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# Genetic Identification of a High-Affinity Ni Transporter and the Transcriptional Response to Ni Deprivation in *Synechococcus* sp. Strain WH8102

**C. L. Dupont,[a,d] D. A. Johnson,[c] K. Phillippy,[c]* I. T. Paulsen,[b,c] B. Brahamsha,[a] and B. Palenik[a]**

Scripps Institution of Oceanography, University of California, San Diego, San Diego, California, USA[a]; Macquarie University, Sydney, Queensland, Australia[b]; J. Craig Venter Institute, Rockville, Maryland, USA[c]; and J. Craig Venter Institute, San Diego, California, USA[d]

**One biological need for Ni in marine cyanobacteria stems from the utilization of the Ni metalloenzyme urease for the assimilation of urea as a nitrogen source. In many of the same cyanobacteria, including *Synechococcus* sp. strain WH8102, an additional and obligate nutrient requirement for Ni results from usage of a Ni superoxide dismutase (Ni-SOD), which is encoded by *sodN*. To better understand the effects of Ni deprivation on WH8102, parallel microarray-based analysis of gene expression and gene knockout experiments were conducted. The global transcriptional response to Ni deprivation depends upon the nitrogen source provided for growth; fewer than 1% of differentially expressed genes for Ni deprivation on ammonium or urea were concordantly expressed. Surprisingly, genes for putative Ni transporters, including one colocalized on the genome with *sodN*, *sodT*, were not induced despite an increase in Ni transport. Knockouts of the putative Ni transporter gene *sodT* appeared to be lethal in WH8102, so the genes for *sodT* and *sodN* in WH8102 were interrupted with the gene for Fe-SOD, *sodB*, and its promoter from *Synechococcus* sp. strain WH7803. The *sodT*::*sodB* exconjugants were unable to grow at low Ni concentrations, confirming that SodT is a Ni transporter. The *sodN*::*sodB* exconjugants displayed higher growth rates at low Ni concentrations than did the wild type, presumably due to a relaxed competition between urease and Ni-SOD for Ni. Both *sodT*::*sodB* and *sodN*::*sodB* lines exhibited an impaired ability to grow at low Fe concentrations. We propose a posttranslational allosteric SodT regulation involving the binding of Ni to a histidine-rich intracellular protein loop.**

As the most abundant photoautotrophs on the planet, the cluster 5.1 marine cyanobacteria *Synechococcus* and *Prochlorococcus* contribute greatly to the global carbon and nitrogen cycles. In addition to the classical macronutrients nitrogen, phosphorus, and carbon, marine cyanobacteria also require several micronutrients such as Fe and Co in appreciable-enough quantities to affect their distribution and abundance in natural marine systems (32, 51). Recently, Ni additions were also shown to prompt growth in select natural populations of *Synechococcus* and *Prochlorococcus* (14), suggesting that Ni also influences the distributions of cyanobacteria in the ocean.

In *Synechococcus* and *Prochlorococcus*, the biological requirements for Ni arise from the usage of two known enzymes, though others may be currently unknown. Marine *Synechococcus* bacteria require a Ni metalloenzyme, urease, to use urea as a nitrogen source (10) and possibly for arginine catabolism (44). Without added Ni, *Synechococcus* cultures provided with urea as a nitrogen source will cease to grow, with growth being restored by the addition of either Ni, resulting in a functional urease, or $NH_4^+$, which does not require Ni for assimilation (13). Some *Synechococcus* and all *Prochlorococcus* genomes sequenced or assembled from metagenomic data contain a gene (*sodN*, *synw1626* in WH8102) coding for a Ni-containing superoxide dismutase (Ni-SOD) (15, 39, 50, 64). Growth without Ni for *Synechococcus* sp. strain WH8102 results in reduced SOD activity, regardless of the nitrogen source used for growth, which is eventually lethal (13, 43). Some *Synechococcus* strains, like CC9311, have a second SOD containing a Cu/Zn active site, which appears to facilitate growth without Ni, but only when supplied with $NH_4^+$ as a nitrogen source. Thus far, the few marine *Synechococcus* sp. strains lacking a Ni-SOD (e.g., strains WH7803 and WH7805) contain the gene for a Fe-containing SOD (*sodB*) in the genomic location corresponding to *sodN* (Fig. 1A). This evolutionary exchange has been hypothesized to result in a loss of an obligate Ni requirement, but with increased Fe requirements as a consequence (15).

In cyanobacteria, nutrient deprivation often induces a stress response specific to the nutrient but also a general stress response due to the perturbations in the intracellular balance of metabolites (52). In both CC9311 and WH8102, declining intracellular Ni concentrations induce an increase in the activity of a Ni-specific high-affinity ($K_m$ = 5 nM) uptake system, maintaining intracellular Ni concentrations at 100 to 300 nM (14). In the simplest sense, this Ni-specific stress response requires a transporter and a regulator. In other bacteria, Ni uptake has been shown to be catalyzed by one of four families of transporters, the Ni/Co permeases with eight transmembrane domains, HupE/UreJ-like permeases, the ABC-type NikABCDE transporters, and the ABC-type NikMNQO transporters (47). In the WH8102 genome, two genes code for proteins in the *hupE/ureJ* permease family. One gene (*synw1628*), called *sodT* in one review (17), colocalizes with *sodN* in all cyanobacterial genomes that contain it but is absent from all

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Address correspondence to B. Palenik, bpalenik@ucsd.edu.
* Present address: K. Phillippy, National Center for Biotechnology Information, National Library of Medicine, National Institutes of Health, Bethesda, Maryland, USA.
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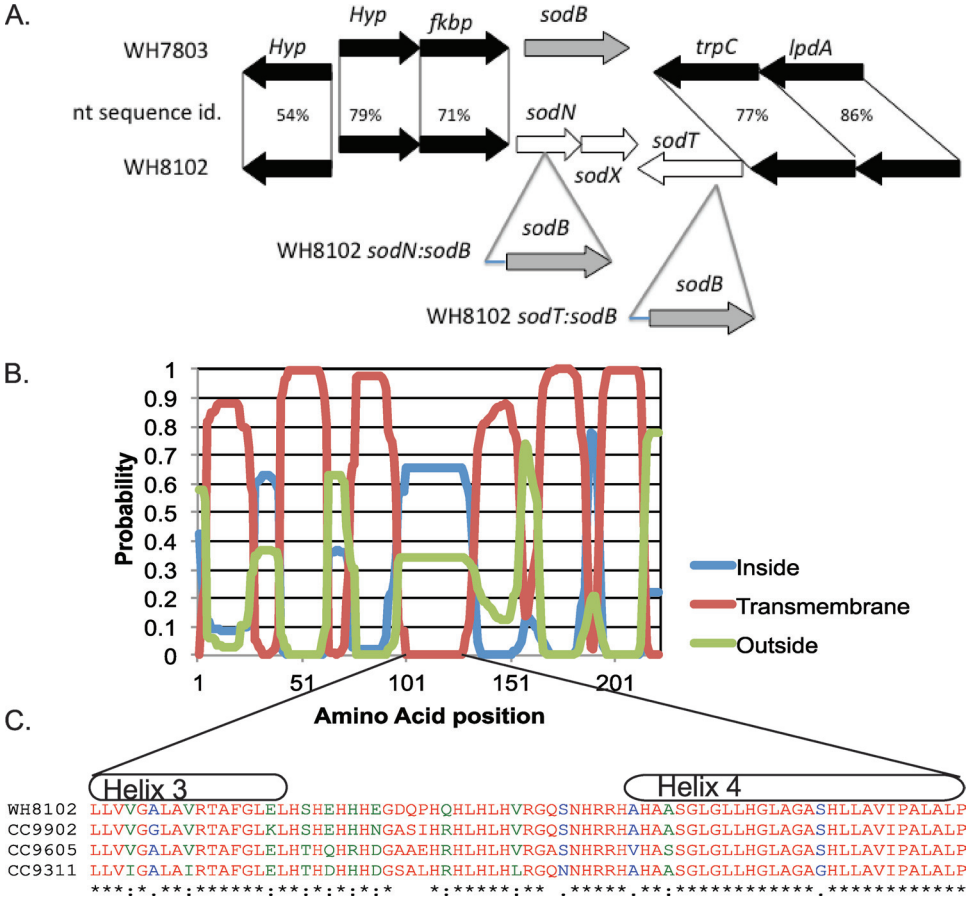


**FIG 1** Genomic locales of *sodN* and *sodT*. (A) A comparison of the genomic locations where *sodB* and *sodN* are found in WH7803 and WH8102, respectively, as well as the nucleotide sequence identities (nt sequence id.) of flanking genes. Also shown are the *sodN*::*sodB* and *sodT*::*sodB* lines developed for this study. The short extension at the N terminus of the *sodB* sequence indicates the 75 bp upstream of this gene in WH7803. Note that Kan$^r$ is also inserted into the indicated site along with *sodB* but is not shown here for clarity. (B) The predicted locations of transmembrane domains in *sodT* using TMHMM (27). (C) The sequences of the intracellular loop found between transmembrane helices 3 and 4 in WH8102, CC9902, CC9605, and CC9311.

*sodB*-containing genomes, indicating a clear linkage between *sodN* and *sodT* (Fig. 1A). *sodT* codes for a protein with six predicted transmembrane domains (Fig. 1B), a topology associated with the HupE/UreJ Ni transporter family (17). Between transmembrane helices 3 and 4, an intracellular domain contains a high percentage of the Ni-binding amino acid histidine (Fig. 1C). The other putative Ni/Co permease in the WH8102 genome also has sequence similarity to the hydrogenase-associated Ni transporter *hupE* (*synw2127*), and yet the presence of a predicted $B_{12}$ riboswitch in the upstream promoter region of the gene led to the conclusion that the WH8102 *hupE* transports Co (47). The WH8102 genome contains a putative transcriptional unit (*synw0708-synw0709*) coding for an ABC transporter in the nickel/peptide/opine family, where *synw0709* codes for the putative periplasmic substrate-binding protein. While a homolog of *sodT* is found exclusively in *sodN*-containing *Synechococcus* and *Prochlorococcus* genomes, homologs of *hupE* (*synw2127*) and *synw0709* are also found in the *sodB*-containing *Synechococcus* genomes such as those of strains WH7803 and WH7805.

Ni uptake in many bacteria is regulated at the transcriptional level by the protein NikR (12). In *Escherichia coli*, NikR is a negative repressor of the ABC-type *nikABCDE* operon expression, thereby controlling Ni uptake activity (12). NikR also controls the expression of a Ni transporter in the Ni/Co permease family in *Helicobacter pylori* (18), indicating a certain evolutionary ability to exchange regulator and transporter pairs. Another Ni regulatory protein, Nur, is a divergent ferric uptake regulator (Fur) family protein that represses Ni uptake in *Streptomyces* when intracellular Ni concentrations are sufficient for growth and Ni-SOD function (1). Homologs of NikR and Nur are absent from the genomes of WH8102 and other *sodN*-containing cyanobacterial genomes, implying a novel form of Ni transport regulation in these organisms.

Little is known about general stress responses to Ni deprivation, though an examination of the hypothetical biochemical consequences of Ni deprivation provides several hypotheses. *A priori*, Ni starvation should induce increased oxidative stress due to the reduced amounts of active Ni-SOD. Ni deprivation could also reduce the amount of active urease in the cell, affecting anabolic (10) and catabolic (44) N cycling and, as a result, the balance between carbon and nitrogen assimilation. The nitrogen source provided for growth should modulate the relative importance of catabolic and anabolic urease roles; growth on $NH_4^+$ should reduce the anabolic role of urease. To elucidate the molecular dynamics behind enhanced Ni uptake and the general stress responses to Ni deprivation, the global changes in gene expression prompted by Ni deprivation were studied using full-genome mi-

**TABLE 1** Organisms and plasmids used in this study

| Strain or plasmid | Relevant characteristic(s) | Reference(s) or source |
|---|---|---|
| Bacterial strains | | |
| *Synechococcus* | | |
| WH8102 | Clade III, used for microarray studies | 39 |
| WH8102 *sodN*::*sodB1* | WH8102 conjugated with pM*sodN*::*sodB* | This work |
| WH8102 *sodT*::*sodB4* | WH8102 conjugated with pM*sodT*::*sodB* | This work |
| WH8102 *sodT*::*sodB5* | WH8102 conjugated with pM*sodT*::*sodB* | This work |
| CC9311 | Clade I, coastal isolate, chromatic adapting | 39a |
| CC9605 | Clade II, abundant serotype in stratified California current | 59a |
| CC9902 | Clade IV, coastal isolate | 57 |
| *E. coli* | | |
| MC1061 | Host for pRK24, pRL528; donor in pMUT conjugations | 17a |
| DH5α | Recipient in transformations | |
| Plasmids | | |
| pMUT100 | $Kan^r$ $Tet^r$; suicide vector | 6, 7 |
| pRK24 | $Tc^r$ $Amp^r$; conjugal plasmid, RK2 derivative | 6, 7 |
| pRL528 | $Cm^r$; helper plasmid, carries *mob* | 6, 7 |
| pCR2.1-TOPO | $Kan^r$ $Amp^r$; PCR product cloning vector | Invitrogen |
| pMSYNW*sodT* | pMUT100 containing *synw1628* fragment of nt[a] 174584 to 174839 | This work |
| pMSYNC*sodT* | As above: *sync0753* fragment of nt 703532 to 703832 | This work |
| pM9902*sodT* | As above: SynCC9902_0871 fragment of nt 842654 to 842947 | This work |
| pM9605*sodT* | As above: SynCC9605_1528 fragment of nt 14748101 to 1478370 | This work |
| pMSYNW*sodN* | As above: *synw1626* fragment of nt 173466 to 173758 | This work |
| pM*sodN*::*sodB* | pMSYNW*sodN* containing WH7803 *sodB* and promoter (nt 1594036 to 1594704) | This work |
| pM*sodT*::*sodB* | pMSYNW*sodT* containing WH7803 *sodB* and promoter (nt 1594036 to 1594704) | This work |

[a] nt, nucleotide.

croarrays. In parallel, gene knockouts were used to determine the identity of the Ni transporter in WH8102.

## MATERIALS AND METHODS

**Strains and culturing techniques.** *E. coli* strains MC1061(pRL528, pRK24) and DH5α (Table 1) were grown in Luria-Bertani medium. When appropriate, ampicillin (100 μg/ml), kanamycin (50 μg/ml), and chloramphenicol (30 μg/ml) were used for the selection and maintenance of plasmids in *E. coli*. *Synechococcus* sp. strains WH8102, CC9605, CC9902, and CC9311 (Table 1) were grown in SOW medium made using the trace metal clean methods described in the work of Dupont et al. (13). Unless indicated, all cultures were grown at a constant light level of 50 μmol photons $m^{-2}$ $s^{-1}$ and a constant temperature of 19°C (CC9311) or 24°C (WH8102, CC9605, and CC9902). In the experiments presented here, variations were made in the nickel concentrations and the nitrogen source added for growth. $NiCl_2$ was added in final concentrations of 500 pM, 1 nM, 5 nM, or 50 nM to achieve $Ni^{2+}$ concentrations of 0.5, 1, 5, and 50 pM, respectively, as determined using MINEQL (63). Another set of experiments varied Fe concentrations from 740 to 7.4 μM total Fe. Chelex-100-purified 1 M $NH_4Cl$, 1 M $NaNO_3$, or 1 M urea was added to a final concentration of 2 mM N for growth on the different N sources.

The cultures for the growth rate and microarray experiments were grown in "semicontinuous" fashion, with constant Ni-limited exponential growth being achieved and maintained through transfer. With macronutrients added in great excess and bioavailable inorganic trace metal concentrations being buffered by EDTA, stable exponential growth rates and intracellular metal concentrations can be achieved, particularly if cultures are maintained at modest cell density (42, 55). When grown in this fashion, the cultures are essentially chemostats, where the limiting nutrient is the free metal concentration (55). Culture phycoerythrin fluorescence (Turner 10-AU; Turner Instruments, CA) was monitored as a proxy for growth, and rates were determined by slope of the linear ln (phycoerythrin fluorescence)-versus-time relationship. Cultures were transferred during exponential growth to media with the same [$Ni^{2+}$] until growth rates stabilized, at which time they were allowed to grow for 4 to 5 more generations prior to harvesting.

One experiment examined the transcriptional and Ni uptake response of Ni-starved *Synechococcus* WH8102 to the addition of Ni. Here, a 2-liter culture of WH8102 was grown on $NH_4^+$ without any added Ni. Following the cessation of growth, increasing additions of $NiCl_2$ were made over the next 96 h. Two-hundred-fifty-milliliter samples were removed using trace metal clean techniques at each time point in a HEPA-filtered laminar flow bench.

**RNA isolation.** Cultures were harvested by centrifugation (10,000 × *g* for 10 min) in acid-washed and autoclaved 250-ml polyethylene bottles. Cells were lysed in TRIzol (Invitrogen, Carlsbad, CA) by incubation at 60°C for 1 h, followed by RNA isolation according to the manufacturer's instructions. DNA was removed, and RNA was further purified using a Qiagen RNeasy kit with DNase digestion according to the manufacturers' instructions.

**Microarray hybridizations and analysis.** The WH8102 whole-genome microarray and the methods for cDNA synthesis, labeling, hybridization, and scanning have been presented previously (54, 58). Here, RNAs from eight urea-grown cultures and six ammonium-grown cultures were used. Fourteen different hybridizations were carried out, of which seven used different RNA pools and seven were replicates of these seven experiments, either dye swapping experiments or direct replicates. Statistical analyses were carried out using the Significance Analysis of Microarrays (SAM) software package (60), with the hybridizations treated as independent experiments. SAM orders the genes by using a modified *t* statistic called relative difference based on the ratio of change in gene expression to standard deviation in the data for that gene. It declares a gene to be up- or downregulated if the difference (*D* value) between the observed relative difference and expected relative difference is above (*D* value $> 0$) or below (*D* value $< 0$) the global cutoff point, respectively (60). This procedure allows estimation of the median of false discovery rates, and a gene was considered down- or upregulated by Ni deprivation if it displayed a negative or positive *D* value beyond the selected 1% me-

**TABLE 2** Primers used in this study

| Name | Sequence | Purpose |
|---|---|---|
| Qsynw1628for | TCTGGTCCATGTTGAGACGA | Q-PCR of *synw1628* |
| Qsynw1628rev | TGACTGTGCAGCTCAAGACC | Q-PCR of *synw1628* |
| Qsynw2127for | GACCAATCTTCTGGCTTCTCC | Q-PCR of *synw2127* |
| Qsynw2127rev | GGGCTGGAGATCAATAGCAA | Q-PCR of *synw2127* |
| Qsynw1709for | TGAAGATTGCAGACCGACAG | Q-PCR of *synw0709* |
| Qsynw1709rev | AGTTGCGAAAGGCTGAAAAG | Q-PCR of *synw0709* |
| Incsynw0635for | GTTGCTGCACCCTCCA | Inactivation of *synw0635* |
| Incsynw0635rev | AGCAGCCCACAAGGATCA | Inactivation of *synw0635* |
| Incsynw1628for | CAGCATCCTCACCGGTTT | Inactivation of WH8102 *sodT* |
| Incsynw1628rev | GGCACCCACCACCAGTAG | Inactivation of WH8102 *sodT* |
| Incsynw1626for | GTGTCGCGGCTGAAGCC | Inactivation of WH8102 *sodN* |
| Incsynw1626rev | CAGTTCCTCAGCTTTGGC | Inactivation of WH8102 *sodN* |
| Incsync0753for | AGCCATGAAGTCAGGAATGG | Inactivation of CC9311 *sodT* |
| Incsync0753rev | CTGGTCTTGGATTGCTCCAT | Inactivation of CC9311 *sodT* |
| IncSyncc9902_1528for | CCAGAAGATGACTCGCACCT | Inactivation of CC9902 *sodT* |
| IncSyncc9902_1528rev | CGATTGGCTTGAAAGACCTC | Inactivation of CC9902 *sodT* |
| IncSyncc9605_0871for | GCAGAGCATCGTCATCTTCA | Inactivation of CC9605 *sodT* |
| IncSyncc9605_0871rev | GATCGAGAGAGCACCTGTCC | Inactivation of CC9605 *sodT* |
| pMUTgibsonFor | CTGTTGCCCGCTTGGCGGGGCTGCTAGCGCTATATGCGTTGATG | Linearization of pMUT vectors with *sodB* sticky ends |
| pMUTgibsonRev | CCAACCTGGCTGCCGCCTGACCGCAAGAGGCCCGGCAGTA | Linearization of pMUT vectors with *sodB* sticky ends |
| Synwh7803_1742gibsonFor | CGCATATAGCGCTAGCAGCCCCGCCAAGCGGGCAACAG | Adding pMUT sticky ends to WH7803 $sodB^+$ promoter sequence |
| Synwh7803_1742gibsonRev | TACTGCCGGGCCTCTTGCGGTCAGGCGGCAGCCAGGTTGG | Adding pMUT sticky ends to WH7803 $sodB^+$ promoter sequence |
| VerSynw1626For | ATGCTGCGCTCGGCCCTCACCG | PCR verification of *sodN*::*sodB* |
| VerSynw1626Rev | GCCCTTGGATTGCCAGAACATGCC | PCR verification of *sodN*::*sodB* |
| Versynw1628 for | GGATCAGGGCTCGTTGCTGGTGA | PCR verification of *sodT*::*sodB* |
| Versynw1628 rev | GTGCAGCTCAAGACCGAAG | PCR verification of *sodT*::*sodB* |

dian false discovery rate. An additional fold change cutoff of |0.5| was used.

**Reverse transcriptase quantitative PCR (RT-Q-PCR).** Gene-specific PCR primers (Table 2) were designed to amplify a 100- to 150-bp internal region of each gene of interest using Primer3. DNA-free RNA (100 ng) was converted to cDNA by reverse transcriptase PCR (SuperScript II reverse transcriptase; Invitrogen, Carlsbad, CA) with random hexamer primers (50 ng per reaction; IDT, Inc.) according to the manufacturer's instructions. Quantitative PCR was carried out with each gene-specific primer pair at a concentration of 400 nM each with 2 μl of the cDNA synthesis reactions using the Brilliant SYBR green Q-PCR Master Mix kit and the Mx3000p real-time PCR thermocycler and fluorescence detection system (Stratagene, Santa Clara, CA). PCRs were carried out in 96-well plates in 25-μl volumes following the activation of the SureStart *Taq* DNA polymerase included in the kit at 95°C for 10 min. The cycling conditions were as follows: 95°C for 30 s, 60°C for 60 s, and 72°C for 60 s for 40 cycles, followed by a single cycle of 95°C for 30 s and 55°C for 30 s and a ramp to 95°C at 0.2°C $s^{-1}$ to determine the melting temperature of the amplified cDNA and to verify the absence of secondary products. Also analyzed for each primer pair was a standard curve of 0.001 to 1 ng genomic DNA from *Synechococcus*. All reactions were run in duplicate. Threshold fluorescences of unknown samples were converted to cDNA concentrations using the standard curves for each primer pair. Agarose gels (1.2%) and Q-PCR disassociation curves were examined to verify single amplicons for each primer pair. At the time of this work, a suitable housekeeping gene was not known for WH8102; therefore, changes were quantified as the ratio of expression between experimental (Ni-deprived) and control (Ni-sufficient) samples.

**Genetic manipulations.** Inactivations were performed and validated according to the methods presented in reference 7. Briefly, for pMSYNW*sodT*, pMSYNC*sodT*, pM9902*sodT*, pM9605*sodT*, and pMSYNW*sodN*, a gene-specific internal fragment for each target (Table 2) was cloned into the EcoRI site (bp 0) of the suicide vector pMUT100 (7). To generate the pM*sodN*::*sodB* and pM*sodT*::*sodB* vectors, the *sodB* gene and 70 bp of the upstream noncoding region were amplified from WH7803 genomic DNA (primers, Synwh7803_1742gibsonFor and Synwh7803_1742gibsonRev, bp 1594036 to 1594704) while the pMSYNW*sodT* and pMSYNW*sodN* vectors were linearized at bp 174 using inverse PCR (primers, pMUTgibsonFor and pMUTgibsonRev). All primers contained 20-bp overhangs to desired cloning sites (Table 2). After isothermal cloning (22), the resulting plasmids were verified by restriction screening and sequencing. Each of the pMUT constructions was transformed into MC1061(pRL528, pRK24) and introduced into *Synechococcus* by conjugation with subsequent selection of exconjugants on pour-plated SN medium (6, 62) supplemented with 25 μg/ml filter-sterilized kanamycin sulfate, unless noted otherwise. Colonies were inoculated into liquid SN supplemented with 25 μg/ml kanamycin and maintained in exponential growth until axenic, as determined by spotting on LB agar plates. The complete segregation of mutant chromosomes was verified using PCR as described in reference 34 with the verification primers from Table 2. In addition, the presence of the *sodB* genes was also examined by PCR. To facilitate the isolation of Ni-uptake-impaired exconjugants, SN-agar medium was amended with 5 nM $NiCl_2$ (10× the amount present in normal SN agar). In the experiments with Cu/Zn-SOD-containing CC9311, CC9902, and CC9605, SN-agar medium was amended with 5 nM $CuCl_2$ and 0.5 mM $NH_4^+$ to mimic the conditions where these strains can grow on $NH_4^+$ without Ni. Cell lines were also screened using native protein gels with SOD activity screening (4), and the different SODs were identified by protein size.

**Ni uptake rates.** Cells were harvested by centrifugation (8,000 × *g* for 10 min) in acid-washed polycarbonate bottles, rinsed twice with SOW

**TABLE 3** Growth rates and nickel uptake rates for cultures used in microarray experiments[a]

| Strain and nitrogen source | 5 pM [$Ni^{2+}$] | | 50 pM [$Ni^{2+}$] | |
|---|---|---|---|---|
| | μ ($day^{-1}$) | Ni uptake (zmol $cell^{-1}$ $min^{-1}$) | μ ($day^{-1}$) | Ni uptake (zmol $cell^{-1}$ $min^{-1}$) |
| WH8102 and $NH_4$ | 0.43 ± 0.03 | 3.5 ± 0.05 | 0.49 ± 0.03 | 0.7 ± 0.05 |
| WH8102 and urea | 0.375 ± 0.25 | 4.0 ± 0.05 | 0.52 ± 0.3 | 1 ± 0.05 |

[a] Ranges are for duplicate cultures. Uptake rates were determined at a Ni concentration of 5 nM for cells that had been precultured at the indicated Ni concentrations.

containing no added EDTA or trace metals, and resuspended in 10 ml SOW with no added EDTA or trace metals in 70-ml acid-washed polycarbonate bottles. To measure high-affinity Ni uptake rates, $^{63}Ni$ was added to a final concentration of 5 nM. Following a 30-min incubation under standard growth conditions, experiments were terminated by filtration onto 0.2-μm-pore-size filters (Supor; polyethersulfone). The filters were rinsed with 5 ml of sulfoxime (a Ni chelator, 1 mM, in SOW; Avocado Biochemicals, Lancashire, United Kingdom) and 5 ml of SOW to remove surface-bound $^{63}Ni$ prior to being added to 15 ml of scintillation fluid (Ecolyte; MP Biomedicals, Solon, OH). To determine the total added $^{63}Ni$, small aliquots (150 μl) of unfiltered medium were added to 15 ml of scintillation fluid. Radioactivity was assayed using standard scintillation counting with quench correction. Disintegrations per minute were converted to $^{63}Ni$ concentrations using a dilution curve of a $^{63}Ni$ of known Ni concentration, as done in reference 13. The single time point uptake rate measurements are possible for this Ni concentration based on more detailed time course experiments published in reference 13, where Ni uptake was constant through 60 min.

**Microarray data accession numbers.** Microarray data have been deposited under GEO accession numbers GSM982627 to GSM982639.

## RESULTS

**Overall physiological and transcriptional effects of Ni deprivation.** Ni deprivation was examined in cultures of WH8102 growing on $NH_4^+$ (here referred to as $NH_4^+$-Ni) and urea (urea-Ni). To induce Ni deprivation, cultures were maintained in exponential growth at constant [$Ni^{2+}$] of 5 and 50 pM until growth rates were stable (at least 20 doublings), at which point they were harvested and mRNA was isolated for use in microarray experiments. At the lower [$Ni^{2+}$], growth rates were suppressed 15 to 20% and Ni uptake rates (with 5 nM Ni) increased 400 to 500% (Table 3). Using a maximum false-positive rate of 1% and a fold change of $>|0.5|$ as selection criteria for the transcriptomic analysis (see Materials and Methods), 50 to 115 open reading frames were determined to be significantly up- or downregulated in response to Ni deprivation in both experiments (see Table S1 in the supplemental material).

The nitrogen source for growth drastically affected the transcriptional response of WH8102 to Ni deprivation (see Fig. S1 in the supplemental material), as only 18 of the 290 differentially expressed genes were shared between treatments. Of this subset, 11 genes were downregulated in both experiments, three were upregulated in $NH_4^+$-Ni and downregulated in urea-Ni, three were downregulated in $NH_4^+$-Ni and upregulated in urea-Ni, and only *synw1592* was upregulated in both experiments. Many (114/290) of the differentially expressed genes colocalize on the genome (see Table S1), though 38 of the genes converge tail to tail from opposite strands and thus are not operons.

**ATP and protein synthesis.** Consistent with the decline in growth rates, genes coding for ATP synthase (*synw0488* to *synw0495*) were downregulated in both experiments (see Table S1 in the supplemental material), but not in a coordinated fashion. Three subunits were downregulated in $NH_4^+$-Ni while four other subunits were downregulated in urea-Ni. All of these genes occur in the same genomic region, and a closer examination reveals that the entire region exhibits negative fold changes in both experiments, though individual genes do not make the statistical cutoff. Two genes coding for ribosomal proteins (50S L7/L12 and L10) were also downregulated in both experiments, with a further one and five repressed in $NH_4^+$-Ni and urea-Ni, respectively. One ribosomal protein (50S protein L28) was upregulated in urea-Ni. No tRNA synthases were downregulated in the $NH_4^+$-Ni experiment, while only one was downregulated in the urea-Ni experiment. Genes coding for two subunits of Clp protease (*synw0938* and *synw1503*) were upregulated by Ni deprivation for cells growing on $NH_4^+$.

**Transcription and regulatory elements.** Sigma factors are global transcriptional regulators. One alternative type II σ factor (*synw1509*) was downregulated in $NH_4^+$-Ni while another (*synw1621*) was downregulated in urea-Ni. The putative principal sigma factor (*synw1783*) was upregulated in urea-Ni. Another system of cellular regulation in cyanobacteria involves histidine kinases and response regulators, of which WH8102 has only 5 and 9, respectively. While no histidine kinases were differentially expressed in these experiments, 5 of the response regulators were differentially expressed, but again not in a shared fashion. One response regulator (*synw2289*) was upregulated in $NH_4^+$-Ni, while *synw0126* was downregulated. Two response regulators (*synw2236* and *synw2246*) were downregulated by urea-Ni, while another was induced (*synw0808*). The only gene upregulated in both experiments is a putative transcription factor (*synw1592*) containing an AraC domain (PFAM 00165). The helix-turn-helix AraC domain is located at the C terminus of the putative protein, while the N terminus is uncharacterized.

**Expression of the *ntc* regulon and carbon assimilation-related genes.** In cyanobacteria, the cyclic AMP (cAMP) receptor protein NtcA controls the expression of genes involved in nitrogen assimilation (19). Recently, the subset of genes regulated in WH8102 by *ntcA* was examined using a combination of bioinformatic and microarray-based approaches (WH8102 *ntcA* regulon [54]). Putative *ntcA* promoter sequences are located upstream of 18 genes differentially regulated in one of the experiments, with 12 in $NH_4^+$-Ni and 7 in urea-Ni. Only one gene was shared: a putative allophycocyanin beta-18 subunit was downregulated in both experiments. Most (11/12) of the differentially expressed *ntcA*-associated genes in $NH_4^+$-Ni were downregulated, with only a putative ferredoxin being induced. In the urea-Ni experiment, two genes involved in molybdenum cofactor biosynthesis were upregulated, while several genes involved in nitrogen assimilation were downregulated. These include a putative ammonium transporter (*synw0253*), a putative cyanate transporter (*synw2487*), and glutamine synthase (*synw1073*). This downregulation of canonical elements of nitrogen assimilation is contrary to the expectation

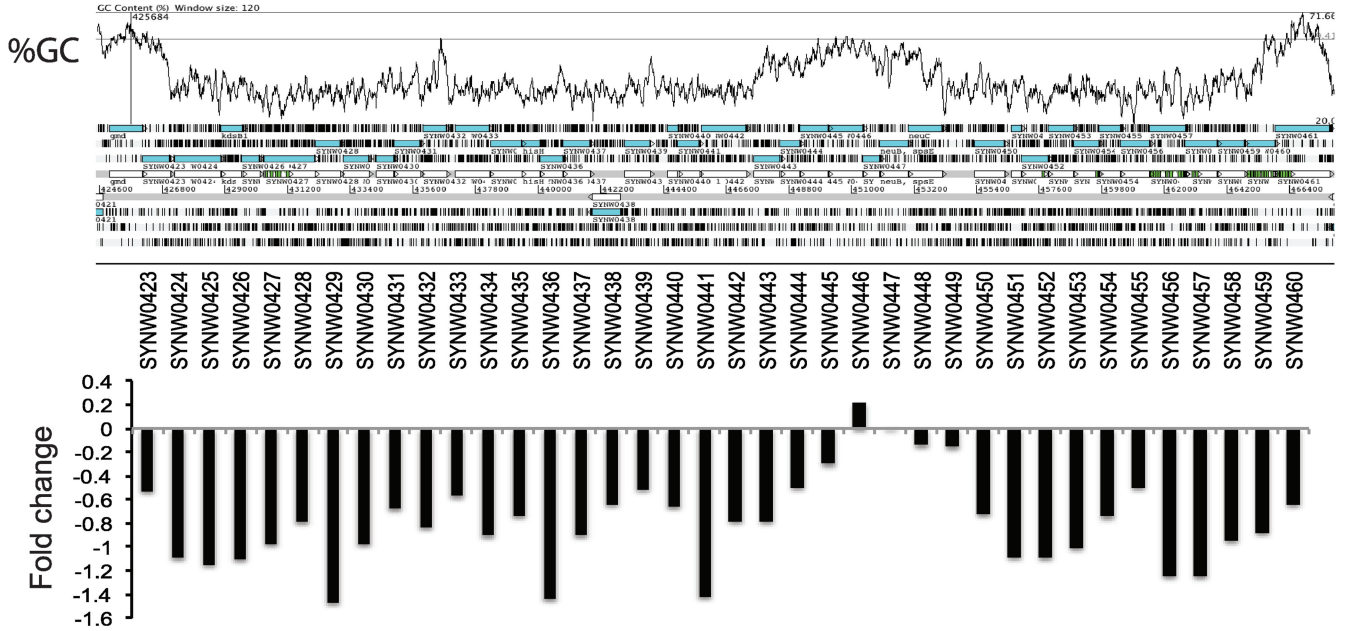


**FIG 2** Genomic island downregulated by Ni deprivation. Shown are the genome organization and percent guanine-cytosine of a 38-kbp region of the WH8102 genome. Also shown is the fold change of each gene within the island associated with Ni deprivation when WH8102 is grown on $NH_4^+$.

that nickel deprivation would induce a nitrogen starvation-like response for growth on urea.

The expression of genes predicted to be part of the carbon fixation pathway in WH8102 by an *in silico* analysis (19) suggests that Ni deprivation affects carbon assimilation in a nitrogen-dependent manner. In the $NH_4^+$-Ni experiment, downregulated carbon fixation genes included *fabF* (*synw0142*), phosphoribulose kinase (*prk*, *synw0785*), *cbbE* (*synw1115*), *hemA* (*synw1117*), and *glyC* (*synw1118*). In contrast, only *fabF* and *hemA* were downregulated in urea-Ni. Together, this suggests that carbon fixation may be repressed in the $NH_4^+$-Ni experiment relative to that in the urea-Ni experiment.

**Photosynthesis, phycobiliproteins, and porphyrins.** Ni deprivation resulted in a downregulation of photosynthetic reaction centers and associated proteins in both experiments but in a more extreme fashion for growth on urea than for growth on ammonium. No photosynthesis genes were upregulated. In all, 13 genes for photosynthetic reaction centers were downregulated in urea-Ni, while only three were downregulated in $NH_4^+$-Ni. In both cases, these were split relatively evenly between photosystems I and II. In both experiments, five genes related to the light-harvesting phycobilisomes were downregulated, with one (*synw1074*, allophycocyanin beta-18 subunit) shared in the two experiments.

Ni deprivation on urea prompted *Synechococcus* WH8102 to downregulate four of the eight genes coding for proteins involved in the synthesis of the precursor of chlorophyll and heme, protoporphyrinogen IX, from glutamate, specifically *hemA*, *hemC*, *hemE*, and *hemL*. *hemA* and *hemE* were also downregulated in the $NH_4^+$-Ni experiment. Mg-chelatase (*chlH*), which catalyzes the first unique step in the synthesis of chlorophyll, was downregulated in both experiments. Ferrochelatase (*hemH*) was downregulated for Ni deprivation on urea only. In both experiments, nearly every gene in the pathway exhibited negative fold changes, though only the genes noted here were statistically significant for the cutoffs used. The downregulation of this pathway can reduce the amount of chlorophyll *a* (Chl *a*) and therefore light absorption at the photosynthetic reaction centers. Alternatively, reduced heme synthesis may indicate a reduced amount of electron transport chaperones in the photosynthetic reaction pathway.

**Electron flow to terminal oxidases.** Several of the WH8102 terminal oxidases were regulated by Ni availability. In the $NH_4^+$-Ni experiment, subunits for a putative cytochrome *c* oxidase were upregulated in response to Ni deprivation (*synw1861* to *synw1863*; see Table S1 in the supplemental material). The *Synechococcus* sp. strain PCC7002 has a pair of functionally characterized cytochrome *c* oxidases; one (*ctaI*) was shown to act as a sink for excess electrons and oxygen in the thylakoids (38). The second, *ctaII*, appears to regulate the cellular response to oxidative stress from one of the cellular membranes (37). A phylogenetic analysis reveals that *ctaDIEIFI* from PCC7002 corresponds to *synw1861* to *synw1863* while PCC7002 *ctaDIIEIIFII* matches *synw1528* to *synw1530*. While on the strand opposite that of *ctaD1E1F1*, the colocalized genes *synw1859-synw1860* were also upregulated. Also upregulated by Ni deprivation for growth on $NH_4^+$ was a gene coding for a putative plastoquinone terminal oxidase (PTOX), based upon the sequence similarity to the well-characterized PTOX found in plant chloroplasts (35).

**Repression of "island" genes by Ni deprivation.** In the $NH_4^+$-Ni experiment, nearly every gene of a 38-kbp low-percentage guanine-cytosine stretch of the genome was downregulated (Fig. 2). Relative to the rest of the genome, this region recruits poorly from the Global Ocean Sampling data set (49), indicating that it is not highly conserved in natural populations of *Synechococcus* with otherwise high nucleotide identity (>90%). Regions with similar characteristics have been identified in *Synechococcus* WH8102 as being similar to pathogenicity islands (39), and similar regions have been found in *Prochlorococcus* (9). In *Prochlorococcus*, these regions have been found to be transcriptionally hy-

**TABLE 4** Ni uptake[a]

| $[Ni^{2+}]$ (pM) | pNi | Ni uptake rate with 5 nM Ni (zmol cell$^{-1}$ min$^{-1}$) |
|---|---|---|
| 0.5 | 12.3 | 4.75 |
| 5 | 11.3 | 1.90 |
| 50 | 10.3 | 1.00 |

[a] The ratios or fold changes of Ni uptake rates at 5 nM total Ni and the expression of the indicated genes were determined for semicontinuous cultures grown on $NH_4^+$ at the indicated $[Ni^{2+}]$ where pNi = $-\log_{10}[Ni^{2+}]$. The values are shown as the ratios of the measured rates and gene transcript abundance for the indicated cultures. Ranges for triplicate Q-PCR measurements on duplicate cultures are shown.

perresponsive to phosphate limitation (33). The observation of Ni influencing the expression of a genomic island is novel, though Fe acquisition systems are often found in the genomic islands of pathogenic bacteria (11). Here, the majority of the island contents are involved in the synthesis of a cell surface polysaccharide or of unknown function, suggesting that the concerted downregulation may be due to an ancillary effect of Ni starvation for growth on $NH_4^+$, like the downregulation of carbon fixation.

**Identification of genes involved in Ni uptake in WH8102.** Ni uptake rates (at Ni concentrations of 5 nM) are greatly enhanced by the decreases in extracellular Ni concentrations (Table 3); therefore, it came as some surprise that none of the three hypothetical Ni transporters, *hupE*, *sodT*, and *synw0709*, were upregulated in response to Ni deprivation in any treatment. *synw0709* was downregulated in the $NH_4^+$-Ni experiment, providing evidence that this gene is unlikely to be involved in high-affinity Ni uptake. Two further experiments were designed to manipulate the biological Ni uptake rates while examining the expression of *sodT* and *hupE* using RT-Q-PCR. One putative sodium ion-bile symporter (*synw0635*) was also examined because it exhibited positive fold changes in both microarray experiments, though not in a statistically significant fashion. In the first experiment, cultures of WH8102 were grown on $NH_4^+$ at constant $[Ni^{2+}]$ of 0.5, 5, and 50 pM. While Ni uptake rates with 5 nM Ni increased nearly 5-fold, *sodT* and *hupE* expression levels were relatively constant (Tables 4 and 5). *synw0635* was upregulated, though not substantially. In a second experiment, WH8102 was grown on $NH_4^+$ without any Ni added to the medium, resulting in a cessation of growth due to Ni limitation (Fig. 3A). At this point, the culture received a Ni addition of 5 nM, stimulating a return to exponential growth (Fig. 3A) and a decline in Ni uptake rates (Fig. 3B). The transcription of *synw0635*, *sodT*, and *hupE* did not follow the changes in Ni uptake rates.

Inactivation of genes in *Synechococcus* WH8102 can be achieved via conjugation with *E. coli* (7), providing a valuable tool

**TABLE 5** Ni transporter expression at constant $[Ni^{2+}]$[a]

| Comparison | Fold change | | |
|---|---|---|---|
| | SYNW1628 | SYNW0635 | *hupE* |
| pNi 12.3 vs pNi 10.3 | 0.80 ± 0.1 | 1.33 ± 0.05 | 0.62 ± 0.1 |
| pNi 11.3 vs pNi 10.3 | 0.86 ± 0.08 | 1.10 ± 0.03 | 0.63 ± 0.07 |

[a] The ratios or fold changes of Ni uptake rates at 5 nM total Ni and the expression of the indicated genes were determined for semicontinuous cultures grown on $NH_4^+$ at the indicated $[Ni^{2+}]$ where pNi = $-\log_{10}[Ni^{2+}]$. The values are shown as the ratios of the measured rates and gene transcript abundance for the indicated cultures. Ranges for triplicate Q-PCR measurements on duplicate cultures are shown.

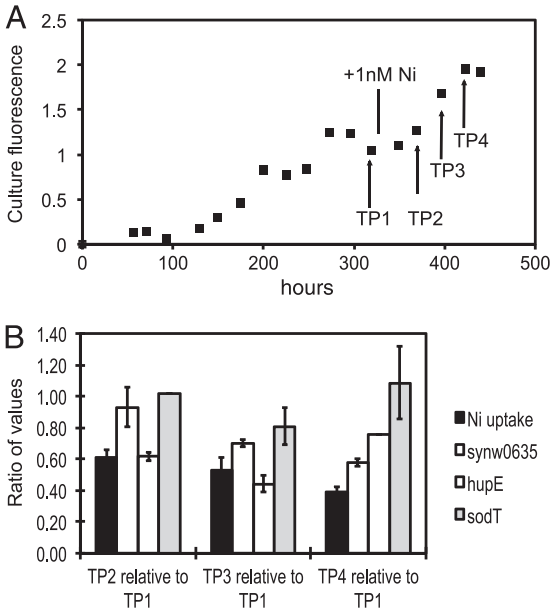


**FIG 3** Time course of Ni limitation and putative Ni transporter expression. (A) The top panel shows the culture fluorescence of a culture of WH8102 grown on $NH_4^+$ without added Ni until limitation (TP1). At this point, Ni was added, resulting in a return to exponential growth. Culture fluorescence is in arbitrary units. (B) Ni uptake rates and the expression of the indicated genes were determined at the indicated time points. Ratios of each time point with the initial Ni-limited values are shown. Ranges are for duplicate cultures.

for functional identification. *synw0635* was targeted despite the modest correlation with Ni uptake rates. While constitutively expressed, *sodT* also was chosen for inactivation due to the lack of functional information, the possibility of posttranslational regulation, and the genomic colocalization with *sodN* in all genomes. Exconjugants with an interrupted *synw0635* were obtained on the first attempt. Like the wild-type *Synechococcus* or the motility-impaired *swmA* knockout (6), this strain exhibited a normal tolerance to Ni deprivation and inducible Ni uptake (data not shown). This provides strong evidence either that *synw0635* is not a nickel transporter or that a functional alternative is present.

Several attempts to insertionally inactivate *sodT* in WH8102 were unsuccessful. Conjugations were performed in parallel with constructions specific for *swmA* that were successful, providing experimental positive controls for conjugation conditions. Given the obligate Ni requirement for growth, the inactivation of a Ni transporter could be fatal to this strain. Exconjugants of *sodT* were also not recovered with supplementation of selective plates with 10× normal Ni concentrations, which is close to the toxicity threshold. Alternatively, *sodT* may be required for *sodN* activity through a non-transport-based mechanism. *Synechococcus* sp. strains CC9311, CC9605, and CC9902 can grow on $NH_4^+$ without Ni, presumably due to the usage of a Cu/Zn-containing SOD, though not on nitrate or urea (13). Therefore, pMUT plasmids were constructed for conjugation-based inactivation of *sodT* in *Synechococcus* CC9311, CC9902, and CC9605 (Table 1), yet exconjugants were not attained. While results were exclusively negative, the consistent lack of success suggests that inactivation of *sodT* in these strains is lethal, potentially due to an interaction of SodT and SodN. Attempts to interrupt *sodN* also were unsuccessful, suggesting that this gene is similarly necessary.

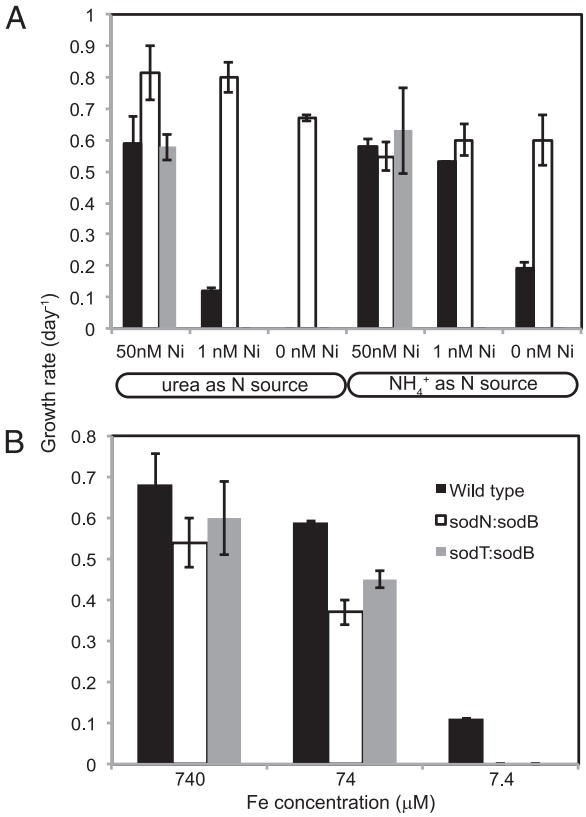


**FIG 4** Growth rates of WH8102 wild type and *sod* knockouts. (A) Growth rates at the indicated nitrogen source and nickel concentration. (B) Growth rates on nitrate at the indicated Fe concentration. Error bars are for triplicate cultures.

Hypothesizing that mature SodT is required for Ni-SOD activity, conjugation vectors were designed to interrupt either *sodN* or *sodT* in WH8102 with a construct containing both Kan$^r$ and the Fe-SOD-encoding *sodB* and its attendant upstream region from WH7803 (8) (Fig. 1). Exconjugants were isolated, and the genomic interruption was validated via PCR (see Fig. S2 in the supplemental material) for both *sodN*::*sodB* and *sodT*::*sodB*. On native SOD activity gels, both knockout strains exhibited SOD activity associated by protein size with Fe-SOD, while only the wild-type strain exhibited Ni-SOD activity (data not shown).

When grown at 50 nM, 1 nM, and 0 nM total added Ni on either urea or $NH_4^+$, each strain exhibited unique growth characteristics (Fig. 4A). The wild-type WH8102 exhibited the previously described characteristics: optimal growth at high Ni concentrations regardless of N source, with declining growth rates at 1 pM where urea levels were lower than $NH_4^+$ levels, and no growth at 0 pM $Ni^{2+}$. The *sodT*::*sodB* line was unable to grow at 1 pM or 0 pM $Ni^{2+}$ regardless of nitrogen source. The *sodN*::*sodB* cell line displayed heightened ability to grow at low Ni concentrations relative to that of the wild type. Near-maximal growth rates were maintained at 1 pM $Ni^{2+}$. With zero added Ni for growth on urea, over 20 generations were required to induce Ni-urea colimitation compared to 5 in the wild type and 1 for the *sodT*::*sodB* line. Ni limitation on $NH_4^+$ was not achieved for the *sodN*::*sodB* line. For Chelex-treated artificial seawater, the background Ni concentrations were determined to be 10 to 20 pM total using competing ligand exchange cathodic stripping voltammetry (10 to 20 fM $Ni^{2+}$ [14]); thus, the *sodN*::*sodB* line has vanishingly low or no Ni requirements for growth on $NH_4^+$.

Given the relaxation of Ni requirements, we examined the converse effects of increased Fe requirements. Here, each line was grown on nitrate at 740 μM, 74 μM, or 7.4 μM total Fe (Fig. 4B). In the wild-type WH8102, 74 μM total Fe results in a 10% reduction in growth rates while 7.4 μM total Fe results in a 90% reduction relative to Fe-replete conditions. In both *sodN*::*sodB* and *sodT*::*sodB* strains, 74 μM total Fe suppressed growth by 25%, and neither strain was able to grow at 7.4 μM total Fe (multiple inoculations). This is consistent with the hypothesis that the usage of *sodB* instead of *sodT* increases Fe requirements, but more detailed experiments directly examining Fe and Ni quotas and uptake rates are necessary for complete validation.

Ni uptake rates in response to low Ni concentrations could not be directly assessed in the *sodT*::*sodB* mutant as the biomass required could not be attained. However, the lack of growth of the *sodT*::*sodB* lines at low Ni concentrations is consistent with the role of *sodT* as a Ni transporter. As with the wild type, *sodN*::*sodB* lines exhibited enhanced Ni uptake when grown at 5 pM $Ni^{2+}$ (see Fig. S3 in the supplemental material). Therefore, the *sodN*::*sodB* line provides ancillary evidence that *sodT* is a Ni transporter; by removing an intracellular sink for Ni while retaining transport function, an increased resistance to Ni starvation could be achieved. While Ni uptake rates were induced in *sodN*::*sodB* bacteria, they were not induced to the extent observed in wild-type WH8102. As discussed below, this is consistent with a competition between urease and Ni-SOD for intracellular Ni; with removal of Ni-SOD, lower uptake rates are required to provide Ni for just urease.

## DISCUSSION

**Influence of Ni deprivation on gene expression in WH8102.** The full-genome microarray analyses provided limited insight into the Ni-specific stress response to Ni deprivation. First, Ni uptake and the transcription of the verified Ni transporter *sodT* are uncoupled. Second, only one gene, *synw1592*, was upregulated in both experiments. This AraC domain-containing putative transcription factor may be involved in sensing Ni concentrations within the cell. However, as there were few genes regulated in a concordant fashion between the two experiments, either the regulon of *synw1592* is modulated by other transcription factors or *synw1592* senses another metabolite. Further supporting the latter hypothesis, homologs of *synw1592* are found in nearly every other cyanobacterial genome, including WH7803, which lacks both urease and Ni-SOD, though Ni-requiring proteins not identified at this time may be present. As *synw1592* is found in strains that apparently do not require Ni homeostasis, it seems unlikely that it is a Ni-sensing regulatory protein, though functional verification is necessary.

The general stress response to Ni deprivation is heavily influenced by nitrogen source (see Fig. S1 in the supplemental material). The only consistency between the two experiments was a downregulation of ATP synthase, porphyrin synthesis, and photosynthesis-related genes, which is consistent with the lower growth rates. The few sigma factors and response regulators found in the WH8102 genome were either divergently expressed or at least not shared. Carbon assimilation and nitrogen assimilation pathways were affected differently. An entire 38-kbp genomic "is-

land" was downregulated in response to Ni deprivation on $NH_4^+$ only. Potentially, this lack of overlap is due to a role of urease in central metabolism and a competition for Ni between urease and Ni-SOD. Ni deprivation for growth on $NH_4^+$ may result in a deficiency of catabolically produced $NH_4^+$ from the cyanobacterial urea cycle, while Ni deprivation on urea reduces both catabolic and anabolic $NH_4^+$ production. By removing the competitive sink for Ni in Ni-SOD, the *sodN*::*sodB* cell line will be invaluable for elucidating the possible catabolic role of urease and the cyanobacterial urea cycle. Specifically, Ni deprivation of the *sodN*::*sodB* line for growth on ammonium or nitrate would limit just urease without the ancillary oxidative stress associated with the Ni-SOD using wild-type WH8102.

One consistent response was the upregulation of terminal oxidases CtaI and PTOX. The upregulation of *ctaI* may be important in detoxifying superoxide anions in the absence of Ni-SOD or reducing the generation of superoxide by acting as a sink for oxygen. While the exact functional role in cyanobacteria remains unknown, in plastids PTOX couples the oxidation of plastoquinol with the reduction of oxygen to water (2). PTOX may act as a "safety valve" preventing the overreduction of photosystem I electron acceptors in plastids (48). In cyanobacteria, PTOX may stabilize the redox state of the plastoquinone pool, which is important in the regulation of NADPH dehydrogenase (NADI)-catalyzed cyclic electron flow around photosystem I in cyanobacteria (31). The physiological trademarks of a water-water cycle have been observed in marine cyanobacteria and evoked as a mechanism to scavenge oxygen radicals (3, 24, 25, 36), yet the machinery involved is not known. Either the thylakoid-localized PTOX or CtaI may catalyze a water-water cyclic electron flow similar to the Mehler reaction, generating ATP while compensating for the reduced Ni-SOD activity.

**A theoretical model of Ni uptake and homeostasis involving *sodT*.** As transcription of *sodT* and high-affinity Ni uptake are uncoupled (Fig. 3 and Tables 4 and 5), the activity of the protein must be controlled at a posttranscriptional or posttranslational level. The posttranscriptional regulation of metal transporter genes has not been observed in bacteria, though positive regulation of genes like *sodB* and ferritin by Fur is achieved indirectly through Fur-dependent repression of an antisense regulatory small RNA that acts posttranscriptionally to reduce translation of *sodB* and ferritin (28). WH8102 does not contain a *nur* homolog, and the *fur* orthologs found in the genome were not differentially expressed.

Alternatively, Ni uptake in WH8102 may be regulated posttranslationally. The protein coded for by *sodT* contains an intracellular loop laden with histidine residues (Fig. 1), which have imidazole side chains with high affinity for Ni. Constitutively expressed, SodT would presumably be present in the cytoplasmic membrane, and with sufficient intracellular Ni concentrations, the His-rich loop would be complexed with Ni. With declining intracellular Ni concentrations, Ni would disassociate from the His-rich loop as it is bound by Ni-SOD and urease. Potentially, the structural changes associated with Ni disassociation would activate the transport activities of the enzyme. The reduction in Ni uptake rates for the *sodN*::*sodB* lines relative to those for the wild type supports this model; by removing Ni-SOD, only urease is removing Ni from SodT, thus resulting in lower Ni uptake rates. The reduction of competition between Ni metalloenzymes has been shown to influence the induction of Ni uptake in *Helicobacter pylori* (5). Similar allosteric regulation of transporter activity has been observed for transporters of methionine (23) and molybdate (21), though not for Ni. In this scenario, the genomic locale displayed in Fig. 1 contains all of the elements required for marine cyanobacteria to use a Ni-containing SOD: (i) *sodN*, coding for apo-Ni-SOD; (ii) ppIase and *sodX*, protein chaperones required for posttranslational modifications of the apo-Ni-SOD following Ni binding (16, 26); and (iii) *sodT*, which we hypothesize both senses and transports Ni. Further, this would represent a form of streamlining, where one protein, SodT, performs roles performed by several proteins in other organisms.

The transcription of specific stress-responsive genes has been suggested as a way to assay the limitation state of communities of marine microbes. For example, within the cyanobacteria, genes that are responsive to nitrogen, phosphate, Fe, or Cu stress have been identified (29, 46, 53, 58, 59). The proposed posttranslational regulatory mechanism would stymie such approaches to studying Ni stress and possibly other stresses in natural cyanobacterial populations.

**Implications for the biogeography of *Synechococcus* and *Prochlorococcus*.** Another implication of this model is the lack of inducible high-affinity Ni uptake in all of the *sodB*-containing strains of marine *Synechococcus*, including WH7803, WH7805, RSS9917, RSS9916, and WH5701. While these strains do not need Ni for SOD activity, all but WH7803 have the genes for a Ni-containing urease. As a low-affinity Ni uptake system may be present, these strains may be able to grow on urea if provided with high-enough Ni concentrations. However, one would expect that strains like WH8102 with high-affinity Ni uptake and lower Fe requirements would have a competitive advantage in oligotrophic conditions where urea features prominently in community metabolism (61). Essentially, the exchange of *sodN-sodX-sodT* for *sodB* may have created a major physiological divide in the marine cyanobacteria.

The usage of Ni-SOD versus Fe-SOD mostly lies along phylogenetic lines of the cluster 5 cyanobacteria. Specifically, all *Prochlorococcus* and most clade 5.1 *Synechococcus* bacteria, including clades I to IV, contain *sodN*. *Synechococcus* bacteria in clusters 5.2 and 5.3 (e.g., WH5701 and RCC307) and cluster 5.1B clades V, VI, VIII, and IX contain *sodB* instead (15). It is generally well accepted that *Prochlorococcus* dominates in the open ocean or stratified nutrient-poor waters, along with *Synechococcus* clades II and III (65). Clades I and IV are often found in mesotrophic waters with intermediate nutrient concentrations (30, 57). In contrast, the *sodB*-containing strains of *Synechococcus* are numerically dominant in upwelling systems (20, 41), where mixing delivers nitrate, phosphate, and Fe to the euphotic zone. Given the shift in Fe and Ni requirements, it is tempting to suggest that the choice of *sodB* constrains some *Synechococcus* strains to either low abundance or specific locations where Fe is enhanced by mixing or dust delivery. From the perspective of geological time, the modern ocean is Fe poor, with concentrations likely falling several orders of magnitude with the shift to large-scale oxic conditions around 500 million years ago (40, 45). Freshwater *Synechococcus* strains such as PCC7942 that are basal to cluster 5 (56) utilize Fe-SOD, suggesting that this is the ancestral state. Potentially, the replacement of a Fe-SOD with a Ni-SOD associated with cluster 5.1 cyanobacteria facilitated the proliferation of these *Synechococcus* lineages in the modern ocean.

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