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Coregulated Genes Link Sulfide:Quinone Oxidoreductase and Arsenic Metabolism in *Synechocystis* sp. Strain PCC6803

Csaba I. Nagy,[a] Imre Vass,[a] Gábor Rákhely,[b,c] István Zoltán Vass,[a] András Tóth,[b,c] Ágnes Duzs,[c] Loredana Peca,[a]* Jerzy Kruk,[d] Péter B. Kós[a,b]

Institute of Plant Biology, Biological Research Centre of the Hungarian Academy of Sciences, Szeged, Hungary[a]; Department of Biotechnology, Faculty of Science and Informatics, University of Szeged, Szeged, Hungary[b]; Institute of Biophysics, Biological Research Centre of the Hungarian Academy of Sciences, Szeged, Hungary[c]; Department of Plant Physiology and Biochemistry, Faculty of Biochemistry, Biophysics and Biotechnology, Jagiellonian University, Krakow, Poland[d]

Although the biogeochemistry of the two environmentally hazardous compounds arsenic and sulfide has been extensively investigated, the biological interference of these two toxic but potentially energy-rich compounds has only been hypothesized and indirectly proven. Here we provide direct evidence for the first time that in the photosynthetic model organism *Synechocystis* sp. strain PCC6803 the two metabolic pathways are linked by coregulated genes that are involved in arsenic transport, sulfide oxidation, and probably in sulfide-based alternative photosynthesis. Although *Synechocystis* sp. strain PCC6803 is an obligate photoautotrophic cyanobacterium that grows via oxygenic photosynthesis, we discovered that specific genes are activated in the presence of sulfide or arsenite to exploit the energy potentials of these chemicals. These genes form an operon that we termed *suoRSCT*, located on a transposable element of type IS*4* on the plasmid pSYSM of the cyanobacterium. *suoS* (sll5036) encodes a light-dependent, type I sulfide:quinone oxidoreductase. The *suoR* (sll5035) gene downstream of *suoS* encodes a regulatory protein that belongs to the ArsR-type repressors that are normally involved in arsenic resistance. We found that this repressor has dual specificity, resulting in 200-fold induction of the operon upon either arsenite or sulfide exposure. The *suoT* gene encodes a transmembrane protein similar to chromate transporters but in fact functioning as an arsenite importer at permissive concentrations. We propose that the proteins encoded by the *suoRSCT* operon might have played an important role under anaerobic, reducing conditions on primordial Earth and that the operon was acquired by the cyanobacterium via horizontal gene transfer.

Despite the oxidizing atmosphere that evolved as a consequence of the abundant oxygenic photosynthesis, anaerobic biochemistry is still present in organisms living under constantly or temporarily anoxic and possibly sulfidic conditions. This represents a relic of the primordial environment that existed when the oceans on Earth were anoxic and sulfidic (1, 2).

A wide variety of living organisms evolved in the presence of sulfide, and they contain enzymes to oxidize sulfide for mitigating its toxic effect or for obtaining electrons for photosynthesis (purple and green sulfur bacteria) or respiration (3, 4). Enzymes with such functions belong to the disulfide oxidoreductase (DiSR) flavoprotein family. The most thoroughly characterized, important representatives of this family are the sulfide:quinone reductases (SQRs), which have been identified in a great variety of organisms from archaea and bacteria to fission yeasts, molluscs, worms, *Drosophila*, and even humans (5). Generally, SQR enzymes catalyze the electron transfer from sulfide directly into a membrane-bound quinone pool as the electron acceptor (6). Beside photosynthesis (7) or respiration (4), SQRs are implicated in heavy metal tolerance (8), in detoxification (9), and also in signal transduction as "gasotransmitters" (10).

Cyanobacteria gain ATP via photophosphorylation in oxygenic photosynthesis, but in the presence of sulfide the photosystem II (PSII) reaction center is inhibited (11). Nonetheless, besides green and purple sulfur bacteria, which drive photosynthesis using electrons from sulfide, some purple nonsulfur bacteria such as *Rhodobacter capsulatus* (12), some extremophile microorganisms and even a few strains of cyanobacteria contain SQR enzymes by which they can shift to anoxygenic, sulfur bacterium-type photosynthesis using sulfide as an electron donor, thus overcoming the deleterious effect of this compound. Although many cyanobacteria have genes for SQRs, only a few of the encoded enzymes have been investigated experimentally. The two cyanobacterial SQR enzymes that are the most thoroughly characterized are those of *Oscillatoria limnetica* and *Aphanothece halophytica* (13).

Synechocystis sp. strain PCC6803 (here referred to as *Synechocystis*) is one of the few organisms that contain different types of SQR genes in its genome: one of them, slr0876, is located on the chromosome and is classified as a type II SQR (2, 14), which could originate from alphaproteobacteria from which mitochondria arose. This can explain why the SQR II sequence shows higher similarity to SQRs from *Schizosaccharomyces pombe*, *Anopheles*, *Drosophila*, and *Caenorhabditis* or to human mitochondrial SQRs than to those from cyanobacteria (2).

The other SQR gene, sll5036, is located on a plasmid of *Synechocystis*, coding for an enzyme that is classified as type I SQR (5, 15), a group that is mostly specific for cyanobacteria and may provide electrons for an alternative photosynthetic pathway using reduced sulfur species. In our investigation, we focused on this enzyme due to its presumed involvement in alternative photosynthetic electron transport.

Although SQR enzymes have been studied extensively (5, 6),

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Address correspondence to Péter B. Kós, kp@brc.hu.
* Present address: Loredana Peca, National Institute of Research and Development for Biological Sciences, Institute of Biological Research, Cluj-Napoca, Romania.

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no regulatory proteins that would control the level of expression of these enzymes have been identified. Constitutive expression has been reported in bacteria thriving in sulfide-rich habitats like purple sulfur bacteria (*Chromatium vinosum* [16]), purple nonsulfur bacteria (*R. capsulatus* [12]), or green sulfur bacteria (*Chlorobium tepidum* [17]) and in bacteria living in sulfide-free niches like the thermophile bacterium *Aquifex aeolicus* (15). Inducible regulatory mechanisms have been suggested for another SQR gene, CT1087 in *C. tepidum* (18) and in *R. capsulatus*, but no regulatory proteins have been identified (6). Cyanobacteria usually do not thrive in sulfidic habitats, but some strains, like *O. limnetica*, can shift to sulfide-based photosynthesis (19) after several hours of induction with sulfide.

Whereas sulfide has long been known to serve as an electron donor for microbial growth, arsenic, despite its toxicity, can serve as a bioenergetic substrate for microbial growth as well (20).

Arsenic has a complex chemistry and can occur in different inorganic forms, such as trivalent As(III) in As_2O_3, AsO_2^-, AsO_3^{3-} (arsenite), or AsH_3 (arsine), or as pentavalent As(V) in AsO_4^{3-} (arsenate), as well as in organic form, such as in arsenobetaine, trimethylarsine, or arsenosugars, among others. Due to its redox-active nature, arsenite can donate electrons to various acceptors, and this feature can be utilized in organisms performing anoxygenic photosynthesis. Indeed, the occurrence of such a type of photosynthesis in primordial life on Earth has been proposed, and recently its existence in a current arsenic-containing anaerobic niche was presented (21). On the other hand, as an analogue of phosphate, arsenate may interfere with several biochemical reactions, including oxidative phosphorylation, rendering it toxic to most organisms, whereas arsenite may bind to thiol groups of proteins, making it even more toxic (22). Due to this toxicity, most microbes have developed defense mechanisms, mainly redox and export systems (23).

The genes involved in arsenic detoxification processes in bacteria are generally regulated by ArsR-type repressor proteins (24). *Synechocystis* is rather tolerant, as it is able to grow in up to 3 mM arsenite and as high as 100 mM arsenate. This raised the question whether the arsenic transport system encoded by the *arsBHC* operon (25) under the regulation of the ArsR protein is exceptionally effective or perhaps some other biochemical process contributes to the detoxification.

In this study, we aimed to investigate arsenic and sulfur metabolisms in *Synechocystis* PCC6803, arguably the most thoroughly investigated cyanobacterial strain with its whole-genomic sequence available. We found a putative *arsR*-type gene, which is part of an SQR-related operon and demonstrated that this repressor has dual specificity for both arsenite and sulfide. This finding, together with the high resistance of this cyanobacterium to both of these toxic compounds, suggests a functional link between the corresponding metabolic or detoxifying pathways, which was investigated here.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *Synechocystis* sp. strain PCC6803 was grown photoautotrophically as described previously (26).

Escherichia coli cells were grown in Luria-Bertani (LB) broth at 37°C in a rotational shaker at 250 rpm. Cultures were supplemented with 50 μg ml^{-1} carbenicillin or 34 μg ml^{-1} kanamycin sulfate when required. *E. coli* strain XL1-Blue was used for cloning purposes and strain BL21(DE3)RIL for protein expression.

Anaerobic treatments. *Synechocystis* cells in exponential growth phase (optical density at 670 nm [OD_{670}], 0.6) were transferred to crimp top vials and flushed with N_2 for 10 min in the presence of 5 μM dichlorophenyl dimethylurea (DCMU) to prevent oxygen production and prevent PSII from reducing the plastoquinone pool. Treatments were started by adding the appropriate solutions kept in anaerobic chambers for at least 2 days.

Assessment of intracellular arsenic content. Intracellular arsenic content was determined in the wild-type (WT) *Synechocystis* and Δ*suoT* and Δ*arsB* mutant strains (25). The cell cultures were treated with $NaAsO_2$ for 3 h in light, collected by centrifugation, washed once with BG-11 solution, and freeze-dried. From 50-ml cultures (43.97 ± 3.15 mg, wet weight; estimated to be the same number of microliters in volume), cell pellets were harvested, from which 8.73 ± 0.34 mg (dry) material was obtained. The samples were decomposed by microwave-assisted digestion (MarsXpress; CEM, Matthews, NC) at 200°C and 1,600 W for 15 min, and the arsenic contents were determined by inductively coupled plasma mass spectrometry (ICP-MS) (X Series II; Thermo Scientific, Asheville, NC).

H_2S measurement by gas chromatography. Anaerobic *Synechocystis* cultures were covered with aluminum foil to inhibit photosynthesis, and 0.5 mM Na_2S was injected into the medium. Cells were preincubated for 1 h in the dark. The H_2S content was measured in the headspace of the vials before and after 24 h of incubation in light. The gas chromatograph (Shimadzu GC-2010) was equipped with a thermal conductivity detector (TCD). An HP-PLOT Q polystyrene-divinylbenzene (DVB) capillary column was used to separate gasses (30 m by 0.53 mm [inner diameter], 40-μm-thick film; Agilent Technologies). N_2 was used as the carrier gas at 7.95 ml min^{-1}. The inlet and detector temperature was maintained at 150°C, while the column temperature was set to 120°C.

Spectroscopic assay of SQR activity. The SQR activity was assessed using isolated thylakoid samples according to the method of Arieli et al. (19) with minor modifications as follows. Anaerobic *Synechocystis* cultures were incubated in the presence of 2 mM Na_2S for 5 h. Thylakoids were isolated according to the method of Komenda and Barber (27). Sulfide-dependent PQ-1 reduction activity was measured at 257 nm using an Evolution 300 UV-Vis spectrophotometer (Thermo Scientific) under anaerobic conditions in N_2-flushed quartz cuvettes sealed with rubber septa. The reaction mixture contained 10 mM K-HEPES, pH 7.4, 10 mM $MgCl_2$, 10 mM KCl, 40 μM PQ-1, and thylakoids containing 10.5 μg chlorophyll · ml^{-1}. To maintain strict anaerobic conditions, 10 U ml^{-1} catalase, 1.0475 U ml^{-1} glucose oxidase, and 20 mM glucose were added. Cuvettes were sealed and flushed with N_2 for 10 min, and 125 μM Na_2S was injected to start the reaction. Na_2S stock solution was freshly prepared in an anaerobic chamber.

Gene expression measurement by quantitative RT-PCR. Gene expression investigations were carried out as presented earlier (28). The PCR primers used for quantitative reverse transcription-PCR (RT-PCR) and cloning purposes are as shown in Table 1.

Cloning procedures and construction of mutant strains of *Synechocystis*. For various investigations, three deletion mutant strains, *Synechocystis* Δ*suoRSCT*, Δ*suoR*, and Δ*suoT*, were constructed by replacing the respective genomic regions with antibiotic resistance cassettes as follows.

Vector NTI Advance 10 software (Life Technologies) was used to design cloning steps and PCR primers. For all cloning purposes, the pBluescript II SK+ (pBluescript) vector was used. All restriction enzymes and T4 ligase were purchased from Thermo Scientific.

PCR amplification of the 3,832-bp DNA fragment containing sll5035, sll5036, and slr5037 coding sequences and slr5038 was performed using the 5038Reg2SalIFw and 5038Reg2NotRev primer pair (Table 1) using *Synechocystis* genomic DNA as the template and Phusion Hot Start polymerase (Finnzymes, Sweden) according to the manufacturer's recommendations. The PCR products were purified with the MinElute PCR purification kit (Qiagen, Germany). The DNA fragment was cloned into pBluescript to generate the p-suoRSCT construct.

TABLE 1 List of PCR primers used in the experiments

Primer	Sequence (5′–3′)[a]
5038Reg2SalIFw	AGGAGTCGACTGGTTAAATTTTGCCAAGGCTA
5038Reg2NotRev	ACTTGCGGCCGCCTTACCAATGACTAATAAA GTC
Km/StuI-Fw	TCAGGCCTAACTAAGTAATCATGAACAATA AAACTGTCTGCTTAC
Km/StuIRv	TCAGGCCTTAGTTATTAGAAAAACTCATCGA GCATC
5035 Sal/EcoF	GTAGTCGACTGGGGATAGCCATCTTTAAT
5035 Sal/EcoR	CATGAATTCAAGCATCTGCAAATATTGCG
5035Pst/NotF	AGGCTGCAGGAGACATTTGCTTGACTTAG
5035Pst/NotR	GAAGCGGCCGCAAATTACCTACATTACTCC TGAACC
KmEco/PstF	AGTGAATTCTTACTTAGTTAGGCGTTTTTC CATAGGCTCC
KmEco/PstR	TGGCTGCAGTAAGTAACTAATGCGCGGAAC CCCTATTTGT
sll5035Exp2Fw	AAGGATCCATGCAAATATCTGTGAATA AAACCC
sll5035Exp2Rv	TGGTAAGCTTTTAGTCTTCGCAGGATGAAA
FoUp5035NotI	AAGCGGCCGCGGGGATAGCCATCTTTAATA AACTGC
ReUp5035BamHI	AAGGATCCTGCGCTCAGTCTTTAATTGA
suoTdwnstrmFv-1	TTTTAAGCTTGGCATTGTCTGTCGCTATTT
suoTdwnstrmRw-577	TTTCTCGAGCCCCTAGTAATAAATAAACTCA
suoCKanamFv-1	TTTGGATCCAAGAACATGTGAGCAAAAGG
suoCKanamRw-1675	TTTTAAGCTTGCGGAACCCCTATTTGTTTA
slr5037up-1F	CGATGTCGACTTAAGGTCTCATGGGTGTAA
slr5037up-410R	TTTATATCTCTGATTTTTATTGAAC
arsBpromot1F	TATCAGAAAAATAGGTTGAATGCCC
arsBpromot265R	AAACAAATTGAGCGTTCCCC
suoS-F	TCCTGAACCCTACATTGGCC
suoS-R	TCTGAGCATTGGTTAACGTGG
suoT-F1	TGATCGCCCTGATGGAAGAA
suoT-R1	GAGCCGTATTTACCGGAAAAGA
suoC-F1	AATGTGGTGGTGCAGGAACA
suoC-R1	CAATCTCCTTTGCCTGGGC

[a] Enzyme restriction sites are underlined.

Deletion of *suoT*. In order to disrupt the slr5038 (*suoT*) gene, an 899-bp kanamycin resistance cassette (Km^{r}c) was PCR amplified from the pET-28a plasmid (Novagen) with the Km/StuI-Fw and Km/StuI-Rv primer pair, to add StuI restriction sites on both ends of the amplicon. The p-suoTKm construct was generated by inserting the Km^r cassette into the EcoRI site of the *suoT* gene in the p-suoRSCT construct.

Deletion of *suoR*. In order to delete the sll5035 (*suoR*) gene, a DNA fragment upstream of *suoR* was amplified using 5035Sal/EcoF and 5035Sal/EcoR primer pairs and inserted into pBluescript to obtain construct p-upsuoR. Similarly, a DNA fragment downstream of *suoR* was amplified with primers 5035Pst/NotF and 5035Pst/NotR to generate the plasmid p-dwnsuoR. The 1,640-bp Km^r cassette was amplified by PCR from the pET-28a vector using KmEco/PstF and KmEco/PstR primers. The Km^r cassette was ligated into the p-upsuoR plasmid, and subsequently the suoR-upstream+Kmr fragment was cut out from the plasmid and inserted into the p-dwnsuoR construct to generate the final p-suoRKm construct.

Deletion of *suoRSCT*. To delete the sll5035-to-slr5038 region from the genome of *Synechocystis*, a 708-bp fragment upstream of sll5035 was amplified by PCR with primers FoUp5035NotI and ReUp5035BamHI and ligated to a 1,662-bp Km^r cassette amplified from a pET-28a plasmid. A 562-bp fragment downstream of slr5038 was amplified with primers suoTdwnstrmFv-1 and suoTdwnstrmRw-577 and ligated to the previous fragments in a pBluescript plasmid. This plasmid was used to create the Δ*suoRSCT* mutant strain.

Transformation. In order to introduce DNA constructs into the cells, electroporation (29) and natural transformation (30) were used.

The transformants were grown initially on 10 μg ml^{-1} kanamycin-supplemented BG-11 agar plates at 30°C under continuous illumination. Subsequently, colonies were passed on BG-11 plates containing increasing amounts of kanamycin sulfate. Finally, kanamycin-resistant samples were inoculated into 50 ml of 50 μg ml^{-1} kanamycin-supplemented BG-11 medium.

The correct integration and complete segregation of the recombinant strains were confirmed experimentally. All three strains were viable, and their growth rates in BG-11 medium were similar to that of the wild type under normal conditions.

Cloning, expression, and purification of SuoR protein. The complete sll5035 (*suoR*) gene was PCR amplified using primers sll5035Exp2Fw and sll5035Exp2Rv to add 5′ flanking BamHI and HindIII sites, respectively, using genomic DNA as the template. Amplified *suoR* was cloned into a pET-28a expression vector (Novagen), providing a His tag for Ni-affinity purification. The SuoR fusion protein was expressed in *E. coli* strain BL21(DE3)RIL as follows: transformed cells were cultured at 22°C and 250 rpm in 250 ml of LB+ medium (LB with 20 mM glucose and 2% ethanol) supplemented with 34 μg kanamycin sulfate ml^{-1}. At an OD_{600} of 0.4 to 0.5, protein production was induced for 5 h using 0.2 mM IPTG (isopropyl-β-D-thiogalactopyranoside). The cells were collected by centrifugation, washed with 20 ml wash buffer (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole, 10% glycerol, pH 8.0), and resuspended in 9 ml wash buffer (containing 5 mg lysozyme, 2 mM phenylmethylsulfonyl fluoride [PMSF], and 2 mM β-mercaptoethanol), and 1 ml CelLytic B 10X solution (Sigma). The mixture was incubated for 15 min at room temperature with shaking. The cells were disrupted by sonication (Branson Sonifier 450), and the cell debris was removed by centrifugation. The supernatant was filtered through Miracloth (Calbiochem) and subjected to column chromatography purification. For this purpose, Bio-Rad's Profinity IMAC Ni-charged resin was used following the manufacturer's instructions.

EMSAs. For electrophoretic mobility shift assays (EMSAs), a 410-bp DNA region containing the putative promoter region of the *suoRSCT* operon was amplified with slr5037up-1F and slr5037up-410R primers. The pBluescript SK+ multiple cloning site (280 bp) was used as a control DNA fragment amplified by M13 universal primers. Binding reactions were carried out in a final volume of 20 μl containing 100 mM Tris-HCl (pH 7.5) 10 mM EDTA, 1 M KCl, 1 mM dithiothreitol (DTT), 50% vol/vol glycerol, 0.1 mg ml^{-1} bovine serum albumin (BSA), 647 ng target DNA, 338 ng control DNA, and increasing amounts of purified SuoR protein. The mixtures were incubated for 30 min at 30°C and loaded on a nondenaturing 8% polyacrylamide gel as described by Hellman and Fried (31). The electrophoresis was carried out at 4°C and 40 V in Tris-acetate-EDTA buffer (pH 9.5).

Online databases and software. Cyanobacterial sequences were obtained from CyanoBase, the database for cyanobacteria (32). Similarity searches were performed with Basic Local Alignment Search Tool (BLAST) (33) using nonredundant databases for a general search and the microbial genomes for the genomic localization of genes. Secondary structures were predicted using the Jpred service (34). Transmembrane helices were primarily identified using the Sosui software (35) linked to the BLAST result in NCBI and verified using the Topcons service (36), which generates a consensus of membrane topologies calculated using various prediction methods. Three-dimensional (3D) structures of SuoR were calculated via homology modeling using Swiss-Model (37).

Proteins showing sequence similarity to the amino acid sequence of Slr5036 with known crystal structures were selected from the Protein Data Bank (38).

Genomic contexts were surveyed using the GeCont-I online service (39).

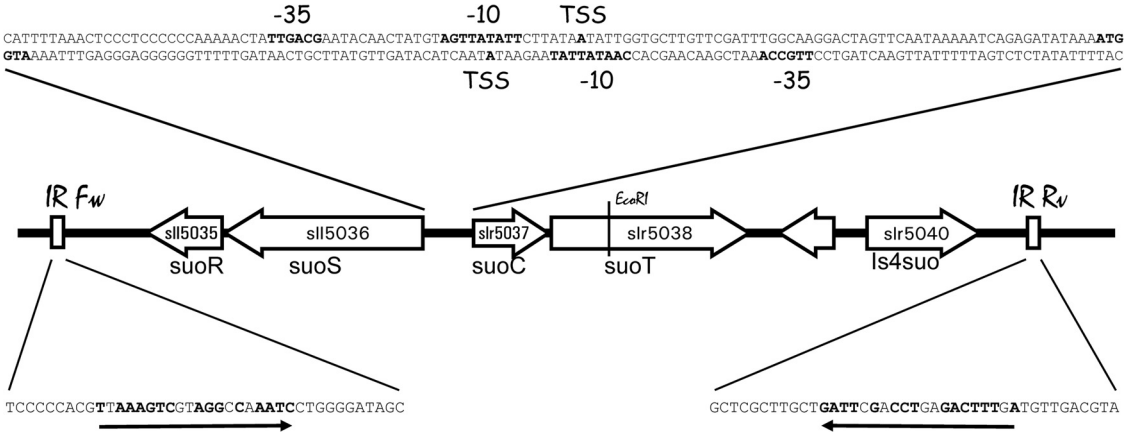

FIG 1 Genetic organization of the *suoRSCT* operon. Gene orientations are shown by arrows. Divergently transcribed *suoSR* and *suoCT* gene pairs share a DNA region for their promoter sequences: putative −35 and −10 promoter elements and transcription start sites (TSS) are underlined. Start codons of *suoS* and *suoC* are shown in bold. The *suoRSCT* operon makes part of an IS*4*-like transposon. The *suo* genes are delimited by a putative gene for IS*4*-like transposase (IS*4arr*) and by terminal imperfect inverted repeat (IR) sequences (underlined by arrows).

Conserved amino acid residues involved in the formation of the active site of SuoS were verified using the RaptorX binding site prediction (40).

RESULTS

***In silico* investigation of the genomic region encoding a type I SQR enzyme.** The gene sll5036 is located on the pSYSM plasmid of the cyanobacterium, and the deduced amino acid sequence shows 52% and 54% identity (66% and 67% similarity) to *O. limnetica* SQR and *A. halophytica* SQRs, respectively. According to the similarity-based annotation, sll5036 encodes a sulfide:quinone oxidoreductase enzyme. It is part of an operon that has a bidirectional promoter region with overlapping −35 and −10 promoter elements for two tandem gene pairs oriented in opposite directions (Fig. 1). The similarity to the organization of heavy metal resistance operons (41) suggested that the syntheses of the two bicistronic mRNA molecules are coregulated by a common repressor protein that binds to the above-mentioned overlapping region. The SQR gene is located adjacent to sll5035, which is predicted to encode a bacterial transcriptional regulatory protein of the ArsR family, involved in arsenic resistance. In the opposite direction, slr5037 encodes a protein with a domain characteristic to the highly conserved DUF302 superfamily of yet-unknown function and no high similarity to any proteins with known function. Nevertheless, it shows 32% identity and 60% similarity ($e = 10^{-22}$) to a hypothetical protein (AT5A_19741) of *Agrobacterium tumefaciens* that has some low similarity to several arsenic oxidase and transport proteins. Downstream of this gene, slr5038 encodes a protein with 8 to 12 transmembrane helices according to predictions by Sosui (35) and Topcons (36). This gene is annotated as a putative chromate transporter based on similarity, but its colocalization with an SQR enzyme and an arsenical repressor suggests that it may be involved in an alternative sulfide-based metabolism or arsenic resistance.

Considering that these genes are related to *su*lfide *o*xidation, we designated them *suoR* (sll5035), *suoS* (sll5036), *suoC* (slr5037), and *suoT* (slr5038) (Fig. 1).

The deduced SuoS SQR protein (sll5036) consists of 428 amino acid residues with a molecular mass of 46.794 kDa, an isoelectric point of 8.7, and a net charge of 4.97 at neutral pH. No transmembrane regions could be identified using Sosui predictions in concordance with the fact that most SQR enzymes are monotopic membrane enzymes (42) located in the periplasm although strongly attached to the membrane. SQRs were described as monomeric enzymes in cyanobacteria, and purified SQRs from *O. limnetica* and *A. halophytica* were functionally active in the monomeric form (13). Nevertheless, dimeric (*Acidianus ambivalens* [43]) and even trimeric (*Aquifex aeolicus* [3]) forms have also been reported.

We noticed that on the pSYSM plasmid, the DNA region of the *suoRSCT* operon is in the vicinity of a sequence that shows the characteristic structure of transposable elements: the slr5040 (denominated as IS*4suo*) open reading frame is located 645 bp downstream of *suoT* (Fig. 1) and shows the conserved domain structure characteristic of the DDE superfamily transposases (44, 45). The majority of IS elements that encode DDE transposases are enclosed by short terminal inverted repeat sequences (IR) of 10 to 40 bp in length (46). We found a 20-bp imperfect inverted repeat delimiting the *suo* operon on the pSYSM plasmid and the presence of IS*4suo*, a putative transposase of type IS*4* (46) (Fig. 1). Cassier-Chauvat et al. (47) have shown that in *Synechocystis* the IS*4* and other insertion elements have played significant roles in plasmid rearrangements as well as in horizontal gene transfer, which seems also possible in the case of the evolution of this plasmid.

***In silico* investigation of the 3D structure of the SuoR and SuoS (SQR) proteins.** Prior to performing "wet lab" experiments on the two proteins, we investigated their putative 3D structure *in silico* in order to see whether these results would support or contradict their presumed functions.

Modeling the *Synechocystis* SuoR protein. Arsenic binding proteins have been investigated extensively, and it has been shown that three conserved cysteine residues contribute to arsenic binding within the active sites (48). In order to see whether an arsenic binding site of similar configuration is present in the arsenic-dependent SuoR repressor, we carried out homology modeling using the Swiss-Model service. The model in Fig. 2 shows the C_{35}-X-C_{37} dyad together with C-41 in a helix-turn-helix structure. These structural features corroborate the assumption of the function of the protein as discussed below.

Modeling the *Synechocystis* SQR enzyme. Site-directed mu-

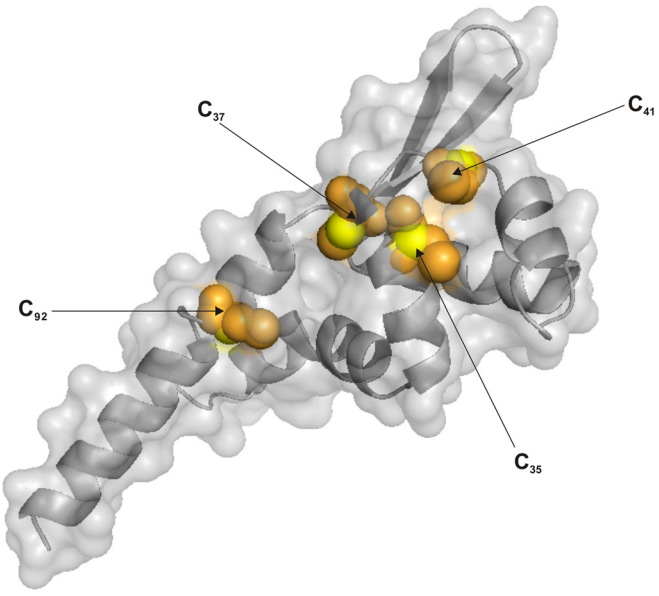

FIG 2 Putative arsenate binding site of the SuoR protein. A homology model was built from the deduced amino acid sequence using the 3F6O structure in the PDB databank. The cysteine residues supposedly involved in the arsenite binding (at positions 35, 37, 41, and 92) are shown in orange and the sulfur atoms in yellow.

tagenesis (49) and investigations of the crystal structure of several SQRs from different bacteria (3, 15, 42, 43, 50) led to the detailed description of the catalytic site of the SQR enzymes. To verify the presence and appropriate positions of all necessary components of an active SQR enzyme in the SuoS protein, we carried out domain parsing using the RaptorX service (40). The ligand binding pockets for flavin adenine dinucleotide (FAD), sulfide, and quinone can be predicted with high significance (P, $\sim 10^{-14}$), firmly supporting the view that the enzyme is functional and worthy of further experimental investigation.

Furthermore, we built a homology model using Swiss-Model (37). We used the *Acidithiobacillus ferrooxidans* SQR (50) as the template, which is a publicly available type I SQR crystal structure (Fig. 3.).

The *in silico* investigations shown above are in concert with the supposition that the *suoS* gene encodes an SQR enzyme; nevertheless, we aimed to get direct experimental proof of its expression and active functioning, as follows.

SuoS sulfide:quinone oxidoreductase is induced when cells are exposed to sulfide. In order to test whether the *suo* operon is inducible by sulfide, we treated the *Synechocystis* culture under anaerobic conditions with Na_2S and followed its expression level. After 30 min of treatment with 0.5 mM Na_2S, we observed an

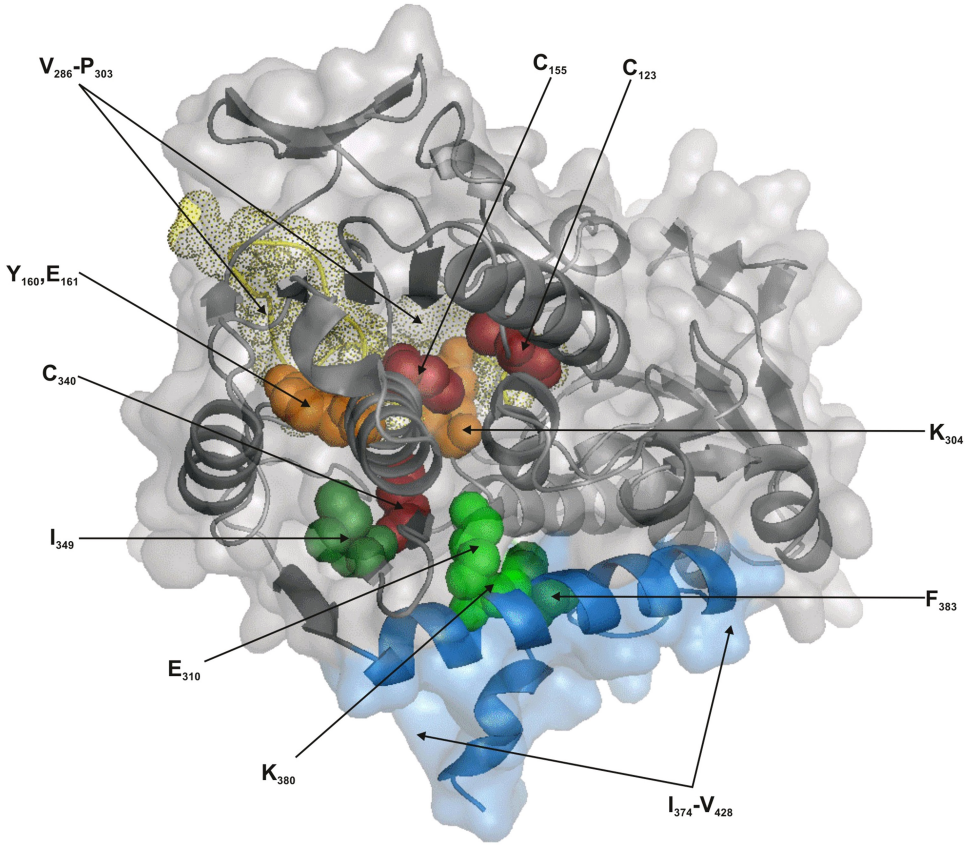

FIG 3 3D model of *Synechocystis* 6803 SuoR SQR enzyme. Conserved amino acids that play basic roles in the enzyme function are colored: the three main cysteine residues (in the active site of the enzyme, C_{123}, C_{155}, C_{340}) that coordinate the FAD cofactor and catalyze the oxidation of H_2S are depicted as red spheres. Auxiliary amino acids (Y_{160}, E_{161}, K_{304}) with substantial roles in the oxidation of H_2S are marked by orange spheres. Yellow dots and the ribbon in the "rear" of the enzyme mark the "capping loop" formed by residues V_{286} to P_{303} with a role in the uptake of H_2S. Dark green spheres are amino acids (I_{349}, F_{383}) that stabilize/bind the plastoquinone, while light green residues (E_{310}, K_{380}) catalyze the reduction of the quinone molecule. Residues I_{374} to V_{428}, depicted in blue, create a hydrophobic double helix. The enzyme is bound to the membrane at this site, and also the plastoquinone is taken up at this part of the protein.

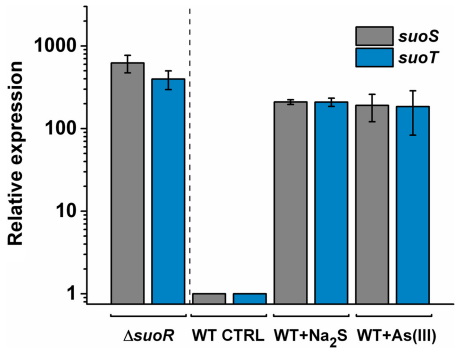

FIG 4 Expression of *suoS* and *suoT* genes. When treated with H_2S or As(III), both *suoS* and *suoT* genes are expressed about 200-fold compared to the WT untreated genes. In the mutant strain lacking *suoR*, the operon is constitutively expressed. Data represent the averages of three independent replicates with standard deviations.

approximately 200-fold induction relative to the untreated control (Fig. 4), indicating that the genes in both directions of the *suo* operon are repressed under normal conditions. It is noteworthy that in the cases where the repressors are parts of the operons that they regulate, the repression is obviously somewhat "leaky," and from the genomic context (see above) this gene organization was suspected in the case of *Synechocystis* as well.

Investigation of the sulfide oxidation by the SQR enzyme. To test whether the SQR enzyme is indeed functional, we monitored the concentration of H_2S in the headspace of the vials of *Synechocystis* WT and Δ*suoRSCT* cultures supplemented with 0.5 mM Na_2S. We found that after 24 h of incubation in light, the WT cells oxidized the sulfide in the culture medium, whereas only a slight decrease of the H_2S concentration could be detected in the culture of Δ*suoRSCT* mutant cells (Fig. 5A). This slight decrease in concentration may be due to the transition metal cations present in the medium (as microelements), since these react with sulfide ions to form insoluble sulfides.

Furthermore, we found that the WT cells incubated without light had only a similarly minor decrease in sulfide concentration, proving that *in vivo* the SQR activity is light dependent. The most feasible explanation of this feature is that the electrons are transferred to quinone acceptors in light, a process that is hindered in darkness due to the more reduced nature of the quinone pool under dark anaerobic conditions. This feature strongly suggests that the SQR enzyme transfers the electrons to the photosynthetic electron transport chain. As a consequence, this path may serve as an alternative electron source.

Investigation of the quinone reduction by the SQR enzyme. Beyond the light-dependent nature of the sulfide oxidation, we tested whether the electron acceptor substrate of the putative SQR enzyme is indeed plastoquinone. Since it is difficult for plastoquinones to enter the cell through the plasma membrane and the periplasmic space, we carried out this investigation using thylakoid membranes isolated from sulfide-induced wild-type and mutant cells. PQ-1, a water-soluble short-isoprenoid-chain plastoquinone, was used, which is an appropriate analogue of the internal quinone in *in vitro* investigations (51). Following the changes in the absorption at 257 nm of the plastoquinol, formed by the reduction of the added plastoquinone, we found plastoquinol formation in thylakoid samples isolated from induced WT cells, whereas this feature could not be observed using the similarly treated thylakoids from the mutant lacking the *suo* operon (Fig. 5B).

The SuoS sulfide-quinone oxidoreductase is regulated by an arsenic-responsive protein. According to the inducibility of the operon, the existence of a regulatory protein controlling the expression of SQR in cyanobacteria is plausible. Due to its position in the close proximity of the *suoS* gene and the similarity of its organization to that of one of the heavy metal transporter systems (41), we suspected that *suoR* is involved in the regulation of this operon. We constructed a mutant Δ*suoR* strain that lacks the SuoR protein and measured the expression of the *suoS* and *suoT* genes. The transcripts of these genes were detected in high abundance irrespectively of the presence or absence of sulfide in the growth medium. This observation strongly supports the idea that this gene encodes a regulatory protein that represses the *suoRSCT* genes (Fig. 4). It is noteworthy that although most of the stress-induced genes are positively regulated in cyanobacteria (52), negative regulation also occurs, mostly for detoxification genes.

The protein encoded by sll5035 belongs to the ArsR family of transcriptional regulators, of one single domain belonging to

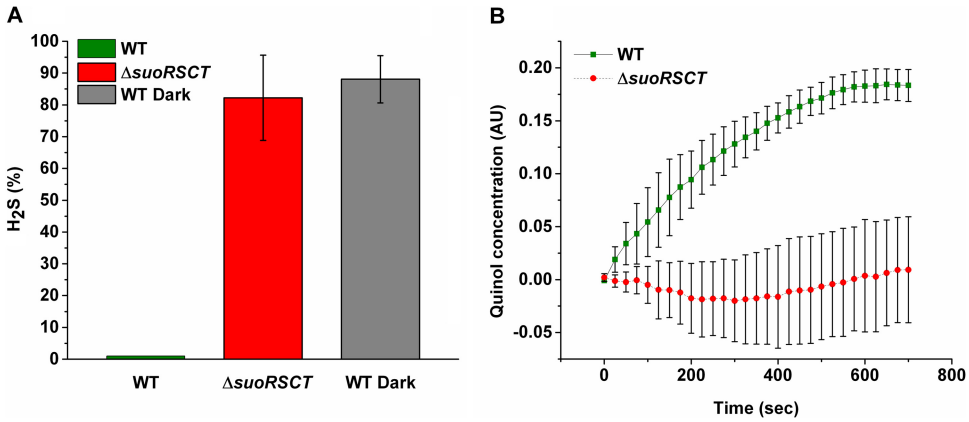

FIG 5 Oxidative (A) and reductive (B) half reactions of the SuoS SQR enzyme of *Synechocystis* PCC6803. (A) Sulfide content of the headspace of WT, Δ*suoRSCT*, and WT Dark cultures relative to the initial 100% value. (B) Plastoquinone reduction in the presence of thylakoids isolated from sulfide-treated WT and Δ*suoRSCT* cells. Plastoquinone reduction can be observed only in the thylakoids isolated from WT cells in which SQR is induced by sulfide pretreatment. Data represent the averages of three independent replicates with standard deviations.

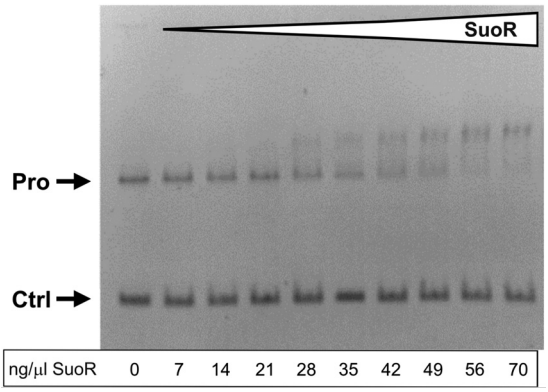

FIG 6 Electrophoretic mobility shift assay of the *suoRSCT* promoter region with increasing quantities of SuoR protein. The SuoR repressor binds the 410-bp promoter fragment (Pro), decreasing its electrophoretic mobility. The mobility of the control DNA (Ctrl) is not altered.

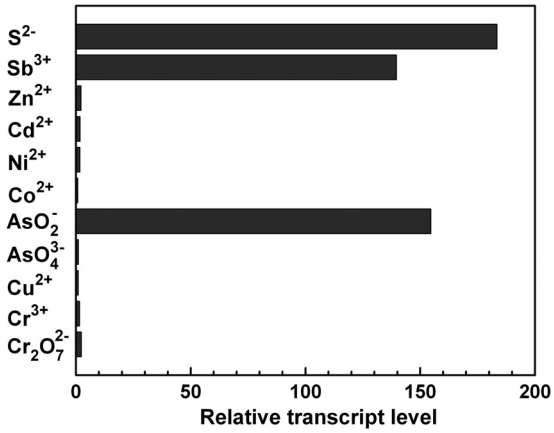

FIG 7 Expression pattern of *suoT* (sll5038) upon exposure to different heavy metals in *Synechocystis*. The putative chromate transporter *suoT* is induced when exposed to sulfide, arsenite, and antimony but does not show any response to the other ions tested.

HTH_ARSR in the Conserved Domains Database (CDD). This is an unexpected association with sulfide:quinone oxidoreductase enzymes since ArsR proteins in general are repressors of arsenic resistance genes (see reviews in references 23, 53, and 54). Three cysteine residues have been shown to have key roles in the active functioning of the related arsenical repressors (48). To verify the presence of the residues necessary for arsenic binding, we built a homology model (Fig. 2), which showed that these cysteines are in the appropriate distance and orientation, corroborating the arsenic-dependent operation of this protein.

We tested the expression of the *suoS* and *suoT* genes upon $NaAsO_2$ treatment and found a 200-fold overexpression of both genes (Fig. 4). In order to clarify whether this protein has a direct or indirect role in regulation of the *suo* operon, we overexpressed it in *E. coli* and purified the His-tagged SuoR protein via Ni-affinity chromatography. This purified protein gel shift assay was performed to assess the binding of SuoR to the putative promoter sequence of the *suoRSCT* operon. To this end, we PCR amplified the intergenic region between *suoS* and *suoC*, which contains the putative promoter sequence and performed gel electrophoresis to estimate the mobility of the free DNA fragment compared to that of the SuoR-DNA complex. Our result shows that SuoR interacted with the target DNA and reduced the mobility of the 410-bp promoter fragment resulting in virtually increased size (Fig. 6). The mobility of the control DNA of unrelated sequence was not altered, indicating the specificity of this binding.

It is noteworthy that no such effect of the purified protein could be found with the *arsB* promoter region (data not shown), demonstrating that the two respective ArsR-type repressors have different sequence specificities. In order to clarify the specificity of SuoR toward various effectors, the expression level of the *suoT* gene was assessed upon exposing the wild-type cells to heavy metal and metalloid ions and sulfide. The expression of the gene was induced only by sulfide and arsenic apart from antimony, the last being a common inducer of arsenic-inducible genes, but does not show any response to the other ions tested (Fig. 7).

Thus, we conclude that the product of *suoR* is a regulatory protein with two specific substrates, sulfide and arsenite (and antimony). This protein specifically binds to the promoter region, repressing the operon, whereas it dissociates in the presence of As(III), allowing the expression of the genes. The conserved cysteine residues necessary for the arsenic binding have been identified in the 3D structure of the modeled protein (Fig. 2), whereas the sulfide sensing of such a repressor is a unique feature and has not been elucidated at the molecular level.

The SuoT protein is involved in arsenic metabolism. ArsB, the membrane-localized protein in the well-documented arsenical resistance operon of *Synechocystis* (25), functions as an arsenite transporter. We aimed to test whether the protein encoded by the *suoT* gene has similar function, since SuoT is also a membrane protein and its gene is induced by arsenic. To test this, we constructed a *suoT*-deficient (Δ*suoT*) mutant and compared it with the Δ*arsB* mutant (a kind gift from J. C. Reyes). After 3 h of exposure to 100 μM and 1 mM arsenite, respectively, under normal light conditions, we assessed the intracellular arsenic content via ICP-MS (Fig. 8A).

Surprisingly, SuoT had an effect opposite to that of ArsB, suggesting that it functions as an arsenite importer. It is also noteworthy that arsenite is not toxic to the cells in this concentration range (up to 1.5 mM); therefore, arsenite uptake is not harmful. In concert with this, the growth of the Δ*suoT* mutant was not hindered compared to the WT (Fig. 8B), with or without arsenite added to the cultures at the applied 1 mM concentration.

Since this kind of link between the arsenic and sulfidic metabolism is unprecedented, we verified whether similar gene clusters can also be found in other organisms. We carried out a gene context survey using GeConT (39) software. Interestingly, while this gene organization is sparse, in some alphaproteobacteria like *Bradyrhizobium japonicum*, *Bradyrhizobium* sp. strain BT, or the purple nonsulfur bacterium *Rhodopseudomonas palustris*, the two genes are in close proximity in the genomes. Furthermore, although whole-genome sequences are not available for *O. limnetica* and *A. halophytica* and the genomic contexts are therefore not known, we found that the putative regulatory proteins in the two strains (13) (accession numbers AF242370 and AF242371, respectively), also belong to the ArsR family of repressors.

It is also important to mention that putative orthologous genes with reciprocal best BLAST hits for all four deduced *suo* protein sequences can be detected in the genome of *Thioalkalivibrio thiocyanodenitrificans*, an alphaproteobacterium that is closely related

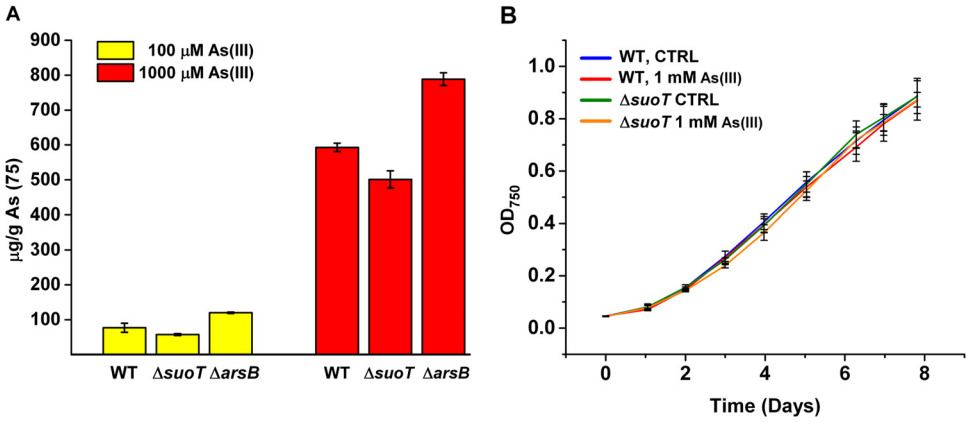

FIG 8 (A) Arsenic content of the dry material of cyanobacterial cells. Cells were incubated for 3 h in BG-11 medium supplemented with $NaAsO_2$. The bars represent the averages and standard deviations from three independent experiments each. (B) Growth of the cultures with and without arsenite. The points are averages with standard deviations from five cultures.

to the salt-tolerant chemolithoautotrophic sulfide oxidizers found in hot spring biofilms at Mono Lake, CA (55).

DISCUSSION

It has been demonstrated that bacteria may use arsenic substances as energy sources for growth (20) or as electron donors for photosynthesis (21). Since in many cases sulfidic environments also contain arsenic compounds, the linked metabolism has been documented: some bacteria use H_2S as an electron donor and concomitantly reduce arsenate to arsenite to obtain energy (56–59). In other sulfide-oxidizing bacterial strains, a different scenario was demonstrated, whereby both arsenite and sulfide are oxidized, presumably for arsenic detoxification, via synthesis of thioarsenates as putative intermediary compounds (55). Nevertheless, in spite of the growing amount of observations regarding linked metabolism, the molecular-level details of the processes are still mainly undiscovered.

In the current study, we found an operon in a cyanobacterium that shows a direct link between arsenic and sulfide metabolism at the level of genes and gene expression. According to the genomic context, it is likely that this operon has been acquired by the freshwater bacterium *Synechocystis* via horizontal gene transfer from some sulfide-oxidizing bacterium. Therefore, the functions of the genes are to be considered with the arsenic- and sulfide-containing saline environments in mind, such as the hot springs at Mono Lake, CA, where cyanobacteria have been reported in the green mat, among other bacteria.

SuoT, which is annotated as a chromate transporter, is actually an arsenite uptake transporter. Although arsenite is not used as an energy source under natural oxygenic photosynthetic conditions, this importer is still functional. Its effect is compensated by the chromosomally encoded ArsB exporter, in concert with the finding that arsenite is not toxic to the cells up to 1 mM concentration, and actually cells can tolerate up to 3 mM arsenite (data not shown). Arsenite oxidation may provide energy in anoxic habitats (60, 61), and for this process arsenite uptake could be of high importance. The molecular background of this oxidation is nevertheless mainly undiscovered. In one possible scenario, it occurs via sulfide-arsenite cometabolism, whereby the arsenate produced is first converted from arsenite to thioarsenic compounds, as also hypothesized by Couture et al. (62). This observation (55) was described using enrichment cultures of sulfide oxidizers closely related to some *Thioalkalivibrio* strains. The whole-genomic sequence of *Thioalkalivibrio thiocyanodenitrificans* ARhD1 is available and makes it possible to assess the presence of genes in it. We found that all four genes forming the *suo* operon have their orthologs in this genome, although not in an operon. In contrast, in *Microcystis aeruginosa*, a close relative of *Synechocystis*, the *suoSCT* genes do not have orthologs and the ortholog of *suoR*, MAE11930, is located adjacent to a transposase gene. This finding again corroborates the assumption of the origin via lateral gene transfer together with the notion that the operon is dispensable in recently established normal habitats.

Still, *Synechocystis* may have benefits conveyed by these functional genes. Volcanic eruptions or other geochemical events may result in temporal exposure to both arsenic and sulfide. As sulfide inhibits the activity of PSII, the SQR enzyme may provide advantages, either providing electrons to the photosynthetic electron transport chain or converting sulfide to less toxic substances.

Likewise, recently it was shown (21) that arsenite can serve as an electron donor for anoxygenic photosynthesis. Although this phenomenon has been characterized in purple sulfur bacteria (63), experimental observations of the green mats from the hot spring pond at Mono Lake suggested that the *Oscillatoria*-type cyanobacteria that dominated the green mat may also be capable of As(III)-supported anoxygenic photosynthesis (21). In the genome of *Synechocystis*, no homologs of known arsenite oxidases can be found either (according to its annotation as well as BLAST searches using bacterial AoxA, AoxB, ArrA, and ArxA sequences as query [data not shown]). The supposition that a so-far-unknown protein takes part in the arsenite oxidation in this strain is in concert with the fact that arsenite oxidases are an ancient (64, 65) and very diverse group of proteins (66) far from being fully explored (67). Although the enzymes involved are not yet identified in *Synechocystis*, it is noteworthy that arsenite oxidation to arsenate via electron transport to plastoquinone is energetically more favorable than the reverse reaction according to the more negative midpoint redox potential of arsenate/arsenite (+60 mV) (20) compared to that of plastoquinones (+80 mV) (68).

SuoC is a conserved protein with no known function yet, and its direct or indirect involvement in arsenic redox reactions is not

known. Nevertheless, similar genes can be found in a wide variety of bacteria, including species like *Anaerophaga thermohalophila*, a moderately thermophilic and strictly anaerobic bacterium and some alkaliphilic, sulfur-oxidizing bacterial strains belonging to the *Thioalkalivibrio* genus. This protein is coregulated in an operon with a sulfide:quinone oxidoreductase, of which the closest ortholog is that of an *Oscillatoria* strain, and in both genomes these genes are located in the vicinity of ArsR-type repressor genes.

Considering the fact that a capability for arsenite-dependent photosynthesis was attributed to bacteria (including not only proteobacteria but also cyanobacteria closely related to *Synechocystis*) found in mats in sulfidic and arsenite-containing anaerobic (microaerobic) ponds, the similar organization of the genes in these bacteria strongly supports the idea that they have a functional link, that both take part in anoxic photosynthesis, and that they have evolved together. The fact that the *suo* genes are localized on a mobile genetic element of type IS*4* led us to assume that *Synechocystis* may have acquired these genes from another microorganism via lateral gene transfer. Nevertheless, under normal conditions *Synechocystis* uses water as the electron donor for photosynthesis, so these genes may represent the remnants of an ancient metabolism or a feature acquired from some bacteria with no functional PSII, and they may have retained their activities due to their utility in sulfide and arsenite detoxification. The functionality of the *suo* genes indicates the presence of some selection pressure even in recent times and points to the possibility that when it grows in nature *Synechocystis* can experience environmental conditions under which the function of the *suo* operon is beneficial.

Because *Synechocystis* is naturally competent for transformation (69, 70) and conjugation (71), the presence of 128 putative transposase sequences in *Synechocystis* (according to CyanoBase) strongly suggests that this organism has been truly involved in the dissemination of IS elements between distantly related species. Of the 39 complete cyanobacterial genome sequences in CyanoBase, 9 possess genes annotated as SQR enzymes based on sequence similarity. It is noteworthy that in the 16S RNA-derived phylogenetic tree (72) cyanobacteria with relatively large genomes (and hence more flexibility with respect to environmental changes) are fully separated from cyanobacterial strains with small genomes. Genes showing high sequence similarities to the ones studied here are present in several representatives of the former group and completely missing from the latter one with small genomes. This indicates either that the genes were acquired right after those two branches diverged or that these were lost during genome reduction.

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There is no conflict of interests to declare.

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