

# An Arsenate Reductase from *Synechocystis* sp. Strain PCC 6803 Exhibits a Novel Combination of Catalytic Characteristics

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The deduced protein product of open reading frame *slr0946* from *Synechocystis* sp. strain PCC 6803, SynArsC, contains the conserved sequence features of the enzyme superfamily that includes the low-molecular-weight protein-tyrosine phosphatases and the *Staphylococcus aureus* pI258 ArsC arsenate reductase. The recombinant protein product of *slr0946*, rSynArsC, exhibited vigorous arsenate reductase activity ( $V_{\max} = 3.1 \mu\text{mol}/\text{min} \cdot \text{mg}$ ), as well as weak phosphatase activity toward *p*-nitrophenyl phosphate ( $V_{\max} = 0.08 \mu\text{mol}/\text{min} \cdot \text{mg}$ ) indicative of its phosphohydrolytic ancestry. pI258 ArsC from *S. aureus* is the prototype of one of three distinct families of detoxifying arsenate reductases. The prototypes of the others are Acr2p from *Saccharomyces cerevisiae* and R773 ArsC from *Escherichia coli*. All three have converged upon catalytic mechanisms involving an arsenocysteine intermediate. While SynArsC is homologous to pI258 ArsC, its catalytic mechanism exhibited a unique combination of features. rSynArsC employed glutathione and glutaredoxin as the source of reducing equivalents, like Acr2p and R773 ArsC, rather than thioredoxin, as does the *S. aureus* enzyme. As postulated for Acr2p and R773 ArsC, rSynArsC formed a covalent complex with glutathione in an arsenate-dependent manner. rSynArsC contains three essential cysteine residues like pI258 ArsC, whereas the yeast and *E. coli* enzymes require only one cysteine for catalysis. As in the *S. aureus* enzyme, these “extra” cysteines apparently shuttle a disulfide bond to the enzyme’s surface to render it accessible for reduction. SynArsC and pI258 ArsC thus appear to represent alternative branches in the evolution of their shared phosphohydrolytic ancestor into an agent of arsenic detoxification.

Arsenate and related compounds (e.g., arsenite, antomite) are naturally occurring, broadly acting toxins frequently encountered at biologically deleterious concentrations in the environment (reviewed in reference 44). The strong chemical parallels between phosphorous and arsenic, which reside in the same column of the periodic table, limit the ability of phosphate transport systems to discriminate between this vital nutrient and arsenate (45), thus exacerbating the latter’s toxic potential. Microorganisms combat the collateral importation of arsenate by a two-step mechanism in which this compound is first reduced to arsenite. Although arsenite is a more potent toxicant than arsenate, the former can be selectively banished from the cell’s interior through the intervention of a dedicated, inducible transporter.

While the basic strategy for conferring arsenical resistance is broadly conserved among microorganisms, the arsenate reductases responsible for catalyzing the conversion of arsenate to arsenite are not. To date, three “detoxifying” arsenate reductases have been identified and characterized in molecular detail: Acr2p from the microbial eukaryote *Saccharomyces cerevisiae* (2), ArsC encoded by plasmid R773 from the gram-negative bacterium *Escherichia coli* (R773 ArsC) (7), and ArsC encoded by plasmid pI258 from the gram-positive bacterium *Staphylococcus aureus* (pI258 ArsC) (20). It should be noted that some microorganisms can utilize arsenate as a terminal electron acceptor for the generation of cellular energy (30).

However, their “respiratory” arsenate reductases differ radically in both structure and function from the detoxifying arsenate reductases (24) and therefore will not be considered further herein.

Acr2p, R773 ArsC, and pI258 ArsC are the exemplars of three distinct, independent families of arsenate reductases that differ from one another in several of their physical and catalytic properties (reviewed in reference 37). Recombinant Acr2p forms a homodimer (38), while R773 ArsC (31) and pI258 ArsC (19) are monomeric. The *E. coli* (15) and yeast (38) enzymes obtain their reducing equivalents from glutathione and glutaredoxin, whereas pI258 ArsC requires thioredoxin (19, 21). While R773 ArsC displayed only a slight hint of sigmoidal kinetic behavior with respect to arsenate (28), Acr2p exhibited strong positive cooperativity (36), and the saturation curve for pI258 ArsC is strikingly biphasic (19, 33). The *S. aureus* enzyme contains three catalytically essential cysteine residues (32), while R773 ArsC (28) and Acr2p (36) each possess only one. While genes encoding homologs of both R773 ArsC (4, 11, 39, 47, 51) and pI258 ArsC (6, 46, 52) have been identified in a variety of bacterial organisms, in general, the functional characteristics of their protein products have yet to be examined in detail.

Perhaps not surprisingly given the chemical similarity of arsenate and phosphate, Acr2p from *S. cerevisiae* and pI258 ArsC from *S. aureus* both are descended from protein phosphatases (14, 48). Acr2p is a derivative of the Cdc25 family of dual-specific protein phosphatases that are responsible for the dephosphorylation of adjacent phosphotyrosine and phosphothreonine residues on cyclin-dependent protein kinases during the eukaryotic cell cycle (40). pI258 ArsC, on the other hand,

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is derived from a family of protein-tyrosine phosphatases sometimes referred to as the low-molecular-weight protein-tyrosine phosphatases (LMW PTPs) (42). Both pI258 ArsC (56) and a homologous protein from *Bacillus subtilis* (1) exhibit vestigial phosphohydrolase activity toward the small phospho-monoester *p*-nitrophenyl phosphate (*p*NPP).

Despite their discrete origins, Cdc25 and the LMW PTPs (along with a third protein phosphatase family, the conventional protein-tyrosine phosphatases) have converged upon a common catalytic mechanism and active-site motif, Cys-Xaa<sub>5</sub>-Arg, sometimes referred to as the P-loop (reviewed in references 18 and 57). The defining feature of this mechanism is the formation of a covalent phosphoenzyme intermediate with the conserved active-site cysteine. The P-loop arginine participates in the binding of phosphoester substrates and the stabilization of the phosphoenzyme intermediate. The Cys-Xaa<sub>5</sub>-Arg active-site signature sequence also is conserved among the respective arsenate-reducing derivatives of these protein phosphatases, where the conserved cysteine forms the analogous thioester with arsenate (reviewed in reference 37). Intriguingly, while R773 ArsC from *E. coli* exhibits no recognizable sequence similarity with either the yeast or *S. aureus* arsenate reductases, it too has converged upon a catalytic mechanism involving the formation of an arsenocysteine intermediate (31).

During the course of our continuing investigations into the protein-serine/threonine/tyrosine phosphorylation-dephosphorylation events that take place in *Synechocystis* sp. strain PCC 6803, we encountered three open reading frames [ORFs] in the cyanobacterium's genome whose deduced protein products contained the Cys-Xaa<sub>5</sub>-Arg-Ser/Thr-Xaa<sub>85-105</sub>-Asp-Pro signature sequence that is conserved among the LMW PTPs and their homologs (48). When analysis of the expressed protein product of one of these ORFs, *slr0946*, revealed it to be a relatively inefficient phosphohydrolase, we explored the possibility that it might be a pI258 ArsC-like arsenate reductase. While this did, indeed, prove to be the case, the catalytic mechanism of the arsenate reductase from *Synechocystis* sp. strain PCC 6803, SynArsC, was observed to deviate in many important respects from that of the *S. aureus* prototype.

## MATERIALS AND METHODS

**Materials.** Glutaredoxin 1, glutathione reductase, thioredoxin, and thioredoxin reductase were all from Sigma (St. Louis, Mo.). Chelating Sepharose Fast Flo was from Pharmacia (Uppsala, Sweden). The pET101/D-TOPO cloning kit was from Invitrogen (Carlsbad, Calif.). Oligonucleotides were from Life Technologies, Inc. (Frederick, Md.). [<sup>35</sup>S]glutathione was from Perkin-Elmer Life Sciences (Boston, Mass.). All general laboratory reagents and culture media were from Sigma or Fisher (Pittsburgh, Pa.), unless otherwise noted.

**Standard procedures.** Protein concentrations were determined by the method of Bradford (3) using premixed reagent and a standardized solution of bovine serum albumin, both from Pierce (Rockford, Ill.). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by Laemmli (25). Gels were stained with Coomassie blue as described by Fairbanks et al. (13).

**Growth of the organism and isolation of genomic DNA.** The cyanobacterium *Synechocystis* sp. strain PCC 6803 was cultivated in 200 ml of BG11 medium (43) at a temperature of 27°C with continuous shaking at 100 rpm and continuous lighting at 10 microeinsteins m<sup>2</sup> s<sup>-1</sup>. Upon reaching the late exponential phase of growth, the cells were harvested by centrifugation, washed three times with type 1 reagent-grade water (resistivity ≥ 17.3 Mohm cm<sup>-1</sup>), and stored at -80°C until needed. Cells were lysed, and genomic DNA was isolated as described by Li et al. (26).

**Cloning of ORF *slr0946* and expression of its recombinant protein product.**

ORF *slr0946* was cloned using the materials provided in the pET101/D-TOPO cloning kit following the manufacturer's protocols. Briefly, *slr0946* was amplified by PCR using genomic DNA (20 ng) as template and 10 pmol each of a forward and a reverse oligonucleotide primer. The sequences of the forward and reverse primers were, respectively, 5'-CACCGTAAAAAGGTAATGTTTCGTTTGC-3' and 5'-GCTAATTTGGCGATCAGGTTTCCACC-3'. The resulting PCR product was then ligated into vector PET101/D-TOPO, which added oligonucleotides encoding a C-terminal extension to the full-length sequence of the wild-type protein that contained a hexahistidine sequence and a recognition epitope for the anti-Xpress antibody. Fusion to the C terminus was selected to avoid potential interference with the predicted active-site sequence of the protein, which was located near the extreme N terminus of the protein. The resulting plasmid was used to transform One Shot TOP 10 chemically competent *E. coli* cells. The orientation and sequence of the cloned gene were verified by DNA sequencing of the isolated plasmid. This plasmid was then used to transform *E. coli* BL21Star (DE3) One Shot cells. The transformed cells were cultured at 37°C in 200 ml of Luria-Bertani medium containing 0.1 mg of ampicillin/ml until the optical density at 600 nm reached 0.6 to 0.8. Isopropyl-β-D-thiogalactopyranoside was then added to a final concentration of 1 mM, and the cells were harvested 3 h later.

Site-directed mutagenesis of ORF *slr0946* was performed using Promega's (Madison, Wis.) Gene Editor in vitro site-directed mutagenesis system according to the manufacturer's instructions. For the mutagenic alteration of Cys<sub>88</sub>, Cys<sub>13</sub>, Cys<sub>80</sub>, and Cys<sub>82</sub> to Ser, the following primers were used: 5'-AATGTTTCGTTA GCAAACGTAAT-3', 5'-ACGTAATCCAGTCGCTCCCA-3', 5'-TAATTTCCCTTAAGTGGCTGTGG-3', and 5'-CCTTTGTGGCAGTGGGGTTAA-3', respectively.

**Purification of rSynArsC.** The cell pellet harvested as described above was resuspended in 5 ml of 50 mM Tris-HCl, pH 7.5, containing 10 mM imidazole, 250 mM NaCl, and 5% (vol/vol) glycerol (lysis buffer). Lysozyme (10 mg) was added, and the suspension was placed on ice for 30 min. The cells then were lysed by sonic disruption, and the lysate was clarified by centrifugation at 10,000 × *g* for 20 min. The supernatant liquid was applied to a 0.5- by 5-cm column of chelating Sepharose Fast Flo that had been charged with NiSO<sub>4</sub> and subsequently equilibrated with lysis buffer. The column was then washed in lysis buffer, and adherent proteins were eluted with lysis buffer containing 150 mM imidazole. Protein-containing fractions were then pooled, dialyzed versus 20 mM Tris-HCl (pH 7.5) containing 0.5 mM EDTA and 1 mM dithiothreitol (DTT), and stored at 4°C. Unless otherwise noted, recombinant SynArsC (rSynArsC) was dialyzed versus 20 mM Tris-HCl (pH 7.5) containing 0.5 mM EDTA to remove DTT prior to each experiment.

**Assay of arsenate reductase activity.** Arsenate reductase activity was measured using a coupled assay system that measures the arsenate-dependent oxidation of NADPH (15). Briefly, the recombinant protein product of ORF *slr0946* (rSynArsC; 25 μg unless otherwise indicated) was incubated at 37°C in 1.0 ml of 100 mM Tris-HCl (pH 7.5) containing, unless otherwise indicated, 0.1 mg of bovine serum albumin/ml, 0.25 mM NADPH, 0.2 μM yeast glutathione reductase, 8 mM reduced glutathione, 40 mM sodium arsenate, and 4 μM *E. coli* glutaredoxin 1. Oxidation of NADPH was monitored spectrophotometrically at a wavelength of 340 nm. The quantity of NADPH oxidized was calculated using an extinction coefficient of 6,200 M<sup>-1</sup> cm<sup>-1</sup>. Kinetic constants were extrapolated using a Hanes-Woolf plot.

**Assay of phosphatase activity.** The phosphatase activity of rSynArsC initially was assayed using *p*NPP as substrate. Briefly, 25 μg of rSynArsC was incubated for 30 min at 37°C in 200 μl of 100 mM Tris-HCl (pH 7.5) containing 5 mM DTT. The concentration of *p*NPP was varied over a range of 10 to 250 mM. The reaction was terminated by the addition of 400 μl of 0.5 M NaOH, and the absorbance of the resulting solution was determined at a wavelength of 410 nm. The quantity of *p*-nitrophenol produced was calculated using an extinction coefficient of 17,800 M<sup>-1</sup> cm<sup>-1</sup>. Kinetic constants were extrapolated using a Hanes-Woolf plot. Inclusion of DTT in the assay buffer resulted in an approximately twofold increase in reaction rate with little effect on the apparent affinity for substrate.

The assay for protein-tyrosine phosphatase activity employed similar incubation conditions to those described above, with the exception that reduced carboxyamidomethylated and maleylated lysozyme that had been phosphorylated on tyrosine residues, at a final concentration of 3 μM protein-bound [<sup>32</sup>P]phosphate, was substituted for *p*NPP. The phosphorylation of reduced carboxyamidomethylated and maleylated lysozyme, using [γ-<sup>32</sup>P]ATP and the *lyn* protein-tyrosine kinase, and the determination of the quantity [<sup>32</sup>P]phosphate hydrolyzed following incubation with rSynArsC both were performed as described in reference 41. The estimated limit of detection of this assay was ≥0.55 pmol/min · mg.



TABLE 1. Comparison of the phosphohydrolase activity of the recombinant protein product of ORF *slr0946*, rSlr0946, with other detoxifying arsenate reductases and several bacterial LMW PTPs

Organism and enzyme	$V_{\max}$ ( $\mu\text{mol}/\text{min}/\text{mg}$ )	$K_m$ (mM)	Reference
<b>Arsenate reductases</b>			
<i>Synechocystis</i> rSlr0946 (rSynArsC)	0.08	77	This paper
<i>S. aureus</i> ArsC	0.04	146	56
<i>B. subtilis</i> ArsC	0.04 <sup>a</sup>	NA <sup>b</sup>	1
<b>LMW PTPs</b>			
<i>S. coelicolor</i> A3(2) PtpA	4.85	0.75	27
<i>S. aureus</i> PtpA	33.6	1.2	50
<i>S. aureus</i> PtpB	1.2	1.5	50
<i>E. coli</i> Wzb	4.6	1	54
<i>A. johnsonii</i> Ptp	9.7	5	16

<sup>a</sup> Rate of *p*NPP hydrolysis measured at a single substrate concentration of 70 mM.

<sup>b</sup> NA = not applicable.

alyzed by gel filtration chromatography (data not shown), exhibited clearly detectable phosphohydrolase activity toward *p*NPP, a general substrate for many phosphohydrolases, including the LMW PTPs, in vitro. However, kinetic analyses indicated that its maximal catalytic efficiency for the hydrolysis of *p*NPP was 1 to 2 orders of magnitude lower and its  $K_m$  was 1 to 2 orders of magnitude higher than those reported for LMW PTPs from other bacteria (Table 1). Moreover, when the protein was challenged with a commonly used phosphoprotein substrate for protein-tyrosine phosphatases, reduced carboxyamidomethylated and maleylated lysozyme that had been phosphorylated with the *lyn* protein-tyrosine kinase (41), no protein-tyrosine phosphatase activity was detected (data not shown).

**The protein product of ORF *slr0946* possesses arsenate reductase activity.** Continued annotation of the genome of *Synechocystis* sp. strain PCC 6803 (22) revealed that an ORF immediately adjacent to *slr0946*, *slr0945*, resembled *arsH*. *arsH* is a gene of unknown function found in a few arsenate-resistant bacteria (39, 47). In pathogenic strains of *Yersinia*, however, its presence in either *cis* or *trans* was essential for arsenate resistance (39). The revelation that ORF *slr0946* was juxtaposed with a potential arsenate resistance gene, coupled with the lack of performance of its recombinant protein product as a phosphatase, stimulated us to directly assess whether it might be an arsenate reductase.

When the recombinant protein was assayed using thioredoxin—the source of reducing equivalents for the homologous arsenate reductase from *S. aureus*, pI258 ArsC (19, 21), thioredoxin reductase, and NADPH—no arsenate reductase activity could be detected. However, when glutathione and glutaredoxin—the source of reducing equivalents for the dissimilar arsenate reductases Acr2p (38) and R773 ArsC (15)—and glutathione reductase were substituted for thioredoxin and thioredoxin reductase, NADPH was oxidized at a relatively rapid rate (Fig. 2). Product formation in this coupled enzyme system was linearly dependent upon time and the quantity of protein present. Pretreatment of the protein with 1 mM DTT had no apparent effect on the observed reaction rate. In the absence of arsenate, little or no NADPH oxidation could be detected, even when an equivalent quantity of either phosphate or sulfate was included, indicating that the protein

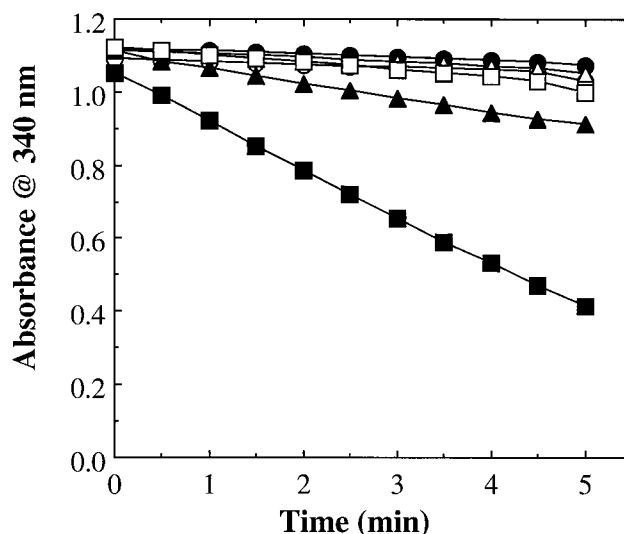


FIG. 2. rSlr0946 possesses arsenate reductase activity. The arsenate reductase activity of the recombinant protein product of ORF *slr0946*, rSlr0946 (25  $\mu\text{g}$ ), was measured spectrophotometrically as described in Materials and Methods (open circles). Also shown are the changes in absorbance observed in control assays in which either the recombinant protein (closed circles), arsenate (open triangles), glutathione (closed triangles), glutaredoxin (open squares), or glutathione reductase (closed squares) was omitted.

product of ORF *slr0946* was acting as an arsenate reductase. We therefore designated the native protein SynArsC and the recombinantly produced fusion protein used for our experiments as rSynArsC. rSynArsC required glutaredoxin for activity (Fig. 2). Significant levels of arsenate-dependent NADPH oxidation, i.e.,  $\geq 25\%$  of maximum, were detectable over a wide range of pH values, i.e., 6.0 to 9.5, with highest activity being achieved around pH 7.5 in this coupled enzyme system.

We next examined the dependence of the measured rate of arsenate reduction by rSynArsC upon the concentration of the individual components of the coupled assay system in order to determine optimal, saturating levels for each (Fig. 3). The arsenate reductase activity of rSynArsC exhibited simple saturation kinetics with respect to both arsenate (Fig. 3A) and glutaredoxin (Fig. 3B) over the entire concentration range examined. Kinetic analysis indicated that rSynArsC was a much better arsenate reductase than phosphohydrolase (Table 2). Its  $V_{\max}$  for the reduction of arsenate was roughly one-fifth that of its sequence homolog, pI258 ArsC, comparable to that of R773 ArsC, and an order of magnitude greater than that of Acr2p (Table 2). The  $K_m$  of rSynArsC for arsenate, 1.25 mM, was 20-fold higher than that reported for pI258 ArsC, although it was significantly lower than that of either Acr2p or R773 ArsC (Table 2). The apparent  $K_m$  of rSynArsC for glutaredoxin, 0.43  $\mu\text{M}$ , was comparable to that of Acr2p, 0.6  $\mu\text{M}$  (38).

**Mutagenic alteration of cysteine residues 8, 13, 80, or 82.** The roles of four of the five cysteine residues in rSynArsC, specifically those that appeared to be physically and/or functionally conserved between this cyanobacterial arsenate reductase and its homologs from *S. aureus* and *B. subtilis*, were examined using site-directed mutagenesis. As expected, mutagenic alteration of Cys<sub>8</sub>, which corresponds to the conserved

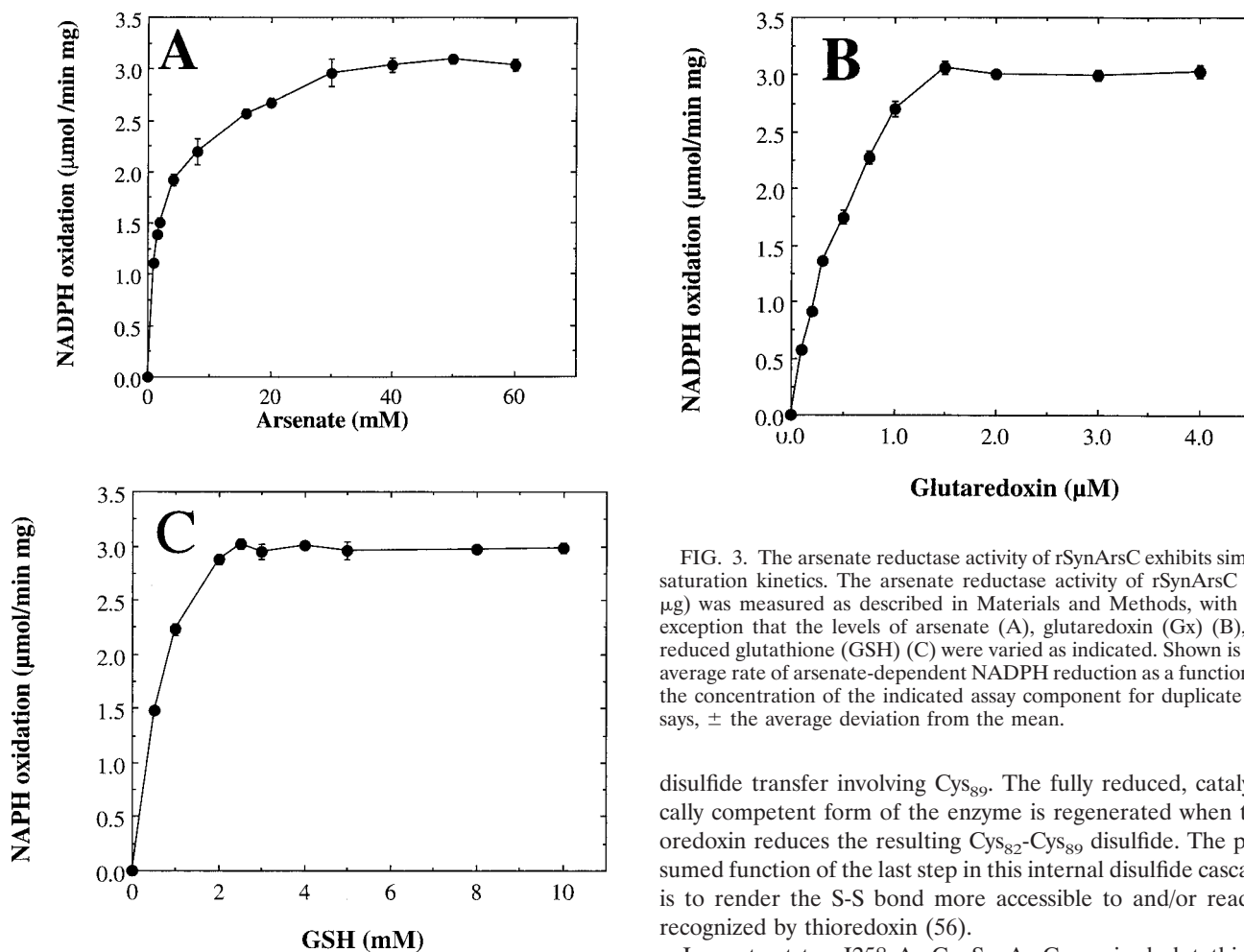


FIG. 3. The arsenate reductase activity of rSynArsC exhibits simple saturation kinetics. The arsenate reductase activity of rSynArsC (25  $\mu\text{g}$ ) was measured as described in Materials and Methods, with the exception that the levels of arsenate (A), glutaredoxin (Gx) (B), or reduced glutathione (GSH) (C) were varied as indicated. Shown is the average rate of arsenate-dependent NADPH reduction as a function of the concentration of the indicated assay component for duplicate assays,  $\pm$  the average deviation from the mean.

active-site cysteine that forms an arsenocysteine intermediate in prototypical arsenate reductases (31) and the phosphocysteine intermediate in homologous LMW PTPs (9, 55), to serine resulted in the complete loss of both arsenate-dependent NADPH oxidation and *p*NPP hydrolysis (Table 3). Mutagenic alteration of Cys<sub>13</sub>, which is conserved in the pI258 ArsC from *S. aureus* and its homolog from *B. subtilis* (Fig. 1) as well as in many bacterial LMW PTPs (27), to serine had little or no effect on arsenate reductase activity (Table 3). Similar behavior was reported for pI258 ArsC (32). Substitution of Cys<sub>13</sub> by serine did, however, abolish the ability of rSynArsC to hydrolyze *p*NPP. This finding was not wholly unexpected, as substitution of the equivalent cysteine residue in a bovine LMW PTP has been variously reported to either reduce (10) or eliminate (8, 9) its phosphohydrolyase activity.

pI258 ArsC from *S. aureus* and its homolog from *B. subtilis* contain a pair of cysteine residues, Cys<sub>82</sub> and Cys<sub>89</sub>, that are not conserved among their phosphohydrolytic counterparts, the LMW PTPs (Fig. 1). These cysteines are essential for the arsenate reductase activity of pI258 ArsC (32). In the enzyme from *S. aureus*, the catalytic arsenocysteine intermediate is reduced by electrons generated via the formation of a disulfide bond between the active-site cysteine residue, Cys<sub>10</sub>, and Cys<sub>82</sub> (34). Cys<sub>10</sub> is restored to its initial, reduced state via an internal

disulfide transfer involving Cys<sub>89</sub>. The fully reduced, catalytically competent form of the enzyme is regenerated when thioredoxin reduces the resulting Cys<sub>82</sub>-Cys<sub>89</sub> disulfide. The presumed function of the last step in this internal disulfide cascade is to render the S-S bond more accessible to and/or readily recognized by thioredoxin (56).

In contrast to pI258 ArsC, rSynArsC required glutathione and glutaredoxin for activity, like the structurally dissimilar arsenate reductases Acr2p and R773 ArsC. These latter enzymes require only a single, active-site cysteine for catalysis (28, 36). SynArsC, however, contains another cysteine residue, Cys<sub>80</sub>, whose position within the protein's amino acid sequence precisely corresponds to that of one of the conserved cysteines, Cys<sub>82</sub>, that participates in the internal disulfide cascade in pI258 ArsC and its homologs (Fig. 1). While no exact duplicate of the third essential cysteine from the *S. aureus* enzyme, Cys<sub>89</sub>, is present in SynArsC, the latter protein does contain another cysteine, Cys<sub>82</sub>, in close proximity to Cys<sub>80</sub>. Their close juxtaposition suggests that Cys<sub>80</sub> and Cys<sub>82</sub> could readily form an internal disulfide analogous to the Cys<sub>82</sub>-Cys<sub>89</sub> disulfide of pI258 and *B. subtilis* ArsC. It was therefore asked whether either Cys<sub>80</sub> or Cys<sub>82</sub> in SynArsC was essential for catalysis. As can be seen in Table 3, mutagenic alteration of either cysteine to serine was sufficient to abolish the arsenate reductase activity of rSynArsC. The effect of these mutagenic alterations appears to have been discrete in nature, as they had little or no effect upon the ability of the enzyme to hydrolyze *p*NPP (Table 3).

**Cys<sub>80</sub> and Cys<sub>82</sub> can form a disulfide bond in rSynArsC.** rSynArsC and mutagenically altered variants thereof were incubated with (5,5'-dithiobis) nitrobenzoic acid (DTNB, also known as Ellman's reagent), which reacts with accessible thiol

TABLE 3. Effect of mutagenically produced alterations on the chemical and catalytic properties of rSynArsC<sup>a</sup>

Mutagenic alteration	Arsenate reductase activity (μmol/min/mg)	Phosphatase activity		[ <sup>35</sup> S]GSH bound (mol/mol)	Reactivity w/DTNB (A <sub>412</sub> )
		V <sub>max</sub> (μmol/min/mg)	K <sub>m</sub> (mM)		
None	3.1	0.08	77	0.06	0.22
Cys <sub>8</sub> to Ser	ND <sup>b</sup>	ND	ND	ND	0.15
Cys <sub>13</sub> to Ser	1.8	ND	ND	0.04	0.15
Cys <sub>80</sub> to Ser	ND	0.07	274	0.05	0.32
Cys <sub>82</sub> to Ser	ND	0.08	98	0.05	0.31

<sup>a</sup> The first column lists the various forms of rSynArsC, designated by the amino acid substitution, if any, that was produced by recombinant means. The right-hand columns give the arsenate reductase activity of each enzyme form, as measured under saturating conditions for all reactants with rSynArsC, the kinetic constants of the enzymes for hydrolysis of pNPP (phosphatase activity), the number of moles of [<sup>35</sup>S]glutathione bound per mole of protein, and the absorbance at 412 nm of the free thionitrobenzoate released when the enzyme was incubated with DTNB. Presented are the results of representative experiments. For further details, see Materials and Methods.

<sup>b</sup> ND = not detectable, below the limits of detection for the relevant assay.

groups to form a mixed disulfide with the concomitant release of the chromophore thionitrobenzoate (12). As can be seen from Table 3, substitution of either Cys<sub>8</sub> or Cys<sub>13</sub> with serine resulted in a net decrease in the quantity of free thionitrobenzoate produced, as expected. By contrast, replacement of either Cys<sub>80</sub> or Cys<sub>82</sub> with serine results in an increase of slightly more than one-third in the quantity of free thionitrobenzoate produced, despite the fact that the total number of cysteine residues in the protein has been reduced from five to four. Since mutagenic alteration of either partner in a disulfide bond would be expected to leave the second cysteine in a free, reduced state, these observations suggest that Cys<sub>80</sub> and Cys<sub>82</sub> are so linked in rSynArsC.

**rSynArsC forms a stable complex with glutathione.** In the glutathione-glutaredoxin-dependent arsenate reductases Acr2p and R773 ArsC, catalysis is thought to proceed via a covalent cysteinyl-arseno-glutathione intermediate that subsequently breaks down to form free arsenite and a mixed disulfide between glutathione and the active-site cysteine (29, 31, 49). Glutaredoxin then reduces the mixed disulfide to regenerate the free, reduced form of the enzyme and a mixed disulfide between glutaredoxin and glutathione. In order to better understand the catalytic mechanism of rSynArsC, [<sup>35</sup>S]glutathione was used to determine whether this cyanobacterial arsenate reductase could form a stable complex with glutathione.

When rSynArsC was incubated with [<sup>35</sup>S]glutathione and sodium arsenate and then analyzed by SDS-PAGE in the absence of reducing agents, an SDS-stable complex with glutathione could be detected (Fig. 4). While the proportion of the enzyme molecules that bound the radiolabeled glutathione was relatively small, ranging from 4 to 11% over several experiments (Table 3), complex formation was dependent upon the presence of both arsenate and the active-site cysteine residue, Cys<sub>8</sub> (Fig. 4). Mutagenic alteration of either of the other essential cysteines, Cys<sub>80</sub> or Cys<sub>82</sub>, to serine had little discernible effect on complex formation, suggesting that—at least initially—the glutathione moiety binds to the enzyme via Cys<sub>8</sub>, as predicted (Table 3).

**Glutaredoxin reduces a mixed disulfide between SynArsC and glutathione.** The immediate effect of reducing arsenate to

TABLE 2. Comparison of rSynArsC with detoxifying arsenate reductases from other microorganisms<sup>a</sup>

Organism	Group	Protein	Class	Subunit structure	Reductant	HASeO <sub>2</sub> <sup>-</sup> K <sub>m</sub> (mM)	Gx K <sub>m</sub> (μM)	V <sub>max</sub> (μmol/min/mg)	Optimal pH	Reference
<i>Synechocystis</i> sp. strain PCC 6803	Cyanobacteria	rSynArsC	LMW PTP-like	Homodimer	GSH/Gx	1.25	0.43	3.1	7.5	This paper
<i>E. coli</i> plasmid R773	Gram-negative bacteria	ArsC	Unique	Monomer	GSH/Gx	8	NR	0.8–1.5	6.3–6.8	15
<i>S. aureus</i> plasmid p1258	Gram-positive bacteria	ArsC	LMW PTP-like	Monomer	Thioredoxin	0.068	NA	14.5	7.5	33
<i>S. cerevisiae</i>	Yeast	Acr2p	Cd25-like	Homodimer	GSH/Gx	35	0.6	0.3–0.4	6.5–8.5	38

<sup>a</sup> Abbreviations: Gx, glutaredoxin; GSH, reduced glutathione; NA, not applicable; NR, not reported. The references listed were the sources for the reported V<sub>max</sub>, K<sub>m</sub>, and optimal pH values.

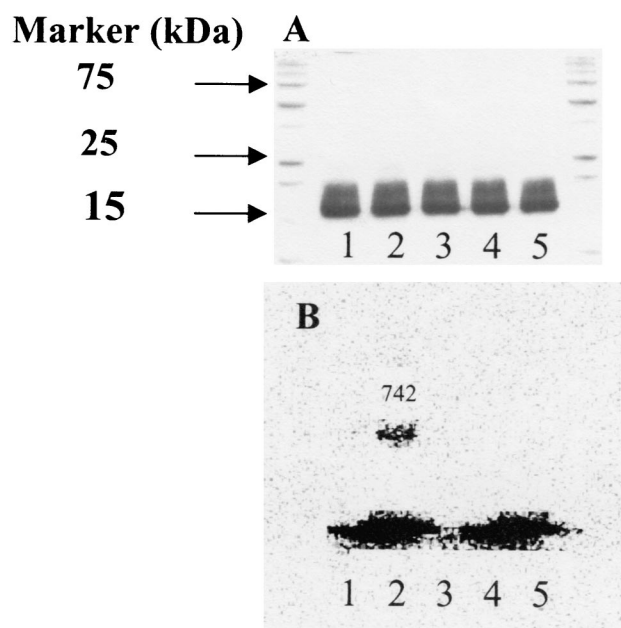


FIG. 4. rSynArsC binds glutathione in an arsenate-dependent manner. rSynArsC or a form in which the predicted active-site cysteine, Cys<sub>8</sub>, was mutagenically altered to serine was incubated with [<sup>35</sup>S]glutathione (4 mM) in either the presence or absence of arsenate as described in Materials and Methods. (A) SDS-polyacrylamide gel stained with Coomassie blue. (B) Electronic autoradiogram of the same gel taken prior to staining. The outer lanes of the gel contain prestained standards, the molecular masses of certain of which are indicated at the left on panel A. Lane 1 contains rSynArsC that was incubated with [<sup>35</sup>S]glutathione (reduced) in the absence of arsenate, while lane 2 contains rSynArsC that was incubated with [<sup>35</sup>S]glutathione in the presence of arsenate. The number given above the upper band in lane 2 lists the number of counts per minute of radioactivity present in the band, as determined by electronic autoradiography (estimated efficiency, ≈2%), relative to that in the corresponding regions of the other lanes. Lane 3 contains rSynArsC, while lanes 4 and 5 contain the Cys<sub>8</sub>-to-Ser variant that was incubated with [<sup>35</sup>S]glutathione in the absence and presence of arsenate, respectively. The radioactive material at the bottom of the gel is free [<sup>35</sup>S]glutathione.

arsenite in detoxifying arsenate reductases is to leave the enzyme in an oxidized state, which must subsequently be reduced to ready the enzyme for the next round of catalysis. In Acr2p and R773 ArsC, glutaredoxin reduces the mixed disulfide formed between the active-site cysteine residue and glutathione, while in pI258 ArsC thioredoxin reduces the internal disulfide formed between Cys<sub>82</sub> and Cys<sub>89</sub> via the enzyme's internal disulfide cascade (1, 56). SynArsC displays features consistent with both mechanisms, i.e., the participation of glutathione and glutaredoxin characteristic of Acr2p and R773 ArsC and the requirement for three, rather than one, cysteine residues characteristic of pI258 ArsC.

Glutaredoxin is capable of reducing both mixed protein-glutathione disulfides and internal protein disulfides. However, while the former requires the presence of only one of the enzyme's two active-site cysteine residues, Cys<sub>11</sub> in the case of glutaredoxin 1 from *E. coli*, the commercially available form of which was used in the assays described above, the reduction of internal protein disulfides requires the participation of both Cys<sub>11</sub> and Cys<sub>14</sub> (5). We therefore cloned the *grx4* gene from

*E. coli* K-12 and used it to produce recombinant forms, as histidine-tagged fusion proteins, of both glutaredoxin 1 and mutagenically altered forms in which either Cys<sub>11</sub> and Cys<sub>14</sub> were replaced with serine. While our recombinantly produced fusion protein was somewhat less efficient than the equivalent quantity of commercial glutaredoxin 1 in supporting catalysis, replacement of Cys<sub>14</sub> by Ser had little effect on the rate of arsenate-dependent NADPH oxidation ( $1.10 \pm 0.05 \mu\text{mol}/\text{min} \cdot \text{mg}$  versus  $1.23 \pm 0.02 \mu\text{mol}/\text{min} \cdot \text{mg}$  with the unaltered glutaredoxin 1), while mutagenic alteration of Cys<sub>11</sub> abolished activity below the limits of detection. These results indicate that glutaredoxin reduces a mixed SynArsC-glutathione disulfide and not an internal cysteine-cysteine disulfide.

## DISCUSSION

Analysis of the catalytic capabilities of the recombinant gene product of ORF *str0946* from *Synechocystis* sp. strain PCC 6803 indicated that it is an arsenate reductase. As has been shown to be the case for the homologous arsenate reductases from *S. aureus* (56) and *B. subtilis* (1), with which it shares 36 and 45% sequence identity, respectively (Fig. 1), rSynArsC also has retained weak, vestigial phosphohydrolase activity indicative of its descent from the LMW PTP family of protein phosphatases. Intriguingly, while SynArsC is clearly a sequence homolog of pI258 ArsC, its catalytic mechanism combines certain features of this *S. aureus* enzyme with others that mimic the structurally dissimilar arsenate reductases from yeast, Acr2p, and *E. coli*, R773 ArsC.

Like Acr2p (38) and R773 ArsC (15), rSynArsC utilizes glutathione and glutaredoxin as its immediate source of reducing equivalents, in direct contrast to pI258 ArsC, which uses thioredoxin (19, 21). As has been postulated for Acr2p and R773 ArsC (29, 31), rSynArsC forms a complex with glutathione. While only small quantities of the complex were detected, its formation was strictly dependent on the presence of both the active-site cysteine residue and arsenate, attributes consistent with catalytic relevance. As is also the case with Acr2p and R773 ArsC, glutaredoxin restores SynArsC to its reduced, catalytically ready state by reducing a mixed disulfide between the side chain of a cysteine residue and glutathione.

The behavior of rSynArsC deviates from that of the yeast and *E. coli* enzymes in at least one important respect. Whereas Acr2p (36) and R773 ArsC (28) require only a single active-site cysteine residue for catalysis, mutagenic alteration of any one of three cysteine residues in rSynArsC eliminated its ability to reduce arsenate at a measurable rate. The two "extra" cysteine residues, i.e., Cys<sub>80</sub> and Cys<sub>82</sub>, appear to play specific, essential roles in the catalysis of arsenate reduction, since (i) their replacement with serine had little or no effect on the ability of the enzyme to hydrolyze *p*NPP and (ii) a similarly positioned pair of cysteine residues play key roles in the catalytic mechanism of pI258 ArsC (1, 32, 56).

In pI258 ArsC and its homolog from *B. subtilis*, the three essential cysteines participate in an internal disulfide cascade (56) that also has been referred to as a redox relay (1). The first step in this cascade is the attack of the sulfhydryl group of Cys<sub>82</sub> in pI258 ArsC on the arsenocysteine intermediate formed between arsenate and Cys<sub>10</sub>, the active-site cysteine. The resulting disulfide constitutes the immediate source of the

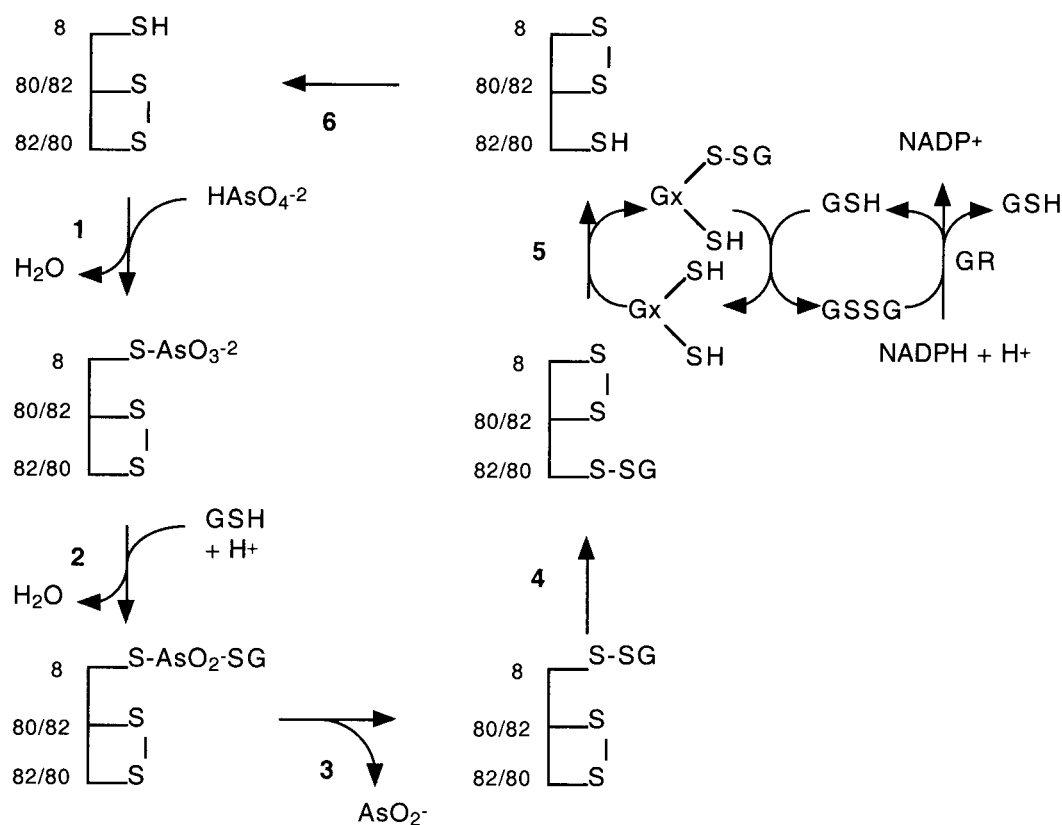


FIG. 5. Schematic description of a potential catalytic mechanism for SynArsC from *Synechocystis* sp. strain PCC 6803. Shown is a potential catalytic mechanism for the SynArsC arsenate reductase consistent with the experimental observations reported herein. Abbreviations: GR, glutathione reductase; GSH, reduced glutathione; GSSG, oxidized glutathione; Gx, glutaredoxin, Cys<sub>8</sub>, the active-site cysteine on rSynArsC; Cys<sub>80/82</sub>, either Cys<sub>80</sub> or Cys<sub>82</sub>; Cys<sub>82/80</sub>, either Cys<sub>82</sub> or Cys<sub>80</sub>.

reducing equivalents necessary to convert arsenate to arsenite. An internal disulfide transfer then occurs that results in the formation of a disulfide bond between Cys<sub>82</sub> and Cys<sub>89</sub>, regenerating the reduced active-site cysteine. The Cys<sub>82</sub>-Cys<sub>89</sub> disulfide, in turn, is reduced by thioredoxin to complete the catalytic cycle. Presumably, the last step in this internal disulfide cascade renders the S-S bond accessible to and/or places it in an environmental context recognizable by thioredoxin. The essential nature of Cys<sub>80</sub> and Cys<sub>82</sub> in rSynArsC suggests that an analogous disulfide cascade takes place in this cyanobacterial arsenate reductase to translocate a mixed cysteine-glutathione disulfide to the enzyme's surface.

Figure 5 displays a possible mechanism for SynArsC that accommodates all of the experimental observations reported herein. Cys<sub>80</sub> and Cys<sub>82</sub> initially are in an oxidized, disulfide-bonded state, as was indicated by titrations with DTNB. Steps 1 to 3 are postulated to reprise the initial steps of the proposed reaction mechanisms of Acr2p and R773 ArsC. Specifically, an arsenoenzyme intermediate is formed in step 1 that then is attacked in step 2 by glutathione to form a cysteinyl-arsenoglutathione intermediate. This intermediate then breaks down, in step 3, to yield arsenite and a mixed disulfide between the active-site cysteine residue, Cys<sub>8</sub>, and glutathione. Steps 4 to 6 represent a modified version of the internal disulfide cascade that takes place in pI258 ArsC. In step 4, an internal disulfide exchange reaction takes place between the mixed disulfide

between Cys<sub>8</sub> and glutathione and the internal disulfide between Cys<sub>80</sub> and Cys<sub>82</sub> that shifts the mixed disulfide with glutathione from within the depths of the active-site pocket to a point nearer the enzyme's surface. The mixed disulfide then is reduced, in step 5, by glutaredoxin. In step 6, a second internal disulfide transfer event restores the active-site cysteine to its reduced, reactive state with the concomitant restoration of the original disulfide bond between Cys<sub>80</sub> and Cys<sub>82</sub>.

Regardless of whether the scheme presented in Fig. 5 proves correct in every detail, it is clear that SynArsC employs a unique combination of mechanistic steps to reduce arsenate to arsenite. How might this novel mechanism have arisen? One possible explanation is that SynArsC initially mimicked the catalytic mechanism of its sequence homolog from *S. aureus*. (Intriguingly, the source of reducing equivalents for the *B. subtilis* homolog of pI258 ArsC has yet to be determined.) However, SynArsC continued to refine its catalytic mechanism until it obtained its contemporary form. The reciprocal also may have occurred, in which case the cyanobacterial enzyme may be a molecular fossil reflecting an intermediate stage in the development of the catalytic mechanism now employed by pI258 ArsC. A third possibility is that cyanobacterial and *S. aureus* enzymes are the products of two independent transformations of LMW PTPs into arsenate reductases. This last possibility appears to be the least likely, as these arsenate reductases are more closely related to each other than they are



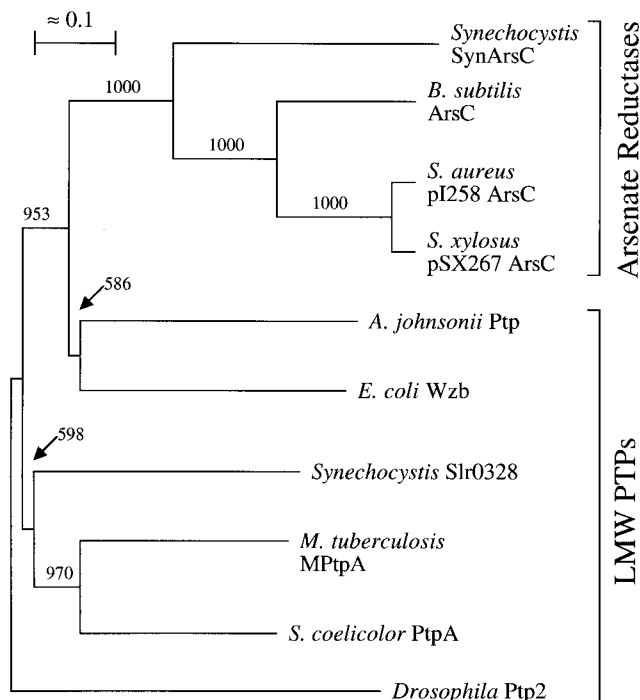


FIG. 6. Phylogenetic relationship between SynArsC and several known or likely arsenate reductases as well as known LMW PTPs from bacterial organisms. The phylogenetic relationships between known or likely pI258 ArsC-like arsenate reductases and known LMW PTPs from bacterial organisms were analyzed using CLUSTAL W version 1.7 (53) as described in Materials and Methods. Known or predicted arsenate reductases utilized for this analysis included the product of ORF *slr0946* from *Synechocystis* sp. strain PC6803 (*Synechocystis* SynArsC [this manuscript]), ArsC from the *skin* element of *B. subtilis* (*B. subtilis* ArsC [52]), ArsC encoded by plasmid pI258 from *S. aureus* (20), ArsC encoded by plasmid pSX267 from *Staphylococcus xylosum* (46), and ArsC encoded by plasmid R773 from *E. coli* (*E. coli* R773 ArsC [7]). LMW PTPs utilized for comparison include Wzb from *E. coli* (54), Ptp from *Acinetobacter johnsonii* (16), the product of ORF *slr0328* from *Synechocystis* sp. strain PC6803 (A. Mukhopadhyay and P. J. Kennelly, unpublished observations), MPTpA from *M. tuberculosis* (23), PtpA from *Streptomyces coelicolor* A3(2) (27), and Ptp2 from *Drosophila melanogaster* (35). The last named was used as the outgroup for these analyses. The scale bar indicates 1 base substitution per 10 amino acid residues. Bootstrap values above 500 (out of 1,000) are indicated at the corresponding nodes.

to bacterial LMW PTPs (Fig. 6). Regardless, the addition of SynArsC to the present library of arsenate reductases and their ancestral LMW PTPs opens exciting new opportunities to explore the mechanisms by which enzymes evolve new catalytic capabilities.

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