PMED4_16011 (PMM1402)	Conserved hypothetical protein	3	-0.23	-0.32	-0.62	**-0.79**	**1.24**
PMED4_16051 (PMM1404)‡	possible high light inducible protein (*hli5*)	19	0.46	**0.65**	**0.84**	**1.00**	**-1.12**
PMED4_16131 (PMM2010)	hypothetical protein	13	-0.09	0.41	0.12	0.12	**-1.06**
PMED4_16161 (PMM2012)	conserved hypothetical protein	9	0.06	-0.45	-0.95	**-1.22**	**1.82**
PMED4_16171 (PMM1412)	conserved hypothetical protein	19	-0.04	**1.13**	**1.67**	**2.08**	**-2.29**
PMED4_16221 (PMM2013)	conserved hypothetical protein	19	-0.08	**1.20**	**1.01**	**1.11**	**-1.77**
PMED4_16311 (PMM1424)	possible Uncharacterized protein family UPF003	19	-0.23	**0.74**	**0.79**	**1.15**	**-1.41**
PMED4_16481 (PMM1439)‡†	ATP synthase, Epsilon subunit (*atpC*)	9	-0.08	-0.24	**-0.66**	**-0.70**	**1.10**
PMED4_17291 (PMM1519)‡†	Photosystem I PsaL protein (subunit XI) (*psaL*)	3	-0.30	-0.46	**-0.67**	**-1.11**	**1.34**
PMED4_17301 (PMM1520)‡	photosystem I subunit VIII (*psaI*)	3	-0.25	-0.37	**-0.53**	**-0.80**	**1.11**
PMED4_18731 (PMM1663)‡†	putative photosystem I assembly related protein Ycf37	3	-0.21	-0.15	-0.52	**-0.64**	**1.10**
PMED4_asRNA_04601		19	0.54	**-0.79**	**1.84**	**1.48**	**-0.56**
PMED4_asRNA_07401		19	-0.22	0.21	**0.82**	**1.23**	**-1.13**
PMED4_ncRNA_Yfr10		3	0.02	-0.10	0.04	-0.70	**1.61**
PMED4_ncRNA_Yfr11		3	-0.63	**-0.69**	**-0.78**	**-1.16**	**1.83**
PMED4_ncRNA_Yfr16		9	-0.03	**-1.27**	**-1.15**	**-0.75**	**1.58**
PMED4_ncRNA_Yfr19		9	0.19	-0.29	-0.36	-0.41	**1.14**
PMED4_ncRNA_Yfr2		16	0.03	0.14	**0.79**	**1.71**	-0.27
PMED4_ncRNA_Yfr20		19	0.45	**1.01**	**0.97**	**1.56**	**-2.21**
PMED4_ncRNA_Yfr4		16	0.32	0.20	**0.80**	**1.50**	-0.12
PMED4_ncRNA_Yfr8		9	0.09	-0.55	-0.46	-0.43	**1.70**
PMED4_pseudo_3		3	-0.20	-0.28	-0.43	**-0.58**	**1.72**
PMED4_pseudo_6		4	-0.26	-0.83	-0.05	-0.54	**1.61**
PMM_tRNA-Ser2		19	0.13	0.29	**1.19**	**1.36**	-0.28

- All values indicate log2(-Fe/+Fe) with the exception of R where values are log2(experimental treatment after iron addition/experimental treatment before iron rescue).
- Values in bold print have q-values<0.01. Values in red bold print have q-values<0.01 and log2(fold change) greater than 1 or less than -1.

* Following iron addition to experimental treatment (R) – See Methods.

° Hierarchical clustering. Gene expression profiles of each cluster shown in Figure 3.

† *Prochlorococcus* core gene.

‡ MED4-MIT9313 shared gene (bi-directional ortholog of MED4 and MIT9313).

Supplementary Table 2: Fold change and cluster membership of MIT9313 genes that are differentially expressed in response to changing iron availability.

Gene	Description	Cluster°	0	16	28	53	R*
P9313_01131 (PMT0107)	conserved hypothetical protein	14	-0.19	0.45	0.06	**-1.24**	0.01
P9313_01141 (PMT0108)	ABC transporter, ATP binding protein	13	0.04	0.27	-0.20	**-1.02**	-0.24
P9313_01161 (PMT0110)	conserved hypothetical protein	13	-0.33	0.37	-0.14	**-1.64**	-0.02
P9313_01731	conserved hypothetical protein	10	-0.09	0.86	0.31	**-1.51**	-0.30
P9313_01981	conserved hypothetical protein	19	0.06	1.71	0.68	-0.05	**-1.28**
P9313_02141		20	-0.54	**1.55**	0.41	0.33	0.37
P9313_02281		19	0.14	**1.65**	0.46	0.07	-0.50
P9313_02851		18	0.25	0.69	0.29	**-1.33**	-0.38
P9313_03041		17	-0.24	-0.40	-0.62	**-1.03**	**2.00**
P9313_03111		26	0.24	-0.39	1.18	**1.10**	-0.24
P9313_03341 (PMT0283)‡†	glycyl-tRNA synthetase, alpha subunit (*glyQ*)	4	-0.11	**1.95**	**1.83**	**1.89**	**-1.71**
P9313_03361 (PMT0284)	possible porin (*som*)	2	-0.19	0.32	0.27	**1.51**	**-1.75**
P9313_03371 (PMT0285)	possible Peptidase family M20/M25/M40	3	-0.05	0.52	0.14	**1.35**	**-1.93**
P9313_03381 (PMT0286)	uncharacterized iron-regulated protein (*piuC*)	4	0.02	1.11	0.74	**1.18**	**-0.99**
P9313_03391 (PMT0287)‡†	putative iron ABC transporter, substrate binding protein (*futA1*)	4	-0.26	1.19	0.81	**2.03**	**-2.72**
P9313_03491		14	0.07	-0.38	0.06	**-1.03**	0.28
P9313_03641 (PMT0303)	possible Kelch motif	38	0.35	0.41	0.70	**1.22**	**-1.01**
P9313_03841	conserved hypothetical protein	30	-0.50	**-1.36**	-0.45	**-0.64**	0.69
P9313_03941 (PMT0328)	possible bromodomain adjacent to zinc finger domain, 2B...	13	-0.17	0.10	-0.16	**-1.13**	-0.11
P9313_05621 (PMT0477)	hypothetical	8	0.02	**1.07**	0.10	0.12	0.04
P9313_05661	conserved hypothetical protein	17	-0.52	0.28	-0.19	**-1.23**	0.08
P9313_05761		8	-0.10	0.93	0.26	**1.07**	0.19
P9313_05891		4	-0.09	**1.57**	**1.89**	**3.59**	**-3.72**

P9313_05901 (PMT0496)	light-harvesting complex protein (*pcbB*)	4	-0.13	**2.04**	**2.38**	**3.78**	**-3.70**
P9313_05911	conserved hypothetical protein	2	-0.15	0.06	1.09	**1.43**	**-1.49**
P9313_05921		4	-0.09	1.77	1.79	**2.02**	**-1.25**
P9313_05941 (PMT0498)	possible Gram-negative pili assembly chaperone	30	-0.11	**-1.74**	**-2.07**	**-1.96**	**2.87**
P9313_05951 (PMT0499)‡†	ferritin	23	-0.05	**1.20**	0.54	0.44	-0.12
P9313_06081 (PMT0510)‡†	GCN5-related N-acetyltransferase (*rimI*)	30	-0.06	**-1.92**	**-2.05**	**-1.61**	**1.28**
P9313_06881	conserved hypothetical protein	38	-0.04	0.75	1.04	**1.06**	-0.28
P9313_08511 (PMT0722)‡†	putative pantetheine-phosphate adenylyltransferase (*coaD*)	2	-0.11	0.34	0.53	**1.10**	**-1.10**
P9313_08731		4	0.05	0.94	0.43	**0.87**	**-1.01**
P9313_09371 (PMT0801)‡†	flavodoxin (*isiB*)	4	0.09	**1.81**	**1.79**	**3.19**	**-2.96**
P9313_09501	conserved hypothetical protein	14	0.16	-0.18	0.38	**-2.46**	-0.14
P9313_09581 (PMT0820)	conserved hypothetical protein	23	-0.03	**1.25**	0.40	0.40	-0.10
P9313_09691		1	-0.20	0.40	-0.16	**1.11**	-0.18
P9313_09901		4	-0.17	0.62	0.44	**1.39**	-0.51
P9313_09911 (PMT0842)	pyrimidine dimer DNA glycosylase	4	-0.07	**1.73**	**1.98**	**2.71**	**-2.91**
P9313_10101 (PMT0855)‡†	isochorismatase hydrolase family	4	-0.23	0.84	0.56	0.40	**-1.32**
P9313_10111 (PMT0856)	possible large-conductance mechanosensitive channel (*mscL*)	4	-0.10	0.20	0.82	**1.02**	**-1.57**
P9313_10171 (PMT0861)	uncharacterized protein conserved in bacteria	3	0.22	-0.21	0.17	**0.81**	**-1.10**
P9313_10181 (PMT0862)‡†	predicted Fe-S oxidoreductases	4	-0.14	0.99	1.00	**1.03**	**-1.21**
P9313_10851	conserved hypothetical protein	4	0.17	0.58	0.83	**0.64**	**-1.40**
P9313_10901 (PMT0911)	hypothetical	4	-0.03	**1.04**	0.50	**0.58**	-0.66
P9313_10911	conserved hypothetical protein	2	-0.56	-0.22	0.99	**1.52**	**-0.97**
P9313_10981 (PMT0916)	hypothetical	18	-0.38	0.54	-0.40	**-1.22**	-0.07
P9313_11171		14	-0.01	-0.69	-0.25	**-1.97**	-0.40
P9313_11641		3	1.22	-0.03	0.14	**1.55**	-0.23
P9313_12191		4	-0.17	0.26	0.61	0.88	**-1.07**

P9313_12361	conserved hypothetical protein	4	0.29	0.54	0.81	**1.28**	-0.60
P9313_12371 (PMT0992)	possible high light inducible protein (*hli7*)	4	-0.16	0.61	-0.07	**1.08**	**-2.78**
P9313_12381		2	0.09	0.15	0.87	**1.38**	-0.90
P9313_12581	conserved hypothetical protein	2	-0.03	0.26	0.88	**1.43**	-0.13
P9313_12771		38	-0.07	0.06	**1.10**	0.53	-0.51
P9313_12781 (PMT1018)	hypothetical	4	-0.07	0.60	0.41	**1.39**	**-0.87**
P9313_12801		4	-0.21	**5.53**	**4.01**	**6.14**	**-6.38**
P9313_12851 (PMT1019)	conserved hypothetical protein	38	0.23	0.11	0.60	**1.16**	**-0.85**
P9313_13161	Conserved hypothetical protein	8	0.06	**1.69**	-0.23	0.14	-0.94
P9313_13251	Conserved hypothetical protein	35	0.65	0.17	-0.10	**1.14**	0.12
P9313_13381		18	0.15	**1.54**	-0.09	-0.45	-0.21
P9313_13611		26	0.06	-0.56	0.53	**1.23**	-0.38
P9313_14621 (PMT1152)	possible high light inducible protein (*hli9*)	3	-0.16	0.05	-0.34	0.55	**-1.29**
P9313_14641 (PMT1154)‡†	possible high light inducible protein (*hli8*)	3	0.00	-0.18	-0.40	0.30	**-1.45**
P9313_14861	cytochrome b6-F complex subunit VII	30	0.12	**-1.31**	-0.65	-0.53	0.48
P9313_14961 (PMT1181)‡†	maf-like protein	30	-0.13	-0.87	-0.53	**-1.04**	**0.86**
P9313_15141	conserved hypothetical protein	17	-0.67	-0.59	-0.47	**-1.30**	0.61
P9313_15331 (PMT1212)‡†	conserved hypothetical protein (with homology to nitrogen regulatory protein P-II)	17	-0.06	-0.35	-0.53	**-1.40**	0.37
P9313_15411 (PMT1220)	possible photosystem I reaction centre subunit XII (*psaM*)	30	0.14	-0.87	-0.41	**-1.12**	**0.92**
P9313_16111 (PMT1277)	hypothetical	4	0.29	0.97	0.98	**1.77**	**-1.43**
P9313_16231	conserved hypothetical protein	2	0.11	0.02	1.39	**1.95**	**-2.18**
P9313_16241	conserved hypothetical protein	3	0.41	0.09	-0.15	**1.24**	**-0.94**
P9313_16251 (PMT1288)	conserved hypothetical	4	0.19	0.65	**1.71**	**1.92**	**-2.25**
P9313_16611	guanylate kinase (*gmk*)	30	-0.18	**-1.44**	-0.78	**-0.99**	**0.81**
P9313_17651	conserved hypothetical protein	17	0.06	-0.76	-0.62	**-1.23**	0.52
P9313_18001		30	-0.17	-1.17	-1.06	**-1.17**	**0.98**
P9313_18021		35	0.15	**-1.63**	-0.30	-0.01	0.51

P9313_18041 (PMT1429)‡†	2Fe-2S Ferredoxin:Ferredoxin (*petF*)	30	0.01	-1.02	-0.71	**-0.84**	**1.27**
P9313_18941‡	conserved hypothetical protein	30	-0.09	-0.98	-0.75	**-1.40**	0.79
P9313_19161	conserved hypothetical protein	38	0.57	0.93	1.19	**2.13**	**-1.07**
P9313_19261	conserved hypothetical protein	18	-0.33	1.23	-0.10	**-1.17**	-0.59
P9313_19501	conserved hypothetical protein	2	-0.05	-0.15	0.24	**1.43**	-0.53
P9313_19551 (PMT1554)‡†	pentapeptide repeats	4	-0.31	**2.52**	**2.49**	**4.50**	**-4.29**
P9313_19831 (PMT1570)	conserved hypothetical protein	13	0.32	0.03	-0.25	**-1.09**	0.01
P9313_19851 (PMT1572)	conserved hypothetical protein	27	0.06	-0.81	-0.11	**-1.50**	0.48
P9313_19981	conserved hypothetical protein	3	0.90	0.70	-0.38	**1.06**	-0.22
P9313_20721	conserved hypothetical protein	3	0.86	0.33	0.20	**1.19**	-0.13
P9313_20761		3	-0.03	-0.80	0.04	0.55	**-1.23**
P9313_21301	conserved hypothetical protein	38	-0.78	0.46	0.64	**1.03**	**-1.52**
P9313_21331		17	-0.12	-0.28	-0.58	**-1.04**	**1.21**
P9313_21571		13	-0.03	0.31	-0.41	**-1.32**	0.21
P9313_21601		39	0.09	-0.05	0.80	**-1.06**	-0.19
P9313_22121	conserved hypothetical protein	16	0.07	-0.70	-0.83	**-1.29**	0.45
P9313_22981	conserved hypothetical protein	16	0.37	-0.05	-0.24	**-1.16**	0.79
P9313_23111 (PMT1827)	hypothetical	17	-0.16	-0.04	-0.33	**-1.06**	0.35
P9313_23481		4	0.03	0.66	0.26	**1.39**	**-0.77**
P9313_24261 (PMT1927)	putative glycosyl transferase, group 1	27	-0.24	**-1.20**	-0.05	-0.43	0.04
P9313_24921	conserved hypothetical protein	35	0.29	**-1.94**	-0.88	0.11	0.34
P9313_25471		13	-0.26	0.15	-0.33	**-1.38**	0.03
P9313_28041		17	-0.18	-0.59	-0.48	**-1.09**	**1.17**
P9313_28051	conserved hypothetical protein	17	-0.11	**-1.09**	**-0.77**	**-1.39**	**1.62**
P9313_28071	conserved hypothetical protein	17	0.11	-0.44	-0.57	**-1.45**	0.59
P9313_28081 (PMT2240)	formate and nitrite transporters	30	-0.31	-0.96	-0.66	**-1.04**	**1.00**
PMT_ffs		35	0.04	**-2.02**	-0.09	1.01	-0.25
tRNA-Ala2		3	0.44	0.44	0.10	**1.91**	-0.81
tRNA-Arg2		3	0.45	-0.02	0.24	**1.28**	-0.62
tRNA-Asn1		35	-0.16	**-2.62**	-0.74	-0.13	-0.26

tRNA-Glu1		2	-0.09	-0.06	0.06	**1.02**	-0.46
tRNA-Leu1		35	-0.40	**-1.25**	-0.63	-0.52	-0.72
tRNA-Thr3		3	0.28	-0.02	0.12	**1.63**	-0.98
ncRNA_Yfr2-5_1		1	-0.03	0.62	**-1.20**	**2.01**	-0.74
ncRNA_Yfr7		2	0.15	-0.18	-0.36	**1.14**	-0.32

- All values indicate log2(-Fe/+Fe) with the exception of R where values are log2(experimental treatment after iron addition/experimental treatment before iron rescue).
- Values in bold print have q-values<0.01. Values in red bold print have q-values<0.01 and log2(fold change) greater than 1 or less than -1.

* Following iron addition to experimental treatment (R) – See Methods.

° Hierarchical clustering. Gene expression profiles of each cluster shown in Figure 3.

† *Prochlorococcus* core gene.

‡ MED4-MIT9313 shared gene (bi-directional ortholog of MED4 and MIT9313).

Supplementary Table 3: Categories of genes differentially-expressed during iron stress and rescue in MED4 and MIT9313.

	Whole genome	Core genome°	Flexible genome*	MED4-MIT9313 shared genes‡	MED4-MIT9313 non-shared genes˙
MED4	**125**/2022	**16**/1223 (**12.8%**)	**109**/799 (**87.2%**)	**20**/1159 (**16.0%**)	**105**/863 (**84.0**%)
MIT9313	**111**/2906	**13**/1223 (**11.7%**)	**98**/1683 (**88.3%**)	**14**/1159 (**12.6%**)	**97**/1747 (**87.4**%)

Bold numbers refer to genes that were significantly differentially-expressed in response to iron starvation or rescue (R) and non-bold refers to the total genes in each category. The top set of numbers indicates the number of genes in each of the categories (differentially-expressed over total in the category). The bottom set of numbers in bold indicates the percentage of the differentially-expressed genes that are in each category.

° Core genome: Genes shared by all 13 sequenced *Prochlorococcus* genomes (Kettler *et al*, 2007 with the addition of MIT9202, this study).

* Flexible genome: Genes in MED4 or MIT9313 that are not shared by all 13 *Prochlorococcus* genomes (Kettler *et al*, 2007 with the addition of MIT9202, this study).

‡ MED4-MIT9313 shared genes: Bi-directional orthologs of MED4 and MIT9313 (Kettler *et al*, 2007; http://proportal.mit.edu/). Note that most, but not all, MED4-MIT9313 shared genes are also *Prochlorococcus* core genes. Also, because a gene can be *Prochlorococcus* core (Kettler *et al*, 2007; http://proportal.mit.edu/) without being a bi-directional ortholog of MED4 and MIT9313, the number of *Prochlorococcus* core genes is greater than the number of MED4-MIT9313 shared genes.

˙ MED4-MIT9313 non-shared genes: Genes that are not bi-directional orthologs of MED4 and MIT9313 (Kettler *et al*, 2007; http://proportal.mit.edu/).