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ORIGINAL PAPER

The ChrA homologue from a sulfur-regulated gene cluster in cyanobacterial plasmid pANL confers chromate resistance

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Abstract The cyanobacterium *Synechococcus elongatus* strain PCC 7942 possesses pANL, a plasmid rich in genes related to sulfur metabolism. One of these genes, *srpC*, encodes the SrpC protein, a homologue of the CHR chromate ion transporter superfamily. The *srpC* gene was cloned and expressed in *Escherichia coli* and its role in relation to sulfate and chromate was analyzed. *srpC* was unable to complement the growth of an *E. coli cysA* sulfate uptake mutant when sulfate was utilized as a sole sulfur source, suggesting that SrpC is not a sulfate transporter. Expression of *srpC* in *E. coli* conferred chromate resistance and caused diminished chromate uptake. These results suggest that the *S. elongatus* SrpC protein functions as a transporter that extrudes chromate ions from the cell's cytoplasm, and further demonstrate the close relationship between sulfate and chromate metabolism in this organism.

Keywords Chromate resistance · CHR superfamily · *Synechococcus* · Plasmid

Introduction

Synechococcus elongatus PCC 7942 (previously *Anacystis nidulans* R2) is a unicellular freshwater cyanobacterium that possesses the pANL plasmid, which encodes several sulfur-regulated genes (Nicholson and Laudenbach 1995; Chen et al. 2008). One of these genes, *srpC,* encodes putative SrpC protein, a homologue of the CHR chromate ion transporter superfamily (Nicholson and Laudenbach 1995; Nies et al. 1998). The CHR superfamily is constituted by hundreds of proteins distributed in the three life domains (Díaz-Pérez et al. 2007; Henne et al. 2009). The best-studied example is the *Pseudomonas aeruginosa* ChrA protein, which functions as a chemiosmotic pump that extrudes chromate from the cell's cytoplasm using the proton motive force (Alvarez et al. 1999; Ramírez-Díaz et al. 2011). Several members of the CHR superfamily have been shown to confer chromate resistance by functioning as chromate transporters (reviewed in Ramírez-Díaz et al. 2008). Nicholson and Laudenbach (1995) reported that a *S. elongatus* transposon insertion mutant disrupting the *srpC* gene exhibited higher chromate resistance as compared to the wild type strain, and suggested that the SrpC protein was involved in chromate uptake. In this work, it was shown that SrpC was able to confer chromate resistance in *Escherichia coli* by a mechanism probably involving efflux of chromate ions.

Materials and methods

Bacterial strains and culture media

Escherichia coli W3110 (prototroph) (Hayashi et al. 2006) was utilized as recipient strain for recombinant plasmids. *E. coli* BW25113 (wild type) and JW2415-1 (*cysA97*), its cysteine auxotroph derivative with a transposon insertion in the *cysA* gene encoding the ATPase subunit of the CysPTWA sulfate permease (obtained from *E. coli* Genetic

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Stock Center; Baba et al. 2006), were employed to measure sulfate requirement. Culture media utilized were: Luria–Bertani broth (LB; 1.5% agar for solid medium); Nutrient broth (NB; Bioxon), and M9 salts minimal medium (M9; Sigma) supplemented with 20 mM glucose, 2 mM $MgSO_4$, and 0.1 mM $CaCl_2$. Varied sulfate concentrations in M9 were achieved by adjusting the amounts of $MgSO_4$.

Genetic techniques

General molecular genetic techniques were employed according to standard protocols (Sambrook et al. 1989).

Cloning of the *srpC* gene

Cosmid 2F10 (a gift from S. Golden, Texas A & M University, Houston, TX) contains a ca. 19 kilobases (kb) fragment from *S. elongatus* PCC 7942 native pANL plasmid (46.3 kb), which includes a sulfur-regulated region (Chen et al. 2008) (Fig. 1). 2F10 DNA was utilized as a template to obtain the *srpC* gene (including 200 pb upstream and 160 pb downstream with respect to start and stop codons, respectively) by Polymerase chain reaction (PCR) with oligonucleotides 5′-GATCGCTTGGGATCC TAAGACTTTAC-3′ (forward) and 5′-CGATCCACAAG CTTAGTCGGTTGAG-3′ (reverse) (Fig. 1), with *BamH*I and *Hind*III restriction endonuclease sites, respectively (underlined). Amplification with *Taq* DNA polymerase (Fermentas) was conducted by the following protocol: first denaturing step at 94°C, 30 s; 30 cycles of denaturation at 94°C, 30 s; primer annealing at 50°C, 1 min; extension at 72°C, 3 min. A 5-min final extension at 72°C was performed. The amplified fragment (1.5 kb) was purified utilizing Wizard SV gel and PCR clean-up system (Promega) and ligated into the pGEM-T vector (Promega). Recombinant plasmids were transferred by electroporation to *E. coli* strains selecting transformants on LB agar plates with 100 μg/ml ampicillin. The cloning process was verified by restriction endonuclease digestions, and by sequencing inserts employing M13 universal primers. The DNA fragment containing the *srpC* gene was recovered by digestion with *BamH*I/*Hind*III endonucleases, and subcloned into the corresponding sites of the pACYC184 vector (Fermentas). *E. coli* W3110 cells were transformed by electroporation, and transformants were selected on LB agar plates with 35 μg/ml chloramphenicol.

DNA sequencing and sequence analysis

DNA sequencing was carried out at the Departmento de Genética, Cinvestav-Irapuato, México. Promoter sequences were searched with the Neural Network Promoter Prediction (http://www.fruitfly.org/cgi-bin/seq_tools/promoter.pl software. Rho-independent terminators were predicted with the FindTerm (Softberry Inc.) program. For Transmembrane segment (TMS) prediction, the TOPpred2 program was employed (http://bioweb.pasteur.fr/seqanal/interfaces/toppred.html).

Chromate susceptibility tests

Overnight cultures, grown at 37°C in NB, were diluted 1:50 in tubes with 4 ml of fresh medium with increasing amounts of K_2CrO_4, and were incubated for 18 h at 37°C. Growth was monitored as Optical density at 590 nm (OD_{590}) with a spectrophotometer.

Measurement of chromate transport

Uptake of chromate was determined in cells grown overnight at 37°C with shaking in M9 medium with standard (2 mM) sulfate concentration. Cultures were then diluted

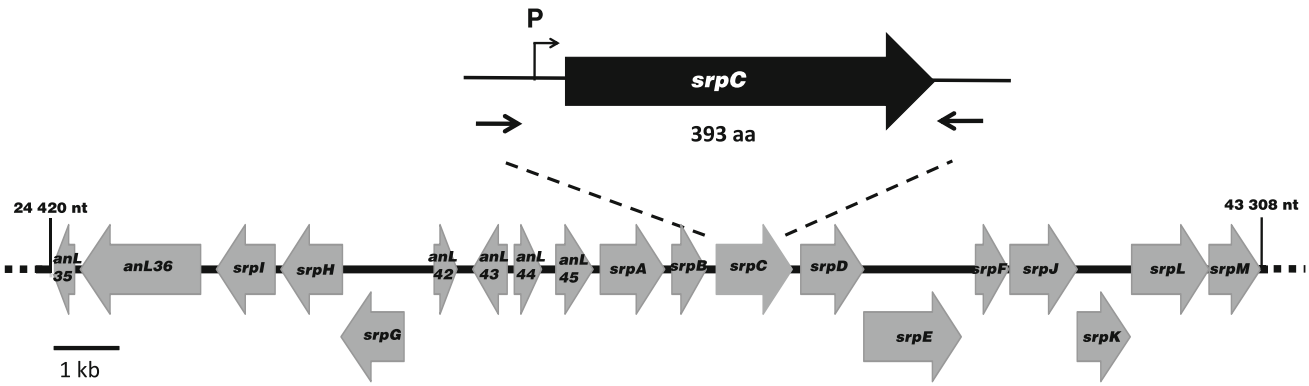

Fig. 1 Schematic representation of the sulfur-regulated region in the *Synechococcus elongatus* pANL plasmid. Thick arrows represent putative ORFs and direction of transcription. The *srpC* gene is highlighted above and location of a putative promoter (*P*) and of the primers employed for cloning (*small arrows*) is indicated. Coordinates based on the pANL plasmid genetic map are shown in nucleotides (*nt*). A *bar scale* indicates 1 kb. Gene names as in Chen et al. (2008)

1:25 in fresh M9 medium with either 0.02 or 0.2 mM sulfate and grown to an OD_{590} of 0.6. Cells were washed twice with 0.1 mM phosphate buffer (pH 7.2), suspended in the same buffer prewarmed to room temperature, and incubated at 37°C. Incorporation of 10 μM (0.2 mCi) or 40 μM $Na_2^{51}CrO_4$ was estimated as reported previously (Aguilar-Barajas et al. 2008).

Results and discussion

Sulfate requirement

The *srpC* gene from *S. elongatus* pANL plasmid encodes the putative SrpC protein, a member of the CHR chromate ion transporter superfamily that shows 62% amino acid (aa) identity to the ChrA protein from the Proteobacterium *Cupriavidus metallidurans* (Nicholson and Laudenbach 1995). Sequence analysis identified a putative promoter and a consensus putative ribosome-binding site in the 5′ region of the *srpC* gene; a potential Rho-independent transcription termination sequence was also identified (data not shown). The TopPred program predicted that the 393-aa SrpC protein contains 11 TMSs, a membrane topology similar to that from other CHR bacterial homologues (Ramírez-Díaz et al. 2008). The *srpC* gene is located within a region of the pANL plasmid that is rich in genes involved in sulfur metabolism (Nicholson and Laudenbach 1995) (Fig. 1). Although sulfate and chromate are analogous oxyanions (Aguilar-Barajas et al. 2011), bacterial CHR homologues had previously not been reported to be linked to sulfur-related genes (Juhnke et al. 2002; Díaz-Pérez et al. 2007; Aguilar-Barajas et al. 2008; Branco et al. 2008; Díaz-Magaña et al. 2009; Henne et al. 2009). A *S. elongatus* transposon insertion mutant strain disrupted in the *srpC* gene was found to be more resistant to chromate than the wild type strain (Nicholson and Laudenbach 1995), thus the SrpC protein was suggested to participate in uptake of chromate. Since chromate uptake would be deleterious to *S. elongatus* cells, SrpC might instead rather be functioning as a transporter of essential sulfate ions. To evaluate this possibility, the *srpC* gene (including its putative promoter and transcriptional termination regions) (Fig. 1) was cloned into the pGEM-T vector. Recombinant plasmid pGEMT-SrpC was then transferred into the *E. coli* cysteine auxotroph mutant strain JW2415-1, affected in sulfate uptake and unable to grow on M9 minimal medium with sulfate as sole sulfur source. After overnight incubation, expression of the *srpC* gene in JW2415-1 did not allow the mutant to grow on M9; a similar absence of growth was observed with the strain transformed only with the vector (Fig. 2). This behavior was maintained even at 10 mM sulfate (data not shown). In contrast, wild type strain BW25113 showed normal growth even at 0.25 mM sulfate, the lowest concentration tested (Fig. 2). This lack of complementation of a sulfate permease indicates that SrpC is not able to take up sulfate in *E. coli* under the conditions tested.

Expression of *srpC* in *E. coli*

E. coli was used as a chromate-sensitive heterologous host to test whether the *S. elongatus srpC* gene confers chromate resistance because its genome does not contain CHR homologues (Nies et al. 1998; Díaz-Pérez et al. 2007). Therefore, *srpC* was subcloned into the pACYC184 vector, as described in the Methods section, rendering recombinant plasmid pACYC-SrpC, which bears the *srpC* gene under its own putative promoter. This plasmid was then transferred into *E. coli* W3110 and chromate susceptibility tests were conducted in NB medium. Transformants expressing the *srpC* gene showed enhanced chromate resistance as compared to control *E. coli* W3110 cells only containing the vector (Fig. 3A). Although this finding contrasts with that reported by Nicholson and Laudenbach (1995), one should consider that the susceptibility assays were not comparable. In the present work, a medium with higher sulfate concentration (NB) was employed for growth of the *E. coli* W3110 derivatives, and the *srpC* gene in the pACYC-SrpC construct has been separated from probable regulatory genes present on the pANL plasmid. These results demonstrated that *S. elongatus* SrpC protein conferred resistance to chromate. CHR superfamily homologues from

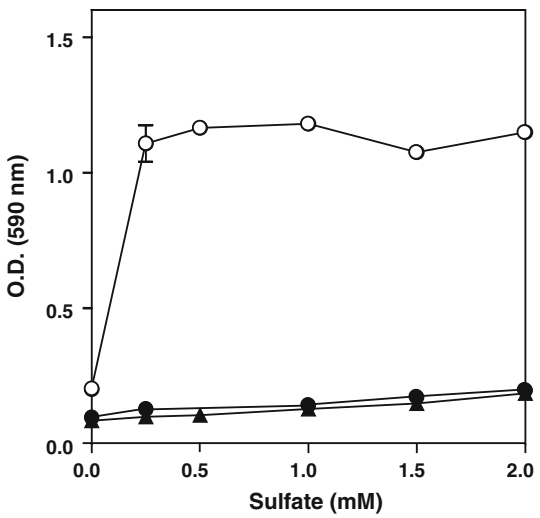

Fig. 2 Requirement of sulfate by *Escherichia coli* strains. Overnight-grown cultures were diluted 1:100 in M9 minimal medium with the indicated concentrations of sulfate. Incubation was for 18 h at 37°C with shaking, and the OD_{590} was recorded. BW25113 (*open circle*), JW2415-1(pGEM-T) (*filled circle*), JW2415-1(pGEMT-SrpC) (*filled triangle*). Data shown are means from duplicates of two independent assays, with standard *error bars* shown

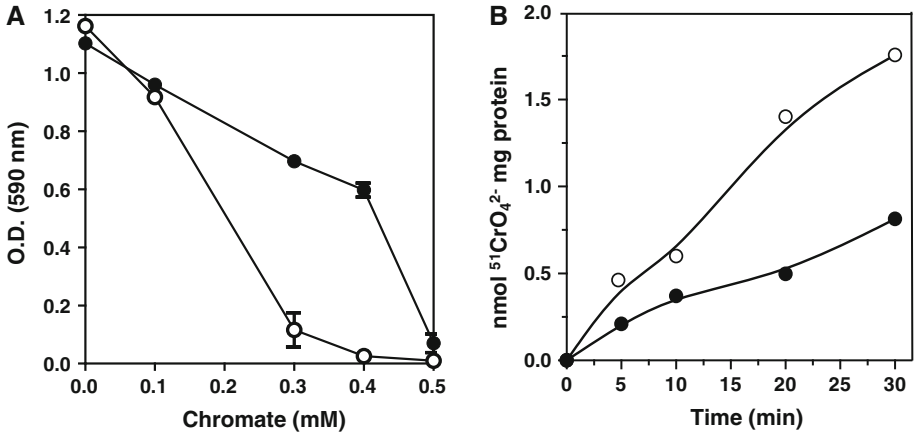

Fig. 3 Chromate susceptibility and uptake by *Escherichia coli* cells expressing the *srpC* gene. **A** Cultures were grown in nutrient broth with the indicated concentrations of K_2CrO_4 for 18 h at 37°C, and the OD_{590} was recorded. Data shown are means from duplicates of three independent assays with standard *error bars* shown. **B** Cultures were grown in M9 medium with 0.2 mM sulfate at 37°C to log phase. Cells were washed, suspended in phosphate buffer, and the incorporation of 10 μM $Na_2^{51}CrO_4$ was measured as described in the "Materials and Methods" section. Data shown are representative of two independent assays with similar results. *E. coli* W3110 (pACYC184) (*open circle*), *E. coli* W3110 (pACYC-SrpC) (*filled circle*)

diverse bacterial species have also been shown to confer chromate resistance when expressed in *E. coli*, including those from *Shewanella* sp. ANA-3 (Aguilar-Barajas et al. 2008), *Ochrobactrum tritici* 5bvl1 (Branco et al. 2008), *Bacillus subtilis* 168 (Díaz-Magaña et al. 2009), and *Burkholderia xenovorans* LB400 (León-Márquez et al. manuscript in preparation).

Chromate resistance mechanism

To gain insight into the mechanism employed by the SrpC protein to confer chromate resistance, transport of chromate was assayed in *E. coli* W3110 cells carrying the pACYC-SrpC plasmid, as described in Methods. Cultures were first grown to log phase in M9 medium with 0.2 mM sulfate, and then cells were washed and suspended in phosphate buffer to conduct short-time chromate uptake assays. Under these conditions, cells expressing the *srpC* gene showed a 2.3-fold decrease in $Na_2^{51}CrO_4$ uptake as compared to a strain bearing only the vector (Fig. 3B). Cells of *E. coli* W3110(pACYC-SrpC) also showed a two-fold decrease in chromate uptake when grown in M9 medium with 0.02 mM sulfate with either 10 or 40 μM $Na_2^{51}CrO_4$ (data not shown). This diminished uptake suggests that the *S. elongatus* SrpC protein is able to extrude chromate from the *E. coli* cytoplasm. Thus, SrpC appears to carry out a similar role as do other homologues of the CHR superfamily that function as membrane transporters able to efflux chromate ions (Ramírez-Díaz et al. 2008). This is the first example of a cyanobacterial species for which an efflux-mediated mechanism of chromate resistance is reported. Our findings extend the spectrum of CHR superfamily members involved in efflux of chromate ions to cyanobacteria. It is noteworthy that in *S. elongatus* pANL plasmid the SrpC protein is encoded within a sulfur-regulated gene cluster (Nicholson and Laudenbach 1995) (Fig. 1). This cluster includes genes involved in cysteine metabolism and transport, as well as genes encoding rhodanese and glutathione-related activities (Chen et al. 2008). Under low-sulfate conditions, such as those found in natural freshwater systems that cyanobacteria use to inhabit (Chen et al. 2008), cells may up-regulate sulfate transport, which would increase uptake of toxic chromate ions. Concerted expression of a chromate efflux system (SrpC) under these conditions would be advantageous for cyanobacterial cells, which may thus avoid chromate accumulation.

In conclusion, our results demonstrate that the *S. elongatus* SrpC protein confers chromate resistance; these data further emphasize the link existing between sulfate and chromate metabolism and transport in cyanobacteria.

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