JOURNAL OF BACTERIOLOGY, Sept. 2003, p. 5363–5371 Vol. 185, No. 18
0021-9193/03/$08.00+0 DOI: 10.1128/JB.185.18.5363–5371.2003


# Arsenic Sensing and Resistance System in the Cyanobacterium *Synechocystis* sp. Strain PCC 6803

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Received 28 March 2003/Accepted 23 June 2003

**Arsenic is one of the most important global environmental pollutants. Here we show that the cyanobacterium *Synechocystis* sp. strain PCC 6803 contains an arsenic and antimony resistance operon consisting of three genes: *arsB*, encoding a putative arsenite and antimonite carrier, *arsH*, encoding a protein of unknown function, and *arsC*, encoding a putative arsenate reductase. While *arsB* mutant strains were sensitive to arsenite, arsenate, and antimonite, *arsC* mutants were sensitive only to arsenate. The *arsH* mutant strain showed no obvious phenotype under the conditions tested. In vivo the *arsBHC* operon was derepressed by oxyanions of arsenic and antimony (oxidation state, +3) and, to a lesser extent, by bismuth (oxidation state, +3) and arsenate (oxidation state, +5). In the absence of these effectors, the operon was repressed by a transcription repressor of the ArsR/SmtB family, encoded by an unlinked gene termed *arsR*. Thus, *arsR* null mutants showed constitutive derepression of the *arsBHC* operon. Expression of the *arsR* gene was not altered by the presence of arsenic or antimony compounds. Purified recombinant ArsR protein binds to the *arsBHC* promoter-operator region in the absence of metals and dissociates from the DNA in the presence of Sb(III) or As(III) but not in the presence of As(V), suggesting that trivalent metalloids are the true inducers of the system. DNase I footprinting experiments indicate that ArsR binds to two 17-bp direct repeats, with each one consisting of two inverted repeats, in the region from nucleotides −34 to + 17 of the *arsBHC* promoter-operator.**

Arsenicals generated from natural and man-made sources are widely distributed contaminants of freshwater, ground water, and seawater (2, 11, 24). Probably because of this, many organisms contain either chromosomal or plasmid-encoded genes involved in arsenical resistance (*ars* genes). Most of the knowledge about *ars* genes and their regulation in prokaryotes comes from studies of *ars* operons in *Escherichia coli* and *Staphylococcus* species (recently reviewed in references 21, 26, and 27). The chromosomal *ars* operon of *E. coli* and the *ars* operons from staphylococcal plasmids pI258 and pSX267 are constituted by three genes, *arsR*, *-B*, and *-C*. *arsR* encodes a *trans*-acting repressor of the ArsR/SmtB family involved in transcriptional regulation, *arsB* encodes a membrane-bound arsenite carrier that exports arsenite but not arsenate, and *arsC* encodes a reductase that converts arsenate to arsenite. In contrast, the *ars* operons of *E. coli* plasmids R773 and R46 encode two additional proteins: ArsA, an arsenite-stimulated ATPase, and ArsD, another metalloid-responsive transcriptional repressor.

Three different families of arsenate reductases have been described (for revisions, see references 18 and 26). The first family to be described was the product of the *arsC* gene from the *E. coli* plasmid R773 (6). This enzyme uses glutaredoxin as a source of reducing equivalents, and it is present in several gram-negative bacteria. The *Staphylococcus aureus* pI258 and the *Bacillus subtilis* ArsC products exhibit no significant similarity with the R773-encoded enzyme (1, 13). This second type of arsenate reductase is related to low-molecular-weight protein tyrosine phosphatases and uses thioredoxin as the source of reducing equivalents. Finally, a third family of arsenate reductases, represented by the Acr2p enzyme from *Saccharomyces cerevisiae*, is also homologous to a different subfamily of protein phosphatases that includes CDC25a (20, 22).

Arsenate has to be reduced by ArsC to the more toxic form arsenite before being exported by the carrier protein ArsB (for a review, see reference 27). Two independently evolving families of arsenite carriers in bacteria have been described. The *arsB* gene, present in the *E. coli* plasmid R773 and in *Staphylococcus aureus* pI258, encodes an integral membrane protein with 12 membrane-spanning segments that can use the membrane potential to extrude arsenite. Interestingly, ArsB proteins can also function as primary ATP-driven arsenite pumps by interacting with ArsA, an arsenite-stimulated ATPase. The metalloid oxyanion antimonite is also a substrate of this family of ArsB proteins and can stimulate ATPase activity of ArsA (28). The second family of arsenite carriers has been much less characterized and includes the ArsB gene of the *B. subtilis ars* operon and the ARR3 protein from *S. cerevisiae* (formerly ACR3) (41). These arsenite transporters (ArsB/ARR3 family) are membrane proteins with 10 predicted membrane-spanning segments. *ARR3*-disrupted cells accumulate arsenite and are sensitive to arsenite and arsenate but not to antimonite, demonstrating that ARR3p specifically exports As(III) (12).

In addition to these factors, another gene, *arsH*, has been found close to or cotranscribed with arsenic resistance genes in *Yersinia enterocolitica* and in *Acidothiobacillus ferroxidans* (3, 23). The function of the ArsH protein is unknown, but it has been shown to be required for resistance to arsenite and arsenate in *Y. enterocolitica*.

Cyanobacteria are photosynthetic prokaryotes that carry out

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oxygenic photosynthesis. Several cyanobacterial species are able to grow in the presence of high concentrations of arsenate (up to 100 mM) and in low-millimolar concentrations of arsenite (36; our unpublished observations). It is worth noting that the maximum contaminant level for arsenic allowed in public water supplies has recently been lowered to 10 μg/liter (approximately 0.13 μM) by the U.S. Environmental Protection Agency (34). The existence of *ars* genes in cyanobacteria has been suggested by gene homology searches of genome databases, but the function and means of regulation of these genes are unknown. In the present work we have characterized the arsenic resistance system of the cyanobacterium *Synechocystis* sp. strain PCC 6803.

## MATERIALS AND METHODS

**Bacterial strains and growth conditions.** *Synechocystis* sp. strain PCC 6803 was grown photoautotrophically at 30°C in BG11 medium (25) supplemented with 1 g of $HCO_3Na$ per liter (BG11C medium) and bubbled with a continuous stream of 1% (vol/vol) $CO_2$ in air under continuous fluorescent illumination (50 μmol of photons per $m^2$ per s; white light from fluorescent lamps). In the BG11C low-phosphate medium, the concentration of $K_2HPO_4$ was reduced to 10 μM. For plate cultures, BG11C liquid medium was supplemented with 1% (wt/vol) agar. Kanamycin and chloramphenicol were added to achieve final concentrations of 50 to 200 and 10 to 40 μg/ml, respectively, when required.

*E. coli* DH5α (Bethesda Research Laboratories) grown in Luria-Bertani (LB) broth medium as described previously (29) was used for plasmid construction and replication. *E. coli* BL21(DE3) grown in LB broth medium was used for expression of the MalE-ArsR protein. *E. coli* was supplemented with 100 μg of ampicillin/ml, 50 μg of kanamycin/ml, or 40 μg of chloramphenicol/ml and glucose 0.2% (wt/vol) when required.

**Insertional mutagenesis of *Synechocystis* genes.** DNA fragments containing loci slr0944, slr0945, slr0946, and sll1945 were amplified by PCR from the cosmids *cs0223* and *cs0205* (provided by Kazusa DNA Research Institute) using the following oligonucleotide pairs: ARSB2-ARSB3, ARSH1-ARSH2, ARSC1-ARSC2, and ARSR1-ARSR2. The oligonucleotide sequences are available on request. DNA fragments were cloned into pGEM-T (Promega) to generate pARSB1, pARSH1, pARSC1, and pARSR1, respectively. The targeting vector pARSB2(+) was generated by replacing a 79-bp *Bst*EII-*Bst*EII fragment by the C.K1 cassette (8) in the same orientation as that of the *arsB* gene. The targeting vectors pARSH2(+) and pARSH2(−) were generated by inserting the C.K1 cassette into the *Eco*RI sites of the *arsH* gene in the same orientation as that of the *ars* operon [pARSH2(+)] or in the inverse orientation [pARSH2(−)]. The targeting vector pARSC2(−) was generated by inserting the C.K1 cassette into the *Sma*I site of the *arsC* gene in the orientation opposite to that of the *ars* operon. The targeting vector pARSR2(+) was generated by inserting the C.C1 cassette into the *Hin*dIII site of the *arsR* gene in the same orientation as that of the *arsR* gene. All targeting vectors were used to transform *Synechocystis* strain 6803 as previously described (9).

The correct integration and complete segregation of the mutant strain were tested by Southern blotting using standard procedures (29). Total DNA from the cyanobacteria was isolated as previously described (5).

**RNA isolation and Northern blot analysis.** Total RNA was isolated from 25-ml samples of *Synechocystis* cultures at the mid-exponential growth phase (3 to 5 μg chlorophyll/ml). RNA extractions were performed as previously described (10).

For Northern blotting, 15 μg of total RNA was loaded per lane and electrophoresed in 1.0% (wt/vol) agarose denaturing formaldehyde gels and transferred to nylon membranes (Hybond N-plus; Amersham). Prehybridization, hybridization, and washes were performed as described in the Amersham instruction manual. Probes were synthesized by PCR with the same oligonucleotide pairs used for cloning of the genes except that the *arsR* probe was synthesized by using oligonucleotides ARSR2 and ARSR3. DNA probes were $^{32}P$ labeled with a random-primer kit (Amersham) with [α-$^{32}P$]dCTP (3,000 Ci/mmol). All of the filters were stripped and reprobed with a 580-bp *Hin*dIII-*Bam*HI probe from plasmid pAV1100 that contains the constitutively expressed RNase P RNA gene (*rnpB*) from *Synechocystis* strain PCC 6803 (38). To quantify the radioactive signals in Northern blot hybridizations, an InstantImager electronic autoradiography apparatus (Packard Instrument Company, Meriden, Conn.) was used.

**Primer extension analysis.** Oligonucleotide ARSB1 end labeled with T4 polynucleotide kinase and [γ-$^{32}P$]ATP (3,000 Ci/mmol) according to standard procedures (29) was used for primer extension analysis of the *arsBHC* operon transcript. Primer extension reactions were carried out as previously described (15). One-half of the reaction mixture was electrophoresed on a 6% polyacrylamide sequencing gel, and the *arsB* promoter region was subjected to a sequencing reaction using the ARSB1 oligonucleotide.

**Cloning and purification of MalE-ArsR.** The complete *arsR* open reading frame (ORF) was PCR amplified with oligonucleotides ARSR2 (which introduces an *Eco*RI site) and ARSR3 (which introduces a *Pst*I site) and cloned in pGEM-T (Promega) to generate pARSR3. This plasmid was sequenced to verify the fidelity of the PCR product. The 452-bp *Eco*RI-*Pst*I fragment containing the *arsR* gene was cloned into *Eco*RI-*Pst*I-digested pMALc-2x (New England Biolabs) to generate pARSR4. The MalE-ArsR recombinant protein was expressed in *E. coli* BL21(DE3) from the plasmid pARSR4. For that process, 500 ml of culture was grown in LB broth supplemented with 0.2% (wt/vol) glucose to an optical density at 580 nm of 0.6, induced with 1 mM isopropyl-β-D-thiogalactopyranoside for 2.5 h, harvested by centrifugation, and resuspended in 8 ml of 50 mM Tris-HCl (pH 8.0)–100 mM KCl (buffer A) supplemented with 1 mM phenylmethylsulfonyl fluoride, 1 mM EDTA, 1 mM EGTA, and 1 mM ε-aminocaproic acid. Cells were broken by sonication, and insoluble debris was pelleted by centrifugation at 18,000 × *g* for 15 min. The supernatant was then diluted to 40 ml with buffer A, and 1 ml of bed volume amylose resin (New England Biolabs) was added and allowed to bind for 1 h a 4°C with gentle agitation. The resin was then transferred to a plastic column, washed with buffer A until no protein was detectable in the flowthrough, and eluted with the same buffer containing 10 mM maltose and 10% glycerol.

MalE-ArsR was digested with factor Xa protease (New England Biolabs) to separate the MalE domain from ArsR as described by the manufacturer. Briefly, 400 μg of purified MalE-ArsR was incubated for 12 h at 25°C in buffer A containing 2 mM $Cl_2Ca$ with 5 μl of factor Xa in a final volume of 500 μl to yield two protein fragments corresponding to MalE (42 kDa) and ArsR (12.5 kDa).

**Gel retardation assays.** The probe was synthesized by PCR using oligonucleotides ARSB7 and ARSB8, containing *Nar*I and *Nco*I restriction sites, respectively, from cosmid *cs0205* (provided by Kazusa DNA Research Institute). The resulting DNA was digested with *Nco*I and end labeled with [α-$^{32}P$]dCTP (3,000 Ci/mmol) by using the Klenow fragment. The size of the resulting probe was 95 bp from nucleotides −59 to +36 with respect to the transcription start point. The binding reaction was carried out in a final volume of 25 μl containing 4 ng of labeled DNA and 5 μg of salmon sperm DNA in 50 mM Tris-HCl (pH 8.0)–100 mM KCl–5 mM dithioreitol, 10% glycerol, and different amounts of purified ArsR. The mixtures were incubated for 25 min at room temperature and loaded on a nondenaturing 6% polyacrylamide gel. Electrophoresis was carried out at 4°C and 180 V in 0.25× Tris-borate-EDTA. Gels were transferred to Whatman 3MM filter paper, dried, and autoradiographed.

**DNase I footprinting.** The probe was synthesized by PCR using oligonucleotides ARSB9 and ARSB10, containing *Nar*I and *Nco*I restriction sites, respectively. The size of the resulting probe was 237 bp from nucleotides −132 to +105 with respect to the transcription start point. For footprinting the coding strand, the DNA was digested with *Nco*I and end labeled with [α-$^{32}P$]dCTP (3,000 Ci/mmol) by using the Klenow fragment. For footprinting the noncoding strand, the DNA was digested with *Nar*I and end labeled in the same way. The binding reaction was carried out as described for gel retardation assays but without glycerol. DNA-protein complexes were incubated for 30 s or 1 min with 1 U of DNase I (Roche) in the presence of $MgCl_2$. Reactions were terminated by the addition of 90 μl of DNase I stop solution (0.4 M sodium acetate, 0.8% sodium dodecyl sulfate, 12.5 mM EDTA, 1 mg of salmon DNA/ml). The samples were extracted with an equal volume of phenol-chloroform (1:1), precipitated with 2 volumes of ethanol, washed with 70% ethanol, and resuspended in formamide loading buffer. After being heated at 80°C for 20 min, the samples were loaded in a denaturing 6% polyacrylamide sequencing gel together with G+A size standards of the end-labeled fragments by the Maxam and Gilbert methods (described in reference 29). After electrophoresis, the gel was transferred to Whatman 3MM filter paper, dried, and autoradiographed.

## RESULTS AND DISCUSSION

**A metalloid-regulated operon in *Synechocystis*.** Within the fully sequenced genome of *Synechocystis* strain PCC 6803 is a cluster of three genes that shows sequence similarity to arsenic resistance genes (Fig. 1A). The first gene of the cluster (the ORF slr0944) encodes a putative arsenite carrier homologous to the *S. cerevisiae* protein ARR3 (35% amino acid identity)

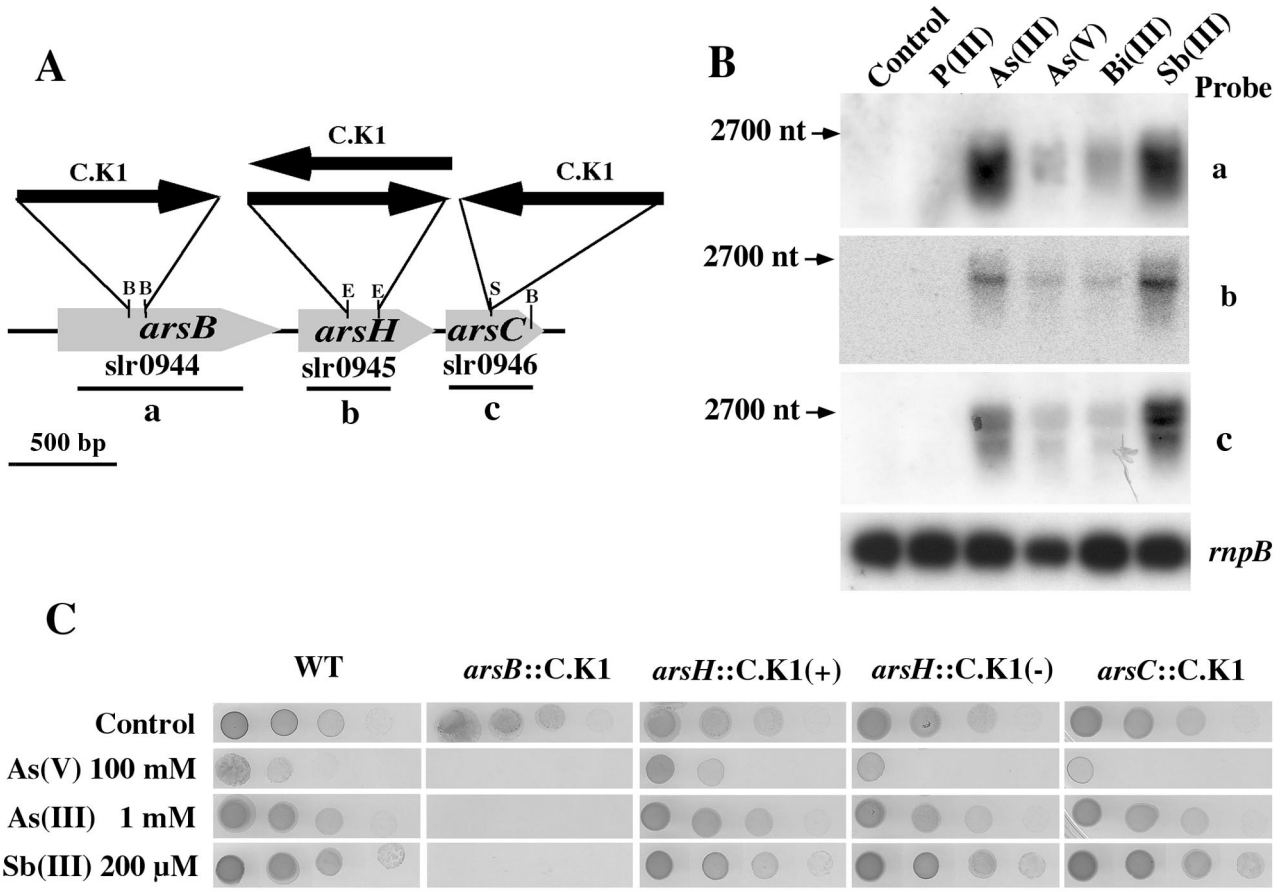


FIG. 1. Metal-dependent regulation and disruption of *Synechocystis ars* genes. (A) Schematic representation of *ars* genomic region in the WT strain and site of insertion of the C.K1 cassette in the *arsB*::C.K1, *arsH*::C.K1(+), *arsH*::C.K1(−), and *arsC*::C.K1 mutants. B, *Bst*EII; E, *Eco*RI; S, *Sma*I. (B) Northern blot analysis of the expression of the *ars* gene cluster. Total RNA was isolated from mid-log-phase *Synechocystis* cells grown in BG11C medium and exposed for 1 h to a 1 mM concentration of either sodium arsenite [As(III)], sodium arsenate [As(V)], potassium antimonyl tartrate [Sb(III)], bismuth nitrate [Bi(III)], and phosphorous acid [P(III)]. Control cells were not exposed to added compounds. Fifteen micrograms of total RNA was denatured, separated by electrophoresis in a 1.2% agarose gel, blotted, and hybridized with probes a, b, and c, indicated in panel A (see Materials and Methods). The filters were stripped and rehybridized with an *rnpB* gene probe as the control. The estimated size of the transcripts (in nucleotides) is indicated. (C) Tolerance of *Synechocystis* WT and *Synechocystis ars* mutants to metalloids. Tenfold serial dilutions were spotted on low-phosphate BG11C plates; supplemented with the indicated concentration of sodium arsenite [As(III)], sodium arsenate [As(V)], or potassium antimonyl tartrate [Sb(III)]; and photographed after 10 days of growth.

and to the *B. subtilis* ArsB protein (26% amino acid identity) (41). The second gene of the group (slr0945), separated by 152 bp of slr0944, is related to the *Y. enterocolitica arsH* gene (67% amino acid identity) (23). Ninety base pairs downstream of slr0945 is the third ORF (slr0946) of the cluster, encoding a predicted protein with sequence similarity to the thioredoxin-dependent arsenate reductases of the *Staphylococcus* (42% amino acid identity) and *Bacillus* (37% amino acid identity) strains (1, 13). Based on their respective homologies, these three genes were named *arsB*, *arsH*, and *arsC*. To find evidence of the role of these three genes in metalloid resistance, we analyzed their levels of expression in the presence of different forms of elements of group 15 of the periodic table. Three probes, one for each of the *ars* genes, were used to hybridize total RNA obtained from mid-log-phase *Synechocystis* cells grown in BG11C medium and exposed for 1 h to a 1 mM concentration of either sodium arsenite ($NaAsO_2$)[As(III)], sodium arsenate ($Na_2HAsO_4$) [As(V)], potassium antimonyl tartrate ($C_4H_4KO_7Sb$) [Sb(III)], bismuth nitrate [$(NO_3)_3Bi$] [Bi(III)], and phosphorous acid ($H_3PO_3$) [P(III)]. Control cells were not exposed to added compounds. As shown in Fig. 1B, the three probes hybridized strongly with RNA obtained from As(III)- and Sb(III)-exposed *Synechocystis* cells and weakly with RNA from As(V)- and Bi(III)-exposed cells. The transcripts were undetected in lanes containing RNA from P(III)-treated or untreated cells. The three probes detected an mRNA fragment of the same size, approximately 2,700 nucleotides, suggesting that *arsB*, *arsH*, and *arsC* form an operon. As(III) was the best inducer of the *arsBHC* operon, followed by Sb(III) and, finally, As(V). As shown in Fig. 2, different levels of induction were determined by using either an *arsB* or an *arsC* probe and suggested a strong polar effect of the gene expression in the *arsBHC* operon. Time course analysis indicated that *arsBHC* mRNA was already induced at the maximal level 15 min after the metalloid addition and that this level was maintained at least during the first 4 h of treatment (data not shown).

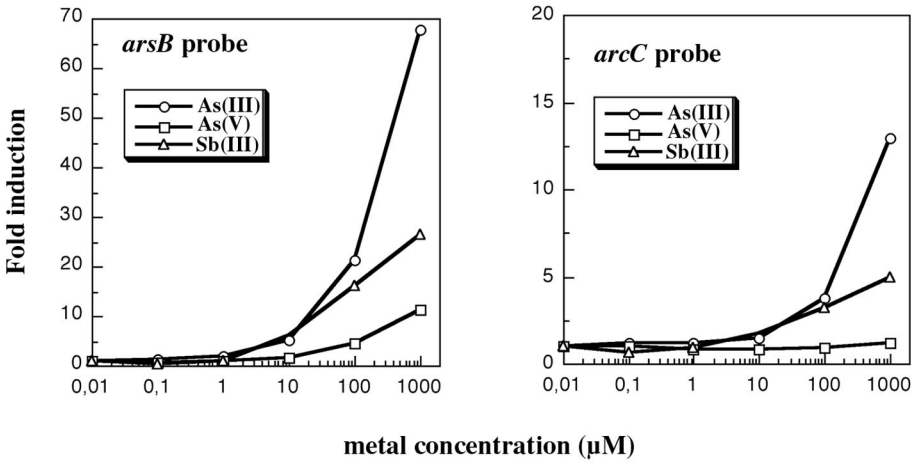


FIG. 2. Effect of metalloid concentration on the amount of transcript. The indicated concentration of sodium arsenite [As(III)], sodium arsenate [As(V)], or potassium antimonyl tartrate [Sb(III)] was added to mid-log-phase *Synechocystis* cells grown in BG11C medium. After 1 h, cells were harvested and total RNA was isolated, processed, and hybridized as described in the legend to Fig. 1 by using an *arsB* gene probe (probe a from Fig. 1A) or an *arsC* gene probe (probe c from Fig. 1A). Radioactive signals of two independent experiments were quantified with an InstantImager electronic autoradiography apparatus. Levels of mRNA were normalized with the *rnpB* signal, and plots of increases in induction (*n*-fold) with increasing doses were drawn. Please note differences in the plotted scales.

***arsB* and *arsC* are involved in arsenic resistance.** The homologies displayed by the ArsB, ArsH, and ArsC proteins together with the pattern of expression of their genes suggested that the *arsBHC* operon is involved in arsenic resistance in *Synechocystis*. In order to verify this hypothesis, mutants were generated by insertion of kanamycin resistance cassettes (C.K1) (8) into each one of the genes of the operon (Fig. 1A). The *arsB*::C.K1, *arsH*::C.K1(+), *arsH*::C.K1(−), and *arsC*::C.K1 *Synechocystis* strains were viable, and their growth rates in BG11C medium were similar to those of the wild-type (WT) strain (data not shown). ArsC-disrupted cells were more sensitive to high concentrations of As(V) than the WT cells (Fig. 1C). This phenotype was more apparent in a medium with a small amount of phosphate, probably due to the fact that arsenate enters the cell by some of the phosphate transport systems (reviewed in reference 26). Resistance to As(III) and Sb(III) was not affected by the *arsC* mutation. This result is in agreement with the notion that *arsC* encodes an arsenate reductase involved in arsenate detoxification. *arsB*-disrupted cells were hypersensitive to both As(V) and As(III), supporting the putative role of ArsB in arsenite export. The *Synechocystis* ArsB protein clusters with the ArsB/ARR3 family of arsenite transporters (41). This family is not related to the ArsB proteins found in many bacterial *ars* operons (including in *E. coli* and *Staphylococcus aureus*) and appears to be the result of convergent evolution (21). In addition to As(III), the *E. coli*-like ArsB proteins are able to carry out Sb(III) export (37). However, *arsB* and *arr3* mutants in *B. subtilis* and *S. cerevisiae*, respectively, are not hypersensitive to Sb(III), suggesting that the proteins encoded by these genes are not able to promote Sb(III) extrusion (30, 41). Interestingly, the *Synechocystis arsB*::C.K1 strain was hypersensitive to Sb(III), indicating that the *Synechocystis* ArsB protein is the first member of the ArsB/ARR3 family able to export both As(III) and Sb(III) and providing a new example of convergent evolution between both families of arsenite transporters.

Finally, disruption of the *arsH* gene showed different phenotypes depending on the orientation of the C.K1 cassette. Since the *npt* gene of the C.K1 cassette lacks a transcription terminator, insertional mutagenesis in the same orientation as that of the *ars* operon [indicated by "(+)"] does not suppress transcription of the *arsC* gene (data not shown). While the *arsH*::C.K1(−) mutants showed a phenotype similar to that of the *arsC*::C.K1 mutants, the *arsH*::C.K1(+) strain did not display any phenotype under the conditions tested (Fig. 1C). This result suggests that the phenotype of the *arsH*::C.K1(−) strain is a consequence of the absence of the expression of the *arsC* gene, not of the mutation of the *arsH* gene. Therefore, the ArsH protein is not required for arsenic or antimony resistance under the conditions tested. Genes homologous to *arsH* have been found close to arsenic resistance genes in *Y. enterocolitica* and in *A. ferroxidans* (3, 23). In *Y. enterocolitica*, the *arsH* product is required for resistance to As(III) and As(V). However, when the *arsH* gene of *A. ferroxidans* was introduced into *E. coli*, it was not required for arsenic resistance, but whether the gene is required for arsenic resistance in *A. ferroxidans* is unknown. There is no correlation between the type of ArsC or ArsB protein present in an organism and the existence of the ArsH protein. For example, *Y. enterocolitica* contains an *E. coli*-like ArsB and a glutaredoxin-dependent ArsC protein; however, *Synechocystis* contains an ArsB/ARR3-type ArsB and thioredoxin-dependent ArsC proteins, but both organisms contain *arsH* genes. A close inspection of the lowest-scored ArsH homologous proteins indicates that the protein shows some similarity with pyridine nucleotide-dependent oxidoreductase. Cysteine residues have been found in the active sites of the three types of arsenate reductases described previously. Furthermore, it is known that these residues participate in the catalysis of the reaction by forming an intermediary arsenate thioester (16, 17, 19, 44). Since *Synechocystis* ArsH lacks cysteine residues, it is unlikely that the function of ArsH is the reduction of arsenate. One possibility is that ArsH works as an alternative electron carrier protein under some specific conditions.

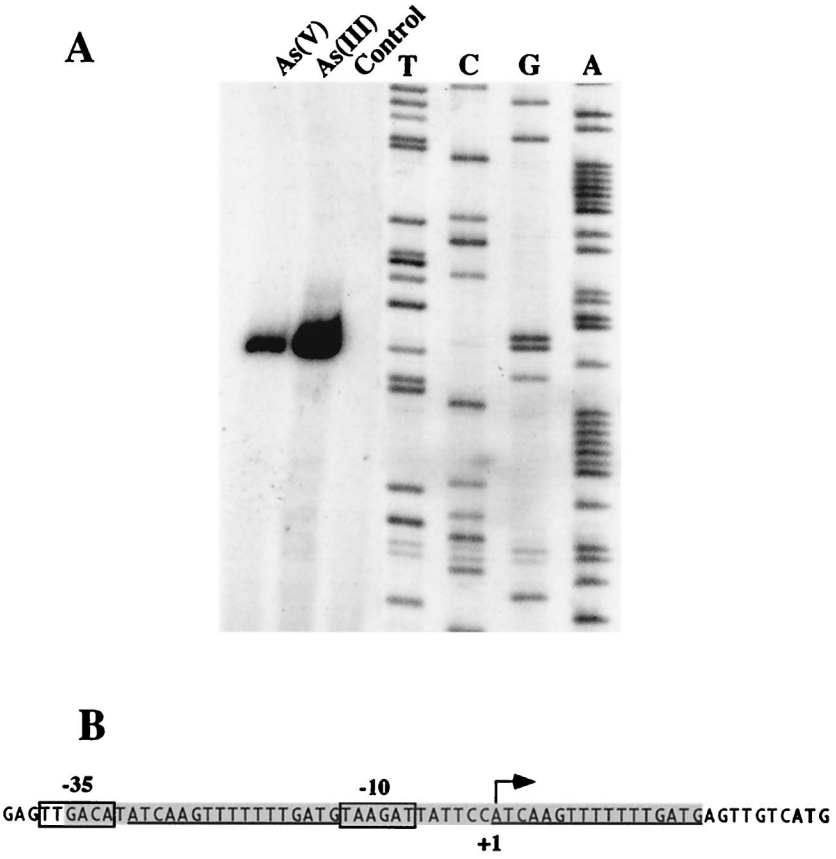


FIG. 3. Primer extension analysis of the *arsBHC* transcripts. (A) Primer extension analysis of the *arsBHC* transcripts from *Synechocystis* cells exposed to 1 mM sodium arsenite [As(III)], and 1 mM sodium arsenate [As(V)], for 1 h or nonexposed cells (control). Sequencing ladders generated with the same oligonucleotide used for the primer extension are also shown. (B) Sequence of the *arsBHC* promoter-operator region. The transcript start point is marked with an arrow. Putative −10 and −35 boxes based on the transcription start site are boxed. Direct repeats are underlined. The shaded sequence indicates the binding site for ArsR as defined by DNase I footprinting. The translation start codon is indicated in boldface type.

*E. coli*-like ArsB transporters are able to function as membrane potential-driven secondary transport systems or as primary ATP-driven transport systems associated with the ATPase ArsA (28). Nothing is known about the energetics of arsenite transport by the ArsB/ARR3 family of proteins. However, *ars* operons encoding members of this family of transporters do not contain genes encoding putative ATPases. The sll0086 ORF of the *Synechocystis* genome has been annotated as a putative ArsA-like arsenical pump-driving ATPase in the CyanoBase (http://www.kazusa.or.jp/cyano/cyano.html). The 583-residue *E. coli* ArsA protein has two homologous halves, A1 and A2 (45); however, the 396-residue *Synechocystis* protein contains only one domain that displays around 27% identity with each one of the domains of the *E. coli* protein. Similarly, one-domain ArsA-like proteins have been found in humans and yeast (14, 31). In order to test the role of this protein in arsenic resistance, we inactivated the sll0086 gene by insertional mutagenesis. While the sll0086-inactivated strain presented some growth defects, its degree of resistance to As(III), As(V), or Sb(III) was indistinguishable from that of the WT strain (data not shown). Therefore, our data indicate that the sll0086-encoded ATPase is not involved in arsenic or antimony resistance.

**Primer extension mapping of the *arsBHC* promoter.** In order to determine the location of the *arsBHC* promoter, the starting point of the *arsBHC* transcript was mapped by primer extension. One single extension product was obtained by using RNA from cells under induced conditions, while no extension product was yielded with RNA from uninduced cells (Fig. 3A). The transcription start site was found 23 bp upstream of the *arsB* ATG start codon. Putative −10 and −35 promoter boxes in the form TAAGAT and TTGACA, respectively, were found at the appropriate positions. Two 17-bp direct repeats (ATC AAGTTTTTTTGATG) separated by 13 bp were found in the region from nucleotides −29 to +17 of the *arsBHC* promoter. Each direct repeat is composed of two inverted repeats in the form ATCAA$(N)_6$TTGAT (Fig. 3B).

**The *arsBHC* operon is repressed by a DNA-binding protein of the ArsR/SmtB family.** We proceeded to investigate the existence in *Synechocystis* strain PCC 6803 of a DNA-binding protein responsible for the metalloid-dependent regulation of the *arsBHC* operon. As previously mentioned, arsenic resistance operons from *E. coli* and *Staphylococcus* are controlled at the transcriptional level by a *trans*-acting repressor of the ArsR/SmtB family (33). Two ORFs encoding proteins of this family exist in the *Synechocystis* genome. The *ziaR* gene en-

codes a transcriptional repressor of the ZiaA Zn-dependent ATPase (35) and therefore is unlikely to be involved in controlling the *arsBHC* operon. We tested whether the other ORF (sll1957) encodes a protein involved in the regulation of the *arsBHC* operon. For that experiment, the sll1957 ORF was interrupted by a chloramphenicol resistance cassette (C.C1) (8). The sll1957::C.C1 *Synechocystis* strain was viable, and its growth rate in BG11C medium was comparable to that of the WT strain (data not shown). Furthermore, resistance to arsenate, arsenite, and antimony was not altered in the sll1957-disrupted strain (data not shown). Then we tested the metalloid-dependent regulation of the *arsBHC* operon in the disrupted strain. Interestingly, the sll1957::C.C1 cells showed high constitutive expression of the *arsBHC* operon independently of the presence of As(V), As(III), or Sb(III). These data suggest that the protein encoded by the sll1957 ORF is a repressor of the *arsBHC* operon, and therefore the protein was named ArsR. The *arsR* gene was expressed at very low levels both in the absence and in the presence of metalloids (data not shown).

In order to verify whether ArsR is able to interact with the *arsBHC* promoter-operator region, we purified a recombinant MalE-ArsR chimeric protein expressed in *E. coli* (Fig. 4A). Proteolysis of the chimeric protein with factor Xa yielded both the MalE protein and a version of the ArsR protein in which the first two amino acids (MK) have been replaced by four amino acids (ISEF). DNA binding was tested by gel retardation assays using a fragment from nucleotide −59 to +36 with respect to the transcription start point of the *arsBHC* operon. As shown in Fig. 4B, ArsR interacted with the DNA, generating different types of ArsR-DNA complexes. The ArsR-dependent band shift was diminished in the presence of a 50-fold excess of the same unlabeled fragment, and it was unaffected by the presence of an excess of an unrelated DNA fragment (data not shown). Control experiments to demonstrate that a purified MalE protein was not able to bind DNA were also performed (data not shown).

The *Synechocystis* ArsR protein contains the proposed metal binding site sequence CVCDLC identified in the *E. coli* ArsR protein (32, 33). Shi et al. (32) found that the three cysteine residues of this sequence were able to interact with arsenite. In vivo, the *arsBHC* operon was derepressed by arsenic(III) and antimony(III) as well as by arsenic(V), albeit to a lesser extent. We investigated the effect of in vivo inducers of the *arsBHC* operon on the in vitro binding activity of ArsR. As shown in Fig. 4C, the ArsR binding activity decreased in the presence of As(III) and Sb(III) but not in the presence of As(V). Similar results have previously been reported for the R773-borne *ars* system (40). The simplest interpretation of these data is that As(III) and Sb(III) are true inducers of the system but that As(V) has to be reduced to As(III) in vivo to induce the system. Interestingly, the *arsBHC* operon was induced by As(V) in vivo in a *Synechocystis* strain where the *arsC* gene had been inactivated (data not shown). This suggests that, in the absence of ArsC, a significant amount of arsenate is reduced in vivo to arsenite by another enzymatic or a nonenzymatic mechanism.

An interesting difference between the *Synechocystis* arsenic resistance system and the well-characterized systems in *E. coli* and *Staphylococcus* is that in *Synechocystis* the *arsR* gene is not autoregulated. Thus, the *Synechocystis arsR* gene is expressed constitutively at very low levels. The autoregulation of a repressor is usually related to the ability of the system to return to the repressed state. This autoregulatory mechanism is probably related to the fact that overexpression of the *E. coli* ArsB protein is toxic for the cell (see, for example, references 4 and

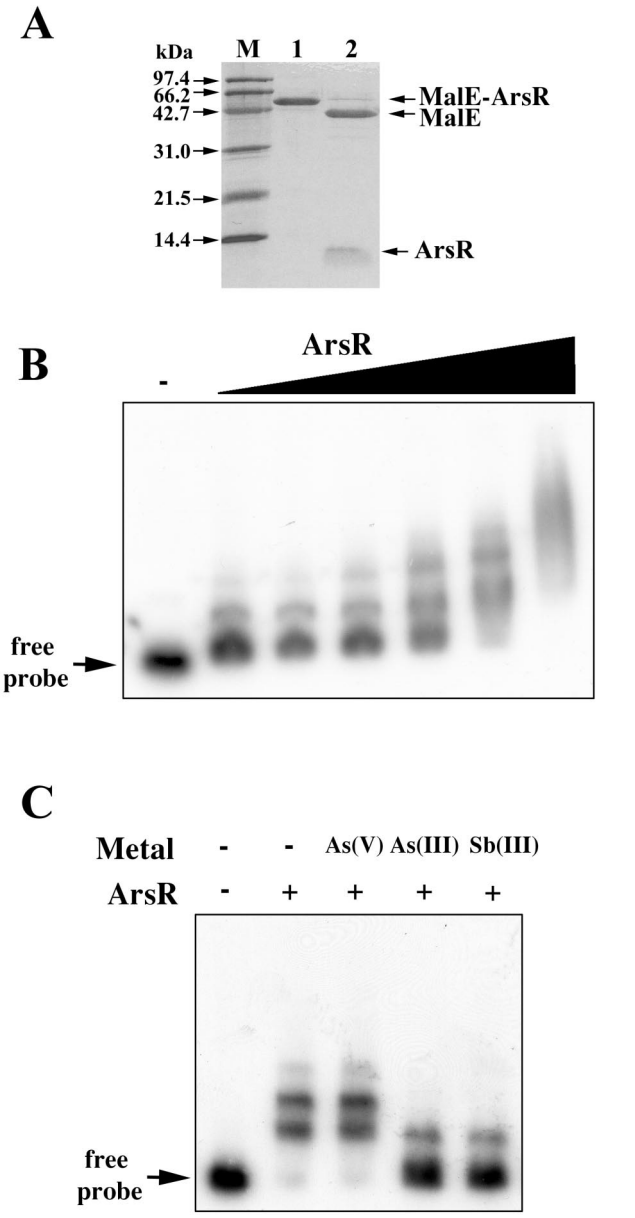


FIG. 4. ArsR binds to the *arsBHC* promoter-operator region. (A) Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of purified MalE-ArsR. Lane M, molecular mass markers; lane 1, purified MalE-ArsR; lane 2, purified MalE-ArsR digested with factor Xa protease to separate the MalE domain from ArsR. (B) Mobility shift assay of the *arsBHC* promoter-operator region with increasing quantities (from 0.095 to 3.8 μM) of ArsR protein. A 95-bp fragment containing the putative *arsBHC* promoter-operator region was used as the probe. (C) Effect of inducers of the *arsBHC* operon on ArsR-DNA complex formation. A 95-bp fragment containing the putative *arsBHC* promoter-operator region was incubated with a 1 μM concentration of the ArsR protein in the presence of 1 mM sodium arsenite [As(III)], sodium arsenate [As(V)], or potassium antimonyl tartrate [Sb(III)] or in the absence of metalloid (−). The binding mixture was analyzed in a mobility shift assay.

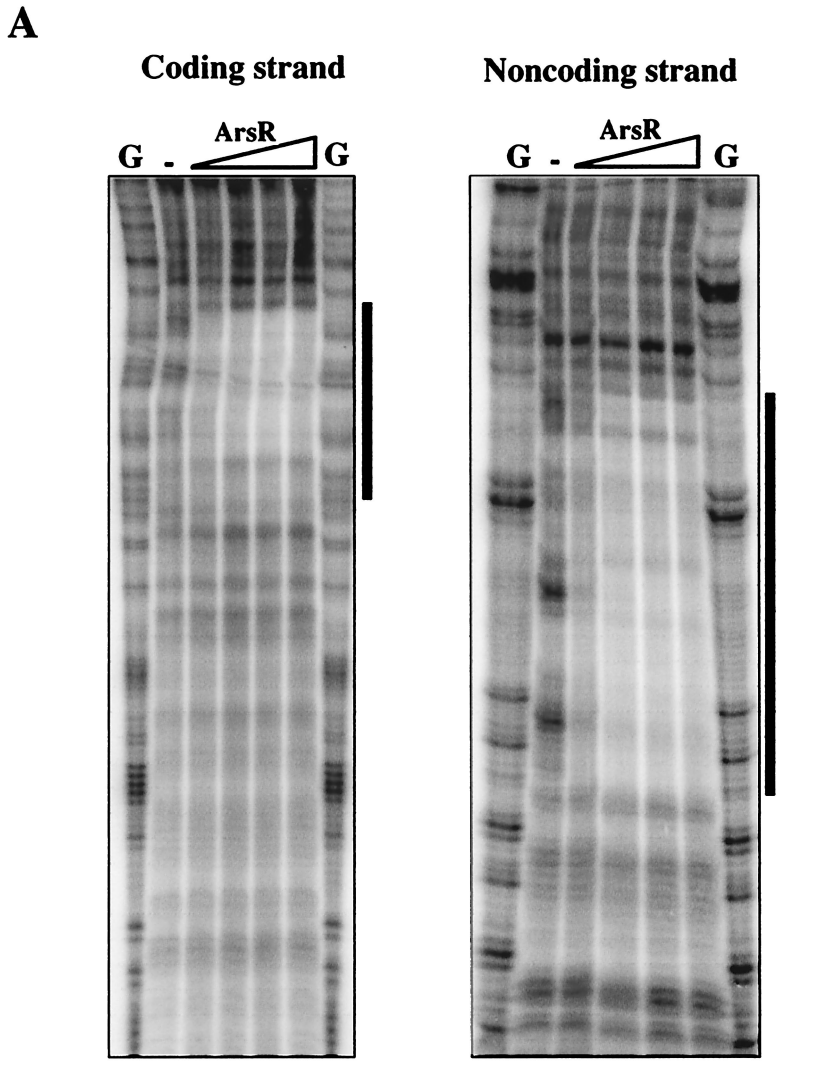


FIG. 5. DNase I footprints of the ArsR protein bound to the *arsBHC* promoter-operator region. (A) DNA fragments of the *arsBHC* promoter-operator region were labeled at positions −132 (coding strand) and +105 (noncoding strand) and subjected to DNase I footprint analysis using increasing quantities of purified ArsR protein (0.025, 0.063, 0.126, and 0.252 μg), as described in Materials and Methods. The regions of the DNA protected by ArsR are indicated by bars. (B) Alignment of ArsR DNA binding sites in the *E. coli* plasmid R773-encoded and chromosomal (chr) *ars* operons and in the *Synechocystis arsBHC* operon. The DNA sequence that was protected in DNase I footprinting experiments is shown in the case of the *E. coli ars* operons. The *Synechocystis* sequence corresponds to one of the two identical direct repeats found in the ArsR-protected region (Fig. 3B). The numbers indicate the position of the sequence with respect to the transcription start point.

39). Therefore, in order to prevent toxicity, it is important to tightly control the expression of the *ars* operon. A more accurate control of the level of induction has been achieved by the five-gene operons from the R773 and R46 plasmids, where an additional transcription factor, ArsD, represses the operon in the presence of low levels of arsenic (7). In agreement with these data, *E. coli arsD* mutants overproduce ArsB in the presence of moderated levels of arsenic that lead to growth inhibition (39). In contrast, the *Synechocystis arsR*-inactivated cells show normal growth parameters in spite of the constitutive expression of the *arsB* gene. This absence of ArsB overexpression-dependent toxicity may explain why ArsR autoregulation has not been a selective advantage for *Synechocystis*.

**Identification of the DNA binding site for the ArsR repressor.** The site of binding of the ArsR protein to the *arsBHC* promoter-operator region was determined by DNase I footprinting analysis (Fig. 5A). A protected region of 51 bp (−34 to +17) in the coding strand and 52 bp (−36 to +16) in the

noncoding strand was observed (Fig. 4B). The protected region overlaps the putative −10 and −35 promoter elements, suggesting that binding of ArsR under noninducing conditions prevents RNA polymerase binding and therefore transcription. This region contains two direct repeats of the sequence ATC AAGTTTTTTTGATG (coding strand), which contains an imperfect dyad symmetry and which may constitute the ArsR binding site. It has been shown that *E. coli* ArsR proteins are dimers in solution and that dimerization is required for DNA binding (40, 42, 43). Therefore, it is tempting to speculate that two ArsR dimers bind to the *arsBHC* promoter-operator fragment. However, more than two DNA-ArsR complexes were obtained in gel retardation assays when large amounts of ArsR were added to the binding reaction mixtures. This result may be due to additional ArsR binding to low-affinity sites or to oligomerization of the ArsR protein by protein-protein interaction. Figure 5B shows an alignment of the putative *Synechocystis* ArsR binding site with those of *E. coli* chromosomal and R773 plasmid-encoded ArsR DNA binding sites. The sequence $TCAT(N)_7TTTG$ has been proposed to be a consensus binding site for both *E. coli* ArsR proteins. The *Synechocystis* ArsR putative DNA binding site can be accommodated to this consensus by introducing two more nucleotides in the spacer region.

In summary, we have characterized for the first time the system for metalloid resistance in a cyanobacterium. The system for resistance in *Synechocystis* strain PCC 6803 combines elements from gram-negative and gram-positive species, as well as interesting particularities. One of the most intriguing questions is the biochemical role of ArsH, which will be the direction of our future research.

## ACKNOWLEDGMENTS

We thank Kazusa DNA Research Institute and S. Tabata for providing *cs0223* and *cs0205* cosmid DNA. We thank Marika Lindahl for critical reading of the manuscript.

Luis López-Maury is a recipient of a fellowship from the Spanish Ministerio de Educación Cultura y Deporte. This work was supported by grant BMC2001-2635 from MCyT and by the Junta de Andalucía (group CV1-802).

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