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Short-Chain Chromate Ion Transporter Proteins from *Bacillus subtilis* Confer Chromate Resistance in *Escherichia coli*▽

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Tandem paired genes encoding putative short-chain monodomain protein members of the chromate ion transporter (CHR) superfamily (*ywrB* and *ywrA*) were cloned from genomic DNA of *Bacillus subtilis* strain 168. The transcription of the paired genes, renamed *chr3N* and *chr3C*, respectively, was shown to occur via a bicistronic mRNA generated from a promoter upstream of the *chr3N* gene. The *chr3N* and *chr3C* genes conferred chromate resistance when expressed in *Escherichia coli* strain W3110. The cloned *chr3N* gene alone did not confer chromate resistance on *E. coli*, suggesting that both *chr3N* and *chr3C* genes are required for function. *E. coli* cells expressing paired *chr3N* and *chr3C* genes demonstrated diminished uptake of chromate compared to that by a vector-only control strain. These results suggest that short-chain CHR proteins form heterodimer transporters which efflux chromate ions from the cytoplasm.

Many membrane proteins are formed by duplicated domains, showing sequence homology between their amino and carboxy halves. These proteins include diverse transporters from the major facilitator superfamily, the resistance-nodulation-division superfamily, and the ATP binding cassette (ABC) superfamily (15). It has been proposed that these proteins evolved by the duplication of ancestral 6-transmembrane-segment (TMS) proteins, followed by a fusion, giving rise to 12-TMS proteins (15). There are several examples of current 6-TMS homologs, including members of the major facilitator (15) and ABC (6) superfamilies.

Chromate resistance systems related to plasmid genes commonly encode membrane transporters which catalyze the efflux of chromate ions from the cytoplasm (14). The best-studied example is the *Pseudomonas aeruginosa* ChrA protein, which functions as a chemiosmotic pump that extrudes chromate from the cytoplasm using the proton motive force (2). ChrA belongs to the chromate ion transporter (CHR) superfamily (11), which includes dozens of homologs from all three domains of life (5). The CHR superfamily is composed of two families of sequences: (i) the short-chain monodomain CHR (SCHR) family, formed by proteins of about 200 amino acid (aa) residues, and (ii) the long-chain bidomain CHR (LCHR) family, comprising proteins of about 400 aa (5). The CHR superfamily is considered to be the first example of the existence of short-unit equivalent ancestral polypeptides, as well as full-length duplicated proteins (5). In fact, some bacterial genomes (e.g., those of *Cupriavidus metallidurans* and *Burkholderia* species) encode multiple SCHR and LCHR homologs of different subfamilies (5). Several proteins of the LCHR family have been demonstrated previously to function as membrane transporters able to extrude chromate ions from the cytoplasm (1, 2, 3, 10), but no function for proteins of the SCHR family has yet been reported.

In the present work, we found that paired genes encoding SCHR proteins from *Bacillus subtilis* confer resistance to chromate by a mechanism involving the efflux of chromate ions from the cytoplasm when expressed in *Escherichia coli*.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *B. subtilis* strain 168 (*trpC2*) was a gift from M. Pedraza-Reyes, University of Guanajuato, Mexico. *E. coli* W3110 (a prototroph) was a gift from C. Rensing, University of Arizona. The culture medium used was Luria-Bertani (LB) broth (1.5% agar for solid medium), nutrient broth (NB; Bioxon, Mexico), or M9 minimal salts medium (Sigma) supplemented with 20 mM glucose, 2 mM $MgSO_4$, and 0.1 mM $CaCl_2$. Liquid cultures were grown for 18 to 20 h at 37°C with shaking.

Genetic techniques. General molecular genetic techniques were used according to standard protocols (16).

Cloning of the *chr3N* and *chr3C* genes. Genomic DNA from the *B. subtilis* 168 strain was obtained as reported previously (4). The *ywrB* (locus tag BSU36120) and *ywrA* (locus tag BSU36130) genes (11, 13), herein renamed *chr3N* and *chr3C*, respectively, were obtained by PCR from *B. subtilis* genomic DNA by using oligonucleotides designed with HindIII (for direct primers) and BamHI (for reverse primers) restriction endonuclease sites (underlined below). For amplifying the *chr3N-chr3C* gene pair, primers Bsu_N_D (5′-GCTCTTAAGCTTGAGGAAGAGC-3′; forward primer corresponding to a sequence located 220 bp upstream of the start codon of *chr3N*) and Bsu_C_R (5′-GAAGGTCCAGGATCCTGTTTGG-3′; reverse primer corresponding to a sequence located 190 bp downstream of the stop codon of *chr3C*) were used (Fig. 1). To obtain the *chr3N* and *chr3C* individual genes, additional primers Bsu_N_R (5′-GAGAATGGATCCTTTGAGAGCC-3′; reverse primer corresponding to a sequence located 130 bp downstream of the stop codon of *chr3N*) and Bsu_C_D (5′-CAATTGTTGCAGGTAAGCTTGGTG-3′; forward primer corresponding to a sequence located 274 bp upstream of the start codon of *chr3C*), respectively, were used (Fig. 1). PCR conditions were as follows: a first denaturing step at 95°C for 2 min; 30 cycles of denaturation at 95°C for 40 s, primer annealing at 54°C for 30 s, and extension at 72°C for 2 min; and a final extension of 5 min. Amplified fragments were purified using the Wizard SV gel and PCR clean-up system (Promega) and cloned into the pGEM-T vector (Promega). Recombinant plasmids were transferred by electroporation into *E. coli* W3110, and transformants were selected on

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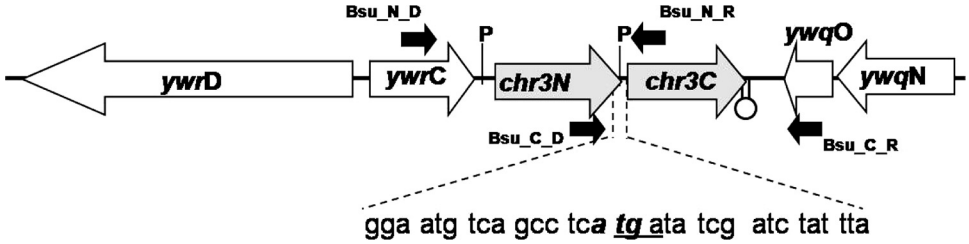

FIG. 1. Arrangement of SCHR protein-encoding genes in the *B. subtilis* genome. White arrows indicate genes and the direction of transcription. The locations of predicted promoter sequences are marked by a "P," and a predicted transcription terminator is indicated by a hairpin. Shaded arrows signal the locations of primers used to amplify the *chr3N-chr3C* pair of genes and the single *chr3N* and *chr3C* genes. The nucleotide sequence at the boundaries of the coding regions of the *chr3N* and *chr3C* genes is shown below. The start codon of *chr3C* (bold) and the stop codon of *chr3N* (underlined) are highlighted. Gene product accession numbers: Chr3N, NP_391493; Chr3C, NP_391494.

LB agar plates with 100 μg/ml ampicillin. The cloning process was verified by restriction endonuclease digestion and by sequencing of the inserts in pGEM-T by using universal primers. The DNA fragments from pGEM-T recombinant plasmids were obtained by digestion with HindIII and BamHI endonucleases and subcloned into the HindIII/BamHI sites of the pACYC184 vector (Fermentas). *E. coli* W3110 cells were transformed with these constructs as described above, except that 35 μg/ml chloramphenicol was used for the selection of transformants.

DNA sequencing and sequence analysis. DNA sequencing was carried out at the Department of Genetics, Cinvestav, Irapuato, Mexico. Potential promoter sequences and probable Rho-independent transcription termination sequences were identified using the Comprehensive Microbial Resource tool (http://cmr.jcvi.org).

Chromate susceptibility tests. Overnight cultures, grown at 37°C in NB or in M9 minimal medium, were diluted 1:100 in tubes with 4 ml of fresh medium with increasing amounts of K_2CrO_4 and incubated for 18 to 20 h with shaking. As the growth of *B. subtilis* in M9 medium was slower than that of *E. coli* strains, NB was used for growth comparisons. Growth was monitored as the optical density at 590 nm by using a spectrophotometer.

RT-PCR. Total RNA from *E. coli* cells grown in LB broth was isolated by using the Tri reagent (Molecular Research Center Inc.). RNA was quantified by spectrophotometric analysis at 260 nm. Reverse transcription-PCR (RT-PCR) was performed with total RNA samples and the one-step master AMP RT-PCR kit according to the instructions of the provider (Epicentre Technologies). Primers used for RT-PCR were as follows: for the *chr3N* gene, primers Chr3N_D (5′-TCTGCCTACATGTCTTGCGATGGT-3′; forward) and Chr3N_R (5′-ATGACCAGGCCGGGATTAATCTGT-3′; reverse) to generate a 240-bp internal *chr3N* transcript; for the *chr3C* gene, primers Chr3C_D (5′-ACCTGCGTCTATCCCGCTAATGTT-3′; forward) and Chr3C_R (5′-GACAGCGTCATGCCTTTGATGACA-3′; reverse) to generate a 276-bp internal *chr3C* transcript; for the intergenic *chr3N-chr3C* region, primers Chr3NC_D (5′-GCTCGGATTCATGGCGTACG-3′; forward) and Chr3NC_R (5′-GGTACGACGGTTGCGATCAGG-3′; reverse) to generate a 476-bp *chr3N-chr3C* overlapping product. RT-PCR positive and negative controls were performed with PCR master mix (Promega) and the set of primers described above using genomic DNA and total RNA, respectively, as templates. The sizes of RT-PCR products were assessed in agarose (1.5%) gels using the 1-kbp-plus DNA ladder (Life Technologies, Rockville, MD).

Measurement of chromate transport. The uptake of chromate was evaluated for cells grown overnight in M9 minimal medium with various sulfate concentrations at 37°C with shaking. Low-sulfate conditions in M9 were achieved by adjusting the amount of $MgSO_4$. Cultures were then diluted 1:25 in fresh medium and grown to an optical density at 590 nm of 0.6. Cells were washed twice with 0.1 mM phosphate buffer (pH 7.2) and resuspended in the same buffer, and the incorporation of 40 μM Na_2-$^{51}CrO_4$ was estimated as reported previously (1).

RESULTS AND DISCUSSION

***B. subtilis chr3N* and *chr3C* genes.** Chromate susceptibility tests showed that *B. subtilis* 168 is more resistant to chromate than *E. coli* W3110 (Fig. 2A), suggesting that the former strain possesses a chromate resistance determinant(s). Chromate pretreatment failed to yield increased chromate resistance in *B. subtilis* 168 (data not shown), suggesting that the resistance phenotype was expressed constitutively. The genome of *B. subtilis* contains a pair of genes probably encoding SCHR proteins, namely, YwrB and YwrA (11, 13), which belong to the SCHR3 subfamily of the CHR superfamily (5). The *ywrB* and *ywrA* genes were renamed *chr3N* and *chr3C* (for *chr* subfamily *3* and *N* or *C* domain), respectively (Fig. 1). The *chr3N* and *chr3C* genes encode polypeptides of 197 and 178 aa (accession numbers NP_391493 and NP_391494), respectively. Comparison of the amino acid sequences of the putatively encoded proteins Chr3N and Chr3C showed that they share 33% identity and 53% similarity. Further sequence analysis identified potential promoter regions (consensus −35 and −10 boxes are shown below in bold, whereas bases conserved relative to general sigma-A type promoter sequences are underlined) in both *chr3N* (**TTGATT**GCCAGATGCTGATCAAA**GATACA**) and *chr3C* (**TTACAG**ATTAATCCCGGCCTGGT**CATTAT**) genes. A probable Rho-independent transcription termination sequence, ATAGAAAAAAGCACCTGGACAGGTGCTTTTTTATTT, with a palindromic GC-rich sequence (underlined) flanked by A/T-rich tracks was identified just downstream of the *chr3C* gene (Fig. 1). Consensus putative ribosome binding sites were also identified in the 5′ regions of both *chr3N* and *chr3C* genes (data not shown). The *ywrC* gene, located just upstream of the *chr3N* gene and transcribed in the same direction (Fig. 1), encodes a probable transcriptional regulator of the Lrp/AsnC family (7, 8), but its function is still unknown. Interestingly, LrpC, the best-studied member of the Lpr family in *Bacillus*, is involved in processes of DNA repair and recombination (8). Other genes depicted in Fig. 1 that are transcribed in the opposite direction with respect to the *chr3N* and *chr3C* genes are considered not to be related to chromate resistance.

Transcriptional analysis of the *chr3N* and *chr3C* genes. The *chr3N* and *chr3C* genes have their coding regions overlapping; the initiation codon of *chr3C* and the stop codon of *chr3N* share four nucleotides (Fig. 1). The overlapping of coding sequences is a conserved feature of paired SCHR3 protein-encoding genes (found in 10 of 12 pairs of sequences analyzed), with intergenic distances between stop and start codons ranging from −4 to +18 bp. These data suggest a possible evolutionary advantage of the close vicinity of coding regions for SCHR proteins, and experiments to evaluate this hypoth-

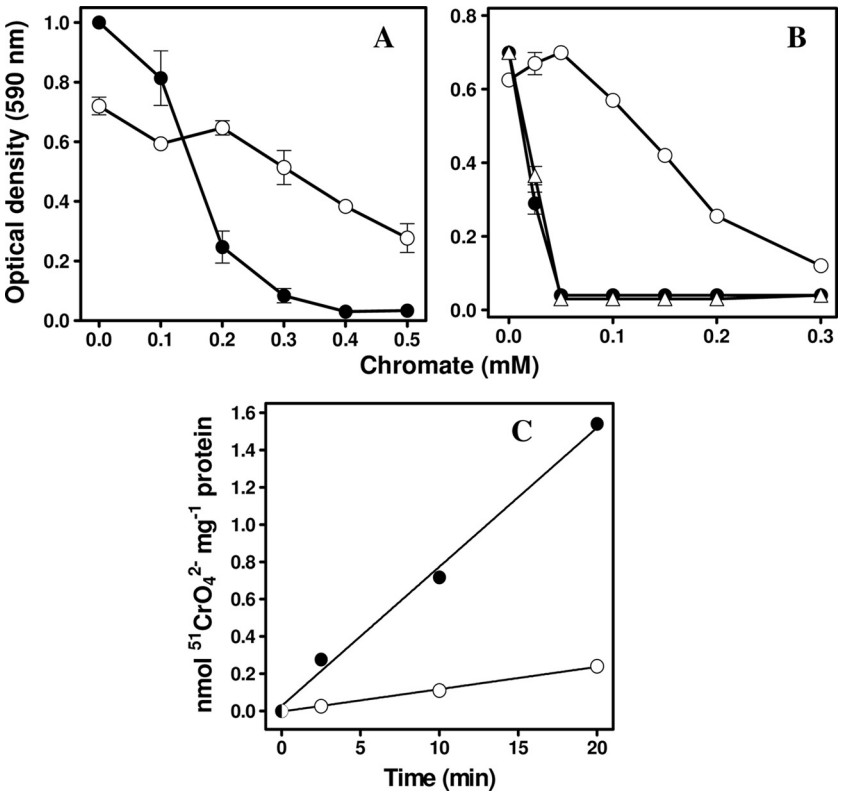

FIG. 2. Chromate susceptibility and chromate uptake by bacterial strains. (A and B) Cultures were grown in NB (A) or in M9 minimal medium (B) with the indicated concentrations of K_2CrO_4 for 18 h at 37°C, and the optical density at 590 nm was recorded. (A) Symbols: ●, *E. coli* W3110, and ○, *B. subtilis* 168. (B) Symbols: ●, *E. coli* W3110(pACYC184); ○, *E. coli* W3110(pAChr3N-C); and △, *E. coli* W3110(pAChr3N). Data shown are means from duplicates of three independent assays, with standard error bars shown. (C) Overnight cultures grown at 37°C in M9 medium with 0.05 mM sulfate were diluted 1:25 in the same medium and grown to an optical density at 590 nm of 0.6. Cells were washed and resuspended in phosphate buffer, and the incorporation of Na_2-$^{51}CrO_4$ was measured as described in Materials and Methods. Symbols are as described in the legend to panel B. Data shown are representative of two assays using the same sulfate concentrations with similar results.

esis are currently under way. The close arrangement of paired *chr3N* and *chr3C* genes suggests that they may be transcribed as a single bicistronic mRNA. However, monocistronic transcripts are also possible, as a putative promoter sequence was identified upstream of the *chr3C* gene (Fig. 1). To investigate a possible transcriptional linkage of *chr3N* and *chr3C* genes, an RT-PCR analysis was performed. Primers were designed to amplify cDNAs synthesized from transcripts originating from single *chr3N* or *chr3C* genes or from a transcript spanning the intergenic *chr3N-chr3C* region (Fig. 3A). When total RNA from an *E. coli* strain possessing paired *chr3N* and *chr3C* genes was probed, in addition to the DNA species produced from single *chr3N* and *chr3C* genes (240- and 276-bp bands shown in Fig. 3B, RT lanes 2 and 3, respectively), a 476-bp DNA fragment, covering the intergenic region, was also detected (Fig. 3B, RT lane 1). No corresponding fragments in negative controls were detected (Fig. 3B, lanes −). These data demonstrate that the *chr3N* and *chr3C* genes are cotranscribed into a bicistronic mRNA.

To determine whether the putative promoters of the *chr3N* and *chr3C* genes are functional, RT-PCR assays with *E. coli* strains expressing the single genes were carried out. RT-PCR analysis of total RNA from a strain bearing only the *chr3N* gene gave rise to the expected 240-bp fragment (Fig. 3C, RT lane 1), confirming that the *chr3N* promoter is functional. In contrast, in RT-PCR assays with RNA from a strain with only the *chr3C* gene, the predicted 276-bp RT-dependent product was undetectable (Fig. 3C, RT lane 2), indicating that the *chr3C* gene lacks a functional promoter, at least under the conditions tested. These results suggest that the *chr3N-chr3C* bicistronic mRNA starts from the promoter upstream of the *chr3N* gene and ends at the predicted termination region in front of the *chr3C* gene. Results from preliminary RT-PCR assays showed that *chr3N* and *chr3C* are also expressed in *B. subtilis* (data not shown).

Expression of *chr3N* and *chr3C* genes in *E. coli*. SCHR protein-encoding determinants are present, with few exceptions, as tandem pairs of genes in bacterial genomes (5), suggesting that both genes are necessary for function and that single genes are probably not functional. Since the *E. coli* genome does not contain CHR homologs (5, 11), which probably relates to its high chromate susceptibility compared to that of *B. subtilis* (Fig. 2A), *E. coli* W3110 was used as a heterologous host to test whether *chr3N* and *chr3C* genes confer chromate resistance. For this purpose, paired *Bacillus chr3N* and *chr3C* genes and a single *chr3N* gene were subcloned into the pACYC184 vector as described in Materials and Methods, rendering recombinant plasmids pAChr3N-C and pAChr3N, respectively, each bear-

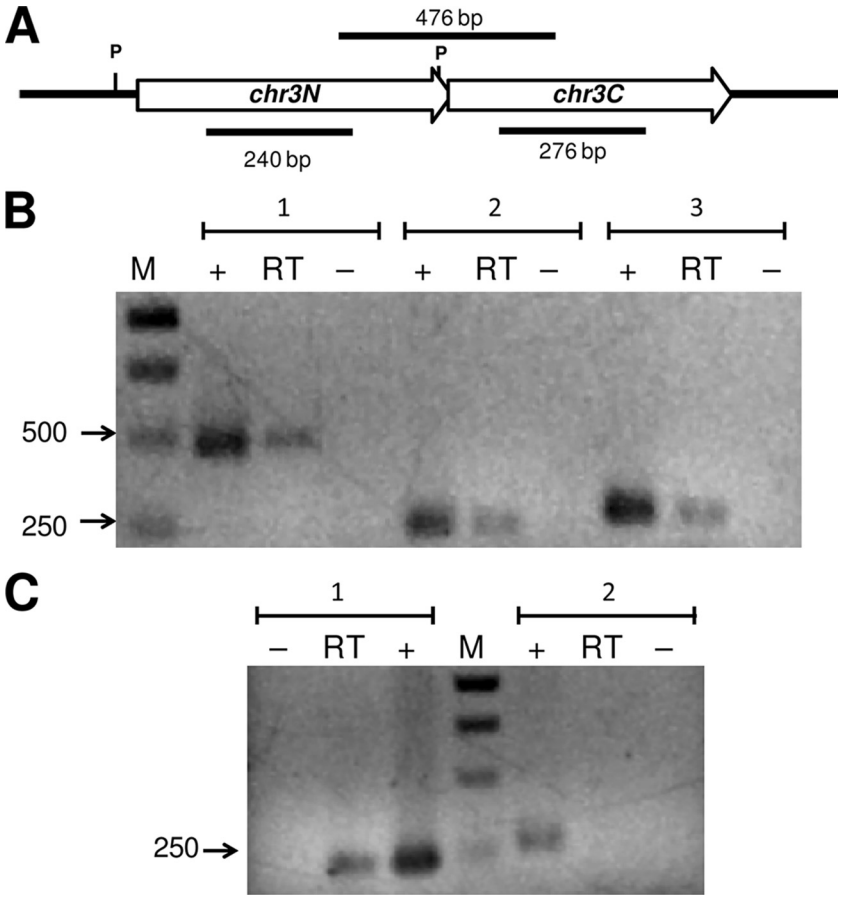

FIG. 3. RT-PCR analysis of the *chr3N* and *chr3C* genes. (A) White arrows indicate the *chr3N* and *chr3C* genes, and the locations of putative promoters (P) are marked. Horizontal lines depict the sizes (given in base pairs) and the locations of predicted cDNA reverse transcripts synthesized from total RNA and amplified by PCR with the designed primers. RT-PCR was carried out as described in Materials and Methods, and amplified fragments were separated in agarose gels. (B) RT-PCR products obtained from *E. coli* W3110(pAChr3N-C) total RNA by using primers for the *chr3N-chr3C* intergenic region (lanes 1), for the *chr3N* gene (lanes 2), or for the *chr3C* gene (lanes 3). (C) RT-PCR products obtained from *E. coli* W3110(pAChr3N) total RNA with primers for the *chr3N* gene (lanes 1) or from *E. coli* W3110(pAChr3C) total RNA with primers for the *chr3C* gene (lanes 2). PCR analyses were performed with genomic DNA templates (+), reverse-transcribed total RNA (RT), and total RNA in the absence of RT (−). M, molecular size markers.

ing the corresponding *chr3* gene(s) under its own putative promoter. These plasmids were then transferred into the *E. coli* W3110 strain. *E. coli* transformants expressing paired *chr3N* and *chr3C* genes showed enhanced chromate resistance compared to the control W3110 strain containing only the pACYC184 vector (Fig. 2B). These data clearly demonstrate that paired SCHR proteins confer resistance to chromate. In contrast, single-*chr3N*-gene *E. coli* transformants were as sensitive to chromate as the control strain (Fig. 2B), suggesting that both *chr3N* and *chr3C* genes are necessary for chromate resistance. Because no additional genes are required to confer chromate resistance on *E. coli*, these data also suggest that paired SCHR proteins form heterodimers in order to be functional. To our knowledge, this is the first time that a function has been experimentally assigned to a member of the SCHR family. Preliminary results showed that paired *chr1N* and *chr1C* genes, encoding SCHR proteins from subfamily 1, from a gram-negative *Burkholderia* strain also confer chromate resistance on *E. coli* (data not shown), thus indicating that the function of SCHR proteins is not restricted to gram-positive bacteria. The fact that both SCHR and LCHR proteins are involved in chromate resistance suggests that this function may be shared by all members of the CHR superfamily. This is not an unexpected possibility given that chromium is abundant on Earth (9) and probably has been since the beginning of life.

Chromate resistance mechanism. To gain insight into the mechanism used for SCHR proteins to confer chromate resistance, the transport of chromate in *E. coli* W3110 cells carrying the pAChr3N-C plasmid was assayed. A three- to sevenfold decrease in $Na_2{}^{-51}CrO_4$ uptake by *E. coli* cells expressing paired *chr3N* and *chr3C* genes compared with that by the strain bearing only the vector was observed when the cells were grown in M9 medium with 0.05 mM sulfate (Fig. 2C). Similar results were obtained with cells grown in 0.2 mM sulfate (data not shown). However, when uptake experiments were carried out with cells grown in M9 medium with excess (2 mM) sulfate, cells expressing the *chr3N* and *chr3C* genes took up levels of chromate similar to or higher than those taken up by control cells (data not shown). This behavior may result from sulfate inhibition of chromate efflux, which has been documented for

the *P. aeruginosa* ChrA transporter activity (12). The diminution in chromate uptake under low-sulfate conditions suggests that SCHR3 proteins participate in the efflux of chromate ions as a basis for the chromate resistance phenotype. This is the first report of an efflux-mediated mechanism of chromate resistance in a gram-positive bacterial species; previously characterized examples are all from gram-negative proteobacteria of the genera *Pseudomonas* (2), *Cupriavidus* (formerly named *Alcaligenes eutrophus*) (10), *Shewanella* (1), and *Ochrobactrum* (3). Thus, our findings further extend the spectrum of CHR superfamily proteins involved in chromate ion efflux.

In summary, our results show for the first time that paired SCHR proteins confer chromate resistance, probably because they form heterodimers in the membrane which expel chromate ions from the cytoplasm.

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