RESEARCH LETTER

Expression of chromate resistance genes from *Shewanella* sp. strain ANA-3 in *Escherichia coli*

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Abstract

The plasmidic chromate resistance genes *chrBAC* from *Shewanella* sp. strain ANA-3 were transferred to *Escherichia coli*. Expression of *chrA* alone, on a high- or low-copy number plasmid, conferred increased chromate resistance. In contrast, expression of the complete operon *chrBAC* on a high-copy number plasmid did not result in a significant increase in resistance, although expression on a low-copy number plasmid made the cells up to 10-fold more resistant to chromate. The *chrA* gene also conferred increased chromate resistance when expressed in *Pseudomonas aeruginosa*. The *chrR* gene from the *P. aeruginosa* chromosome was necessary for full chromate resistance conferred by *chrA*. A diminished chromate uptake in cells expressing the *chrA* gene suggests that chromate resistance is due to chromate efflux.

Introduction

The widespread use of chromium in diverse industrial processes has made it a serious contaminant of air, soil and water (Cervantes & Campos-García, 2007). The biological effects of Cr depend on its oxidation state; Cr(VI) is highly soluble and is considered the most toxic form of chromium (Cervantes *et al.*, 2001). Numerous bacterial species have developed resistance to chromate that can be associated with chromosomal or plasmid-encoded genes (Ramírez-Díaz *et al.*, 2008). Resistance systems related to plasmid genes encode membrane transporters, which mediate the efflux of chromate ions across the cytoplasmic membrane. This mechanism has been widely studied in *Pseudomonas aeruginosa*, where the chromate transporter ChrA functions as a chemiosmotic pump that extrudes chromate using the proton motive force (Alvarez *et al.*, 1999). The ChrA protein belongs to the CHR superfamily that includes dozens of putative homologs from all three domains of life (Díaz-Pérez *et al.*, 2007). The *Cupriavidus metallidurans* plasmid pMOL28 harbors the *chrBAC* genes plus other less-studied genes; *chrB* is proposed to play a regulatory role for expression of the ChrA transporter, and *chrC* encodes a putative superoxide dismutase (Nies *et al.*, 1990; Juhnke *et al.*, 2002). These chromate resistance determinants were only functional in their respective hosts but not in *Escherichia coli* (Cervantes *et al.*, 1990; Nies *et al.*, 1990).

Members of the Gram-negative genus *Shewanella* comprise a diverse group of facultative anaerobic bacteria widely distributed in marine and freshwater environments. *Shewanella* species are able to reduce several metals, among them Cr(VI) (Chourey *et al.*, 2006). For this reason, bioremediation strategies that involve *Shewanellae* have been proposed, including the cleanup of contaminated terrestrial environments and groundwater (Hau & Gralnick, 2007).

In this work it was found that plasmid 1 of *Shewanella* sp. strain ANA-3 harbors a *chr* operon that could be functionally expressed in *E. coli*. *chrA* alone was sufficient to confer resistance by a mechanism probably involving the efflux of chromate ions from the cytoplasm. The complete operon was required for full resistance, indicating that additional Cr transformations or pathways might participate.

Materials and methods

Bacterial strains, plasmids and culture conditions

Shewanella sp. strain ANA-3 containing megaplasmid 1 (accession number NC008573) was used for DNA isolation

(Saltikov *et al.*, 2003); *E. coli* W3110 and *P. aeruginosa* PAO1 were used for heterologous expression. The *P. aeruginosa* PAO1 transposon-insertion mutant ID44395 (Jacobs *et al.*, 2003) was used to test the effect of the disrupted ORF PA4288 (GenBank GeneID:881641), named here as the *chrR* gene. pGEM-T Easy (Promega) and pACYC184 plasmids were used as cloning vectors with a high- and low-copy number, respectively. pUCP20 is an *Escherichia*/*Pseudomonas* binary vector (West *et al.*, 1994). Cells were grown in M9 minimal medium (Sigma) supplemented with 20 mM glucose, 2 mM $MgSO_4$ and 0.1 mM $CaCl_2$ for 18–20 h at 37 °C with shaking.

Genetic techniques

Molecular genetic techniques were used according to standard protocols (Sambrook *et al.*, 1989). Plasmid DNA was purified using the Spin Miniprep kit (Qiagen) according to the manufacturer's instructions. *Shewanella chr* genes (GenBank GeneID:4476026, 4476025 and 4476024) were PCR amplified from plasmidic DNA with primer pairs designed with XbaI (direct primer) and BamHI (reverse primers) restriction endonuclease sites (underlined): 5′-GGCAA CCTTGATGAATCTAGAATGATTCGG-3′ and 5′-CTTGA TTTGCGCGGATCCGAATGGTATG-3′ (for *chrBAC*) and 5′-GCTCGATCATCTAGATTAACGCGCTTGGG-3′ and 5′-CCTTTAGGTGCTGGATCCGACGATTCAGC-3′ (for *chrA*). The fragments were amplified with Taq DNA polymerase (Fermentas) using the following protocol: denaturing, 94 °C for 2 min; annealing, 55 °C for 1 min; and elongation, 72 °C for 4 min. The amplified fragments were purified and cloned in the pGEM-T Easy vector before their subcloning into the XbaI/BamHI sites of the pACYC184 or pUCP20 vectors.

$^{51}CrO_4^{2-}$ uptake

Overnight cultures grown at 37 °C with shaking were diluted 1 : 30 in 30 mL of M9 minimal medium, grown to exponential phase and harvested by centrifugation (6000 ***g*** for 10 min at room temperature). The cells were washed once with prewarmed buffer A (10 mM Tris-HCl, 10 mM Na_2HPO_4, pH 7) and resuspended in 2 mL of the same buffer. $^{51}CrO_4^{2-}$ (0.5 μCi, Perkin-Elmer) was added to a final concentration of 10 μM to start the reaction. The cells were incubated at room temperature and 0.1 mL aliquots were drawn at different times and filtered through 0.45 μm nitrocellulose filters (Millipore Corp., Bedford, MA) presoaked in buffer B (buffer A plus 10 mM K_2CrO_4) and immediately washed with 10 mL of buffer B. The filters were dried and the radioactivity was quantified in an Ames Gammaccord II radiation counter. A blank value, obtained by filtering 0.1 mL of assay mixture without cells, was subtracted from all points.

Protein determination

Protein concentration was determined by the method of bicinchoninic acid (BCA) (Pierce), with bovine serum albumin as a standard.

Results and discussion

Plasmid 1 of *Shewanella* sp. ANA-3 contains a *chr* operon

Plasmid 1 of *Shewanella* sp. strain ANA-3 contains the *chrBAC* operon organized in a similar arrangement as in plasmids pMOL28 of *C. metallidurans* (Nies *et al.*, 1990) and pUM505 of *P. aeruginosa* (Díaz-Pérez *et al.*, 2007). The *chrB* gene encodes a protein of 312 amino acid residues (YP_863879) that is 44% and 46% identical to ChrB of pMOL28 and pUM505, respectively. The ChrA protein (YP_863878) of ANA-3 contains 455 amino acids and is 29% and 28% identical to the ChrA proteins of pMOL28 and pUM505, respectively. The *chrC* gene encodes a 203 amino acid protein (YP_863877) that is 49% identical to ChrC of pMOL28 plasmid; *chrC* of pUM505 is truncated (Cervantes *et al.*, 1990) and probably not functional.

Expression of *chrA* and *chrBAC* genes conferred chromate resistance in *E. coli*

To determine whether ChrA from *Shewanella* sp. ANA-3 alone is able to confer chromate resistance, the *chrA* gene from plasmid 1 was amplified and cloned into the high-copy number pGEM-T Easy and low-copy number pACYC184 vectors. Because the ChrA protein of *C. metallidurans* was only functional in the presence of ChrB (Nies *et al.*, 1990), the whole operon *chrBAC* was also amplified and cloned into both pGEM-T Easy and pACYC184. These constructs were subsequently transferred into *E. coli* W3110 and tested for their ability to confer increased resistance to chromate.

The expression of *chrA* alone conferred a high level of resistance to chromate both in the high-copy number vector pGEMT-Easy and in the low-copy number vector pACYC184 (Fig. 1a). These data show that, unlike the ChrA proteins from plasmids pMOL28 and pUM505, ChrA from *Shewanella* sp. ANA-3 can be functionally expressed in *E. coli*. In contrast, expression of the complete operon in *E. coli* W3110 (pGEMT-ChrBAC) did not give resistance, whereas the strain with the low-copy pACYC-ChrBAC construction showed a 10-fold increased level of resistance to chromate as compared with the sensitive strain (Fig. 1a). It therefore appears that expression of ChrA alone on a high-copy number plasmid was not toxic, as has been reported for other membrane proteins (Kurland & Dong, 1996). However, the level of chromate resistance was higher when the complete operon was expressed on a low-copy number

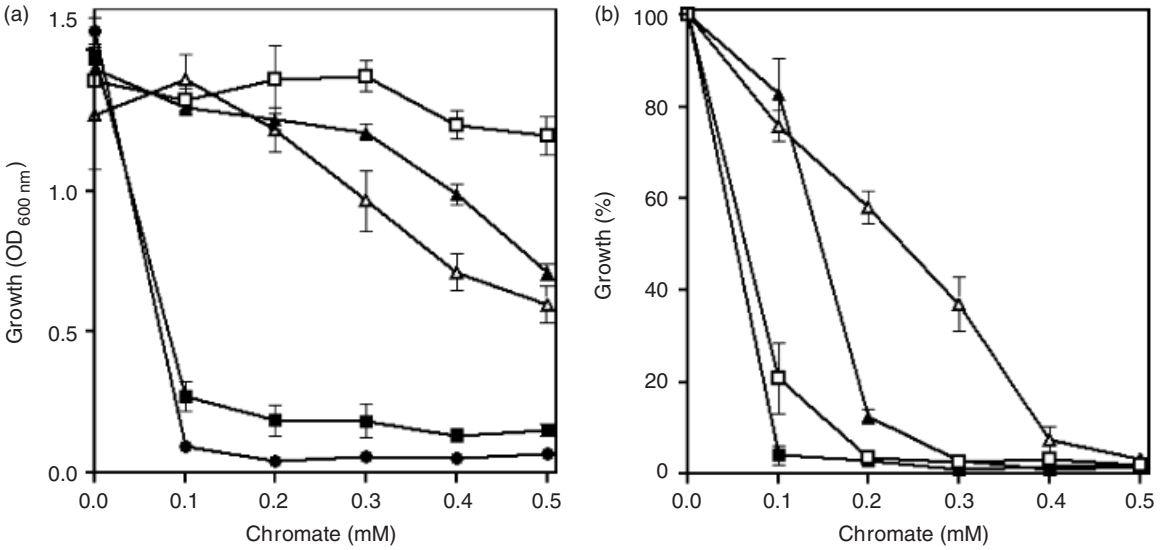

Fig. 1. Chromate resistance of *Shewanella chr* genes in (a) *Escherichia coli* and (b) *Pseudomonas aeruginosa*. Overnight cultures were diluted 1 : 100 into fresh M9 medium with the indicated concentrations of K_2CrO_4. Cell growth was monitored at $OD_{600\,nm}$ after 18–20 h incubation at 37 °C with shaking. (a) *Escherichia coli*, (●) W3110, (▲) W3110 (pGEMT-ChrA), (■) W3110 (pGEMT-ChrBAC), (△) W3110 (pACYC-ChrA) and (□) W3110 (pACYC-ChrBAC). The bars of SD are shown ($n = 4$). (b) *Pseudomonas aeruginosa*, (□) PAO1 (pUCP20), (△) PAO1 (pUCP20-ChrA), (■) PAO1-ChrR$^-$ and (▲) PAO1-ChrR$^-$ (pUCP20-ChrA). Percentage of growth is shown because the strains displayed different levels of growth. The bars of SD are shown ($n = 8$).

plasmid than with the *chrA* gene alone (Fig. 1a). This increased chromate resistance conferred by the complete operon was more pronounced under low-sulfate growth conditions (0.05 mM sulfate; data not shown). Because the *chrAC* genes conferred a resistance level similar to that of *chrA* alone (data not shown), *chrB* is required for maximum resistance to chromate. The function of ChrB is not known at this point; however, ChrB contains a rhodanese-like domain, which is also found in the arsenate reductase Acr2p of *Saccharomyces cerevisiae*. The consensus sequence of the rhodanase domain is $C(X)_5R$, and in Acr2p is part of the active site (Mukhopadhyay & Rosen, 2002). One possible function of ChrB might therefore be reduction of Cr(VI) before extrusion by ChrA, in analogy to the arsenic resistance operons, where the ArsC arsenate reductase converts arsenate [As(V)] into arsenite [As(III)], which is then extruded from the cytoplasm by the ArsB membrane transporter (Mukhopadhyay *et al.*, 2002). Because ChrA alone can function in conferring partial chromate resistance, other proteins must be responsible for additional functions in *E. coli*.

ChrA of *Shewanella* could be functionally expressed in *Pseudomonas*

The ability of the ChrA protein of *Shewanella* sp. to function in *P. aeruginosa* PAO1 was evaluated. The pUCP20-ChrA plasmid conferred resistance to chromate in *P. aeruginosa* PAO1, although at a lower level than in *E. coli* (Fig. 1b).

The *chrR* gene (ORF PA4288) encoded on the PAO1 chromosome (Stover *et al.*, 2000) was shown to play an essential role in the function of ChrA of the pUM505 plasmid, because when *chrR* was disrupted, *chrA* could no longer confer chromate resistance (M.I. Ramírez-Díaz and C. Cervantes, pers. commun.). *chrR* encodes a putative transcriptional regulator of the AraC family. When the pUPC20-ChrA plasmid, bearing the *chrA* gene from *Shewanella*, was transferred into *P. aeruginosa* PAO1 ID44395 (*chrR*$^-$) mutant, the level of chromate resistance was lower than *chrA* expressed in wild-type *P. aeruginosa* PAO1. However, chromate resistance was not completely abolished as when *chrA* from *P. aeruginosa* was expressed in the *chrR*$^-$ mutant (Fig. 1b). These data indicate that the *chrR* gene enhances the chromate resistance phenotype conferred by ChrA from *Shewanella*. Possibly, genes involved in chromate reduction and detoxification are regulated by ChrR and are necessary for full chromate resistance conferred by ChrA from both *Shewanella* sp. ANA-3 and *P. aeruginosa* plasmid pUM505.

Chromate uptake

The uptake of chromate was quantified in *E. coli* strains expressing *chr* genes from *Shewanella* sp. ANA-3. A decreased initial rate of $^{51}CrO_4^{2-}$ uptake by strains harboring the low-copy number plasmids pACYC-ChrA or pACYC-ChrBAC was found when compared with the control *E. coli* W3110 (Fig. 2). Chromate uptake by cells expressing the

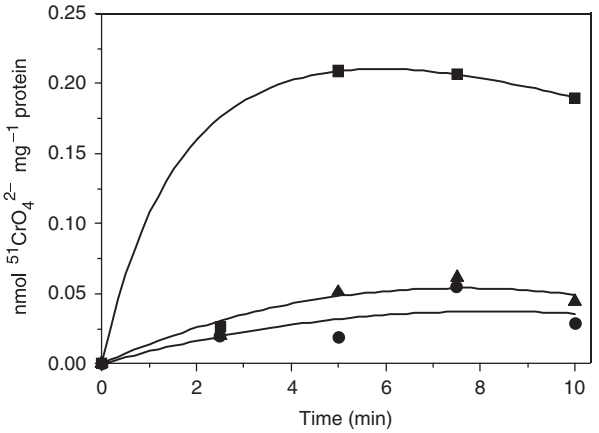

Fig. 2. $^{51}CrO_4^{2-}$ uptake by *Escherichia coli* cells expressing *Shewanella chr* genes. Overnight cultures in M9 medium were diluted 1 : 30 into fresh M9 medium and grown to 0.6–0.8 at $OD_{600\,nm}$. The cells were washed and the incorporation of $^{51}CrO_4^{2-}$ was determined as described in Materials and methods. *Escherichia coli*, (■) W3110, (▲) W3110 (pACYC-ChrBAC) and (●) W3110 (pACYC-ChrA). Data shown are representative of at least three assays with similar results.

ChrA protein was at least 4.5-fold lower than uptake of the plasmidless control strain. The difference in chromate uptake between the chromate-sensitive plasmidless and resistant strains is higher than that reported for ChrA from *P. aeruginosa* that showed a threefold lower chromate uptake (Pimentel *et al.*, 2002). These data suggest that the ChrA protein of *Shewanella* sp. ANA-3 confers resistance to chromate by a mechanism involving the efflux of chromate similar to that of the well-characterized *P. aeruginosa* and *C. metallidurans* ChrA homologs.

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References

Alvarez AH, Moreno-Sánchez R & Cervantes C (1999) Chromate efflux by means of the ChrA chromate resistance protein from *Pseudomonas aeruginosa*. *J Bacteriol* **181**: 7398–7400.

Cervantes C & Campos-García J (2007) Reduction and efflux of chromate by bacteria. *Molecular Microbiology of Heavy Metals* (Nies DH & Silver S, eds), pp. 407–420. Springer-Verlag, Berlin.

Cervantes C, Ohtake H, Chu L, Misra TK & Silver S (1990) Cloning, nucleotide sequence, and expression of the chromate resistance determinant of *Pseudomonas aeruginosa* plasmid pUM505. *J Bacteriol* **172**: 287–291.

Cervantes C, Campos-García J, Devars S, Gutiérrez-Corona F, Loza-Tavera H, Torres-Guzmán JC & Moreno-Sánchez R (2001) Interactions of chromium with microorganisms and plants. *FEMS Microbiol Rev* **25**: 335–347.

Chourey K, Thompson MR, Morrell-Falvey J, VerBerkmoes NC, Brown SD, Shah M, Zhou J, Doktycz M, Hettich RL & Thompson DK (2006) Global molecular and morphological effects of 24-hour chromium (VI) exposure on *Shewanella oneidensis* MR-1. *Appl Environ Microbiol* **72**: 6331–6344.

Díaz-Pérez C, Cervantes C, Campos-García J, Julián-Sánchez A & Riveros-Rosas H (2007) Phylogenetic analysis of the chromate ion transporter (CHR) superfamily. *FEBS J* **274**: 6215–6227.

Hau HH & Gralnick JA (2007) Ecology and biotechnology of the genus *Shewanella*. *Annu Rev Microbiol* **61**: 237–258.

Jacobs MA, Alwood A, Thaipisuttikul I *et al.* (2003) Comprehensive transposon mutant library of *Pseudomonas aeruginosa*. *Proc Natl Acad Sci USA* **100**: 14339–14344.

Juhnke S, Peitzsch N, Hubener N, Große C & Nies DH (2002) New genes involved in chromate resistance in *Ralstonia metallidurans* strain. *Arch Microbiol* **179**: 15–25.

Kurland CG & Dong H (1996) Bacterial growth inhibition by overproduction of protein. *Mol Microbiol* **21**: 1–4.

Mukhopadhyay R & Rosen BP (2002) Arsenate reductases in prokaryotes and eukaryotes. *Environ Health Perspect* **110**: 745–748.

Mukhopadhyay R, Rosen BP, Phung LT & Silver S (2002) Microbial arsenic: from geocycles to genes and enzymes. *FEMS Microbiol Rev* **26**: 311–325.

Nies A, Nies DH & Silver S (1990) Nucleotide sequence and expression of a plasmid-encoded chromate resistance determinant from *Alcaligenes eutrophus*. *J Biol Chem* **265**: 5648–5653.

Pimentel BE, Moreno-Sánchez R & Cervantes C (2002) Efflux of chromate by *Pseudomonas aeruginosa* cells expressing the ChrA protein. *FEMS Microbiol Lett* **212**: 249–254.

Ramírez-Díaz MI, Díaz-Pérez C, Vargas E, Riveros-Rosas H, Campos-García J & Cervantes C (2008) Mechanisms of bacterial resistance to chromium compounds. *Biometals* **21**: 321–332.

Saltikov CW, Cifuentes A, Venkateswaran K & Newman DK (2003) The *ars* detoxification system is advantageous but not required for As(V) respiration by the genetically tractable *Shewanella* species strain ANA-3. *Appl Environ Microbiol* **69**: 2800–2809.

Sambrook J, Fritsch EF & Maniatis T (1989) *Molecular Cloning: A Laboratory Manual*, 2nd edn. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Stover CK, Pham XQ, Erwin AL *et al.* (2000) Complete genome sequence of *Pseudomonas aeruginosa* PAO1, an opportunistic pathogen. *Nature* **406**: 959–964.

West SEH, Schweizerb HP, Dall C, Sample AK & Runyen-Janecky LJ (1994) Construction of improved *Escherichia-Pseudomonas* shuttle vectors derived from pUC18/l9 and sequence of the region required for their replication in *Pseudomonas aeruginosa*. *Gene* **128**: 81–86.