

The Glutathione/Glutaredoxin System Is Essential for Arsenate Reduction in *Synechocystis* sp. Strain PCC 6803^{∇†}

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Arsenic resistance in *Synechocystis* sp. strain PCC 6803 is mediated by an operon of three genes in which *arsC* codes for an arsenate reductase with unique characteristics. Here we describe the identification of two additional and nearly identical genes coding for arsenate reductases in *Synechocystis* sp. strain PCC 6803, which we have designed *arsI1* and *arsI2*, and the biochemical characterization of both ArsC (arsenate reductase) and ArsI. Functional analysis of single, double, and triple mutants shows that both ArsI enzymes are active arsenate reductases but that their roles in arsenate resistance are essential only in the absence of ArsC. Based on its biochemical properties, ArsC belongs to a family that, though related to thioredoxin-dependent arsenate reductases, uses the glutathione/glutaredoxin system for reduction, whereas ArsI belongs to the previously known glutaredoxin-dependent family. We have also analyzed the role in arsenate resistance of the three glutaredoxins present in *Synechocystis* sp. strain PCC 6803 both in vitro and in vivo. Only the dithiolic glutaredoxins, GrxA (glutaredoxin A) and GrxB (glutaredoxin B), are able to donate electrons to both types of reductases in vitro, while GrxC (glutaredoxin C), a monothiolic glutaredoxin, is unable to donate electrons to either type. Analysis of glutaredoxin mutant strains revealed that only those lacking the *grxA* gene have impaired arsenic resistance.

Arsenic is a ubiquitous pollutant that can produce cancer and cause serious health problems in certain parts of the world (36). Because of the wide distribution of arsenic compounds, arsenic resistance is widespread among living organisms (47). Most resistance systems reduce arsenate to arsenite and export the latter to the outside of the cell or transport it to a vacuole (31). Arsenate reduction to arsenite is catalyzed by arsenate reductase, an enzyme at least three families of which have been described (25, 30, 43). The first family is exemplified by *Escherichia coli* ArsC, a protein that uses the glutathione (GSH)/glutaredoxin system as a reducing system and has a single catalytic cysteine. The second family is represented by *Staphylococcus aureus* and *Bacillus subtilis* arsenate reductases (also named ArsC). These enzymes are related to low-molecular-weight protein phosphotyrosine phosphatases and use thioredoxin as a reducing system through an intramolecular redox cascade requiring three cysteines for arsenate reduction (25). The last family is present only in eukaryotic organisms and was initially described in *Saccharomyces cerevisiae* and, more recently in *Leishmania major* and *Arabidopsis thaliana* (6, 32, 53). These arsenate reductases also use the GSH/glutaredoxin system as a reductant, but they are related not to the *E. coli*

reductase but rather to the Cdc25 family of protein phosphatases.

Arsenic resistance in *Synechocystis* sp. strain PCC 6803 is mediated by an operon of three genes that is regulated by an unlinked *arsR* homolog. The operon includes an arsenite transporter gene, *arsB*; an *arsH* homolog without a clear function in arsenic resistance; and an arsenate reductase gene, *arsC* (23), whose product is referred to below as ArsC_{syn}. ArsC_{syn} belongs to a new type of hybrid arsenate reductases that, though related to thioredoxin-dependent arsenate reductases, use the GSH/glutaredoxin system for reduction (19).

Glutaredoxins are small proteins first discovered as electron donors for ribonucleotide reductase but subsequently shown to be able to reduce other proteins, such as *S*-phosphoadenosyl-sulfate reductase and arsenate reductase. They are also able to reduce mixed disulfides between proteins and GSH (10). Two families of glutaredoxins have been described: dithiolic glutaredoxins (which have two cysteines in their catalytic site) and monothiolic glutaredoxins (which only have one cysteine in their catalytic site). To date, despite extensive biochemical characterization of different glutaredoxin isoforms from plants (44), little is known about glutaredoxin function in photosynthetic organisms, although recent biochemical approaches have shed some light (2, 17, 18, 38, 52).

Here we report the identification of two additional and nearly identical genes coding for arsenate reductases. We have designated them *arsI1* and *arsI2*, because these two genes code for identical proteins belonging to the *E. coli* family of arsenate reductases. They are shown to play secondary roles in arsenate resistance in *Synechocystis*, since deletion of either of these genes has an effect only in combination with the absence of *arsC*. We have also performed biochemical characterization of the ArsC_{syn} and ArsI proteins and have found that both proteins have glutaredoxin-dependent arsenate reductase activity.

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TABLE 1. Strains used in this work

Strain	Genotype	Parental strain	Mutated ORF(s)	Plasmid used for transformation	Antibiotic resistance
WT	<i>Synechocystis</i> sp. strain PCC 6803				
SARS4	<i>arsC::C.K1</i>	WT	slr0946	pARSC2	Km
SARS5	<i>arsR::C.C1</i>	WT	sl11957	pARSR2	Cm
SARS6	<i>arsI1::SpΩ</i>	WT	sl15104	pARSI2	Sp
SARS7	<i>arsI2::SpΩ</i>	WT	slr6037	pARSI2	Sp
SARS8	<i>ΔarsI2::SpΩ</i>	WT	slr6037	pARSI8	Sp
SARS9	<i>arsI1::SpΩ ΔarsI2::C.C1</i>	SARS6	slr6037, sl15104	pARSI9	Sp Cm
SARS10	<i>arsC::C.K1 arsI1::C.C1</i>	SARS4	slr0946, sl15104	pARSI4	Km Cm
SARS11	<i>arsC::C.K1 ΔarsI2::SpΩ</i>	SARS4	slr0946, slr6037	pARSI8	Km Sp
SARS12	<i>arsC::C.K1 arsI1::C.C1 ΔarsI2::SpΩ</i>	SARS10	slr0946, slr6037, sl15104	pARSI8	Km Cm Sp
SGRXA	<i>grxA::C.C1</i>	WT	ssr2061	pGRXA2	Cm
SGRXB	<i>grxB::SpΩ</i>	WT	slr1562	pGRXB2	Sp
SGRXC	<i>grxC::C.K1</i>	WT	slr1846	pGRXC2	Km
SGRXAB	<i>grxA::C.C1 grxB::SpΩ</i>	SGRXA	ssr2061, slr1562	pGRXB2	Cm Sp
SGRXAC	<i>grxA::C.C1 grxC::C.K1</i>	SGRXA	ssr2061, slr1846	pGRXC2	Cm Km
SGRXBC	<i>grxB::SpΩ grxC::C.K1</i>	SGRXB	slr1562, slr1846	pGRXC2	Sp Km
SGRXABC	<i>grxA::C.C1 grxB::SpΩ grxC::C.K1</i>	SGRXAB	ssr2061, slr1562, slr1846	pGRXC2	Cm Sp Km

Since both proteins are capable of using the GSH/glutaredoxin system, we have studied the role of this system in arsenate reduction. *Synechocystis* sp. strain PCC 6803 contains three genes encoding glutaredoxins. Two of these genes code for prokaryotic dithiolic glutaredoxins, while the third codes for a monothiolic glutaredoxin. In this study we have analyzed the roles of these glutaredoxins in arsenate resistance and have found that only one of the dithiolic glutaredoxins, GrxA, is essential in vivo. Nevertheless, both of the dithiolic enzymes are active in the arsenate reductase reaction in vitro.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *Synechocystis* sp. strain PCC 6803 was grown photoautotrophically at 30°C in BG11 medium supplemented with 1 g per liter of HCO₃Na (BG11C) (40) and bubbled with a continuous stream of 1% (vol/vol) CO₂ in air under continuous fluorescent illumination (50 μmol of photons per m² per s; white light). For plate cultures, BG11C liquid medium was supplemented with 1% (wt/vol) agar. Antibiotics were added to final concentrations of 50 to 200 μg/ml for kanamycin, 20 to 40 μg/ml for chloramphenicol, or 2 to 10 μg/ml for spectinomycin when required. BG11C medium was supplemented with different concentrations of Na₂HAsO₄ in the presence or the absence of phosphate at the standard concentration as indicated for BG11C.

Escherichia coli DH5α (Bethesda Research Laboratories) grown in Luria-Bertani medium as described in reference 45 was used for plasmid construction and replication. *E. coli* BL21(DE3) and *E. coli* XL1-Blue grown in Luria-Bertani medium or Luria-Bertani medium supplemented with 2% glucose, respectively, were used for protein expression. *E. coli* cultures were supplemented with 100 μg/ml ampicillin, 40 μg/ml chloramphenicol, 50 μg/ml kanamycin, or 100 μg/ml spectinomycin when required.

Insertional mutagenesis of *Synechocystis* genes. All the oligonucleotides used for cloning and insertional or site-directed mutagenesis are described in Table S1 in the supplemental material. For the sl15104 and slr6037 insertional mutants, a 1,063-bp PCR fragment was amplified from total genomic DNA using oligonucleotides ARSI1 and ARSI2 and was cloned into pGEMT to generate pARSI1. Then an SpΩ (39) or C.C1 (8) cassette was inserted into a PflMI site in the *arsI1* and *arsI2* open reading frames (ORFs), generating pARSI2 and pARSI4, respectively. For double mutant construction, a 970-bp DNA fragment lacking the slr6037 ORF was constructed by two-step PCR using oligonucleotide pairs ARSI1–ARSI8 and ARSI7–NIY3, and antibiotic resistance cassettes were inserted into an NcoI site upstream of the slr6037 ORF, generating pARSI8 (SpΩ cassette) and pARSI9 (C.C1 cassette). For inactivation of *grxA* (ssr2061), a 1,269-bp DNA fragment was amplified using the oligonucleotide pair GRXA1–GRXA4 and cloned into pGEMT, and the C.C1 cassette was inserted into an SspI site located in the *grxA* ORF, generating pGRXA2. For inactivation of *grxB* (slr1562), an 845-bp DNA fragment was amplified using the oligonucleotide pair

GRXB1–GRXB2 and cloned into pGEMT, and the SpΩ cassette was inserted into an RsaI site located in the *grxB* ORF, generating pGRXB2. For inactivation of *grxC* (slr1846), a 1,107-bp DNA fragment was amplified using the oligonucleotide pair GRXC1–GRXC2 and cloned into pGEMT, and the C.K1 cassette (8) was inserted into a BstEII site, blunt ended with Klenow enzyme, and placed in the *grxC* ORF, generating pGRXC2. Wild-type (WT) *Synechocystis* sp. strain PCC 6803 or different mutants were transformed with the plasmids listed in Table 1 to generate all the mutants used in this study.

Correct integration and complete segregation of the mutant strain were analyzed by Southern blotting. To this end, total DNA from cyanobacteria was isolated as previously described (4). DNA was digested with appropriate enzymes, electrophoresed on 0.7% agarose gels in a Tris-borate-EDTA buffer system (45), and subsequently transferred to nylon Z-probe membranes (Bio-Rad). DNA probes were ³²P labeled with a random-primer kit (Amersham Biosciences) using [α-³²P]dCTP (3,000 Ci/mmol).

Cloning and expression of ArsC_{syn}, ArsI, GrxA, GrxB, and GrxC. Proteins were expressed with either a carboxy-terminal (ArsC_{syn} and ArsI) or an amino-terminal (GrxA, GrxB, and GrxC) six-histidine tag. The *arsC* gene was amplified from cosmid cs0223 (provided by Kazusa DNA Research Institute) using oligonucleotides ARSC3 and ARSC5, which introduced NdeI and XhoI restriction sites, respectively. The DNA fragment obtained was digested with NdeI-XhoI and cloned into NdeI-XhoI-digested pET24. The *arsI* gene was amplified using oligonucleotides ArsI1 and ArsI3 (which introduces an XhoI restriction site) and was cloned into NcoI-XhoI-digested pET24. The NcoI site is already included in the genomic sequence of *arsI*. *grxA* was amplified using GRXA5 (which introduces a BamHI restriction site) and GRXA4 from total genomic DNA. The corresponding DNA fragment was digested with BamHI and cloned into BamHI-digested pQE80, generating pGRXA4. *grxB* was amplified from total genomic DNA using oligonucleotides GRXB3 and GRXB5, which introduced BamHI and SalI restriction sites, respectively. The resulting DNA fragment was digested with BamHI and SalI and was cloned into BamHI-SalI-digested pQE80, generating pGRXB4. *grxC* was amplified using GRXC3 (which introduces a BamHI restriction site) and GRXC2 from total genomic DNA. The resulting DNA fragment was digested with BamHI and HindIII and was cloned into BamHI-HindIII-digested pQE80, generating pGRXC4.

ArsC_{syn} and ArsI were produced in *E. coli* BL21, and GrxA, GrxB, and GrxC in *E. coli* XL1-Blue, transformed with the plasmids described above. Cells were grown at 37°C to an optical density at 600 nm of 0.5; 1 mM IPTG (isopropylthio-β-D-galactopyranoside) was added; and the cultures were grown for 3 h at 30°C. The cells were harvested and frozen. Pellets were thawed on ice and resuspended in 20 ml of 50 mM Tris HCl (pH 8.0)–0.5 M NaCl (buffer A) per g (fresh weight). The cells were broken by sonication, and cell debris was removed by centrifugation at 50,000 × g for 20 min. The liquid supernatant was applied to a 1-ml HiTrap chelating column (Amersham Biosciences) charged with NiSO₄ and was subsequently equilibrated in buffer A. The column was first washed with 100 ml of buffer A and then with 50 ml of buffer A supplemented with 25 mM imidazole, and adherent proteins were eluted by a 40-ml linear gradient from 0.025 to 0.5 M imidazole in buffer A. Imidazole and salts were removed by gel

filtration using HiTrap desalting columns (Amersham Biosciences) run in 50 mM Tris-HCl pH (8.0)–150 mM NaCl.

Arsenate reductase assay. Arsenate reductase activity was measured as described previously in a coupled assay with GSH and glutathione reductase (19). Assays for glutaredoxin K_m determination were carried out in 50 mM Tris-HCl (pH 7.5), 10 mM GSH, 50 mM Na_2HAsO_4 , 1 U of yeast glutathione reductase (Sigma), 5 μM of ArsC_{syn} or 1 μM of ArsI , and different amounts of glutaredoxins. Activity was monitored spectrophotometrically at 340 nm and was calculated using a ϵ of 6.22 mM for NADPH. We calculated kinetic parameters using double-reciprocal plots.

ArsC_{syn} site-directed mutagenesis. The *arsC* gene was mutated by two overlapping PCR fragments introducing the desired changes using oligonucleotide pairs ARSCmut1–ARSC3 and ARSCmut2–ARSC5 in the first PCR and ARSC3–ARSC5 in the second. The PCR product was digested with *NcoI* and *XhoI* and was then cloned into the *NcoI*-*XhoI*-digested vector pET24. The mutant ArsC_{syn} protein was expressed and purified by the same procedure described for wild-type ArsC_{syn} .

ArsC_{syn} -TrxA(C35S) interaction. Interaction assays were carried out by incubating 10 μg of WT or mutant ArsC_{syn} with 10 μg of TrxA(C35S), prepared as described in reference 20, in 50 mM Tris-HCl (pH 8.0) in the presence or absence of 100 mM Na_2HAsO_4 at room temperature for 30 min. Reactions were stopped by the addition of 1 volume of Laemmli buffer without reductant. Samples were boiled for 5 min, and 1 μg was used for Western blotting using 12% sodium dodecyl sulfate (SDS)–nonreducing polyacrylamide gel electrophoresis (PAGE) and anti-TrxA antibodies (35) at a 1:10,000 dilution.

RNA isolation and Northern blot analysis. Total RNA was isolated from 25-ml samples of *Synechocystis* cultures at the mid-exponential phase (3 to 5 μg chlorophyll/ml). Extractions were performed by vortexing cells in the presence of phenol:chloroform and acid-washed baked glass beads (diameter, 0.25 to 0.3 mm; Braun, Melsungen, Germany) as previously described (12). For Northern blotting, 15 μg of total RNA was loaded per lane, electrophoresed in 1.0% agarose denaturing formaldehyde gels, and transferred to nylon membranes (Hybond N-plus; Amersham Biosciences). Prehybridization, hybridization, and washes were performed as described in the Amersham instruction manual. Probes for Northern blot hybridization were synthesized by PCR using oligonucleotide pairs ARSC1–ARSC2, ARSI3–ARSI4, GRXA250F–GRXA250R, GrxB331F–GrxB331R, and GrxC295F–GrxC295R (see Table S1 in the supplemental material) for *arsC*, *arsI*, *grxA*, *grxB*, and *grxC*, respectively. As a control, in all cases the filters were stripped and reprobed with a 580-bp HindIII–BamHI probe from plasmid pAV1100 containing the constitutively expressed RNase P RNA gene (*mnpB*) from *Synechocystis* sp. strain PCC 6803 (49). DNA probes were ^{32}P labeled with a random-primer kit (Amersham Biosciences) using [α - ^{32}P]dCTP (3,000 Ci/mmol).

RESULTS

Identification and mutagenesis of two new additional arsenate reductase genes. Sequence inspection of the four major *Synechocystis* plasmids allowed us to identify two putative genes (*slI5104* and *slr6037*) encoding an arsenate reductase related to that of *E. coli* (49% identity to *E. coli* *ArsC*) (15). We designated these genes *arsI1* and *arsI2*, respectively. The *arsI* genes are almost identical at the nucleotide level (99% identity) and encode identical proteins, probably reflecting a recent duplication event. They are located in a 2.5-kb region duplicated between pSYSM and pSYSX that contains three additional ORFs: one encoding a putative cytidine deaminase (*slI5107* and *slr6034*), one small ORF encoding a protein with an unknown function (*ssr5106* and *ssl6035*), and the third ORF encoding a ParA homolog (*slr5105* and *slI6036*) (Fig. 1; note that although this region is duplicated, its orientation is reversed). The *arsI* genes are expressed at very low levels, and neither is regulated by the presence of arsenic in the medium (Fig. 2) or by *arsR* (data not shown).

To investigate whether the presence of these additional arsenate reductases could explain the highly arsenate resistant phenotype of a *Synechocystis arsC* mutant, strain SARS4 (previously named *arsC::CK1* [23]) (Table 1), we carried out inser-

tional mutagenesis of the *arsI1* and *arsI2* genes. After transformation of the WT strain or SARS4 with plasmid pARSI2 or pARSI4, we obtained mutants with alterations in either *arsI1* or *arsI2* (66% *arsI1* versus 33% *arsI2*). Subsequently, strains with *arsI1* or *arsI2* inactivated (SARS6, SARS7, and SARS10) were used to generate double or triple mutants using plasmids with an insertion of a different antibiotic resistance cassette in the other *arsI* gene. After several attempts, we still were unable to obtain mutant strains with both *arsI* genes inactivated, since the gene already inactivated was always partially or totally replaced by the new allele, even if both antibiotics were used in the selection step. In order to obtain double *arsI* mutants, we constructed plasmids in which the *arsI2* downstream flanking region (not present in pSYSM [Fig. 1]) was included but the entire *arsI2* ORF was deleted (from 46 bp upstream to 45 bp downstream of the *arsI2* ORF) and a resistance cassette was inserted 205 bp before the starting ATG of the *arsI2* ORF (pARSI8 and pARSI9). This target integration to pSYSX as an *arsI2* downstream flanking region, which is included in this plasmid, is not found in pSYSM. Transformation of the WT strain, SARS4, or SARS10 with plasmid pARSI8 and of SARS6 with pARSI9 resulted in deletion of the *arsI2* gene, allowing us to study the role of each arsenate reductase in arsenic resistance in *Synechocystis* sp. strain PCC 6803. Single mutants of any of the *arsI* genes or double mutants of both *arsI* genes, independently of the alleles (Fig. 3 and data not shown), displayed the same resistance to the presence of arsenate as the WT strain, probably due to the presence of *ArsC* (Fig. 3). In contrast, inactivation of either of the two *arsI* genes in an *arsC::CK1* background rendered the cells more sensitive to arsenate in the medium (Fig. 3, strains SARS10 and SARS11). We have previously studied arsenate toxicity in media with reduced amounts of phosphate (23) (a condition that does not affect the growth of any of the strains tested [Fig. 3]), since this compound has a protective effect against arsenate toxicity, probably affecting arsenate transport (37, 48) (Fig. 3). The strain in which all genes encoding arsenate reductases are inactivated (SARS12) was sensitive to arsenate even in the presence of the standard phosphate concentration in BG11; under the same conditions, none of the other strains were affected (Fig. 3). This indicates that the *arsI* genes are essential only in the absence of ArsC_{syn} and that both *arsI* genes are active in arsenate resistance.

Biochemical characterization of ArsC_{syn} and *ArsI*. In order to study both types of arsenate reductases, we overexpressed and purified them by metal affinity chromatography. First we confirmed that ArsC_{syn} presents GSH-dependent arsenate reductase activity by using *E. coli* Grx1 as previously described (19) (Fig. 4A). *ArsI* displays the same activity (Fig. 4A), as predicted by its high similarity to *E. coli* *ArsC* and its conservation of residues essential for arsenate reductase activity. Although a biochemical characterization of ArsC_{syn} has been published previously, that study was carried out using *E. coli* Grx1, a heterologous electron donor (19). We aimed at characterizing the biochemical properties of ArsC_{syn} by using its physiological electron donor, and to that end we have used *Synechocystis* glutaredoxins. *Synechocystis* sp. strain PCC 6803 contains three ORFs coding for glutaredoxin proteins: *ssr2061*, *slr1562*, and *slr1846* (16). The first two ORFs code for proteins that belong to the bacterial dithiolic family, and we have des-

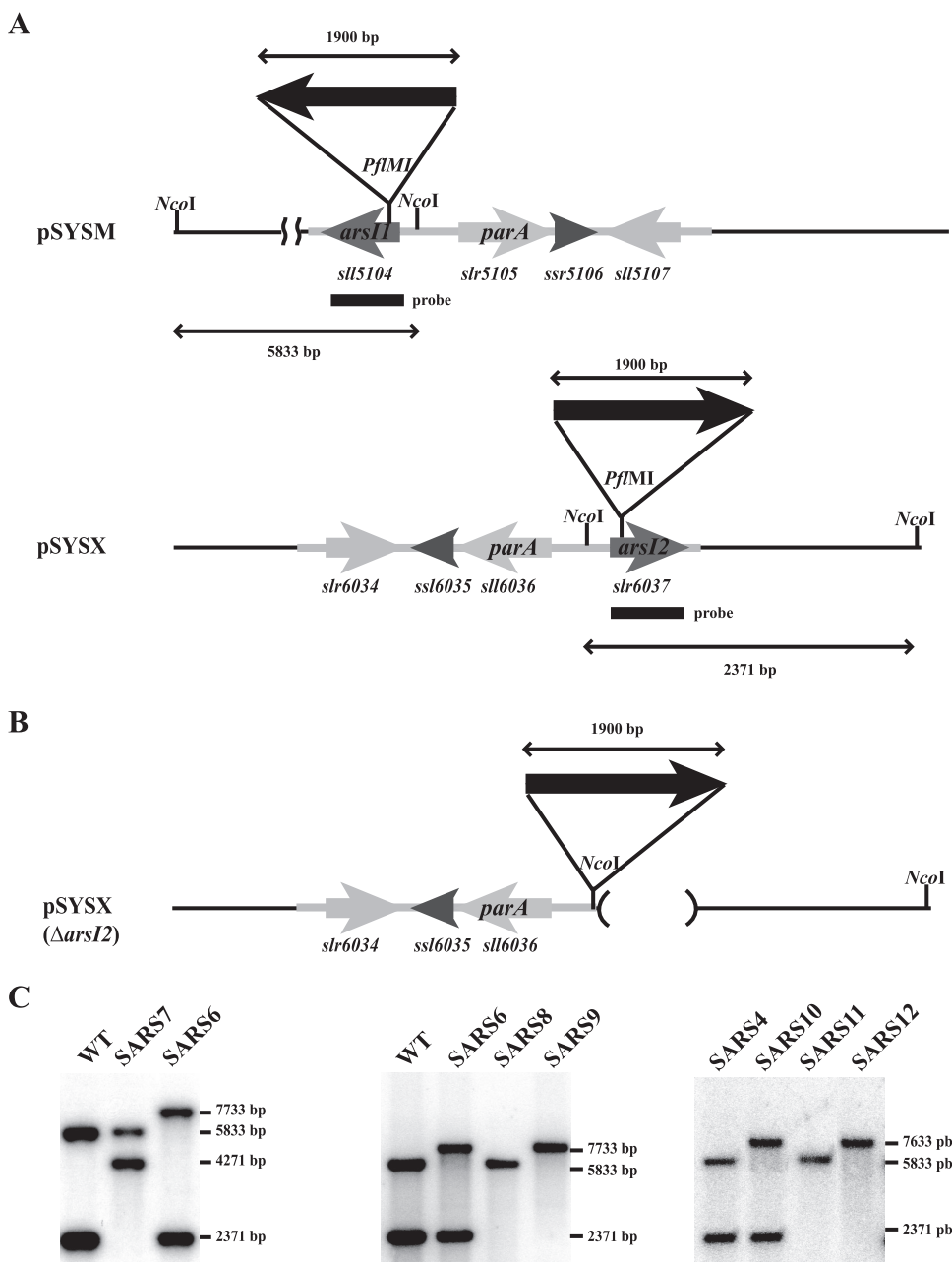


FIG. 1. ORF organization of *arsI*-containing regions in pSYSM and pSYSX. (A) Schematic representation of the pSYSM and pSYSX regions containing *arsI*. The repeated sequence is shaded. Arrows indicate the direction of transcription. Filled arrows represent antibiotic resistance cassettes, although for simplicity, only SpΩ is shown. (B) Schematic representation of the pSYSX $\Delta arsI2$ region present in strains SARS18, SARS19, SARS111, and SARS12. (C) Southern blot analysis of strains SARS4, SARS6, SARS7, SARS8, SARS9, SARS10, SARS11, and SARS12. Genomic DNA was digested with *NcoI* and hybridized using the same DNA fragment indicated in panel A as a probe. The sizes of the hybridizing bands are given on the right of the gels.

ignated them GrxA and GrxB, respectively, while the third ORF encodes a protein, here designated GrxC, that belongs to the newly described monothiolic family (28, 41). We purified these glutaredoxins fused to an amino-terminal histidine tag and analyzed whether they were able to reduce $ArsC_{syn}$ and $ArsI$. We found that only the dithiolic glutaredoxins (GrxA and GrxB) are able to reduce both arsenate reductases, while GrxC is totally inactive with both reductases. In this regard, $ArsI$ showed a clear preference for GrxA, as reflected by a much

lower K_m (60 nM for GrxA versus 1.3 μM for GrxB), while $ArsC_{syn}$ seemed to be able to use both GrxA and GrxB with almost the same efficiency, since it displayed similar K_m s for the two glutaredoxins (0.25 μM and 0.994 μM , respectively) (Table 2). Since GrxA was the best reductant for both arsenate reductases, we used it to determine kinetic parameters for $ArsC_{syn}$ and $ArsI$. Michaelis-Menten constants for arsenate were 5.74 mM and 11 mM for $ArsC_{syn}$ and $ArsI$, respectively. The V_{max} values were 2.81 and 1.3 $\mu mol/min$, and the k_{cat}

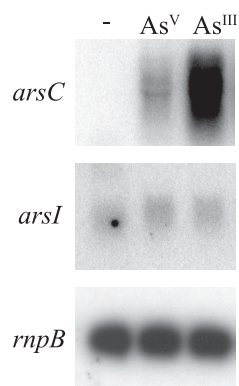


FIG. 2. Northern blot analysis of the expression of the *arsC* and *arsI* genes. Total RNA was isolated from mid-log-phase *Synechocystis* cells grown in BG11C medium and exposed for 1 h either to 1 mM sodium arsenite [As(III)] or to 10 mM sodium arsenate [As(V)]. Control cells were not exposed to added compounds. Fifteen micrograms of total RNA was denatured, separated by electrophoresis on a 1% agarose gel, blotted, and hybridized with probes for *arsC* and *arsI*. The filters were stripped and rehybridized with an *mpB* gene probe as a control (see Materials and Methods).

values were 33.72 s^{-1} and 79.2 s^{-1} , for ArsC_{syn} and ArsI , respectively. These kinetic data for ArsC_{syn} and ArsI are in agreement with those previously published for ArsC_{syn} using a heterologous glutaredoxin (19) and for *E. coli* ArsC (21, 22) (Table 2).

ArsC_{syn} belongs to a new family of arsenate reductases. Amino acid sequence comparison showed that ArsC_{syn} is related to ArsC from *S. aureus* and *B. subtilis* (42% and 37% identity, respectively). However, although they are clearly related, their biochemical properties are markedly different: *S. aureus* ArsC uses thioredoxin as an electron donor, while ArsC_{syn} uses GSH/glutaredoxin (Fig. 4A) (19, 26). The phylogenetic analysis suggests that the ArsC_{syn} type of arsenate reductase is restricted to freshwater cyanobacteria and that no

related sequences are found in other organisms, while the group of marine cyanobacteria contains only the glutaredoxin-dependent type of arsenate reductase represented by *E. coli* ArsC (see Table S2 in the supplemental material). ArsC_{syn} contains three catalytic cysteines: C8, C80, and C82 (19), corresponding to C10, C82, and C89 of *S. aureus* ArsC (Fig. 4B). These three cysteines are essential for enzyme activity (3, 13, 24, 26) and are strictly conserved in all cyanobacterial homologs. We speculated that the difference in spacing between the second and the third catalytic cysteines might explain why ArsC_{syn} uses GSH and glutaredoxin as electron donors instead of thioredoxin, since thioredoxin would be unable to reduce the disulfide bridge formed between these two adjacent cysteines. In order to verify this hypothesis, we constructed a modified *arsC* gene where the spacing between active cysteines C80 and C82 was changed from 1 to 6 residues, as is the case for *S. aureus* ArsC (Fig. 4B). We minimized the number of changes introduced into ArsC_{syn} to those needed to change the spacing between the two catalytic cysteines, which led to a model with a loop, instead of an α -helix, in its oxidized state (see Discussion). The mutated protein was expressed and purified and was found to display abnormally reduced mobility in SDS-PAGE (Fig. 4C). The mutated protein was totally inactive in the coupled arsenate reductase assay using either the thioredoxin or the GSH/glutaredoxin reducing system (data not shown).

We also analyzed the capacity of ArsC_{syn} to interact with the most abundant thioredoxin (TrxA) in *Synechocystis* sp. strain PCC 6803 by using a modified version of TrxA in which the second active-site cysteine has been replaced by Ser [$\text{TrxA}(\text{C35S})$], a mutation that allows the trapping of thioredoxin targets as mixed disulfides (20). We examined the abilities of WT and mutant versions of ArsC_{syn} to interact with $\text{TrxA}(\text{C35S})$ in vitro and asked if the presence of arsenate has any effect on this interaction. To this end, we incubated ArsC_{syn} for 15 min with or without 50 mM arsenate; then $\text{TrxA}(\text{C35S})$, at an equimolar concentration, was added to the reaction mixture, which was incubated for another 30 min. The reactions

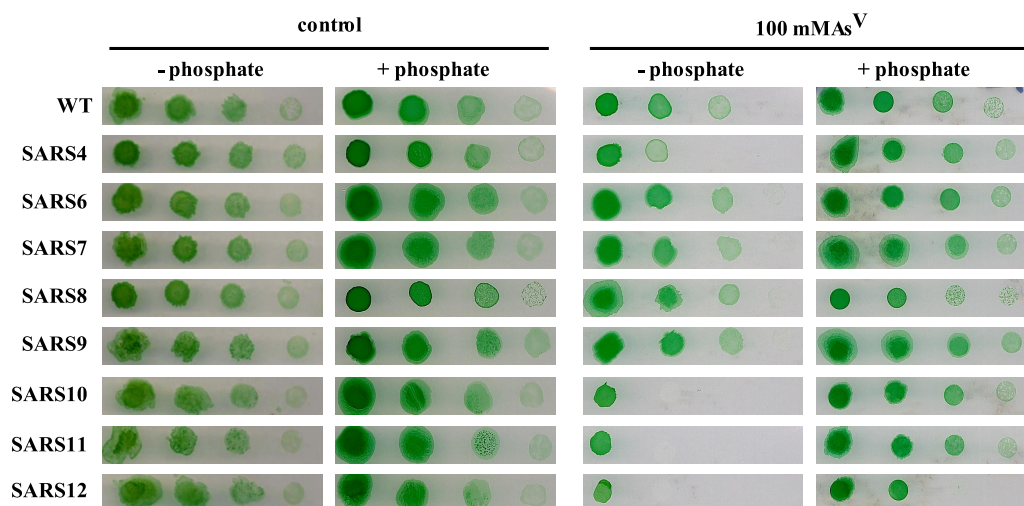


FIG. 3. Phenotypic characterization of *arsI* mutants. The tolerance of WT *Synechocystis* sp. strain PCC 6803 and strains SARS4, SARS6, SARS7, SARS8, SARS9, SARS10, SARS11, and SARS12 to arsenate was examined. Tenfold serial dilutions were spotted onto low-phosphate BG11C plates (– phosphate) supplemented with 100 mM sodium arsenate [As(V)], BG11C plates (+ phosphate) supplemented with 100 mM sodium arsenate [As(V)], or low-phosphate BG11C plates or BG11C plates without additions. Plates were photographed after 10 days of growth.

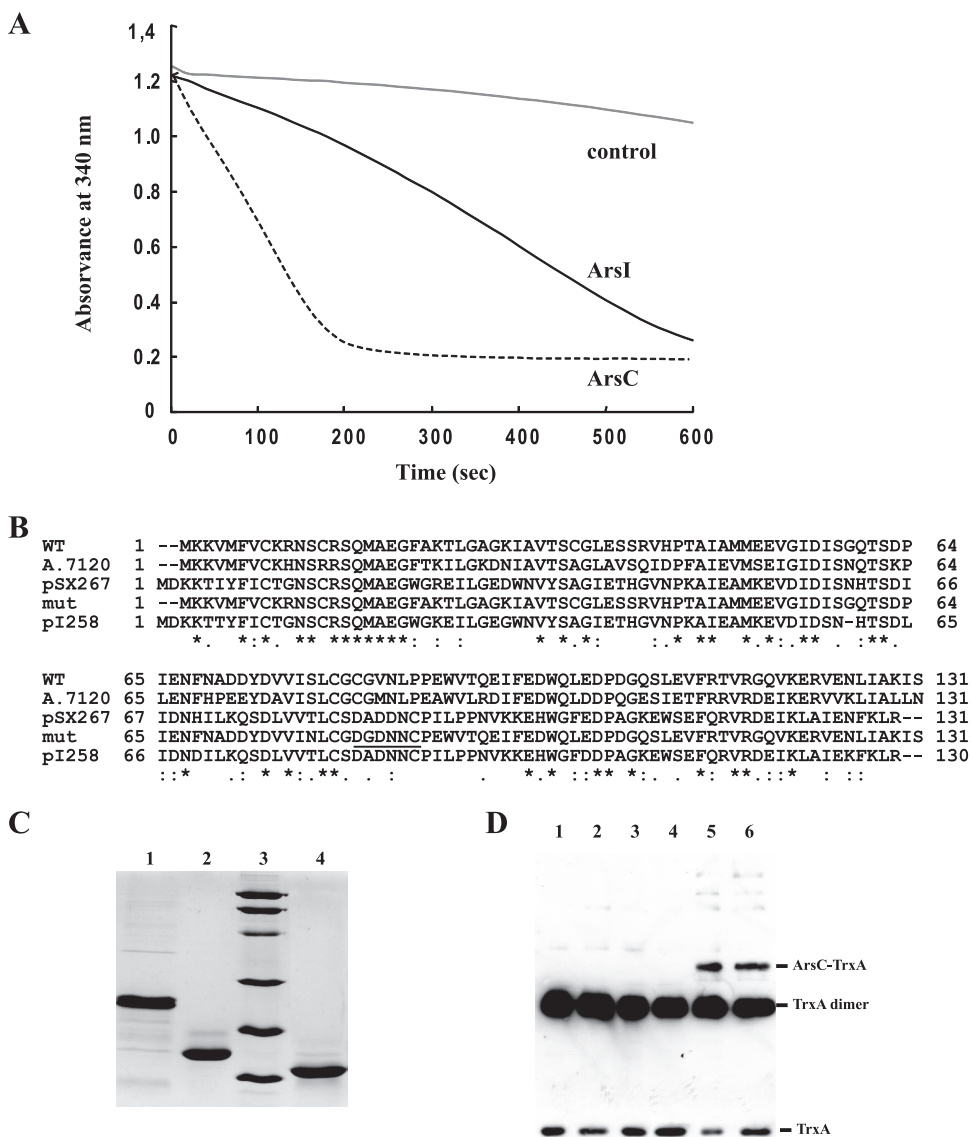


FIG. 4. ArsC has a unique catalytic site. (A) Glutaredoxin-dependent arsenate reductase activities of ArsC and ArsI. Portions (25 μ g) of purified ArsC and ArsI were assayed with 1 μ g of *E. coli* Grx1 following the couple reaction described in Materials and Methods. A control assay without glutaredoxin addition was carried out. (B) Sequence alignment of ArsC_{syn} (WT), mutant ArsC_{syn} (mut), Alr1105 from *Anabaena* sp. strain PCC 7120 (A.7120), and ArsC from *Staphylococcus xylosum* plasmid pSX267 or from *S. aureus* plasmid pI258. Identical amino acids are asterisked; conservative changes are marked with “.” or . as defined by CLUSTAL X. Changes to ArsC_{syn} in the mutant are underlined. (C) SDS-PAGE of purified mutant ArsC_{syn} (lane 1), WT ArsC_{syn} (lane 2), and TrxA(C35S) (lane 4). Lane 3, molecular mass markers. (D) Western blot analysis of interaction experiment between ArsC_{syn} and TrxA(C35S). Proteins were incubated as described in Materials and Methods. Lanes 1 and 2, reaction mixtures containing TrxA(C35S); lanes 3 and 4, reaction mixtures containing TrxA(C35S) and WT ArsC_{syn}; lanes 5 and 6, reaction mixtures containing TrxA(C35S) and mutant ArsC_{syn}. Proteins were incubated in the presence of 100 mM Na₂HAsO₄ (lanes 1, 3, and 5) or in the absence of Na₂HAsO₄ (lanes 2, 4, and 6). The membrane was incubated with anti-TrxA antibodies as described in Materials and Methods.

TABLE 2. Catalytic properties of ArsC_{syn} and ArsI

Arsenate reductase	K_m (μ M)		Kinetic parameter determined with GrxA		
	GrxA	GrxB	K_m for AsO ₃ ²⁻ (mM)	V_{max} (μ mol/min)	k_{cat} (s ⁻¹)
ArsC	0.25	0.994	5.74	2.81	33.72
ArsI	0.063	1.369	11	1.32	79.2

were stopped, and an aliquot was subjected to nonreducing SDS-PAGE and analyzed by Western blotting using anti-TrxA antibodies. While WT ArsC_{syn} was unable to interact with TrxA(C35S) (Fig. 4D, lanes 3 and 4), the mutated version of ArsC_{syn} was clearly able to form a mixed disulfide with TrxA(C35S) (Fig. 4D, lanes 5 and 6).

Glutaredoxin specificity in vivo. To address whether the glutaredoxins had any specificity in vivo, we studied glutaredoxin expression in response to the presence of arsenate in the

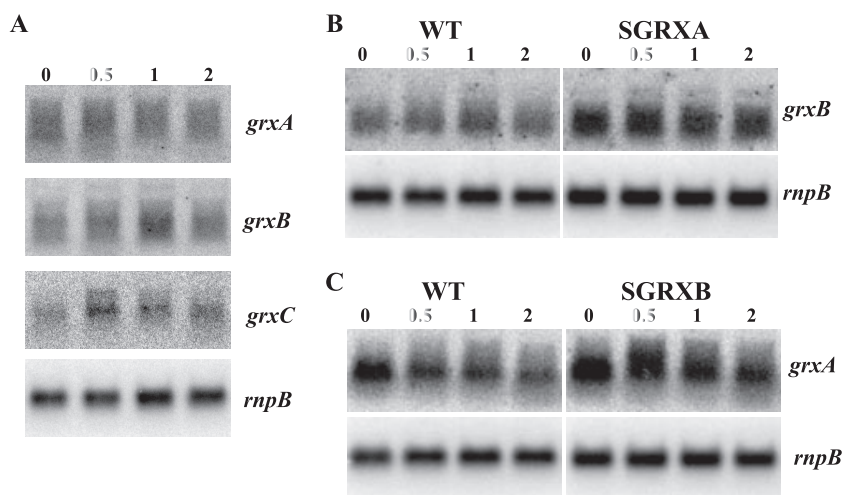


FIG. 5. Analysis of the expression of glutaredoxin genes in response to arsenate. (A) Northern blot analysis of the expression of the *grxA*, *grxB*, and *grxC* genes. Total RNA was isolated from mid-log-phase *Synechocystis* cells grown in BG11C medium and was exposed to 100 mM sodium arsenate for 0.5, 1, and 2 h. Control cells were not exposed. Fifteen micrograms of total RNA was denatured, separated by electrophoresis on a 1% agarose gel, blotted, and hybridized with probes for the *grxA*, *grxB*, and *grxC* genes. The filters were stripped and rehybridized with an *rnpB* gene probe as the control (see Materials and Methods). (B and C) Northern blot analysis of the expression of *grxB* in strain SGRXA (B) and of *grxA* in strain SGRXB (C) after arsenate exposure as described for panel A.

medium. Total RNAs from mid-exponential-phase growing cells treated with 100 mM arsenate for 0.5, 1, and 2 h were analyzed by Northern blotting using probes against the *grxA*, *grxB* and *grxC* genes. While the levels of *grxA* and *grxB* transcripts did not change significantly after arsenate addition, even in the SGRXB or SGRXA mutant strain, respectively, *grxC* showed induction in its mRNA (Fig. 5).

In order to further analyze the *in vivo* specificities of different glutaredoxins, we constructed single, double, and triple mutant strains with inactivated glutaredoxin genes. All mutants were viable, and their growth rates were comparable to those of the WT strain both in the presence and in the absence of phosphate (Fig. 6 and our unpublished results). However, analysis of their phe-

notypes in the presence of arsenate indicated that all strains carrying an insertion in the *grxA* gene were less resistant to the presence of arsenate than the WT strain (Fig. 6). Further inactivation of the *grxB* or *grxC* gene had no effect on arsenate resistance, indicating that GrxA is the main electron donor for both ArsC_{syn} and ArsI *in vivo*. These results are in agreement with our *in vitro* data, in which ArsI and ArsC_{syn} showed strong and moderate specificities for GrxA, respectively.

DISCUSSION

Here we describe the arsenate-reducing system in *Synechocystis* sp. strain PCC 6803, which consists of two different ar-

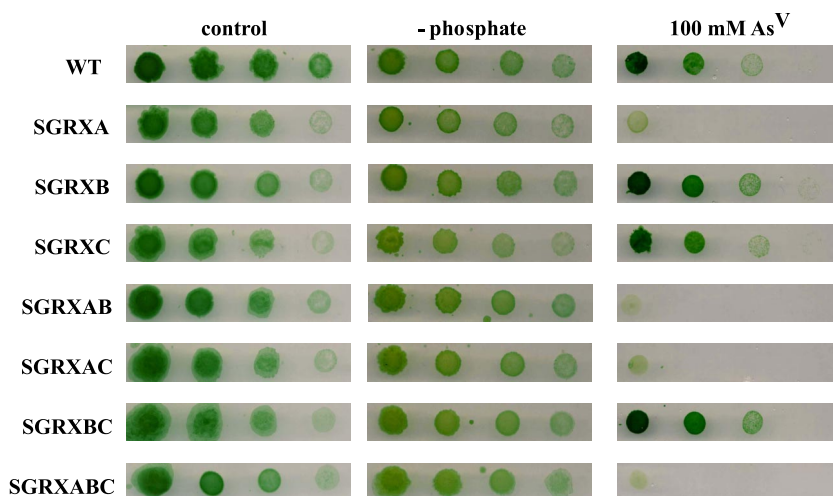


FIG. 6. Phenotypic characterization of the *grx* mutants. The tolerance of WT *Synechocystis* sp. strain PCC 6803 and strains SGRXA, SGRXB, SGRXC, SGRXAB, SGRXAC, SGRXBC, and SGRXABC was tested. Tenfold serial dilutions were spotted onto low-phosphate BG11C plates supplemented with 100 mM sodium arsenate [As(V)], low-phosphate BG11C plates (– phosphate), or BG11C plates. Plates were photographed after 10 days of growth.

senate reductases and implies the GSH/glutaredoxin system as an electron donor. The presence of two different arsenate reductases has also been reported for other bacterial strains by genome analysis (5), but no in vivo experimental data have been reported so far. Although the main resistance determinant in *Synechocystis* sp. strain PCC 6803 involves the highly inducible $ArsC_{syn}$ (23), *ArsI* is here shown to play a role in its absence. This enzyme could allow cells to tolerate small amounts of arsenate, because its gene is expressed at low and constitutive levels (Fig. 2). The fact that the differences in phenotype between SARS10 and SARS11 (double mutants with mutations of *arsC* and *arsI*), on the one hand, and SARS12 (triple mutant with all arsenate reductase genes inactivated), on the other, are observed only in media devoid of phosphate probably reflects the entry into the cells of small amounts of As(V), which are reduced by *ArsI* in strains SARS4, SARS10, and SARS11.

Three families of arsenate reductases have been described to date (25). Here we show that $ArsC_{syn}$, although related to thioredoxin-dependent arsenate reductases, can be considered a member of a new arsenate reductase family, since it clusters together with sequences from other cyanobacteria, forming an independent branch in phylogenetic trees (Fig. 7). The branch that includes $ArsC_{syn}$ has probably shifted from a thioredoxin-dependent to a GSH-dependent reduction mechanism, and the signature for this can be found in the C80–C82 pair of cysteines (Fig. 4B). The arsenate reduction mechanism in the *B. subtilis* and *S. aureus* *ArsC* enzymes is well described and involves a disulfide cascade leading to conformational changes in the α -helix containing the reactive cysteines C82 and C89 (3, 24–27). These cysteines are present in an α -helix that is buried in the *ArsC* structure, and oxidation of these cysteines to a disulfide bridge leads to a change from an α -helix to a loop conformation. This structural reorganization exposes the disulfide bridge and allows it to be reduced by thioredoxin (3, 13, 24, 26). The change in the spacing between these two cysteines in $ArsC_{syn}$ may explain why this protein is reduced by GSH but not by thioredoxin. Two reasons can be argued. The first is that a disulfide bridge between C80 and C82 probably would not cause a conformational switch from an α -helix to a loop conformation, because these cysteines are closely situated and will not disturb the α -helix. The second reason is that the spacing is not adequate for thioredoxin interaction. The site-directed mutagenesis of $ArsC_{syn}$ supports the second explanation, although modeling of WT and mutant $ArsC_{syn}$ enzymes using the SWISS-MODEL server with the sole constraint that the C80 and C82 cysteines form a disulfide bridge results in a model with an α -helix in the WT and a loop in the mutant (data not shown). Thus, a combination of both effects can be claimed to explain the difference in reduction mechanisms between *S. aureus* *ArsC* and $ArsC_{syn}$. Although a change in the spacing between the cysteines is essential for thioredoxin interaction (Fig. 4) and probably also for the conformational change induced upon oxidation of the cysteines, changes other than those introduced in this work may be needed to obtain thioredoxin-dependent arsenate reduction activity, since there are other residues conserved in *S. aureus* *ArsC* that are not conserved in $ArsC_{syn}$. The change from a thioredoxin- to a GSH-dependent mechanism in cyanobacterial arsenate reductase could be due to an easier availability of GSH than of

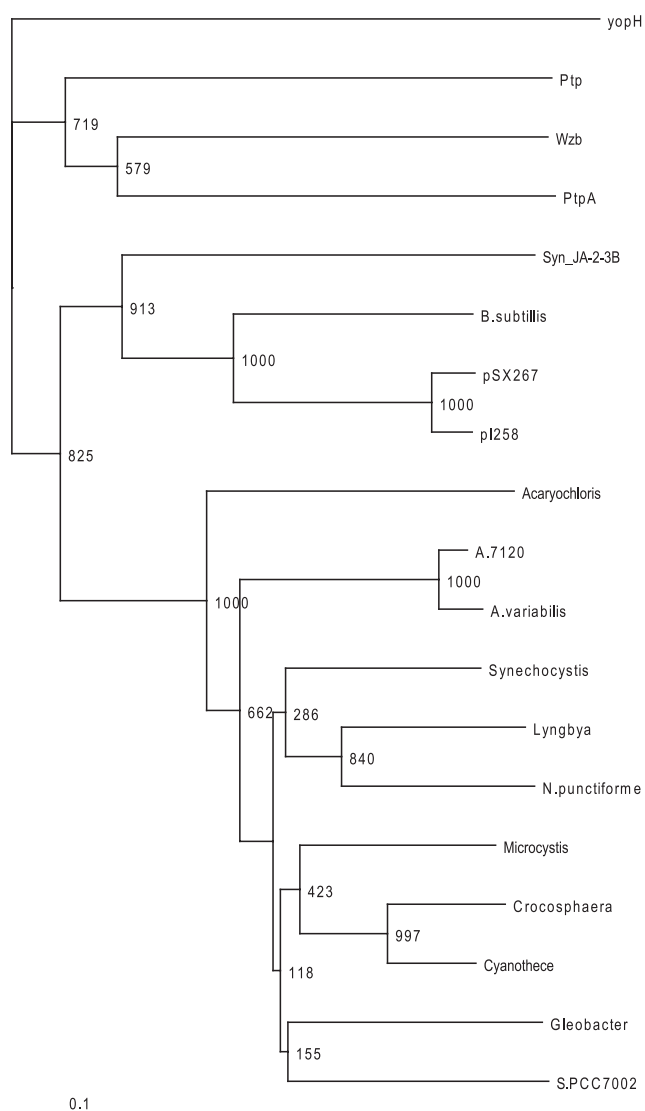


FIG. 7. Phylogenetic analysis of $ArsC_{syn}$. Shown is a neighbor-joining tree of full-length amino acid sequences of the following arsenate reductases (sequences are from the ENTREZ protein database): $ArsC_{syn}$ (*Synechocystis*), Alr1105 from *Anabaena* sp. strain PCC 7120 (A.7120; accession no. NP_485148), Ava 3712 from *Anabaena variabilis* (ZP_00159236), Glr0004 from *Gloeobacter violaceus* (NP_922950.1), and *ArsC* enzymes from *Nostoc punctiforme* (ZP_00108511), *Crocosphaera watsonii* WH 8501 (ZP_00349942), *Lyngbya* sp. strain PCC 8106 (ZP_01620002), *Synechococcus* sp. strain JA-2-3B'a (Syn_JA-2-3B; YP_478568), *Acaryochloris marina* MBIC11017 (YP_001514958), *Synechococcus* sp. strain PCC 7002 (S.PCC7002; YP_001733851), *Microcystis aeruginosa* NIES-843 (YP_001655268), *Cyanothece* sp. strain ATCC 51142 (YP_001804412), *Bacillus subtilis* (BAA12434), *Staphylococcus xylosus* plasmid pSX267 (Q0125), and *S. aureus* plasmid pI258 (P30330). Low-molecular-weight phosphatases are Wzb from *E. coli* (ECU38473), PtpA from *Streptomyces coelicolor* A3 (NP_628106), Ptp from *Acinetobacter johnsonii* (CAA75430), and YopH from *Yersinia pseudotuberculosis* (P08538). YopH was used as the outgroup. The scale bar corresponds to 0.1 estimated amino acid substitution per site. Bootstrap values for 1,000 permutations are shown.

reduced thioredoxin. In this regard, the more abundant thioredoxin (TrxA) is essential in *Synechocystis* and in other cyanobacteria (11, 33, 34), probably indicating its main role in regulating cell metabolism. Furthermore, other thioredoxins

present in *Synechocystis* are expressed at lower levels and do not respond to the presence of arsenate in the growth medium (our unpublished observations). This is also supported by the fact that all cyanobacterial arsenate reductases use GSH as a reducing system (since they are homologs of ArsC_{syn} or *E. coli* ArsC [see Table S2 in the supplemental material]), except for *Synechococcus* sp. strain JA-3-3Ab, which contains a gene coding for an ArsC protein of the *S. aureus* family (Fig. 7).

Interestingly, inactivation of all glutaredoxin genes does not seem to affect the growth of *Synechocystis* sp. strain PCC 6803. In most organisms, inactivation of all glutaredoxin genes is not possible (7, 9). Recently it has been reported that GrxA inactivation impairs H₂O₂ resistance in *Synechocystis*, that GrxA is reduced by a thioredoxin reductase, and that reduced GrxA is able to reduce GrxB (18). In addition, these investigators identified several putative targets that overlap with those of the thioredoxin system in *Synechocystis*. These data suggest that the two systems have redundant roles and that in the absence of the glutaredoxin system, thioredoxins can reduce some of its targets. Particularly striking is the lack of a clear phenotype of the SGRXC mutant, since all homologs of *grxC* either are essential or exhibit a drastic phenotype (2, 9, 41, 51). *Synechocystis* GrxC has been shown to contain an iron-sulfur group (38), like all other monothiolic glutaredoxins (9, 42), and can partially complement the *grx5* mutation in *Saccharomyces cerevisiae* (28), suggesting that GrxC is involved in iron-sulfur assembly, as are plant and yeast monothiolic glutaredoxins (2, 42). Monothiolic glutaredoxin has been proposed to be a scaffold, storage, or regulatory protein during Fe-S cluster assembly in plant chloroplasts (2). Two Fe-S cluster assembly pathways, Suf and Isc, have been described for cyanobacteria (1, 14, 29, 50). The Suf system is involved in Fe-S cluster assembly for photosystem I (with the help of a NifU like protein), while the role of the Isc system is less well characterized. Further experiments are required to clarify if *Synechocystis* GrxC has roles similar to those of its plant homologs in Fe-S cluster assembly and to determine its relation to the Suf and Isc pathways.

Our *in vivo* experiments demonstrate that GrxA has a central role in arsenate reduction, since all strains lacking GrxA are less tolerant of the presence of arsenate in the medium than strains with GrxA. This phenotype is not due to a low level of expression of the *gshB* gene, encoding GSH synthetase, which is just downstream of the *grxA* gene and forms an operon with it (our unpublished observations). Moreover, SGRXA and WT strains contain the same levels of GSH (data not shown). Our results indicate that at least in *Synechocystis* sp. strain PCC 6803, and for ArsC_{syn}, there is a specific dependence on GrxA as an electron donor, and GrxB cannot compensate for the lack of GrxA *in vivo*. This is further supported by the lack of induction of the *grxB* or *grxA* gene in strain SGRXA or SGRXB, respectively, in response to the presence of arsenate (Fig. 5B and C). These data also contrast with data reported on arsenic resistance in *E. coli*, where resistance to arsenate was not affected in any single glutaredoxin mutant or in a triple mutant with all dithiolic glutaredoxin genes interrupted, suggesting that there is another reductant for *E. coli* ArsC (46). The strong specificity of ArsC_{syn} for GrxA *in vivo* contrasts with the much smaller differences observed between the specificities for GrxA and GrxB *in vitro*. Two reasons might explain this apparent discrepancy. First, GrxA protein may be

much more abundant than GrxB. Second, the compartmentalization or spatial localization of GrxB may be different from that of ArsC_{syn}. Further experiments will be required to investigate these possibilities.

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