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“ANÁLISIS FUNCIONAL DE HOMÓLOGOS BACTERIANOS DE LA SUPERFAMILIA CHR”

TESIS

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1.-RESUMEN

El transportador ChrA confiere resistencia a cromato en *Pseudomonas aeruginosa* por medio de la expulsión del ion del citoplasma. La expulsión de cromato se inhibe por sulfato, lo que sugiere que ChrA interacciona con el sulfato. Se ha analizado previamente el papel de ocho aminoácidos básicos de ChrA de *P. aeruginosa*, y se identificaron algunos residuos esenciales que posiblemente interaccionan directamente con cromato, sin embargo, la proteína ChrA contiene 28 aminoácidos básicos totales lo que sugiere que más residuos básicos pueden ser esenciales para su función. ChrA pertenece a la superfamilia CHR constituida por cientos de homólogos, entre ellos ChrA de *Shewanella sp.* ANA-3 y SrpC de *Synechococcus elongatus*, cuya función no se ha descrito en relación con cromato. Ningún transportador CHR se ha analizado en relación con sulfato. El objetivo general de este trabajo fue caracterizar la función de los transportadores CHR en relación con cromato y sulfato.

Se evaluó la importancia de algunos residuos básicos de ChrA de *P. aeruginosa* en la resistencia a cromato empleando mutagénesis dirigida. Mutaciones por alanina en los residuos R54, R68 localizados en el asa periplásmica P1 y R340, en el asa citoplásmica C5, causaron una disminución en el nivel de resistencia a cromato, lo cual sugiere que estos residuos probablemente interactúan con el cromato durante su expulsión del citoplasma.

El transporte de sulfato se evaluó empleando suspensiones celulares de las cepas de *P. aeruginosa* PAO1 (pUCP20) (vector), y PAO1 (pUChrA) que contiene el gen *chrA* del plásmido pUM505. El nivel de captación de sulfato fue similar en ambas cepas. También se analizó el transporte de sulfato en vesículas invertidas de membrana empleando las mismas cepas. De igual forma, no hubo una diferencia significativa en los niveles de captación de sulfato entre las vesículas de la cepa únicamente con el vector y las vesículas de la cepa con *chrA*, con o sin la adición de NADH, por lo que se concluyó que ChrA no transporta sulfato.

Los genes *chrBAC* y *chrA* de *Shewanella* se amplificaron y se clonaron en los vectores pGEMT, pACYC184 y pUCP20. El gen *chrA* confirió un elevado nivel de resistencia a cromato en *E. coli* y *P. aeruginosa*, independientemente del vector en el

2.-ABSTRACT

The *Pseudomonas aeruginosa* ChrA protein confers resistance to chromate by expelling chromate ions from the cytoplasm. The efflux of chromate is inhibited by sulfate, suggesting that ChrA binds sulfate. The role of eight basic residues of ChrA from *P. aeruginosa* has been analyzed previously, identifying some essential residues that probably bind chromate. However, ChrA has 28 total basic residues indicating that more basic residues may be essential for ChrA function. ChrA belongs to the CHR superfamily of transporters that currently comprises hundreds of homologs, including ChrA from *Shewanella sp.* ANA-3 and SrpC from *Synechococcus elongatus*, whose function on chromate resistance have not been reported. The CHR transporters have not been analyzed with respect to sulfate. The objective of this work was to analyze of the function of CHR transporters related to chromate and sulfate.

The importance of the basic residues for the chromate resistance phenotype conferred by ChrA from *P. aeruginosa* was tested by site-directed mutagenesis. Residues R54 and R68 located in the large periplasmic P1 loop, and R340 in cytoplasmic loop C5, which are the most conserved, abolished chromate resistance when they were changed by alanine, suggesting that these residues interact directly with chromate.

Sulfate transport through ChrA was evaluated using cell suspensions of *P. aeruginosa* PAO1 (pUCP20) (vector), and PAO1 (pUChrA), with *chrA* gene of pUM505. The levels of sulfate uptake were similar in both strains. The transport of sulfate was also analyzed in everted membrane vesicles from the *P. aeruginosa* strains. In a similar way, there was not difference in the level of sulfate uptake between the vesicles of the strain only with the vector and the vesicles of the strain with ChrA, in the presence or absence of NADH, suggesting that ChrA does not transport sulfate.

The *chrBAC* and *chrA* genes from *Shewanella* were amplified and cloned in the pGEMT, pACYC184 and pUCP20 vectors. The *chrA* gene conferred increased chromate resistance in *Escherichia coli* and *P. aeruginosa*, in all the vectors tested. The expression of *chrBAC* genes made the cells up to 10-fold more resistant to

que fue clonado. Los genes *chrBAC* incrementaron la resistencia a cromato hasta 10 veces más que el control sensible. Las cepas de *E. coli* transformadas con el gen *chrA* de *Shewanella* presentaron una captación disminuida del cromato, lo que sugiere que esta proteína expulsa el cromato de la célula. El gen *chrC* de *Shewanella* restauró el crecimiento de la mutante de *E. coli* con mutaciones en los dos genes que codifican las enzimas superóxido dismutasa, la cual es incapaz de crecer en medio mínimo sin isoleucina y valina, lo que sugiere que ChrC es una superóxido dismutasa (SOD). Los genes *chrA* y *chrBAC* de *Shewanella* se transfirieron a una mutante de *E. coli* afectada en la síntesis de glutatión. Las transformantes de la cepa mutante fueron menos resistentes a cromato que las transformantes de la cepa silvestre, indicando la importancia del glutatión en la resistencia a cromato. *E. coli* fue 10 veces más resistente a cromato en condiciones de anaerobiosis que en aerobiosis, confirmando la generación de estrés oxidativo como un mecanismo de toxicidad del cromato. En anaerobiosis, las cepas que expresan ChrA fueron más resistentes que el control, indicando que la proteína ChrA es funcional en estas condiciones.

También se evaluó el transporte de sulfato en suspensiones celulares de *E. coli* W3110 (pACYC184) (vector) y W3110 (pACYC-ChrBAC) con el gen *chrA* de *Shewanella*. No se observó diferencia en el nivel de captación entre ambas cepas.

El gen *srpC* de *S. elongatus* se amplificó y se clonó en los vectores pGEMT y pACYC184. La expresión de *srpC* en una cepa de *E. coli* mutante en el transportador de sulfato, auxótrofa a cisteína, no complementó su crecimiento empleando sulfato como fuente de azufre, lo que sugiere que SrpC no funciona como un transportador de sulfato. El gen *srpC* confirió resistencia a cromato en cepas de *E. coli*, y además originó una captación disminuida del cromato, indicando que SrpC expulsa el cromato de la célula.

Los genes *chr3N-chr3C* de *Bacillus subtilis*, que confieren resistencia a cromato en *E. coli*, presentaron una captación disminuida de cromato en comparación de la cepa únicamente con el vector, lo que sugiere que las proteínas pequeñas SCHR funcionan por un mecanismo de expulsión de cromato.

Los resultados en cromato sugieren que los transportadores de la superfamilia CHR confieren resistencia a cromato por un mecanismo similar de expulsión de cromato.

chromate than the control. The expression of *chrA* from *Shewanella* caused a diminished chromate uptake in cells, suggesting that chromate resistance is due to chromate efflux. The expression of c*hrC* gene from *Shewanella* restored the growth of an *E. coli* mutant strain that possesses mutations in the two genes that encode the superoxide dismutase enzymes, which is unable to grow in minimal medium without valine and isoleucine, suggesting that ChrC is a superoxide dismutase (SOD).

The *chrA* and *chrBAC* genes were transferred to an *E. coli* mutant strain with deletions of the genes encoding enzymes involved in glutathione synthesis. The mutants were more sensitive to chromate than the wild-type strain, showing the importance of glutathione in chromate resistance. Chromate resistance was tested in anaerobic conditions, and an *E. coli* strain was 10-fold more resistant in anaerobic than under aerobic conditions, showing that the generation of oxidative stress is a mechanism of toxicity of chromate. Under anaerobic conditions, the strains expressing ChrA were more chromate resistant than the control only with the vector, indicating that the protein is functional in anaerobiosis.

The sulfate transport was also evaluated in cell suspensions of *E. coli* W3110 (pACYC184) (vector) and W3110 (pACYC-ChrBAC) (with *chrA* gene from *Shewanella*) strains. The level of sulfate uptake was similar in both strains.

The *srpC* gene from *S. elongatus* was amplified and cloned in the pGEMT and pACYC184 vectors. The expression of the *srpC* gene from *S. elongatus* was unable to complement the growth using sulfate as a sulfur source of an *E. coli* mutant strain that is cysteine auxotroph, which suggests that SrpC is not a sulfate transporter. However, the expression of *srpC* in *E. coli* conferred chromate resistance and caused a diminished chromate uptake, indicating that SrpC effluxes chromate ions from the cytoplasm.

The *chr3N-chr3C* genes from *Bacillus subtilis*, that confer chromate resistance in *E. coli*, showed a diminished uptake of chromate as compared to the control only with vector, suggesting that the SCHR proteins efflux chromate ions.

The results on chromate suggest that the transporters of the CHR superfamily confer chromate resistance by a similar mechanism of chromate efflux.

3.-INTRODUCCIÓN

3.1.-Características generales del cromo

El cromo es un metal de transición y es el séptimo elemento más abundante en la tierra; aunque presenta diferentes estados de oxidación las formas estables son el Cr(III) y el Cr(VI) (McGrath y Smith, 1990). El Cr(VI) se encuentra presente principalmente como los oxianiones cromato (CrO_4^{2-}) y dicromato ($Cr_2O_7^{2-}$), siendo el cromato la forma predominante a pH>6. El cromato es un fuerte agente oxidante que puede ser reducido por la materia orgánica y otros agentes reductores del suelo y ambientes acuáticos a Cr(III). En contraste, el Cr(III) se encuentra principalmente formando óxidos, hidróxidos o sulfatos que son compuestos menos solubles (Cervantes *et al.*, 2001).

El cromo tiene varias aplicaciones, siendo las más comunes el curtido de pieles, elaboración de pinturas, y en la industria metalúrgica (Barceloux, 1999). La presencia de cromo en el ambiente puede ser debida a fuentes naturales; sin embargo, su amplia gama de aplicaciones, así como la inadecuada disposición de sus desechos han convertido al cromo en un contaminante de aire, suelos y aguas (Armienta-Hernandez y Rodriguez-Castillo, 1995, Cervantes *et al.*, 2001).

3.2.-Transporte del cromato en bacterias

El cromo, al igual que otros metales tóxicos, es transportado al interior celular empleando el sistema de transporte de un ion esencial (Ballatori, 2002). En el oxianión cromato, el cromo se encuentra unido a cuatro oxígenos adoptando una estructura tetraédrica similar al oxianión esencial sulfato, con radios atómicos y distancia de enlace semejantes. Debido a esta característica el cromato es introducido al citoplasma bacteriano empleando el sistema de transporte del sulfato Ambos oxianiones son transportados al interior celular por medio de las permeasas de sulfato, las cuales pertenecen a las familias de transportadores SulT (CysPTWA), SulP, Pit (CysP) o CysZ (Ver anexo 2, al final de la tesis) (**Fig. 1**).

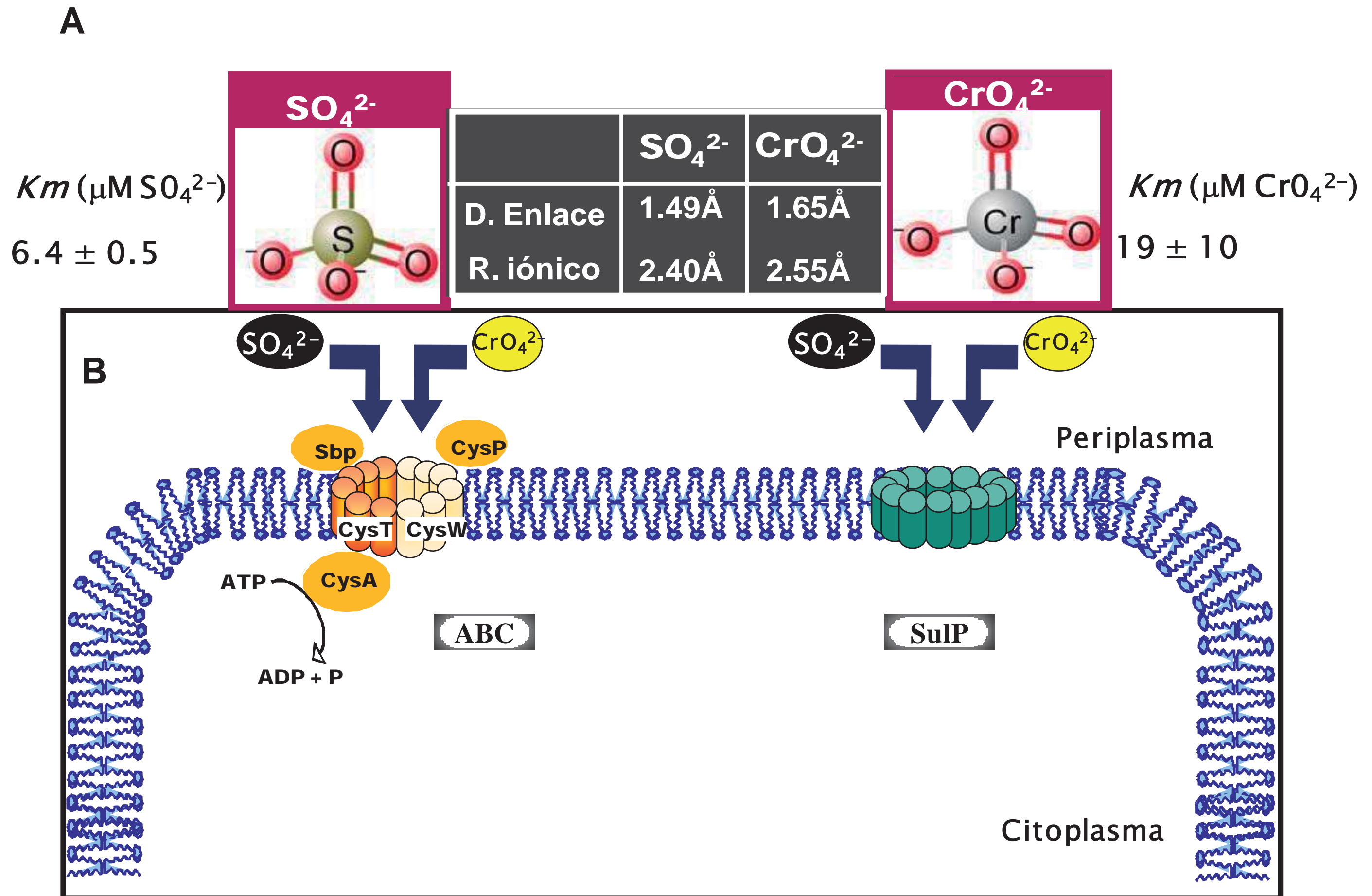


Figura 1. Captación del cromato en bacterias. A) Se muestran las estructuras tetraédricas del sulfato (SO_4^{2-}) y el cromato (CrO_4^{2-}), se indican los valores de distancia de enlace y radio iónicos de ambos oxianiones. Se indican también los valores de *Km* para la captación de ambos oxianiones, determinado en *P. fluorescens* (Ohtake et al., 1987). **B)** Se muestran: el complejo de la permeasa tipo ABC (ATP-binding cassette) constituida por las proteínas periplásmicas de unión al sustrato, Sbp y CysP, las proteínas que forman el canal de membrana, CysT y CysW, y CysA, la ATPasa que energiza al sistema, y la permeasa tipo SulP constituida por una sola proteína.

3.3.-Toxicidad del cromato

La toxicidad del cromo depende de su estado de oxidación, el Cr(III) es considerado como un elemento traza esencial únicamente en mamíferos ya que participa en el metabolismo de lípidos y de la glucosa. En contraste, el Cr(VI) es tóxico para la mayoría de los organismos, se ha reportado como carcinogénico en animales y humanos, causante de necrosis y clorosis en plantas, y como mutagénico en bacterias y hongos (Cervantes *et al.*, 2001).

El cromato ejerce diferentes efectos tóxicos, entre los que encuentra la inhibición competitiva del transporte de sulfato (Ohtake *et al.*, 1987, Nies y Silver, 1989). Intracelularmente el Cr(VI) es reducido con la formación de estados intermediarios Cr(V), Cr(IV) y finalmente Cr(III), la forma final y más estable (Zhitkovich *et al.*, 2002). Durante el proceso de reducción se forman radicales libres, siendo el daño oxidativo al DNA probablemente el responsable de los efectos genotóxicos del cromato. El Cr(III) tiene la capacidad de unirse a los grupos fosfatos del DNA, generando aductos Cr-DNA principalmente de complejos ternarios generados por la formación de enlaces cruzados de cisteína e histidina con Cr-DNA. Además, el Cr(III) también puede ejercer sus efectos tóxicos por su capacidad de unirse a los grupos carboxilos y sulfhidrilos de las proteínas, o compitiendo con el transporte de hierro por la transferrina. En la levadura *Saccharomyces cerevisiae* se ha reportado el daño oxídativo a proteínas como uno de los principales mecanismos de toxicidad del cromo (Revisado en Ramírez-Díaz *et al.*, 2008).

3.4.-Resistencia bacteriana a cromato

Los determinantes bacterianos de resistencia a cromato pueden ser codificados por genes cromosómicos o plasmídicos, generalmente los genes localizados en plásmidos codifican transportadores de membrana que median la expulsión del cromato del citoplasma. Por otra parte, los genes localizados en cromosomas están relacionados con la reducción específica o inespecífica del cromato, sistemas de destoxificación de radicales libres, sistemas de reparación de DNA y sistemas asociados con homeostasis de hierro y azufre (Cervantes y Campos-García, 2007) (**Fig. 2**).

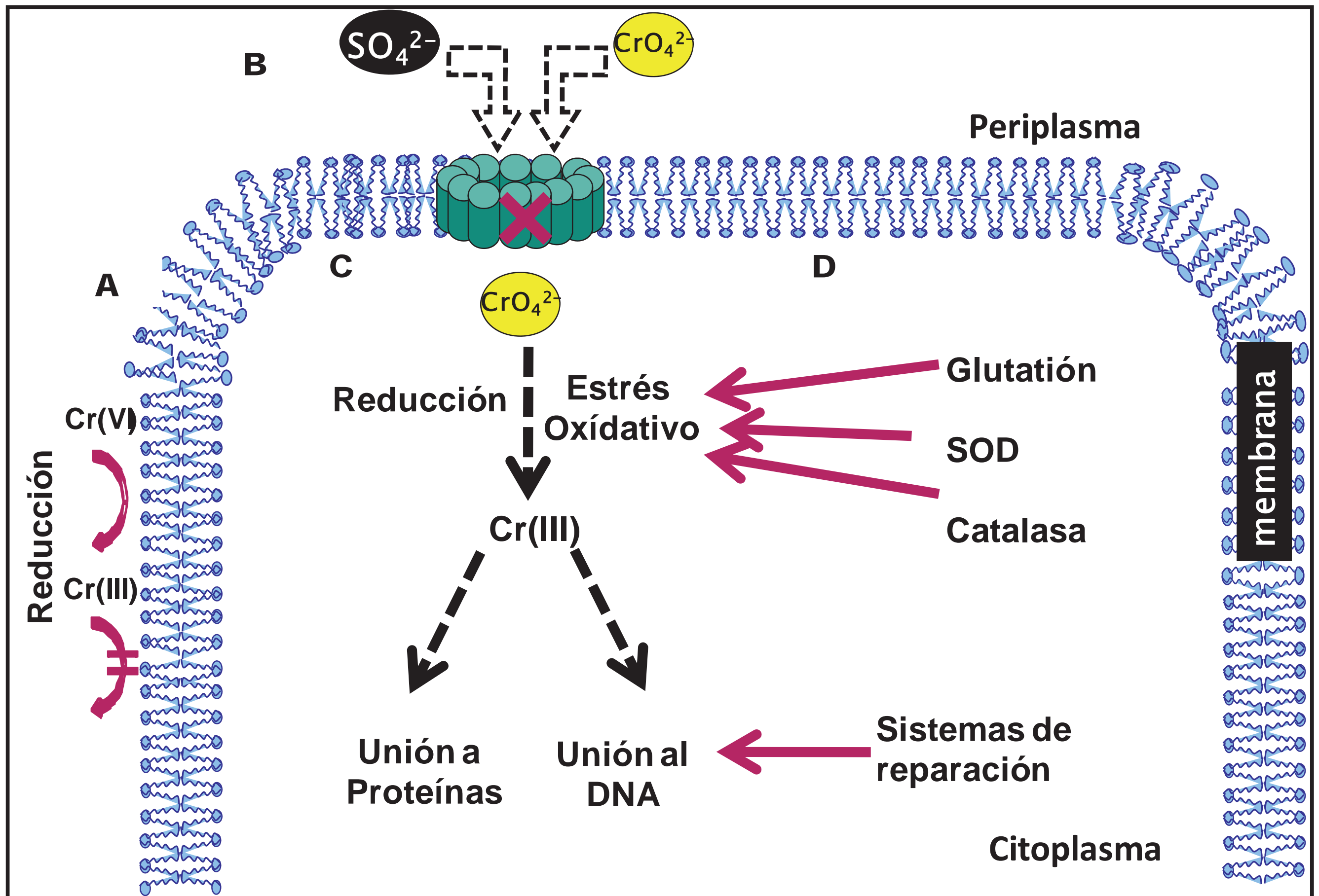


Figura 2. Toxicidad y resistencia a cromato en bacterias. Los mecanismos de daño se indican por líneas punteadas y los mecanismos de resistencia se indican por líneas continuas. **A)** Reducción extracelular de Cr(VI) a Cr(III), el cual no ingresa a la célula. **B)** Mutaciones en el transportador de sulfato (**X**) disminuyen la entrada del cromato. **C)** La reducción intracelular de Cr(VI) a Cr(III) que genera estrés oxidativo, además de daño al DNA y a las proteínas. **D)** Sistemas de protección contra el estrés oxidativo y sistemas de reparación del daño al DNA causado por cromo (Modificado de Ramírez-Díaz *et al.*, 2008).

3.5.-Expulsión de cromato

Uno de los mecanismos de resistencia a cromato mejor caracterizado es la expulsión de cromato por la proteína ChrA de *Pseudomonas aeruginosa* (Cervantes *et al.*, 1990). ChrA es una proteína de 416 aminoácidos codificada por el gen *chrA* el cual se identificó en el plásmido conjugativo de 120 kilobases pUM505 aislado de una cepa de *P. aeruginosa* de origen clínico (Cervantes *et al.*, 1990).

La proteína ChrA de *P. aeruginosa* consta de 13 segmentos transmembranales, con el extremo amino orientado al citoplasma y el carboxilo al periplasma (Jiménez-Mejía *et al.*, 2006). El mecanismo de resistencia a cromato conferido por ChrA se basa en la expulsión activa del cromato que utiliza el potencial eléctrico transmembranal como fuente de energía (**Fig. 3**). Esto se determinó en un inicio con el empleo de vesículas invertidas de membrana (Álvarez *et al.*, 1999), y posteriormente empleando suspensiones celulares de la cepa de *P. aeruginosa* PAO1. La expulsión presentó una cinética típica de saturación con una *Km* de 82 μM de cromato y una *Vmax* de 0.133 nmol cromato min^{-1} mg $proteína^{-1}$ (Pimentel *et al.*, 2002). El transporte de cromato por ChrA en vesículas de membrana invertidas es inhibido hasta en un 80% por sulfato (Álvarez *et al.*, 1999). De igual forma se observó la inhibición del transporte al emplear células completas, lo que sugiere que el sulfato también puede unirse y/o ser transportado por la proteína ChrA (Pimentel *et al.*, 2002) (**Fig. 3**). Un sistema de expulsión similar se localiza en la bacteria resistente a metales *Cupriavidus metallidurans*, la cual posee dos genes *chrA* que codifican las proteínas $ChrA_1$, codificada por el plásmido pMOL28, y $ChrA_2$, codificada por un gen cromosómico (Nies *et al.*, 1990, Juhnke *et al.*, 2002).

3.6.-La superfamilia CHR de transportadores de cromato

La proteína ChrA pertenece a la superfamilia CHR clasificada como TC # 2.A.51 (Saier, 2000). En un inicio por medio de un análisis filogenético se identificaron 135 secuencias de homólogos de la proteína ChrA probablemente involucrados en el transporte de cromato o sulfato (Díaz-Pérez *et al.*, 2007) (**Fig. 4**); sin embargo, debido al aumento en el número de genomas secuenciados actualmente está constituida por cientos de homólogos distribuidos en arqueas, bacterias, y hongos.

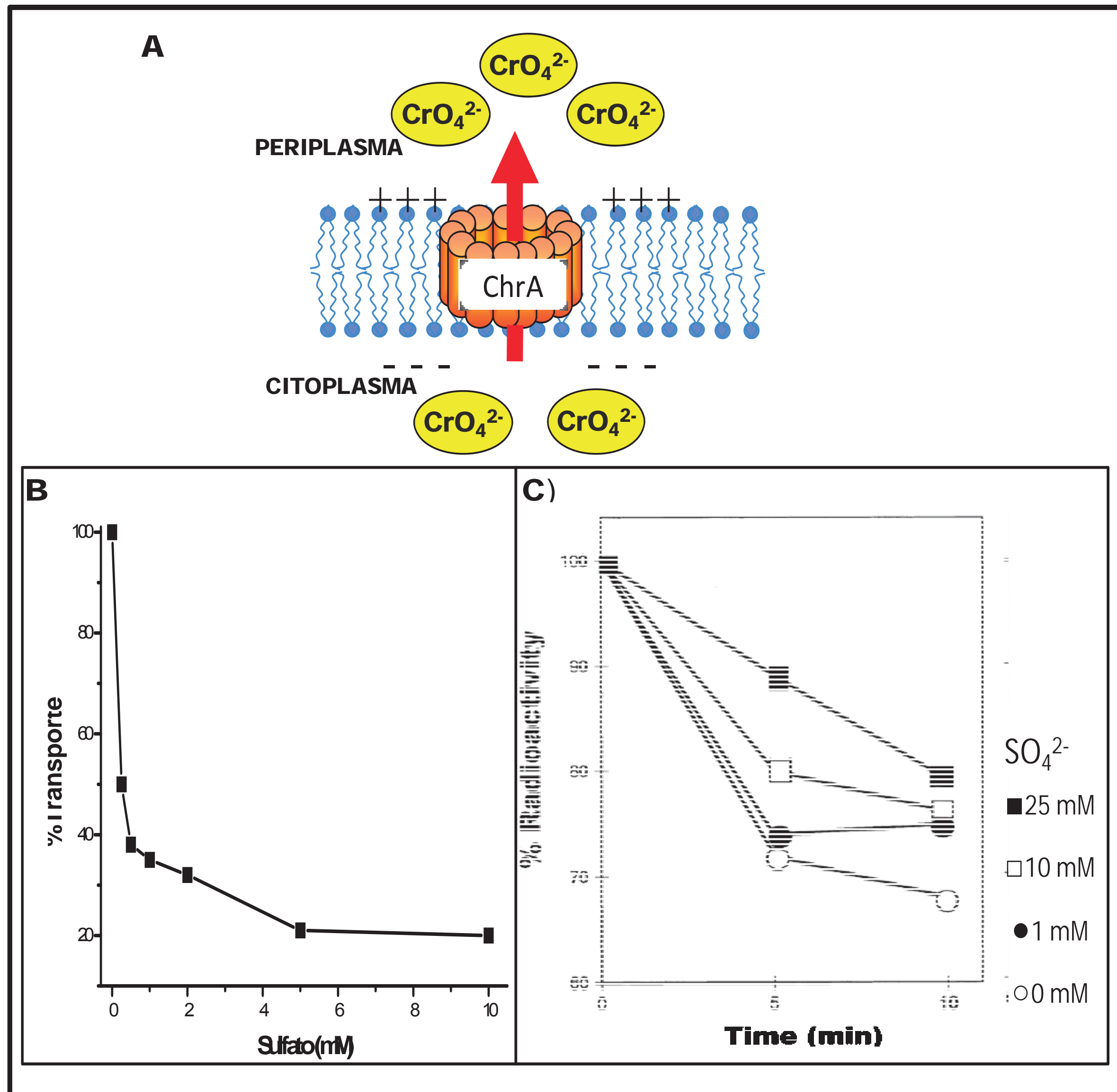


Figura 3. Expulsión de cromato en *P. aeruginosa*. A) Mecanismo de expulsión de cromato (CrO_4^{2-}) por la proteína ChrA del citoplasma empleando el potencial eléctrico transmembranal. **B)** Inhibición de la expulsión de cromato por sulfato en vesículas de membrana invertidas (Álvarez, 1999). **C)** Inhibición de la expulsión de cromato por sulfato en células completas (Pimentel *et al.*, 2002).

La superfamilia CHR está compuesta de dos familias de proteínas, la familia SCHR de proteínas pequeñas (200 aa) que poseen sólo un dominio, y la familia LCHR de proteínas grandes (400 aa, excepto proteínas de eucariotes de 500-600 aa) con dos dominios homólogos (Díaz-Pérez *et al.*, 2007) (**Fig. 4**).

La función en la resistencia a cromato sólo estaba reportada en las proteínas LCHR de *P. aeruginosa* (Cervantes *et al.*, 1990) y *C. metallidurans* (Nies *et al.*, 1990). Sin embargo, se ha reportado un sistema similar en la α-proteobacteria *Ochrobactrum tritici* 5bvl1, aislada de un sitio contaminado (Branco *et al.*, 2008), y *Arthrobacter* sp. FB24 (Henne *et al.*, 2009). Recientemente se reportó la función de las proteínas SCHR de *Bacillus subtilis* y de *Burkholderia xenovorans*, codificadas por los genes adyacentes *chr3N* y *chr3C*, y $chr1_2N$ y $chr1_2C$, respectivamente (Díaz-Magaña *et al.*, 2009). Se determinó que los genes *chr3N* y *chr3C* de *B. subtilis* son transcritos a través de un RNA mensajero bicistrónico y que ambos genes son necesarios para la resistencia a cromato cuando se expresan en *E. coli.* Se ha propuesto que las proteínas SCHR forman heterodímeros en la membrana para formar el canal de expulsión de cromato, funcionando en una forma similar a las proteínas grandes LCHR (Díaz-Magaña *et al.*, 2009).

Algunas bacterias presentan varios homólogos CHR en sus genomas, los cuales confieren resistencia a cromato, tal es el caso de *B. vietnamiensis* (5 LCHR, 2 SCHR) y *B. xenovorans* (4 LCHR, 2, SCHR) (León-Márquez, 2009).

Otro homólogo de la proteína ChrA es SrpC de la cianobacteria *Synechococcus elongatus* PCC 7924 (anteriormente *Synechococcus* sp.) (Nicholson y Laudenbach, 1995). La expresión de esta proteína es regulada por sulfato y se ha propuesto que funciona en la captación de cromato

En la γ-proteobacteria *Shewanella oneidensis* (Díaz-Pérez *et al.*, 2007), la cual es una de las bacterias más empleadas para analizar los mecanismos de resistencia a cromato (Viamajala *et al.*, 2004, Chourey *et al.*, 2006) se localiza otro homólogo de ChrA, sin embargo, no se ha determinado su función.

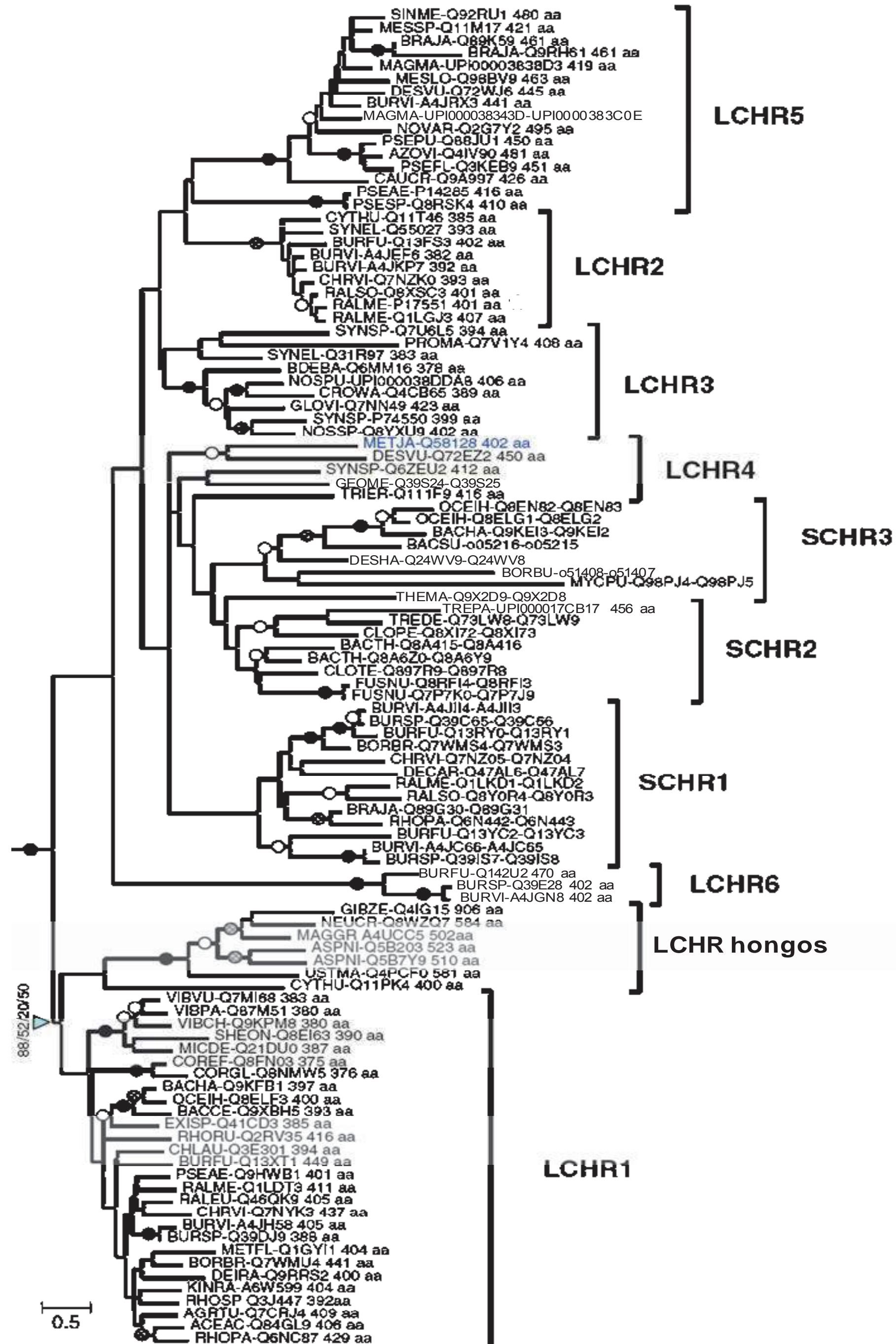


Figura 4. Árbol filogenético de la superfamilia CHR. Se indica la distribución de las 77 proteínas LCHR y de las 58 proteínas SCHR. Los nombres de las secuencias se indican por los nombres abreviados de las especies, seguidos del número de acceso de la base de datos UniProt y del tamaño de la secuencia de la proteína (aa).(Díaz-Pérez *et al.*, 2007).

4.-JUSTIFICACIÓN

La proteína ChrA de *P. aeruginosa* posee diferentes residuos neutros, ácidos y básicos esenciales para su función (Aguilera *et al.*, 2004, Córtes, 2005, Díaz-Pérez, 2005, Moreno, 2006), los cuales se han identificado empleando la estrategia de mutagénesis sitio-dirigida. Dada la naturaleza del oxianión cromato (CrO_4^{2-}), probablemente los residuos básicos de ChrA sean los que participen directamente en la unión del cromato. No obstante, únicamente se han analizado ocho de los 28 residuos básicos totales de la proteína, lo que sugiere que más residuos básicos probablemente son esenciales para la función.

La función en la resistencia a cromato se ha analizado a detalle únicamente en los transportadores ChrA de *P. aeruginosa* y de *C. metallidurans* (Cervantes *et al.*, 1990, Nies *et al.*, 1990), aun cuando la superfamilia de transportadores de cromato CHR está constituida por cientos de homólogos (Díaz-Pérez *et al.*, 2007). *Shewanella* sp. ANA-3 posee un homólogo de ChrA presente en el plásmido 1. Las especies de *Shewanella* son de gran importancia en procesos de biorremediación de sitios contaminados debido a su capacidad de reducir diferentes metales, entre los que se encuentra el Cr(VI). Se ha analizado el efecto del cromato en *Shewanella* y se han identificado varios genes cuya expresión se ve alterada durante la exposición a cromato (Brown *et al.*, 2006, Chourey *et al.*, 2006, Hau y Gralnick, 2007); sin embargo, no se ha reportado la función del homólogo CHR.

Otro homólogo que no se ha analizado a detalle es la proteína SrpC, codificada por el gen *srpC*, localizado en una región con genes relacionados con el metabolismo o transporte de azufre (Chen *et al.*, 2008). Una mutación en el gen *srpC* ocasionó mayor resistencia a cromato comparado con la cepa silvestre (Nicholson y Laudenbach, 1995), un resultado contrario al esperado.

Recientemente se determinó la función de las primeras proteínas pequeñas SCHR de la superfamilia CHR. *Bacillus subtilis* posee una pareja de proteínas SCHR codificadas por los genes adyacentes *chr3N* y *chr3C*, transcritos a través de un RNA mensajero bicistrónico, siendo ambos genes necesarios para conferir resistencia a cromato en *E. coli* (Díaz-Magaña, 2009). Sin embargo, no se ha determinado el mecanismo de resistencia a cromato conferido por las proteínas SCHR de *B. subtilis*.

El transporte de cromato por ChrA es inhibido por sulfato en vesículas de membrana invertidas (Álvarez *et al.*, 1999), y en células completas (Pimentel *et al.*, 2002), y se ha sugerido que el sulfato puede unirse y/o ser transportado por la proteína ChrA. Sin embargo, no se ha reportado información acerca de los transportadores CHR en relación con sulfato.

Por lo que el estudio de las interacciones de los transportadores CHR con cromato podrá aportar conocimiento más detallado acerca del mecanismo de resistencia de los miembros de la superfamilia. Además, aunque se ha descrito la expulsión de cromato por la proteína ChrA de *P. aeruginosa*, no se tiene información en relación con el sulfato.

5.-HIPÓTESIS

Los homólogos de la superfamilia CHR confieren resistencia a cromato por un mecanismo similar a la proteína ChrA de *Pseudomonas aeruginosa* los cuales además pueden transportar sulfato.

6.-OBJETIVOS

OBJETIVO GENERAL

Determinar la función de los transportadores CHR en relación con cromato y sulfato.

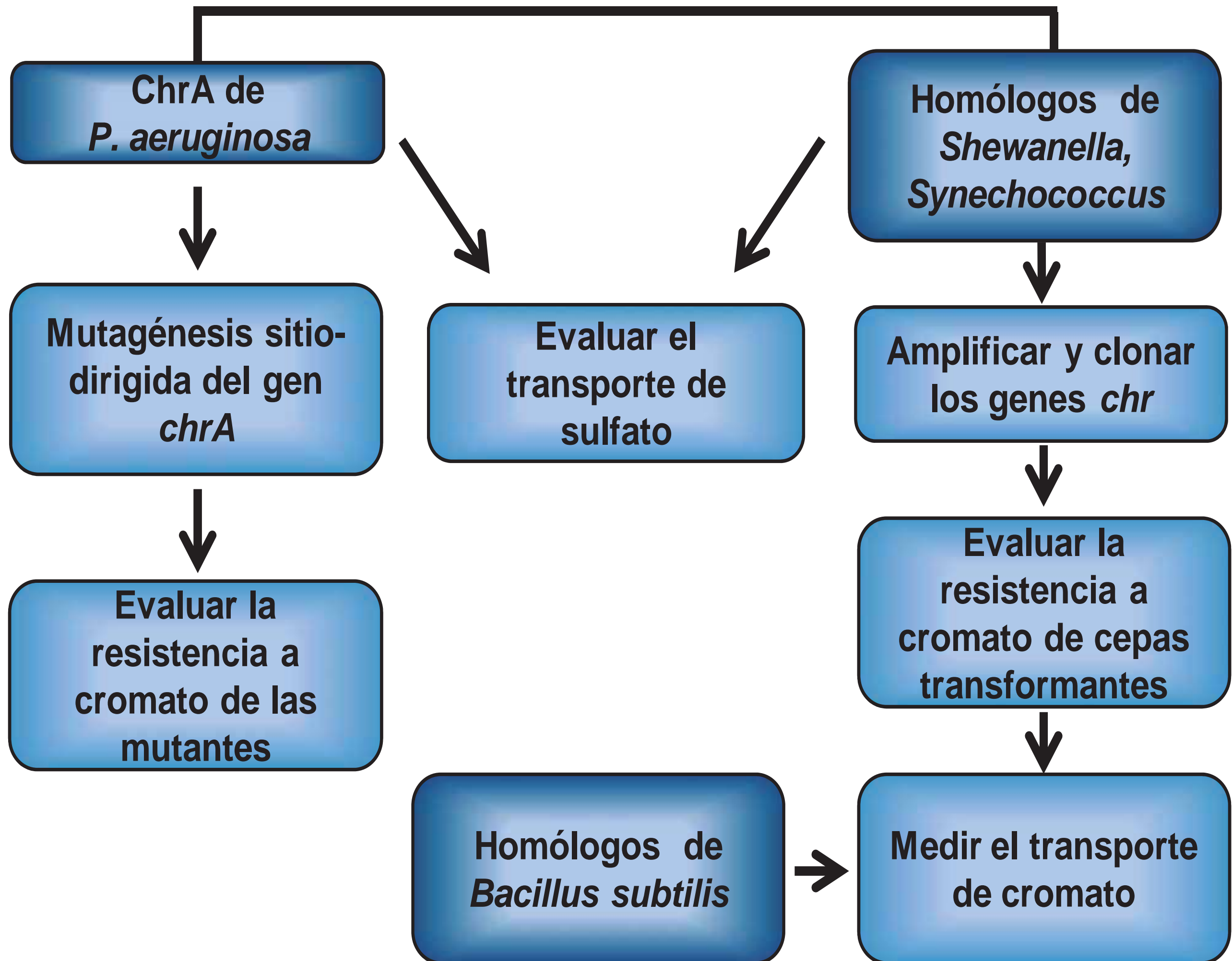
OBJETIVOS ESPECÍFICOS

1.-Identificar los aminoácidos básicos esenciales del transportador ChrA de *P. aeruginosa* que interaccionan con cromato.

2.-Determinar el transporte de sulfato por los transportadores CHR.

3.-Determinar la resistencia a cromato de homólogos de la superfamilia CHR.

7.- METODOLOGÍA GENERAL



8.-RESULTADOS

Capítulo I

1.-Identificación de los aminoácidos básicos esenciales del transportador ChrA de *P. aeruginosa* que interaccionan con cromato.

Capítulo II

2.-Transporte de sulfato por los transportadores CHR.

Capítulo III, IV y V

3.-Resistencia a cromato conferida por homólogos de la superfamilia CHR.

CAPÍTULO I

Identificación de los aminoácidos básicos esenciales del transportador ChrA de *P. aeruginosa* que interaccionan con cromato.

RESUMEN

La proteína ChrA de *Pseudomonas aeruginosa* es una proteína de membrana que confiere resistencia a cromato por medio de la expulsión del oxianión del citoplasma. La importancia de algunos residuos básicos en la resistencia a cromato se evaluó empleando mutagénesis dirigida. Los residuos básicos que se mutaron, seleccionados sin tomar en cuenta el porcentaje de conservación, fueron: Arg10, Lys13, Arg54, Lys67, Arg68, Lys71, Lys96, Lys227, Arg271, Arg340 y Lys392. Los residuos R54 y R68, localizados en el asa periplásmica P1, y R340, localizado en el asa citoplásmica C5, que son los más conservados, al ser mutados por alanina causaron una disminución en el nivel de resistencia a cromato. Estos resultados sugieren que estos residuos básicos interactúan directamente con el cromato durante el proceso de expulsión.

ABSTRACT7

The *Pseudomonas aeruginosa* ChrA protein is a membrane protein that confers chromate resistance by efflux of the chromate ions from the cytoplasm. The importance of basic residues for the chromate resistance phenotype conferred by ChrA was tested by site-directed mutagenesis. The basic residues Arg10, Lys13, Arg54, Lys67, Arg68, Lys71, Lys96, Lys227, Arg271, Arg340 y Lys392 were mutated. Residues R54 and R68, located in the large periplasmic P1 loop, and R340, in cytoplasmic loop C5, that are the most conserved, abolished chromate resistance when they were mutated for alanine. These results suggest that these cationic residues interact directly with chromate oxyanions in the efflux of chromate by ChrA.

INTRODUCCIÓN

El transportador ChrA es una proteína de 44 kDa formada de 13 segmentos transmembranales (Jiménez-Mejía *et al.*, 2006) que confiere resistencia a cromato en *Pseudomonas aeruginosa* por medio de la expulsión del ion del citoplasma,

empleando para ello el potencial eléctrico transmembranal (Álvarez *et al.*, 1999, Pimentel *et al.*, 2002). La proteína ChrA de *P. aeruginosa* pertenece a la superfamilia CHR de transportadores de cromato (Díaz-Pérez *et al.*, 2007), que actualmente posee cientos de homólogos, algunos de los cuales se ha demostrado que confieren resistencia a cromato (Nies *et al.*, 1990, Aguilar-Barajas *et al.*, 2008, Branco *et al.*, 2008, Díaz-Magaña *et al.*, 2009, León-Márquez, 2009). En un análisis inicial se identificaron residuos esenciales para la función de ChrA de *P. aeruginosa* por medio de un análisis mutagénico al azar; dichos residuos se localizaron en su mayoría en la mitad amino de la proteína (Aguilera *et al.*, 2004). Por un análisis de mutagénesis sitio-dirigida de residuos altamente conservados se identificaron los residuos esenciales neutros G44, G45, W63, P80, G81, P82, G92, G100 y V269 (Aguilar-Barajas, 2005, Córtes, 2005, Díaz-Pérez, 2005), y acídicos E56, E83, E86, D162, D346 y D362. De igual forma, se identificaron los residuos básicos R98, R201, R154, K248 y K394 que son esenciales para la función de la proteína, posiblemente participando de manera directa en la interacción con el ion (Moreno, 2006). Dado que la proteína ChrA de *P. aeruginosa* posee 28 residuos básicos, de los cuales sólo se han analizado ocho, es importante analizar algunos de los 20 residuos restantes con la finalidad de identificar otros residuos básicos que interactúen directamente con el cromato en el proceso de expulsión. En este trabajo se describen los resultados del análisis de mutagénesis sitio-dirigida de los residuos básicos como parte del estudio estructura-función de la proteína ChrA. Se identificaron algunos residuos básicos esenciales para la función que probablemente interaccionen directamente con el ión tóxico.

MATERIALES Y MÉTODOS

Cepas y condiciones de crecimiento. Se emplearon las cepas de *Escherichia coli* XL1-Blue (Stratagene) y JM101 (Yanisch-Perron *et al*. 1985) como huéspedes de los plásmidos. La cepa de *P. aeruginosa* PAO1 fue empleada como receptora de transformación (Holloway, 1969). Se emplearon los medios de cultivo: caldo Luria Bertani (LB: NaCl 1%, peptona de caseína 1% y extracto de levadura 0.5%) o caldo

nutritivo (Bioxon, México). Los cultivos líquidos se incubaron por 18-20 h a 37°C con agitación. El K_2CrO_4 fue de los laboratorios Merck.

Plásmidos. El plásmido pGEMT-ChrAHis se empleó como molde para la mutagénesis, es el vector pGEM-T (Promega) con el gen *chrA* con una etiqueta de 6-His en el extremo carboxilo (Aguilar-Barajas, 2005). El vector binario *E. coli/Pseudomonas* pUCP20 (West *et al*., 1994) se empleó para subclonar el gen *chrA* con las mutaciones para ser expresado en *P. aeruginosa*.

Técnicas genéticas. Para el aislamiento del DNA plasmídico se empleó el método de lisis alcalina, y se analizó por medio de un corrimiento electroforético en geles de agarosa (Sigma) al 1% en amortiguador TAE (Tris-acetato 0.04 M y EDTA 0.001 M) (Sambrook *et al*., 1989). Las endonucleasas empleadas se adquirieron de Invitrogen o Promega y los DNA plasmídicos se digirieron de acuerdo a procedimientos estándar. La reacción en cadena de la polimerasa (PCR), ligaciones del DNA y transformación de *E. coli* se realizaron de acuerdo a protocolos estándar (Sambrook *et al*., 1989). La transformación de *Pseudomonas* se realizó por el método de electroporación de *P. aeruginosa* sembradas recientemente en placa (Enderle y Farwell, 1998).

Mutagénesis sitio-dirigida. Para la mutagénesis sitio-dirigida se empleó el método mutagénico basado en PCR y digestion con *Dpn*I (Fisher y Pei, 1997) con algunas modificaciones. Los oligonucleótidos empleados se enlistan en el **Cuadro 1.** Las condiciones del PCR fueron las siguientes: 94ºC/1 min (desnaturalización inicial), 94°C/1 min (desnaturalización), 60-62°C/1 min (alineamiento), 72°C/ 7.5 min (extensión) y 72°C/7.5 min (extensión final); las muestras se sometieron a 16 ciclos de amplificación empleando la DNA polimerasa *Pfu* (Stratagene). Después de la amplificación de la cadena mutada, la reacción se digirió con la enzima de restricción *Dpn*I (New England Biolabs) para la eliminación del DNA molde, y la mezcla total de digestión se empleó para transformar células competentes de *E. coli* XL1-Blue. Las transformantes se seleccionaron en cajas de agar LB con 100 µg/ml de ampicilina. Se seleccionaron al azar algunas transformantes obtenidas y las regiones correspondientes al gen *chrA* se secuenciaron para verificar la mutación.

Expresión y detección. Con la finalidad de purificar la proteína ChrA, el gen *chrA* se amplificó y clonó en diferentes vectores de expresión (**Cuadro 3**). Los plásmidos se transfirieron a diferentes cepas de *E. coli*: BL21, BL21 (DE3) (pLysS), XL10-Gold y BL21 (DE3)-RP codon plus, para la sobreexpresión de ChrA. Además se emplearon diferentes detergentes para tratar de solubilizar la proteína: sarcosil, Tritón-X10 y n-dodecil-β-D-maltósido. También se realizó la purificación en condiciones desnaturalizantes en presencia de urea. Para la purificación se partió de cultivos crecidos durante 18 h obtenidos a partir de una colonia recién transformada de las diferentes cepas empleadas, y se inocularon matraces con 1 L de LB, y se incubaron en agitación hasta una densidad óptica de 0.4-0.6 a 590 nm. Posteriormente se adicionó el inductor, y se incubaron los cultivos de 2 a 18 horas, a temperatura ambiente, 30 ó 37°C dependiendo la cepa empleada. Posteriormente se obtuvó la fracción membranal, la cual se trató con los diferentes detergentes. Las muestras se centrifugaron nuevamente para obtener la fracción solubilizada y el sobrenadante se pasó por una columna de afinidad con resina de cobalto (BD-Talon Clontech). Posteriormente la columna se lavó y se eluyó la proteína con amortiguador de elución. Las diferentes fracciones obtenidas se sometieron a un corrimiento SDS-PAGE. Después las proteínas se transfirieron a una membrana de nitrocelulosa y se realizó la detección empleando el reactivo para histidinas His-Probe (Pierce).

Cuadro 1. Oligonucleótidos empleados en la mutagénesis dirigida.

Mutación	Secuencia de los oligonucleótidos directos	Secuencia de los oligonucleótidos reversos
Arg10→Ala	GAA-GAG-TCG-TAT-GCC-CCG-AGC-AAG-GCG-	CGC-CTT-GCT-CGG-GGC-ATA-CGA-CTC-TTC
Lys13→Ala	GTA-TCG-CCC-GAG-CGC-GGC-GAC-GGA-TG	CAT-CCG-TCG-CCG-CGC-TCG-GGC-GAT-AC
Arg54→Ala	CAG-ATC-GAC-ATG-TTG-GCC-CGC-GAG-CTC	GAG-CTC-GCG-GGC-CAA-CAT-GTC-GAT-CTG-
Arg67→Ala	CTG-GAT-CTC-CAG-CGC-ACG-CTT-CAA-CAA-GC	GCT-TGT-TGA-AGC-GTG-CGC-TGG-AGA-TCC-AG-
Arg68→Ala	CTGGATCTCCAGCAAAGCCTTCAACAAGCTGC	GCAGCTTGTTGAAGGCTTTGCTGGAGATCCAG
Arg71→Ala	GCA-AAC-GCT-TCA-ACG-CGC-TGC-TTG-CGG-TG	CAC-CGC-AAG-CAG-CGC-GTT-GAA-GCG-TTT-GC
Lys96→Ala	GGC-ATC-CGA-GCA-GCG-GGG-CGG-CTG-GGG	CCC-CAG-CCG-CCC-CGC-TGC-TCG-GAT-GCC
Lys227→Ala	GCA-CCG-ACA-GCG-GCG-CTT-GTG-GAA-GCG	CGC-TTC-CAC-AAG-CGC-CGC-TGT-CGG-TGC
Arg271→Ala	AAC-GAC-GCC-GTC-GGG-GCC-GGA-TGG-ATG-ACG	CGT-CAT-CCA-TCC-GGC-CCC-GAC-GGC-GTC-GTT
Arg340→Ala	GTC-GTG-GAG-AAC-AAA-GCG-CTG-CAC-GCC-TTT-C	GAA-AGG-CGT-GCA-GCG-CTT-TGT-TCT-CCA-CGA-C
Lys392→Ala	CCT-TTA-TGC-CTG-GGC-GAA-CAA-GCT-CAA-CGT-C-	GAC-GTT-GAG-CTT-GTT-CGC-CCA-GGC-ATA-AAG-G

RESULTADOS Y DISCUSIÓN

Mutagénesis de los residuos básicos. Del análisis filogenético de la superfamilia CHR se seleccionaron las proteínas clasificadas en las subfamilias LCHR2 y LCHR5, donde se localizan las proteínas ChrA de *Cupriavidus metallidurans* (anteriormente *Alcaligenes eutrophus* y *Ralstonia metallidurans*) y de *P. aeruginosa*, respectivamente, y se determinó el porcentaje de conservación de cada uno de los residuos básicos de ChrA (**Fig. 1**).

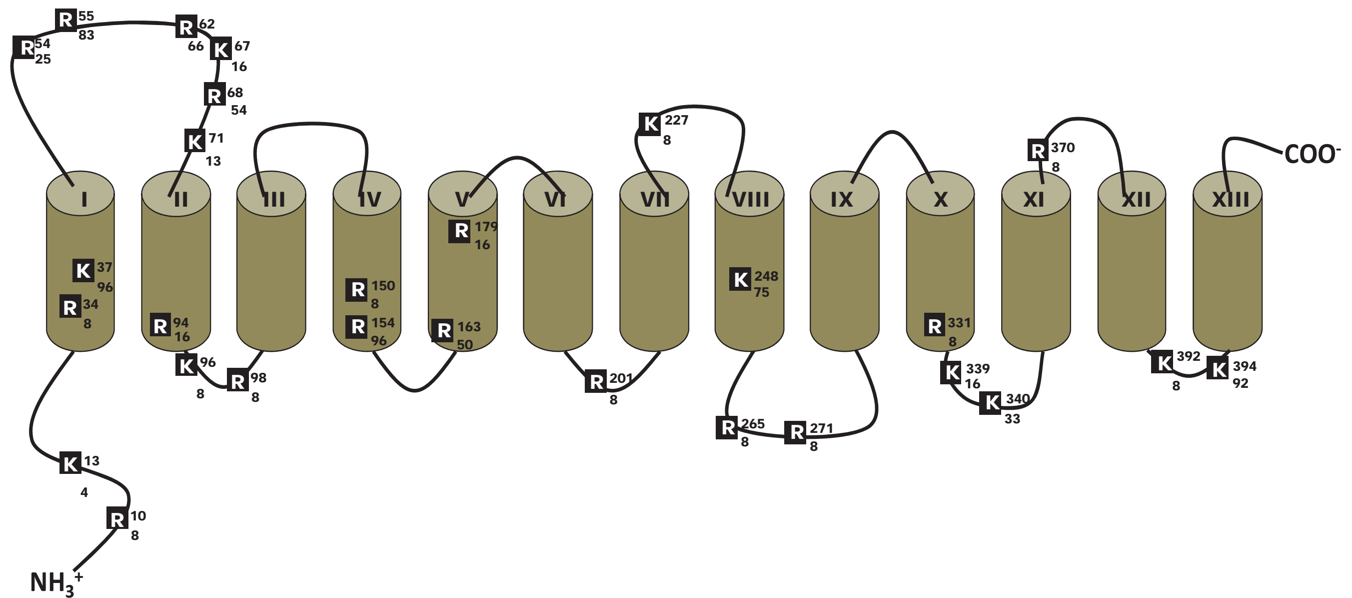


Figura 1. Localización de los residuos básicos de ChrA. Se muestra el modelo topológico de ChrA donde se indican los residuos básicos, arginina (R) y lisina (K). El número superior indica su posición en la proteína y el número inferior el porcentaje de conservación en las subfamilias LCHR2 y LCHR5. P, asa periplásmica. C, asa citoplásmica.

Dado que solamente se ha estudiado la importancia de ocho de los residuos básicos de ChrA, se analizó por mutagénesis sitio-dirigida la importancia de los residuos básicos restantes, independientemente de su porcentaje de conservación (**Cuadro**

2). Como un primer escrutinio, los residuos básicos seleccionados se mutaron por alanina, para determinar la importancia de la carga del aminoácido. Para ello, se diseñaron oligonucleótidos para introducir la mutación de cada uno de los residuos (**Cuadro 1**) y se llevó a cabo la mutagénesis como se describe en Materiales y Métodos. Después de corroborar la presencia de la mutación, el gen *chrA* se subclonó en el vector binario *Escherichia*/*Pseudomonas* pUCP20, para posteriormente transferirse a la cepa PAO1 de *P. aeruginosa* para evaluar el nivel de resistencia a cromato conferido por las mutantes.

Se obtuvieron mutantes en los residuos R10, K13, R54, K67, R68, K71, K96, K227, R271, R340 y K392, ubicados a lo largo de la proteína, tanto en asas periplásmicas como citoplásmicas (**Fig. 1**). Las mutantes en los residuos R10, K13, K67, K71, K96, K227, R271, y K392, presentaron un nivel de resistencia a cromato similar al conferido por la proteína ChrA silvestre, por lo que se clasificaron como resistentes (**Cuadro 2**). Este resultado sugiere que estos residuos probablemente no participen en el transporte de cromato, ya que la eliminación de la carga no afectó el nivel de resistencia. En las mutantes afectadas en los residuos R54, R68 y R340, que son los más conservados, el nivel de resistencia a cromato se vio disminuida en diferentes niveles, por lo que se clasificaron como esenciales (**Cuadro 2**); (**Fig. 2**). Esto indica que probablemente los residuos R54, R68 y R340 interaccionan directamente con el cromato durante el proceso de expulsión. Sin embargo, no se puede descartar que su papel sea contribuir a la correcta estructura de la proteína.

Los residuos básicos son importantes en otros transportadores de aniones, como el transportador PcaK de *P. putida*, en el cual se han identificado los residuos básicos R124 y R328 localizados en los STM 4 y STM 11, respectivamente, y la R386, localizada en el asa citoplásmica entre el STM 10 y STM 11, los cuales son esenciales para el transporte de 4-Hidroxi-benzoato (Ditty y Harwood, 2002). El transportador de oxalato/formato OxlT de *E. coli* posee la K355 localizada en el centro del STM 11, la cual participa en la unión del oxalato (Fu *et al.*, 2001).

Cuadro 2. Susceptibilidad a cromato de las mutantes de ChrA de *P. aeruginosa*

Residuo	Posición	%Conservación*	Fenotipo
Arg68	P1	54	Sensible
Arg340	C5	33	Sensible
Arg54	P1	25	Sensible
Lys67	P1	16	Resistente
Lys71	P1	13	Resistente
Arg10	N	8	Resistente
Lys96	C1	8	Resistente
Lys227	P4	8	Resistente
Arg271	C4	8	Resistente
Lys392	C6	8	Resistente
Lys13	N	4	Resistente

*C, asa citoplásmica; P, asa periplásmica; N, extremo amino.

La R54 y R68 se localizan en el asa periplásmica P1, en la cual se localizan además otros residuos básicos, neutros y acídicos que son esenciales para la función de ChrA (Díaz-Pérez, 2005, Moreno, 2006). El asa periplásmica P1 se encuentra formando una hélice alfa que une los segmentos transmembranales (STM) I y II, y se ha propuesto que funciona controlando la apertura y cierre del canal, en forma similar a lo que ocurre en canales iónicos (Díaz-Pérez, 2005).

El otro residuo identificado como esencial en este trabajo, la R340 localizada en el asa citoplásmica C5, al igual que los residuos básicos esenciales mutados anteriormente R98, R201, y K394, que se localizan en las asas citoplásmicas C1, C3, y C6, respectivamente (Moreno, 2006); si bien estos residuos pueden participar en el reconocimiento inicial del cromato para que pueda ser transportado, también pueden ser requeridos para el correcto ensamble de la proteína en la membrana, dado que los residuos básicos en asas hidrofílicas de proteínas de membrana se localizan preferencialmente en el lado citoplásmico, de acuerdo a la regla del “positivo adentro” (von-Heijne, 1992). Se ha propuesto que los STMs IV y VIII forman parte de la ruta

de translocación del cromato del citoplasma al periplasma, ya que allí se localizan los residuos básicos esenciales R154 y K248, respectivamente, los cuales probablemente interactúan directamente con el cromato (Moreno, 2006). La K248 se localiza en el centro del STM VIII, en una forma similar al transportador de oxalato/formato OxlT que posee el residuo básico K135 en el centro del SMT XI, el cual se une a un grupo carboxilo del sustrato divalente aniónico durante el proceso de transporte (Fu *et al.*, 2001).

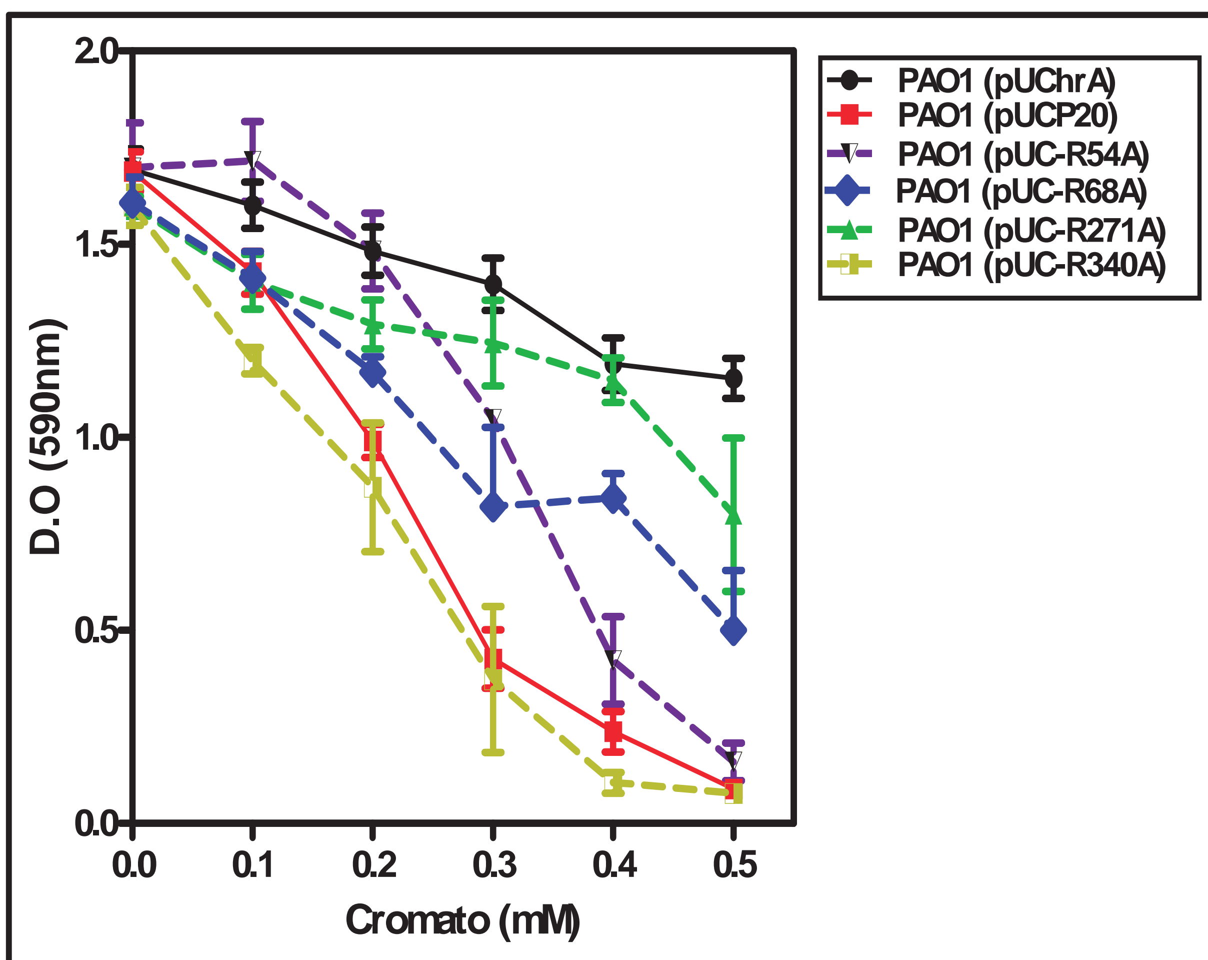


Figura 2. **Susceptibilidad a cromato de las mutantes de ChrA de *P. aeruginosa*.** Cultivos crecidos durante toda la noche se diluyeron 1:100 y se crecieron de 18-20 h a 37°C en C.N. con las concentraciones de cromato indicadas. El crecimiento se determinó midiendo la densidad óptica a 590 nm. Se empleó PAO1(pUChrA), (con el gen *chrA* silvestre) como control resistente, y PAO1(pUCP20) como control sensible. Se muestra el resultado de dos ensayos por duplicado. Se muestran las barras del error estándar de la media.

En este trabajo (**Cuadro 2**), y de igual forma anteriormente (Moreno, 2006), se ha realizado mutagénesis de residuos básicos localizados tanto en segmentos transmembranales, asas citoplásmicas y periplásmicas que al mutarse no afectan la resistencia a cromato, por lo cual probablemente su función sea contribuir a la correcta estructura de la proteína y no tienen una interacción directa con el cromato.
Como perspectivas del trabajo sería ahora determinar la función en la proteína de los residuos básicos esenciales de ChrA. Una de las técnicas que permite analizar la importancia de los aminoácidos en los transportadores ya que permite identificar residuos que están en el sitio de unión del sustrato o en la ruta de translocación del sustrato es realizar mutagénesis de los posibles residuos por cisteína y mediante pruebas bioquímicas evaluar en presencia del sustrato la protección de los residuos a reaccionar con reactivos específicos para grupos tiol. Con esta finalidad se ha empleado esta técnica en los transportadores LacY (Frillingos *et al.*, 1998), Oxlt (Fu *et al.*, 2001, Ye y Maloney, 2002) y UhpT (Yan y Maloney, 1995)

Expresión y purificación de la proteína ChrA. Para determinar el nivel de expresión de la proteína ChrA en las mutantes, se realizaron varias estrategias para la purificación de la proteína para posteriormente obtener anticuerpos. Existen diferentes factores que dificultan la purificación de proteínas de membrana, como son: la sobreexpresión de una proteína de membrana muchas veces resulta tóxica para la célula huésped, otro factor es la solubilidad, ya que la proteína se encuentra inmersa en la membrana que es un ambiente hidrofóbico por lo que es necesario emplear detergentes para solubilizarlas. Para tratar de eludir estas dificultades el gen *chrA* se clonó en diferentes vectores de expresión (**Cuadro 3**). Se emplearon además diferentes cepas de *E. coli* para la sobreexpresión, y diferentes detergentes para la solubilización (ver Materiales y Métodos). Durante las diferentes pruebas de solubilización y detección, se detectó una banda de aproximadamente 30 kDa, un tamaño menor al esperado (44KDa) (**Fig. 3**); sin embargo, este tipo de migración anómala es observado para algunas proteínas de membrana. Se realizó la secuenciación de la banda de 30 kDa, la secuencia obtenida no correspondió a la proteína ChrA. Aunque se exploraron diferentes variables para la detección y sobreexpresión de la proteína ChrA, los cuales se han empleado para otros

transportadores (Wang *et al.*, 2003), ésta no se logró detectar o purificar. Una situación similar sucedió con la proteína ChrA de *C. metallidurans*, la cual tampoco ha podido ser purificada (Nies D. comunicación personal).

Cuadro 3. Vectores empleados para la sobreexpresión de la proteína ChrA.

Vector / Plásmido	Etiqueta / localización	Características
pTrcHis2 (Invitrogen) pChrAES pHisChrA	6XHis Carboxilo Amino	Promotor híbrido para una mayor expresión
pET28 (Novagen) pET-HisChrA	6XHis Amino	Promotor fuerte. Altamente regulado.
pQE30 (Qiagen) pQE-ChrA	6XHis Amino	Fuerte represión.
pGEX (Amersham) pGST-ChrA[2]	Glutation-S-transferasa Amino	Fusión de una proteína reportera de 26 kDa.
pASK-3 pASK-ChrA	Avidina Carboxilo	Represión total en ausencia del inductor

También se trató de detectar la proteína ChrA en extractos de las cepas de *P. aeruginosa* PAO1 (pUChrA), en la cual el gen *chrA* se encuentra fusionado a una etiqueta de histidinas en el extremo carboxilo. Sin embargo, aun cuando en esta cepa se observó la presencia del RNA mensajero correspondiente a *chrA* por medio

de RT-PCR (datos no mostrados), y además de que se observa el fenotipo de resistencia a cromato, no se pudo detectar ChrA empleando anticuerpos anti-His. Es probable que la proteína ChrA se encuentre en la membrana en muy baja concentración, razón por la cual no ha sido posible su detección y purificación.

Una de las perspectivas del trabajo y que no se exploró en este trabajo, es el empleo de péptidos sintéticos de varias regiones de la proteína para la síntesis de anticuerpos, y de esta manera aumentar la posibilidad de detectar la proteína. Los anticuerpos sintetizados a partir de péptidos sintéticos se ha empleado para la detección de los transportadores de zinc, ZnT-1 (McMahon y Cousins, 1998), y de lactosa LacY (Seckler *et al.*, 1983).

CONCLUSIÓN

Los residuos Arg54, Arg68 y Arg340 de la proteína ChrA de *P. aeruginosa* son esenciales para su función y probablemente interaccionan directamente con el cromato durante la expulsión.

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CAPÍTULO II

Determinación del transporte de sulfato por los transportadores CHR

RESUMEN

La expulsión de cromato por la proteína ChrA es inhibida por sulfato en una forma dependiente de su concentración por lo que se evaluó el transporte de sulfato a través de ChrA. La captación de sulfato en células completas de *Pseudomonas aeruginosa* PAO1 que expresan el gen *chrA* fue similar al de la cepa control sin el gen *chrA*. Un resultado similar se obtuvo al evaluar la captación de sulfato en células completas de *Escherichia coli* W3110 que expresan el gen *chrA* de *Shewanella*, comparado con el control únicamente con el vector. De igual forma vesículas invertidas de membrana de la cepa de *P. aeruginosa* PAO1 expresando ChrA no presentaron un mayor nivel en la captación de sulfato, por lo que se concluyó que ChrA no transporta sulfato.

ABSTRACT

The efflux of chromate is inhibited by the oxyanion sulfate in a concentration-dependent fashion, therefore sulfate transport through the ChrA protein was evaluated. Sulfate uptake in cell suspensions of *Pseudomonas aeruginosa* PAO1 expressing *chrA* was similar to the control without the *chrA* gene. A similar sulfate uptake level was obtained when the transport was evaluated in cell suspensions of *Escherichia coli* W3110 expressing the *chrA* gene from *Shewanella*, as compared with the strain only with the vector. Everted membrane vesicles of *P. aeruginosa* did not have a higher level of sulfate uptake, indicating that ChrA does not transport sulfate.

INTRODUCCIÓN

El cromato, un oxianión con estructura tetraédrica, es análogo estructural del ión esencial sulfato; ambos oxianiones poseen distancias de enlace muy similares, S-O de 1.47 Å y Cr-O de 1.65 Å (Dudev y Lim, 2004). Dada esta alta similitud el cromato se ha identificado como inhibidor competitivo del transporte de sulfato (Ohtake *et al.*,

1987). La expulsión de cromato del citoplasma por el transportador ChrA que confiere resistencia a cromato (Cervantes *et al.*, 1990, Nies *et al.*, 1990, Aguilar-Barajas *et al.*, 2008) es inhibida en presencia de sulfato. En experimentos *in vitro* con el uso de vesículas de membrana invertidas se observó una inhibición de hasta un 80% del transporte de cromato por sulfato (Álvarez *et al.*, 1999). De igual forma, se observó la inhibición del transporte de cromato *in vivo* al emplear células completas (Pimentel *et al.*, 2002), sugiriendo la posibilidad de que el sulfato también puede unirse y/o ser transportado por la proteína ChrA. Por lo tanto el objetivo de este trabajo fue determinar el transporte de sulfato por los transportadores de cromato CHR.

MATERIALES Y MÉTODOS

Cepas y condiciones de crecimiento. Se emplearon las cepas de *P. aeruginosa* PAO1 (pUCP20) y PAO1 (pUChrA), que tiene el gen *chrA* de *P. aeruginosa* en el vector pUCP20 (Aguilar-Barajas, 2005). De igual forma se emplearon las cepas de *E. coli* W3110 (pACYC184) y W3110 (pACYC-ChrBAC) que tiene el gen *chrA* de *Shewanella* sp. ANA-3 en el vector pACYC184 (Aguilar-Barajas *et al.*, 2008). Los cultivos se crecieron en medio mínimo M9 (Sigma) adicionado de glucosa 20 mM, $MgSO_4$ 2 mM ó 0.2 mM y $CaCl_2$ 0.1 mM. El K_2CrO_4 fue de los laboratorios Merck.

Captación de $^{35}SO_4^{2-}$ en células completas. Un cultivo crecido durante toda la noche en medio mínimo M9 se diluyó 1:25 en medio M9 fresco y se incubó a 37°C hasta la fase logarítmica. Se centrifugaron los cultivos para colectar la pastilla celular (8000 rpm/10 min/temperatura ambiente). Se lavó la pastilla con amortiguador de fosfatos 0.1 M y se resuspendieron las células en 2 ml del mismo amortiguador precalentado a 37°C. Se adicionó $^{35}SO_4^{2-}$ a una concentración final de 10 μM (0.115 μCi). Las células se incubaron a 37°C y alícuotas de 0.1 ml se filtraron a través de membranas de nitrocelulosa de 0.45 μM de diámetro (Millipore Corp., Bedford, MA) previamente humedecidos con una solución de amortiguador de fosfatos 0.1 M con 10 mM de sulfato de potasio (K_2SO_4). Los filtros con las células se lavaron inmediatamente con 10 ml de la solución de amortiguador de fosfatos 0.1 M con 10 mM de K_2SO_4. Los filtros se secaron y la radioactividad incorporada se cuantificó en un contador de radiación gamma (Ames Gammaccord II). Se filtró por separado una

alícuota de medio radioactivo sin células y los valores se restaron a los valores experimentales. Se tomaron 50 μl de medio para estimar la radioactividad total.

Captación de $^{35}SO_4^{2-}$ en vesículas. Las vesículas de membrana invertidas de *P. aeruginosa* se prepararon como se describe en Álvarez *et al.* (1999). El transporte se realizó a 25°C en amortiguador de fosfatos 0.1 M (pH 7.0) adicionado de NADH 2 mM, $MgCl_2$ 5 mM y $^{35}SO_4^{2-}$ a una concentración final de 0.1 mM (0.1 μCi). La reacción se inició con la adición de las vesículas de membrana (0.2 mg/ml). Se filtraron alícuotas de 0.4 ml a través de membranas de nitrocelulosa de 0.22 μM de diámetro (Millipore Corp., Bedford, MA) previamente humedecidos con una solución de amortiguador de fosfatos 0.1 M con 10 mM de K_2SO_4. El tratamiento de los filtros y la determinación de la radioactividad se realizaron como se describió arriba.

Determinación de proteína. La concentración de proteína se determinó por el método de Lowry (Lowry *et al.*, 1951).

RESULTADOS Y DISCUSIÓN

Captación de $^{35}SO_4^{2-}$ en células completas de *P. aeruginosa*. En un inicio se evaluó la captación de sulfato en células de *P. aeruginosa* para lo cual se determinó la incorporación de $^{35}SO_4^{2-}$ en células completas de cultivos crecidos en medio mínimo M9 con 2 mM de sulfato. La cepa que expresa el gen *chrA*, PAO1 (pUChrA) presentó una captación de sulfato similar al de la cepa únicamente con el vector PAO1 (pUCP20) (**Fig. 1A**). Se obtuvo un resultado similar cuando las células se crecieron en presencia de niveles más bajos de sulfato (0.2mM) (**Fig. 1B**). Este resultado sugiere que no hay transporte de sulfato por ChrA.

Captación de $^{35}SO_4^{2-}$ en vesículas invertidas de membrana de *P. aeruginosa*. Dado que en el genoma de *P. aeruginosa* hay varios genes que codifican probables transportadores de sulfato (Stover *et al.*, 2000), que posiblemente interfieran con el transporte de sulfato por ChrA, se emplearon vesículas invertidas de membrana. Esta es una técnica que presenta menos interferencia y permite evaluar el transporte a través de la proteína ChrA (Álvarez *et al.*, 1999). La captación de sulfato por las vesículas de la cepa PAO1 (pUChrA) con y sin la adición de NADH fue similar al de las vesículas de la cepa únicamente con el vector PAO1 (pUCP20) (**Fig. 2**). Estos

resultados confirman que la proteína ChrA de *P. aeruginosa* no transporta al ion sulfato.

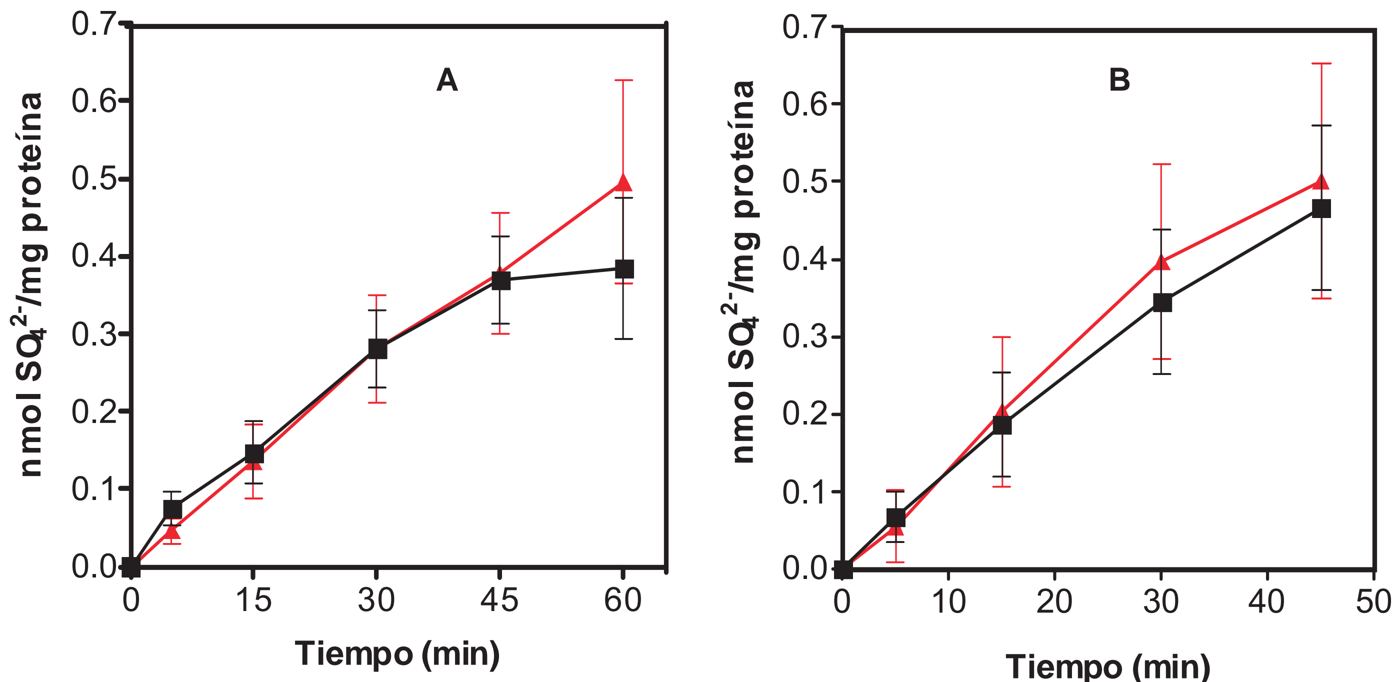

Figura 1. Captación de $^{35}SO_4^{2-}$ en las cepas de *P. aeruginosa* que expresan la proteína ChrA. Cultivos crecidos durante toda la noche en medio mínimo M9 se diluyeron 1:25 en medio fresco y se crecieron hasta la fase logarítmica. Las células se lavaron y la incorporación de $^{35}SO_4^{2-}$ se determinó como se describe en Materiales y Métodos. Se emplearon las cepas PAO1 (pUCP20) (▲) y PAO1 (pUChrA) (■). A) Células crecidas en 2 mM de sulfato, se muestra el resultado de una n=10. B) Células crecidas en 0.2 mM de sulfato, se muestra el resultado de una n=7.

Captación de $^{35}SO_4^{2-}$ en células completas de *E. coli*. Ya que no se observó transporte de sulfato en las células de *P. aeruginosa* que expresan ChrA se determinó el transporte de sulfato en células de *E. coli* que expresan la proteína ChrA de *Shewanella*. La captación de sulfato en células de W3110 (pACYC-ChrA) fue similar al de la cepa únicamente con el vector W3110 (pACYC184) (**Fig. 3**), al igual que las cepas de *P. aeruginosa*. Se midió también la captación de 10 mM de $^{35}SO_4^{2-}$

con resultados similares (datos no mostrados). Los resultados obtenidos indican claramente que aun cuando es estructuralmente similar al cromato, y además ser un inhibidor de la expulsión de cromato, el sulfato no es transportado a través de la proteína ChrA.

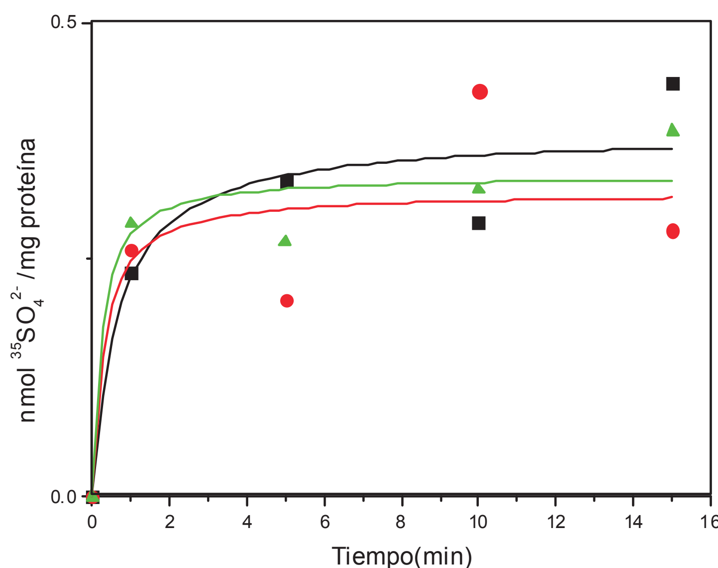

Figura 2. Captación de $^{35}SO_4^{2-}$ en vesículas invertidas de membrana de *P. aeruginosa*. Vesículas de membrana se resuspendieron en amortiguador de fosfatos con 0.1 mM de $^{35}SO_4^{2-}$ y NADH 2 mM y se incubaron 25°C, y se determinó la incorporación de $^{35}SO_4^{2-}$ en las vesículas de PAO1 (pUCP20) (▲), y PAO1(pUChrA) (●). Se midió la captación de PAO1(pUChrA) sin la adición de NADH (■). Experimento representativo de dos repeticiones.

Estos resultados sugieren que la proteína ChrA puede discriminar entre los oxianiones cromato y sulfato, aun cuando éstos presentan una carga, geometría y tamaño similares. Sin embargo, se han reportado proteínas que pueden discriminar entre moléculas muy parecidas. Tal es el caso de la proteína periplásmica de unión a molibdeno, ModA, la cual une específicamente al molibdeno o al tungsteno (Imperial *et al.*, 1998), que presentan distancia de enlace prácticamente iguales (Mo-O de 1.75 Å y W-O de 1.78 Å) (Dudev y Lim, 2004); aun cuando la proteína ModA es estructuralmente similar a la proteína SBP de unión a sulfato, ModA no une al sulfato (S-O de 1.47 Å), siendo capaz de discriminar entre estos oxianiones tan parecidos

(Imperial *et al.*, 1998, Lawson *et al.*, 1998). Además del tamaño también pueden participar otros factores como interacciones específicas del cromo el cual, a diferencia del azufre, es un metal que pueden determinar la especificidad de ChrA por el cromato. Esta es una ventaja para la célula ya que el sulfato es un oxianión esencial cuya expulsión por ChrA podría tener un efecto perjudicial para la célula bacteriana.

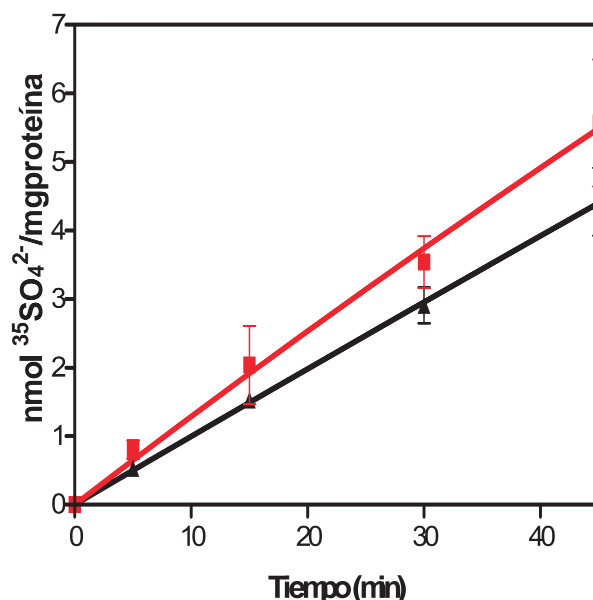

Figura 3. Captación de $^{35}SO_4^{2-}$ en las cepas de *E. coli* que expresan la proteína ChrA de *Shewanella*. La incorporación de 1 mM de $^{35}SO_4^{2-}$ se determinó como se describe en Materiales y Métodos. Se emplearon las cepas W3110 (pACYC184) (■) y W3110 (pACYC-ChrBAC) (▲). Experimento representativo por duplicado de dos repeticiones.

CONCLUSIÓN

Los transportadores de cromato ChrA de *P. aeruginosa* y ChrA de *Shewanella* sp. no transportan al oxianión sulfato.

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CAPÍTULO III

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

Las especies de *Shewanella* forman un grupo diverso de bacterias anaeróbicas facultativas ampliamente distribuidas en ambientes acuáticos. Estas bacterias son capaces de reducir diferentes metales, entre los que se encuentra el Cr(VI) (Chourey *et al.*, 2006), por lo que puede ser de una gran importancia en procesos de biorremediación de sitios terrestres y/o acuáticos contaminados (Hau y Gralnick, 2007). Por ello se consideró de importancia analizar la función de los genes *chr* localizados en el plásmido 1 de *Shewanella sp* ANA-3. Este plásmido posee el operón *chrBAC*, el cual presenta un arreglo similar al reportado para pUM505 de *P. aeruginosa* (Díaz-Pérez *et al.*, 2007).

Se diseñaron oligonucleótidos para amplificar por PCR los genes *chrBAC* y se clonaron en vectores de diferente número de copias. El gen *chrA* confirió un elevado nivel de resistencia a cromato en *E. coli.* La expresión de los genes *chrBAC* en el plásmido pACYC184 incrementó la resistencia a cromato hasta 10 veces más que el control sensible. A diferencia de las proteínas ChrA de los plásmidos pMOL28 de *C. metallidurans* y pUM505 de *P. aeruginosa*, que sólo son funcionales en sus respectivos huéspedes, la proteína ChrA de *Shewanella sp*. ANA-3 es funcional en *E. coli*. El gen *chrA* de *Shewanella* de igual forma confirió resistencia a cromato en *P. aeruginosa.* Las cepas que expresan *chrA* presentaron una captación disminuida del cromato, lo que sugiere que ChrA de *Shewanella* expulsa el cromato de la célula, funcionando en una forma similar a ChrA de *P. aeruginosa*.

RESEARCH LETTER

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

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chromate; *Shewanella*; efflux.

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

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(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

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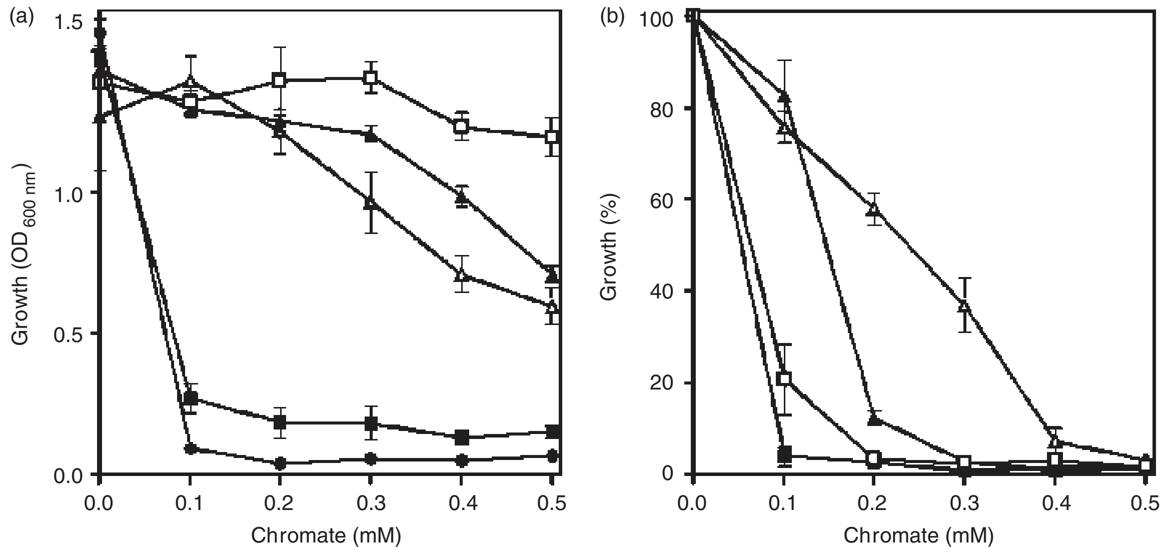

Fig. 1. Chromate resistance of *Shewanella chr* genes in (a) *Escherichia coli* and (b) *Pseudomonas aeruginosa*. Overnight cultures were diluted 1 : 100 into fresh M9 medium with the indicated concentrations of K_2CrO_4. Cell growth was monitored at $OD_{600\,nm}$ after 18–20 h incubation at 37 °C with 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

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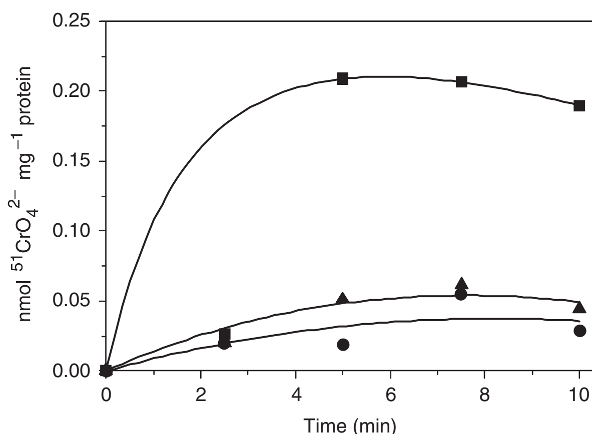

Fig. 2. $^{51}CrO_4^{2-}$ uptake by *Escherichia coli* cells expressing *Shewanella chr* genes. Overnight cultures in M9 medium were diluted 1 : 30 into fresh M9 medium and grown to 0.6–0.8 at $OD_{600\,nm}$. The cells were washed and the incorporation of $^{51}CrO_4^{2-}$ was determined as described 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.

Acknowledgements

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RESULTADOS ADICIONALES RELACIONADOS CON LOS GENES *chr* DE *Shewanella* sp. ANA-3.0

Una vez que se demostró la función de la proteína ChrA de *Shewanella* en la expulsión de cromato, se analizaron también los genes *chr* adyacentes a *chrA* para determinar su posible participación en la resistencia a cromato. Para ello se evaluó la función del gen *chrC*. También se estudio la participación del glutatión en la resistencia a cromato y la participación del estrés oxidativo en la toxicidad de cromato, en cepas con los genes *chrA* y *chrBAC*.

1.-El gen *chrC* codifica una superóxido dismutasa

Aledaños al gen *chrA* de *Shewanella* se localizan los genes *chrB* y *chrC* (**Fig.3**), que se ha reportado participan en la resistencia a cromato en otras bacterias (Nies *et al.*, 1990, Juhnke *et al.*, 2002, Branco *et al.*, 2008). El gen *chrC* de *Shewanella* codifica una proteína de 203 aminoácidos que tiene 49% de identidad con ChrC del plásmido pMOL28 (Juhnke *et al.*, 2002); *chrC* en *P. aeruginosa* se encuentra truncado por lo cual probablemente no es funcional (**Fig.3**). El gen *chrC* codifica una probable superóxido dismutasa (SOD), enzima encargada de eliminar el anión superóxido de la célula (**Fig.3**) (Juhnke et al., 2002, Branco et al., 2008).

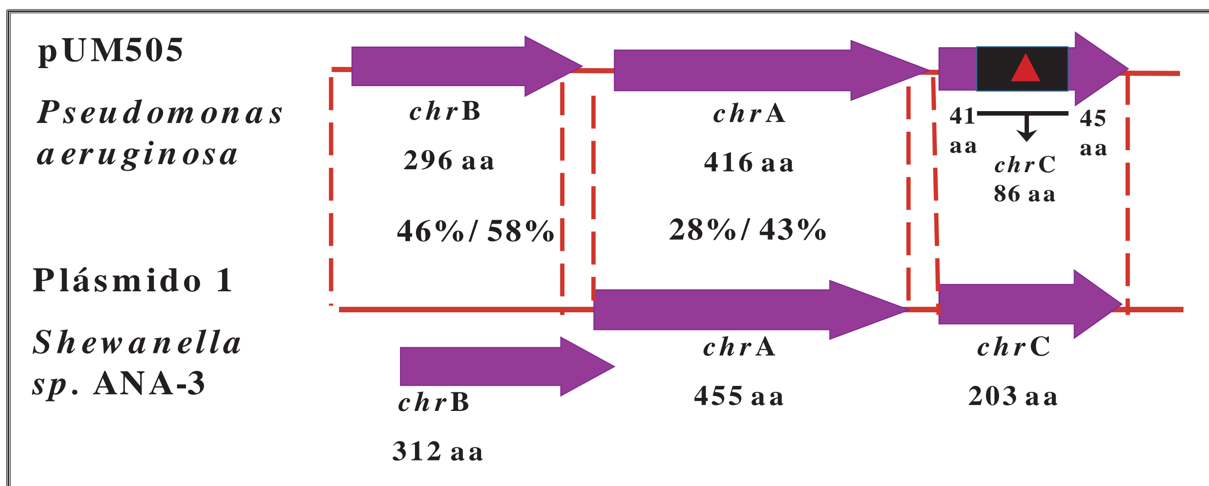

Figura 3. Arreglo de los genes *chr* del plásmido 1 de *Shewanella* sp. ANA-3. Comparación de los genes *chr* del plásmido pUM505 de *P. aeruginosa* y del plásmido 1 de *Shewanella*. Se indica el arreglo de los genes *chrB*, que es un posible regulador transcripcional, *chrA*, un transportador de cromato y *chrC*, una superóxido dismutasa. Se muestra el tamaño de los productos génicos (aa) y el porcentaje de identidad/similitud entre ellos.

Por otra parte, una cepa de *E. coli* con mutaciones en los genes que codifican las enzimas Mn-SOD y Fe-SOD presenta un crecimiento normal en condiciones aeróbicas únicamente en medio rico, o en un medio mínimo suplementado de los 20 aminoácidos. El requerimiento de los aminoácidos es debido a que el anión superóxido inhibe la actividad de la enzima dihidroxi-ácido deshidratasa que participa en la síntesis de aminoácidos ramificados leucina, valina e isoleucina (Kuo *et al.*, 1987). También causa el requerimiento de aminoácidos que contienen azufre, debido a que el anión superóxido causa indirectamente una perdida de sulfito de la célula (Benov *et al.*, 1996), y de aminoácidos aromáticos.

Para determinar la función del gen *chrC* se usó la cepa de *E. coli* PN134 que posee mutaciones en los genes *sodA* y *sodB* que codifican las enzimas Mn-SOD y Fe-SOD, la cual presenta deficiencia en la síntesis de aminoácidos ramificados, aminoácidos que contienen azufre y aminoácidos aromáticos (Carlioz y Touati, 1986, Benov *et al.*, 1996). El gen *chrC* se amplificó por PCR a partir de DNA total de la cepa de *Shewanella* sp. ANA-3 y el fragmento amplificado se recuperó en el vector pGEMT-Easy (Promega). El plásmido recombinante pGEMT-ChrC se empleó para transformar la mutante PN134. Se realizaron curvas de crecimiento en medio mínimo M9 bajo diferentes condiciones. La cepa silvestre de *E. coli* AB1157, la mutante PN134 y la transformante PN134 (pGEMT-ChrC) presentaron un crecimiento similar en medio M9 cuando se adicionaron los 20 aminoácidos (aa) (**Fig. 4**). Sin embargo, cuando los cultivos se crecieron en M9 sin la adición de los aminoácidos aromáticos (fenilalanina, tirosina y triptófano), la cepa PN134 presentó una velocidad de crecimiento menor comparado con la transformante PN134 (pGEMT-ChrC) que presentó un crecimiento similar al de la cepa silvestre AB1157 (**Fig. 5A**). El efecto fue más drástico al crecer las cepas sin la adición de los aminoácidos ramificados (isoleucina y valina); en estas condiciones, la mutante fue incapaz de crecer comparado con las cepas silvestre y la cepa transformante que presentaron un crecimiento normal (**Fig. 5B**). Estos resultados demuestran que ChrC funciona como SOD, ya que la expresión del gen *chrC* fue capaz de complementar la falta de dicha enzima en la mutante de *E. coli* PN134.

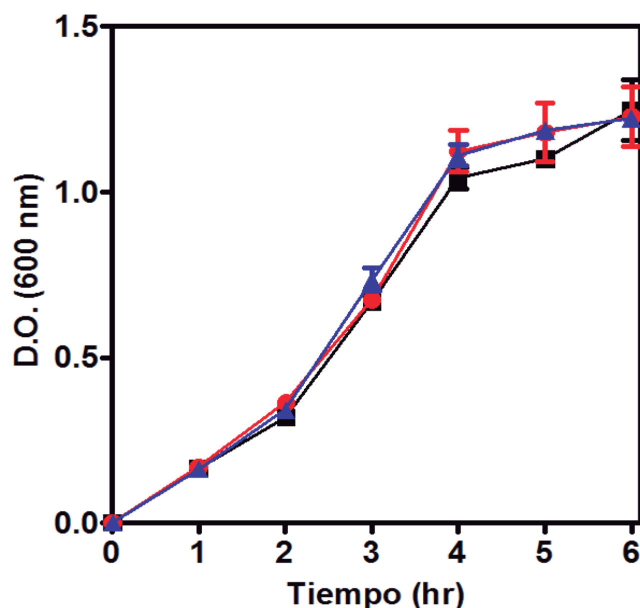

Figura 4. Crecimiento de cepas de *E. coli* en M9 en presencia de los 20 aminoácidos. Cultivos crecidos durante toda la noche en medio mínimo M9 con los 20 aminoácidos se diluyeron 1:25 en medio fresco y se incubaron con agitación constante a 37°C. El crecimiento se determinó midiendo la densidad óptica a 600 nm. Se emplearon las cepas AB1157 (■), PN134 (●) y PNA134 (pGEMT-ChrC) (▲).

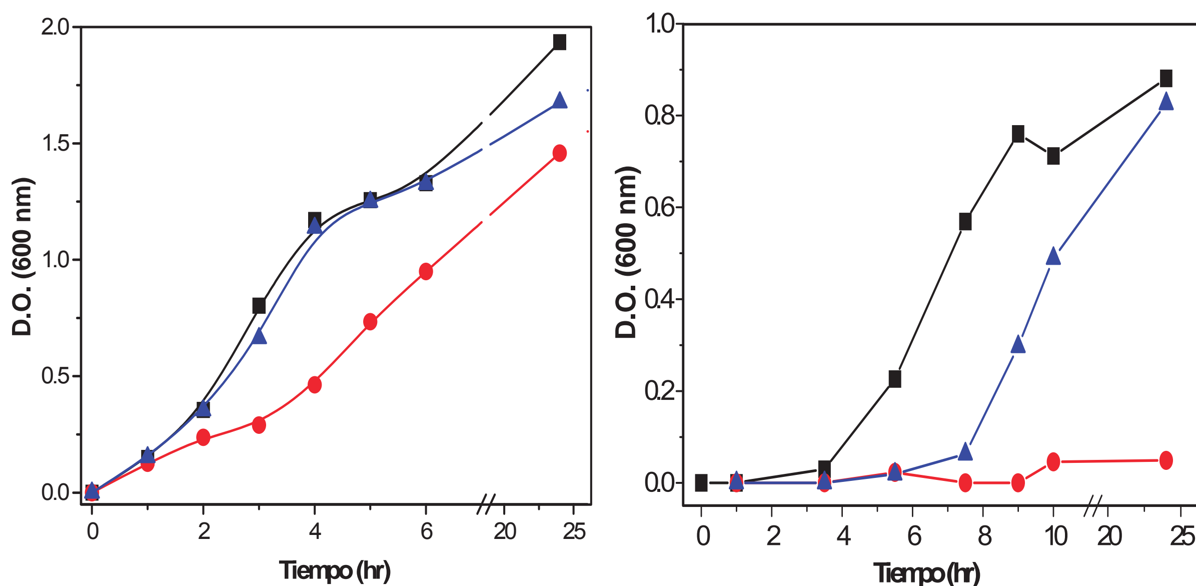

Figura 5. Complementación de la mutante PN134 *sod*$^-$ con el gen *chrC*. Cultivos crecidos durante toda la noche en medio mínimo M9 con los 20 aminoácidos se diluyeron 1:25 en M9 fresco bajo diferentes condiciones y se incubaron con agitación constante a 37°C. El crecimiento se determinó midiendo la densidad óptica a 600 nm. A) Los cultivos se crecieron en medio M9 sin aa aromáticos, B) En medio M9 sin la adición de aa ramificados. Se emplearon las cepas AB1157 (■), PN134 (●) y PNA134 (pGEMT-ChrC) (▲).

En *Shewanella*, ChrC (SOD) representa un mecanismo adicional de resistencia a cromato que probablemente protege a la célula del estrés oxidativo originado por los radicales libres formados durante la reducción del Cr(VI) a Cr(III) (Ramírez-Díaz *et al.*, 2008).

2.- Participación del glutatión en la resistencia a cromato

El cromo hexavalente (Cr(VI)) es reducido intracelularmente a estados de oxidación menores y finalmente a cromo trivalente (Cr(III)), el cual ejerce los principales efectos tóxicos en la célula (Ramírez-Díaz *et al.*, 2008). Uno de los agentes reductores intracelulares del cromato es el glutatión (L- -glutamil-L-cisteinil-glicina) (Aiyar *et al.*, 1991, O'Brien *et al.*, 2001). Durante este proceso de reducción se pueden formar aductos glutatión-Cr(III)-DNA, los cuales son mutagénicos (Voitkun *et al.*, 1998). Por otra parte, se ha reportado que la presencia de compuestos con grupos tiol disminuyen los efectos tóxicos ocasionados por el cromato (Susa *et al.*, 1994). Ackerley (2006) encontró que la resistencia a cromato no es afectada en cepas mutantes de *E. coli* afectadas en la síntesis de glutatión, sin embargo, también se ha propuesto en *E. coli* que este compuesto es necesario para la resistencia a cromato (Helbig *et al.*, 2008). Dado el posible papel dual del glutatión en relación con la toxicidad del cromato, se procedió a analizar la participación del glutatión en el nivel de resistencia a cromato.

Para ello se empleó la cepa de *E. coli* W3110 Δ*gshA* Δ*gshB* que posee deletados los genes *gshA* (-glutamil-cisteína sintetasa) y *gshB* (glutatión sintetasa), por lo que sus niveles de glutatión se encuentran disminuidos (Helbig *et al.*, 2008). La cepa silvestre W3110 y la mutante W3110 Δ*gshA* Δ*gshB* se transformaron con los plásmidos pACYC-ChrA y pACYC-ChrBAC que poseen los genes *chrA* y *chrBAC* de *Shewanella*, respectivamente. La mutante transformada con *chrA* presentó un nivel de resistencia a cromato menor comparado con la cepa silvestre con *chrA* (**Fig. 6A**). Este resultado indica que el glutatión es necesario para la resistencia a cromato. Por otra parte, la mutante que expresa los genes *chrBAC* de igual forma presentó un nivel de resistencia menor que el de la cepa silvestre; sin embargo, a bajas concentraciones las cepas fueron menos afectadas (**Fig. 6B**), lo cual se debe

probablemente a la participación de los genes *chrB* y *chrC* aledaños a *chrA*, en la resistencia a cromato.

Se evaluó la resistencia a cromato en medio sólido, la cepa W3110 (pACYC184) presentó una concentración mínima inhibitoria (MIC) de 0.024mM, el cual fue similar al de la mutante W3110 Δ*gshA* Δ*gshB* que presentó un MIC de 0.018 mM. Sin embargo, la cepas W3110 transformadas con los genes *chrA* y *chrBAC* presentaron un MIC de 0.8 mM y 1.0 mM de cromato, respectivamente, comparado con 0.36 mM y 0.64 mM en la mutante W3110 Δ*gshA* Δ*gshB* (**Cuadro 1**).

Los resultados obtenidos indican que el glutatión es esencial para alcanzar un nivel máximo de resistencia a cromato ya que al encontrarse disminuido sus niveles intracelulares (como en las mutantes), las cepas presentan una mayor sensibilidad a cromato.

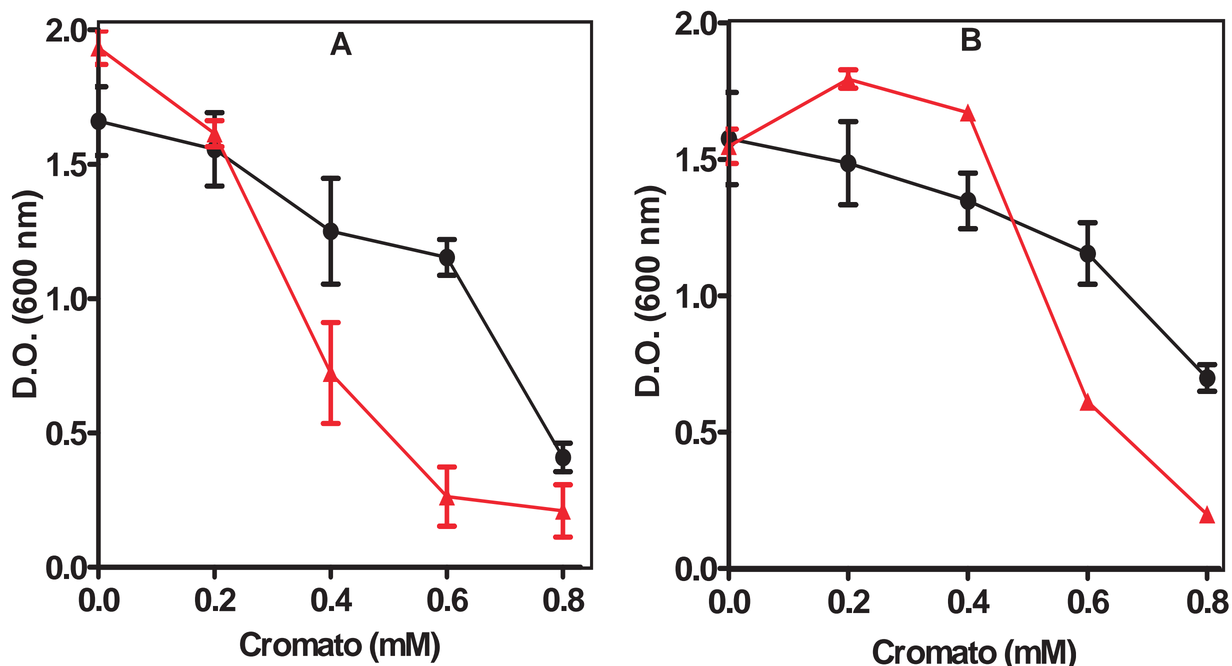

Figura 6. Sensibilidad a cromato en las cepas de *E. coli* mutantes en genes de glutatión. Los cultivos se crecieron de 18-20 h con agitación constante a 37°C en medio mínimo M9 con las concentraciones de cromato indicadas. El crecimiento se determinó midiendo la densidad óptica a 600 nm. A) Susceptibilidad de las cepas W3110 (pACYC-ChrA) (●) y W3110 Δ*gshA*Δ*gshB* (pACYC-ChrA) (▲); B) Susceptibilidad de las cepas W3110 (pACYC-ChrBAC) (●) y W3110 Δ*gshA*Δ*gshB* (pACYC-ChrBAC) (▲). Se muestra el resultado del promedio de una n=5.

El papel principal del glutatión el cual se encuentra en altas concentraciones en la cepa silvestre W3110, aproximadamente 13 mg por g de proteína total (Helbig *et al.*, 2008), es contrarrestar el estrés oxidativo que se forma durante la reducción de cromato (Ramírez-Díaz *et al.*, 2008), y al disminuir su concentración las célula son más susceptibles a dicho estrés.

Cuadro 1. Susceptibilidad a cromato de cepas de *E. coli*

Concentración mínima inhibitoria de cromato (mM)		
Cepas	Aerobiosis	Anaerobiosis
W3110		
pACYC184	0.024 ± 0.008	0.28 ± 0.109
pACYC-ChrA	0.8 ± 0.141	0.56 ± 0.089
pACYC-ChrBAC	1.0 ± 0	0.56 ± 0.089
W3110 Δ*gshA*Δ*gshB*		
pACYC184	0.018 ± 0.002	ND
pACYC-ChrA	0.36 ± 0.089	ND
pACYC-ChrBAC	0.64 ± 0.16	ND

Cultivos crecidos durante toda la noche en medio mínimo M9 se diluyeron 1:100 en medio fresco y se inocularon en cajas de medio mínimo M9 con diferentes concentraciones de cromato. Las placas en aerobiosis se incubaron 24 h., o se colocaron en sistema de anaerobiosis GasPak de BD, y se incubaron 48h a 37°C. ND, no determinado. Se muestra el promedio ± la desviación estándar de un experimento n=4.

3.-Toxicidad del cromato en condiciones anaerobias

Se ha reportado que entre los efectos tóxicos del cromato se encuentra la generación de especies reactivas de oxígeno (Ackerley *et al.*, 2006), por lo que se evaluó la toxicidad del cromato en condiciones de anaerobiosis, ya que en estas condiciones las especies reactivas de oxígeno se encuentran disminuidas. Para ello se emplearon cepas de *E. coli* transformadas con los genes *chrA* o *chrBAC* de *Shewanella*.

La cepa W3110 que sólo contiene el vector presentó un MIC aproximadamente 10 veces mayor en condiciones de anaerobiosis comparado con aerobiosis (**Cuadro 1**). Esto sugiere que el estrés oxidativo originado por las especies reactivas de oxígeno generadas en condiciones aeróbicas incrementa la toxicidad del cromato.

Las cepas que expresan los genes *chrA* y *chrBAC* presentaron un MIC de 0.56 mM de cromato en condiciones de anaerobiosis comparado con 0.8 y 1mM en aerobiosis (**Cuadro 1**). Esto probablemente debido a que la proteína ChrA no se encuentra funcionando en condiciones óptimas ya que ésta emplea el potencial eléctrico transmembranal como fuente de energía para la expulsión de cromato. En anaerobiosis las cepas que expresan la proteína ChrA fueron más resistentes que la cepa sensible sólo con el vector, lo que indica que aún en estas condiciones la proteína ChrA es funcional. Sin embargo, el nivel de resistencia a cromato fue similar entre las cepas que expresan los genes *chrA* y *chrBAC*, indicando que la presencia de los genes aledaños *chrB* y *chrC* no participan en el nivel de resistencia a cromato en condiciones de anaerobiosis.

CONCLUSIONES

-ChrC de *Shewanella* es una superóxido dismutasa (SOD) que representa un mecanismo adicional de resistencia a cromato.

-El glutatión es esencial para un máximo nivel de resistencia a cromato en *E. coli*.

La generación de estrés oxidativo participa en la toxicidad del cromato.

-La proteína ChrA es funcional en anaerobiosis.

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CAPÍTULO IV

Análisis funcional de la proteína SrpC de *Synechococcus elongatus* PCC 7942

RESUMEN

La función del gen *srpC* del plásmido pANL de la cianobacteria *Synechococcus elongatus* PCC 7942 en relación con sulfato y cromato se evaluó en *Escherichia coli*. La expresión del gen *srpC* en la mutante de *E. coli* JW2415-1, auxótrofa a cisteína, no complementó su crecimiento cuando se empleó sulfato como fuente de azufre, lo que sugiere que la proteína SrpC no funciona como un transportador de sulfato. Por otra parte, la expresión del gen *srpC* en la cepa W3110 confirió resistencia a cromato, y originó una captación disminuida del cromato, lo que sugiere que SrpC expulsa el cromato de la célula. La función de SrpC es similar a la de los transportadores de cromato de la superfamilia CHR ya descritos.

ABSTRACT

The *srpC* gene of plasmid pANL from cyanobacterium *Synechococcus elongatus* PCC7942 was expressed in *Escherichia coli* and its role with relation to sulfate and chromate was analyzed. Expression of *srpC* gene was unable to complement the growth of the *E. coli* mutant JW2415-1 strain which is auxotroph to cysteine, when sulfate was used as a sulfur source, which suggests that SrpC is not a sulfate transporter. The expression of *srpC* in W3110 strain conferred chromate resistance and caused a diminished chromate uptake, suggesting that SrpC effluxes chromate ions from the cytoplasm. The function of SrpC is thus similar to that of the chromate ion transporters of the CHR superfamily already described.

INTRODUCCIÓN

Synechococcus elongatus PCC 7942 (anteriormente *Anacystis nidulans* R2) es una cianobacteria unicelular de agua dulce, que posee los plásmidos pANS y pANL. En el plásmido pANL (46,366 bp) se han identificado varios genes cuya expresión es regulada por los niveles de azufre (Nicholson y Laudenbach, 1995, Chen *et al.*, 2008). El gen *srpC* codifica la proteína SrpC, un homólogo de las proteínas ChrA que confieren resistencia a cromato (Nicholson y Laudenbach, 1995). SrpC pertenece a la subfamilia LCHR2 de la superfamilia CHR de proteínas transportadoras de

cromato, la cual está constituida por varias docenas de proteínas distribuidas en los tres dominios de la vida (Díaz-Pérez *et al.*, 2007). Sin embargo, Nicholson y Laudenbach (1995) encontraron que una mutante en el gen *srpC* presentó mayor resistencia a cromato comparado con la cepa silvestre, indicando que posiblemente SrpC participe en la captación de cromato al interior celular. Por otra parte, el gen *srpC* se localiza en una región del plásmido pANL en el que se encuentran genes relacionados con el metabolismo o transporte de azufre (Chen *et al.*, 2008), en contraste con los genes *chrA* homólogos los cuales tienen aledaños genes relacionados con la resistencia a cromato (Juhnke *et al.*, 2002, Díaz-Pérez *et al.*, 2007, Aguilar-Barajas *et al.*, 2008, Branco *et al.*, 2008). Por lo tanto, el objetivo del trabajo fue determinar la función de SrpC en relación con cromato y sulfato.

MATERIALES Y MÉTODOS

Cepas y condiciones de crecimiento. Se empleó la cepa de *E. coli* W3110 para expresión heteróloga del gen *srpC*. La cepa de *E. coli* auxótrofa a cisteína JW2415-1, y la cepa silvestre BW25113 (obtenidas del Genetic Stock Center de *E. coli*) se emplearon para determinar el transporte de sulfato a través de SrpC. Se emplearon los medios de cultivo: caldo Luria Bertani (LB) o medio mínimo M9 (Sigma) adicionado de glucosa 20 mM, $MgSO_4$ 2 mM y $CaCl_2$ 0.1 mM. El K_2CrO_4 fue de los laboratorios Merck.

Técnicas genéticas. Se emplearon técnicas moleculares de acuerdo a protocolos estándar (Sambrook *et al.*, 1989). El cósmido 2F10 que forma parte de una biblioteca genómica de *S. elongatus* (Chen *et al.*, 2008) se empleó como molde para la amplificar el gen *srpC*. Se diseñaron los oligonucleótidos directo 5'-GATCGCTTGGGATCCTAAGACTTTAC-3'¿ y reverso 5'-CGATCCACAAGCTTAGTCGGTTGAG-3', con los sitios de restricción *BamH*I y *Hind*III, respectivamente. Los fragmentos se amplificaron con la enzima *Taq* DNA polimerasa (Fermentas) mediante el siguiente protocolo: desnaturalización 94°C/ 0.5 min, alineamiento 50°C/1 min, elongación 72°C/3min por 30 ciclos. Los fragmentos amplificados se purificaron y se clonaron en el vector pGEM-T Easy (Promega) y posterior subclonación en el vector pACYC184.

Pruebas de susceptibilidad a cromato. Cultivos crecidos durante toda la noche se diluyeron 1:100 en medio fresco con concentraciones variables de cromato y se incubaron por un periodo de 18-20 h a 37°C con agitación constante. La susceptibilidad se midió en función de la absorbencia de los cultivos a 600 nm.

Captación de $^{51}CrO_4^{2-}$ en células completas. Cultivos crecidos durante toda la noche en medio mínimo M9 se diluyeron 1:25 en medio fresco y se incubaron a 37°C hasta fase logarítmica. Se centrifugaron los cultivos para colectar la pastilla celular (5000 rpm/10 min/temperatura ambiente) y se lavó con amortiguador de fosfatos 0.1 M. La pastilla celular se resuspendió en 2 ml del mismo amortiguador precalentado a temperatura ambiente. Se adicionó $^{51}CrO_4^{2-}$ a una concentración final de 10 μM (0.2 mCi). El transporte se determinó como se describe en Aguilar-Barajas *et al.* (2008).

RESULTADOS Y DISCUSIÓN

Requerimiento de sulfato. Dado que el gen *srpC* se encuentra en una región del plásmido pANL con genes involucrados en el metabolismo de azufre (Chen *et al.*, 2008), y que aparentemente participa en la captación de cromato, un oxianión tóxico para la célula que es estructuralmente similar al oxianión esencial sulfato, se evaluó la posibilidad de que la proteína SrpC de *S. elongatus* participe en el transporte de sulfato al interior de la célula. Para ello el gen *srpC* del plásmido pANL se amplificó y clonó en el plásmido pGEMT-Easy. El plásmido recombinante pGEMT-SrpC se transfirió a la cepa de *E. coli* JW2415-1, la cual tiene una mutación en el gen *cysA*, que codifica la ATPasa que energiza la permeasa de sulfato CysPTWA (revisado en Aguilar-Barajas *et al.*, 2010, anexo 2) por lo que es incapaz de emplear sulfato como fuente de azufre. La expresión del gen *srpC* en la cepa mutante JW2415-1 fue incapaz de complementar el crecimiento de la mutante en medio mínimo con sulfato como fuente de azufre (**Fig. 1**); su comportamiento fue similar al de la cepa transformada únicamente con el vector. En contraste, la cepa silvestre BW25113 presentó un crecimiento normal desde las concentraciones más bajas de sulfato empleadas (**Fig.1**). El comportamiento de las cepas fue similar aun en concentraciones más elevadas de sulfato (10mM) (datos no mostrados). Estos resultados indican que SrpC no transporta al interior de la célula el oxianión esencial

sulfato, contrario a lo que se sugirió anteriormente de que podría funcionar en la captación de sulfato dado la similitud estructural que tiene este ion con el cromato.

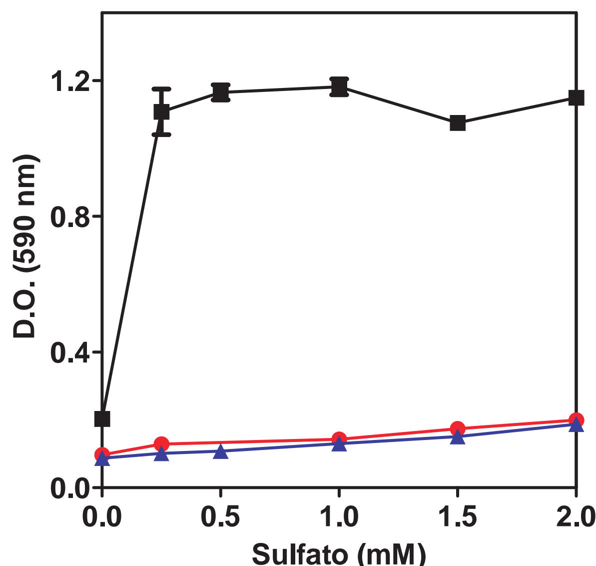

Figura 1. Requerimiento de sulfato en cepas de *E. coli*. Cultivos crecidos durante toda la noche en medio LB se diluyeron 1:100 en medio M9 con concentraciones variables de sulfato y se incubaron por 18-20 h a 37°C con agitación constante. El crecimiento se midió en función de la absorbencia de los cultivos a 590 nm. Se emplearon las cepas BW25113 (■), JW2415-1 (pGEMT) (●) y JW2415-1 (pGEMT-SrpC) (▲).

Resistencia a cromato. Para determinar la función de SrpC en relación con cromato se clonó el gen *srpC* en el vector de bajo número de copias pACYC184. Este vector se empleó anteriormente para demostrar la participación en la resistencia a cromato del gen homólogo *chrA* del plásmido 1 de *Shewanella* sp ANA-3 (Aguilar-Barajas *et al.*, 2008). El plásmido pACYC-SrpC se transfirió a la cepa de *E. coli* W3110 y se evaluó la resistencia a cromato. La expresión de SrpC confirió moderadamente un mayor nivel de resistencia a cromato comparado con la cepa transformada únicamente con el vector (**Fig. 2A**). El nivel de resistencia conferido por la proteína SrpC fue menor al conferido por las proteínas homólogas ChrA de *P. aeruginosa* y de *Shewanella* (Cervantes *et al.*, 1990, Aguilar-Barajas *et al.*, 2008).

Captación de cromato. Para evaluar el mecanismo de acción de la proteína SrpC se determinó la captación de cromato en células de *E. coli*. La cepa W3110 (pACYC-SrpC) presentó una captación de cromato menor en comparación con la cepa control únicamente con el vector W3110 (pACYC184) (**Fig. 2B**). La captación de cromato

por la cepa W3110 (pCYC-SrpC) fue tres veces menor que el control sensible, una diferencia en captación que al igual que el nivel de resistencia es menor al reportado para otras proteínas ChrA (Pimentel *et al.*, 2002, Aguilar-Barajas *et al.*, 2008). La cepa W3110 (pACYC-SrpC) de igual forma presentó una captación de cromato menor al variar la concentración de sulfato del medio de crecimiento, y la concentración de cromato empleada en el transporte. Este resultado sugiere que aunque con menor eficiencia, la proteína SrpC expulsa al ion cromato del citoplasma.

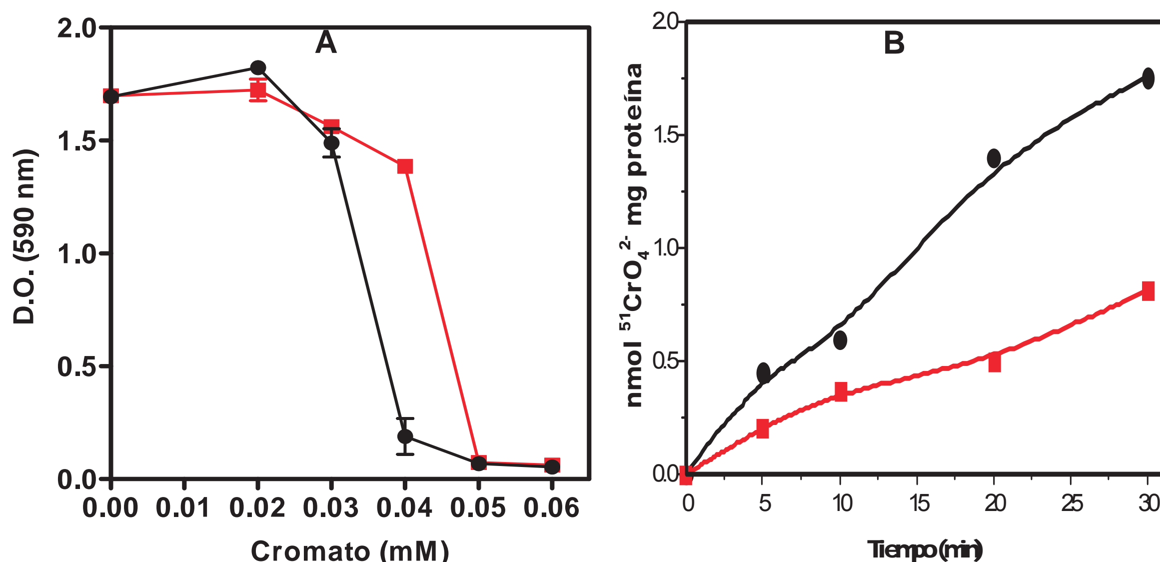

Figura 2. Resistencia y captación de cromato de celulas de *E. coli* expresando el gen *srpC*. Se emplearon las cepas W3110 (pACYC184) (●) y W3110 (pACYC-SrpC) (■). A) La resistencia a cromato se determinó como se describe en Materiales y Métodos. Experimento representativo por duplicado. B) Captación de cromato. Cultivos crecidos toda la noche se diluyeron 1:25 en medio fresco y se incubaron hasta fase logarítmica. El transporte de $^{51}CrO_4^{2-}$ se determinó como se describe en Materiales y Métodos. Datos representativos de dos repeticiones con resultados similares.

El cromato ingresa a la célula empleando el sistema de transporte del sulfato por lo que cuando hay elevadas concentraciones de cromato puede disminuir la cantidad sulfato que entra a la célula, ocasionando que experimente una condición de deficiencia de azufre (Brown *et al.*, 2006, Pereira *et al.*, 2008), haciendo que la célula sea más susceptible al cromato. El hecho de que el gen *srpC* se encuentra en una región con genes relacionados con el metabolismo de azufre (Chen *et al.*, 2008),

probablemente indica que la expresión de SrpC puede estar regulada en conjunto con dichos genes por los niveles de azufre, ayudando de esta forma a contrarrestar los efectos tóxicos del cromato. De igual forma en la bacteria hiperresistente a cromato *Pseudomonas corrugata* 28, se identificó el gen *oscA* localizado río arriba del grupo de genes *sbp, cysTWA* que codifican la permasa de sulfato, cuya mutación abate la resistencia a cromato (Viti *et al.*, 2009).

CONCLUSIONES

La proteína SrpC no funciona como un transportador de sulfato ya que fue incapaz de complementar una mutante de *E. coli* auxótrofa a cisteína.

La proteína SrpC promueve una baja captación del cromato por lo que probablemente expulsa el cromato del citoplasma.

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CAPÍTULO V

Introducción al articulo: Short-chain chromate ion transporter proteins from *Bacillus subtilis* confer chromate resistance in *Escherichia coli*

La superfamilia CHR de transportadores de cromato está compuesta de dos familias de proteínas: la familia SCHR, constituida de proteínas pequeñas monodominio (200 aa), y la familia LCHR de proteínas grandes bidominio (400-600 aa) (Díaz-Pérez *et al.*, 2007). Las proteínas SCHR son parejas de proteínas que están codificadas por genes localizados en tandem (Díaz-Pérez *et al.*, 2007). El genoma de *Bacillus subtilis* posee una pareja de proteínas SCHR, las cuales son codificadas por los genes adyacentes *chr3N* y *chr3C*. Recientemente se encontró que los genes *chr3N* y *chr3C* de *B. subtilis* son transcritos a través de un RNA mensajero bicistrónico y ambos genes son necesarios para conferir resistencia a cromato cuando se expresan en *E. coli* (Díaz-Magaña, 2009). El objetivo de este trabajo fue determinar el mecanismo de resistencia a cromato de las proteínas SCHR de *B. subtilis*.

Para determinar el mecanismo de resistencia a cromato conferido por las proteínas SCHR, se midió el transporte de cromato $^{51}CrO_4^{2-}$ en la cepa de *E. coli* (pAChr3N-C), que contiene los genes *chr3N* y *chr3C* clonados en tándem en el vector pACYC184. La expresión de los genes *chr3N-chr3C* en las células de *E. coli* ocasionó una captación disminuida de cromato en comparación de la cepa únicamente con el vector, cuando se crecieron en medio mínimo M9 con 0.05 mM ó 0.2 mM de sulfato. La menor captación de cromato en las células que expresan los genes *chr3N-chr3C* sugiere que las proteínas pequeñas SCHR funcionan por un mecanismo de expulsión de cromato similar al descrito para los homólogos LCHR ya caracterizados (Pimentel *et al.*, 2002, Aguilar-Barajas *et al.*, 2008).

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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′-GCTCTTAAGCTTGA GGAAGAGC-3′; forward primer corresponding to a sequence located 220 bp upstream of the start codon of *chr3N*) and Bsu_C_R (5′-GAAGGTCCAGGA TCCTGTTTGG-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′-GAGAATGGAT CCTTTGAGAGCC-3′; reverse primer corresponding to a sequence located 130 bp downstream of the stop codon of *chr3N*) and Bsu_C_D (5′-CAATTGTTGC AGGTAAGCTTGGTG-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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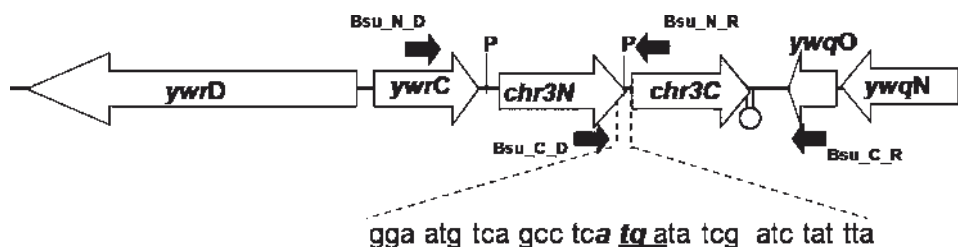

FIG. 1. Arrangement of SCHR protein-encoding genes in the *B. subtilis* genome. White arrows indicate genes and the direction of transcription. The locations of predicted promoter sequences are marked by a "P," and a predicted transcription terminator is indicated by a hairpin. Shaded arrows signal the 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-

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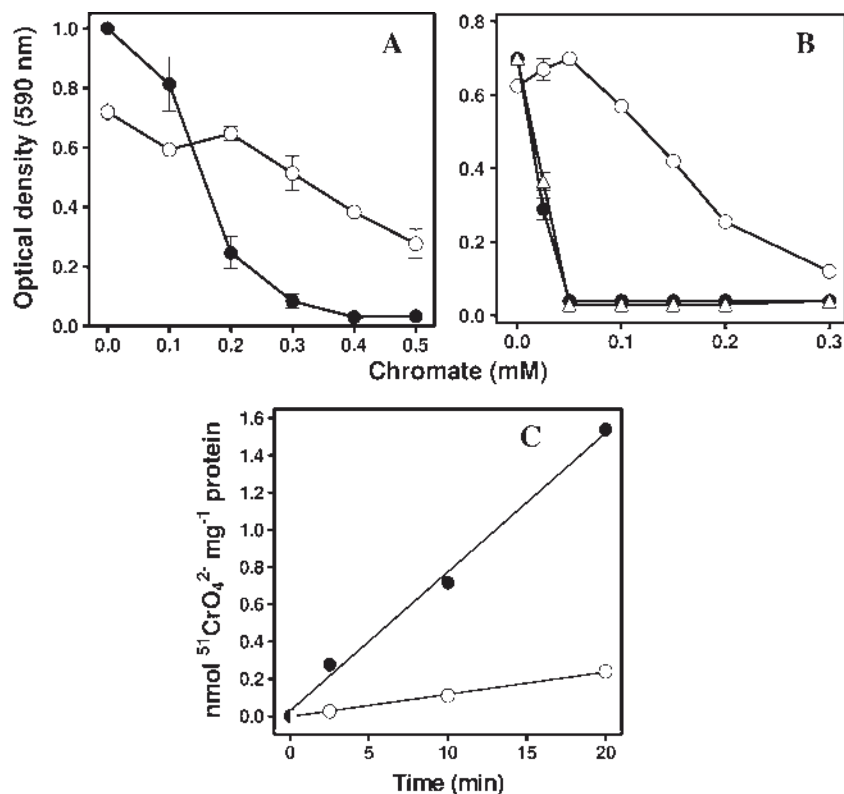

FIG. 2. Chromate susceptibility and chromate uptake by bacterial strains. (A and B) Cultures were grown in NB (A) or in M9 minimal medium (B) with the indicated concentrations of K_2CrO_4 for 18 h at 37°C, and the optical density at 590 nm was recorded. (A) Symbols: ●, *E. coli* W3110, and ○, *B. subtilis* 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-

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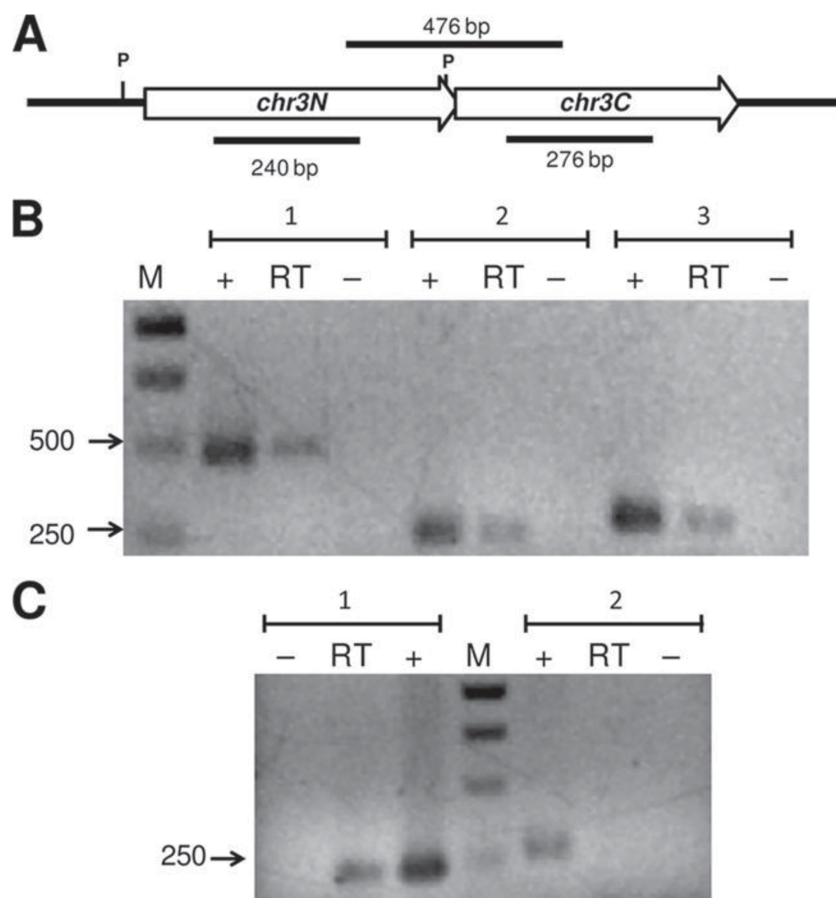

FIG. 3. RT-PCR analysis of the *chr3N* and *chr3C* genes. (A) White arrows indicate the *chr3N* and *chr3C* genes, and the locations of putative promoters (P) are marked. Horizontal lines depict the sizes (given in base pairs) and the locations of predicted cDNA reverse transcripts synthesized from total 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

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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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9.-RESUMEN GENERAL DE RESULTADOS

1. De los 11 residuos básicos de ChrA de *P. aeruginosa* mutados, Arg54 y Arg68, localizados en el asa periplásmica P1, y Arg340, localizado en el asa citoplásmica C5, se identificaron como esenciales para la resistencia a cromato (identificandose hasta el momento ocho residuos básicos esenciales).
2. La proteína homóloga ChrA de *Shewanella* sp. ANA-3 confiere resistencia a cromato en un amplio rango de huéspedes, en contraste con ChrA de *P. aeruginosa* y ChrA de *C. metallidurans* que sólo funcionan en el huésped nativo.
3. Las cepas de *E. coli* que expresan la proteína SrpC de *Synechococcus elongatus* presentaron un moderado nivel de resistencia a cromato y una menor captación de cromato.
4. Las cepas de *E. coli* que expresan las proteínas Chr3N y Chr3C de *B. subtilis* presentaron una acumulación disminuida de cromato.
5. Los transportadores CHR de *P. aeruginosa*, *Shewanella* sp. y *S. elongatus* no transportan sulfato.

10.-CONCLUSIÓN

Los transportadores de la superfamilia CHR confieren resistencia a cromato por un mecanismo similar que expulsa el oxianión del citoplasma, y no transportan al sulfato.

11.- PERSPECTIVAS

ChrA de *P. aeruginosa*.

Una de las perspectivas más importantes relacionadas con la proteína ChrA de *P. aeruginosa* es determinar la expresión de la proteína ChrA en las diferentes mutantes, ya que aun cuando se han identificado un gran número de aminoácidos esenciales no se puede concluir acerca de su papel en la proteína.

Debido a que a pesar de haber empleado diferentes metodologías no se ha purificado la proteína ChrA, la siguiente estrategia a emplear es el diseño y síntesis de diferentes péptidos sintéticos de diferentes regiones de la proteína, los cuales se emplearan para la síntesis de anticuerpos y así evaluar la expresión de ChrA en las mutantes.

Posterior a la determinación de la expresión, el siguiente paso es determinar la función de los aminoácidos esenciales en la proteína con pruebas más sensibles comparadas con las curvas de susceptibilidad. Una de las estrategias ampliamente usadas es mutagenizar los residuos a analizar por cisteína (cysteine scanning), y posteriormente evaluar la reactividad de la cisteína en presencia y/o ausencia del sustrato con diferentes reactivos específicos para grupos tiol (Yan y Maloney, 1995, Frillingos *et al.*, 1998, Fu *et al.*, 2001, Ye y Maloney, 2002). Mediante esta técnica se pueden determinar residuos localizados en el sitio de unión del sustrato y en la ruta de translocación, además permite obtener una amplia información acerca de la estructura de la proteína en la membrana, ya que se pueden determinar interacciones entre las hélices transmembranales y si existen cambios conformacionales en la proteína posteriores a la unión del sustrato. Esta estrategia es una alternativa para dilucidar la estructura de una proteína de membrana cuando se dificulta su purificación y cristalización.

ChrA de *Shewanella* sp. ANA-3.

Como parte de la caracterización de la proteína ChrA de *Shewanella*, determinar la topología de la proteína, para tener más información acerca de la estructura de ChrA. Dado que no se tienen datos acerca del transporte de cromato por ChrA de *Shewanella*, determinar los parámetros cinéticos del transporte, empleando

vesículas invertidas de membrana ó células completas, para establecer si ChrA de *Shewanella* transporta cromato con mayor eficiencia. Con los resultados de estos análisis hacer una comparación con ChrA de *P. aeruginosa* y de *C. metallidurans* que permitan dilucidar más acerca de ChrA de *Shewanella,* por ejemplo, su capacidad de funcionar en varios huéspedes.

Aun cuando se ha propuesto que ChrC es una superóxido dismutasa, falta medir directamente la actividad de la enzima, ya sea en gel ó espectrofotométricamente, y determinar a que clase pertenece, si es Mn-SOD o Fe-SOD:

Hasta el momento, el estudio se ha enfocado en los genes *chrA* y *chrC* de *Shewanella*, y no se ha analizado la función del gen *chrB,* otra perspectiva sería determinar su función. En *C. metallidurans* el gen *chrB* es esencial para la resistencia a cromato y se ha propuesto que funciona como un activador dependiente de cromato (Juhnke *et al.*, 2002), en contraste, en *P. aeruginosa* (Cervantes *et al.*, 1990), y *Shewanella* sp. ANA-3 (Aguilar-Barajas *et al.*, 2008), el gen *chrB* no es esencial para la resistencia a cromato.

Los genes *chrBAC* clonados en el vector de alto numero de copias pGEMT, presentaron mayor sensibilidad a cromato en comparación con el gen *chrA* y *chrAC* clonados en el mismo vector, por lo tanto, evaluar la resistencia a cromato de los genes *chrBA* en vectores de alto y bajo número de copias para evaluar la influencia de ChrB en el nivel resistencia. Determinar de igual forma la expresión del gen *chrA* en presencia y ausencia del gen *chrB*, y en presencia y ausencia de cromato, para establecer el papel de *chrB*.

Por otra parte, ChrB posee un motivo similar a una rodanasa (**C**XXXXX**R**) (Bordo y Bork, 2002), una enzima encargada de detoxificar el cianuro, y que se ha especulado pude participar en la disociación de los complejos cromo-glutatión (Juhnke *et al.*, 2002). Dicho motivo de igual forma se encuentra en la proteína arsenato reductasa Acr2p (Mukhopadhyay y Rosen, 2002). Determinar si ChrB es una rodanasa, una de las formas de determinar la actividad es medir espectrofotométricamente la formación del complejo colorido tiocianato férrico.

De igual forma, analizar la secuencia de ChrB para establecer la presencia de posibles regiones de unión a membrana, ya que se ha especulado que la proteína

ChrB posiblemente se encuentra unida a la membrana. Establecer las condiciones de purificación de ChrB de *Shewanella*, purificar la proteína y realizar ensayos para evaluar su posible unión a membrana, al ión cromato, y a la proteína ChrA.

SrpC de *Synechococcus*

Se demostró la función de la proteína SrpC como un transportador de cromato, sin embargo, falta determinar la relación del sulfato con esta proteína. Por lo tanto evaluar la resistencia a cromato y la expresión del gen *srpC* de células crecidas en diferentes concentraciones de sulfato.

Evaluar si SrpC es un transportador especifico de cromato, para lo cual medir resistencia a otros compuestos.

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ANEXO 1

Resistencia a metales pesados en Pseudomonas

omo arte de las a t dades a ad m as del rograma de Do torado se me as gn la reda n de una re s n b bl ogr f a sobre la res sten a a metales esados en seudomonas. n esta re s n art aron la Dra. art a . am re D a , el Dr. tor eros osas y el Dr. arlos er antes, asesor de la tes s.

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Chapter 9
Heavy Metal Resistance in Pseudomonads

Esther Aguilar-Barajas, Martha I. Ramírez-Díaz, Héctor Riveros-Rosas, and Carlos Cervantes

9.1 Introduction

The metabolic diversity of the genus *Pseudomonas* (and related bacterial species, called collectively pseudomonads) has attracted researchers to study this versatile microbial group. The ability to thrive in hostile environments, aided by a notable capacity to degrade or tolerate a wide variety of natural and synthetic compounds, results from the possession of highly adapted genomes. About 25 genomes from pseudomonad strains have been sequenced to date, representing eight different species from varied habitats. Genomic analyses confirm that pseudomonads evolved complex enzymatic strategies, delicate genetic regulatory switches, and efficient transport systems, to keep pace in ever-changing environments. These adaptive mechanisms include those conferring resistance to toxic compounds such as antibiotics and the ions derived from heavy metals and metalloids. Transport systems able to actively efflux metal ions out from the cytoplasm or the periplasmic space are a key strategy to withstand heavy metal toxicity. As with other bacteria, heavy metal resistance genes in pseudomonads may reside either in the chromosome or within plasmids.

In this chapter, the information concerning the strategies used by pseudomonads to tolerate heavy metals is summarized. It should be noted that some of these resistance mechanisms have been assigned to pseudomonads only by the finding of homologous genes and operons when compared with characterized genes from sequenced genomes of different bacteria. In other cases, biochemical evidence for specific heavy metal resistance systems has been directly provided by the analysis of genes from pseudomonads.

A first compilation on heavy metal resistance mechanisms in pseudomonads appeared almost two decades ago [1], but an overwhelming amount of information has accumulated since, notably by the advent of the genomic era. More recently, a monograph book covered the interactions of heavy metals with the wider microbial

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world [2]; reference to specific chapters will be given below for studies related to pseudomonads.

For this review, the heavy metal protagonists have been divided into three main groups: (i) micronutrient cations (copper, cobalt, nickel and zinc), (ii) toxic cations (cadmium, lead and mercury), and (iii) toxic oxyanions (arsenate/arsenite, chromate and tellurite). A final recount includes other less-studied toxic ions (silver, tin, selenium) for which some information exists in pseudomonads. For each case, a brief account on the metal(loid) toxicity mechanisms is followed by a description of the resistance strategies reported in pseudomonads or (when missing) the best-studied systems uncovered in related bacteria. A scrutiny of the genomes of *Pseudomonas aeruginosa* [3, 4] and *Pseudomonas putida* [5, 6] already showed the presence of numerous metal resistance determinants, including members of the main transporter families: resistance-nodulation-cell division (RND), cation diffusion facilitator (CDF), major facilitator superfamily (MFS), and P-type ATPases able to efflux toxic metal cations or oxyanions. Regulatory systems for bacterial heavy metal resistance, as for other adaptive strategies, are of paramount importance for cell economy. Expression of the corresponding genetic determinants is subjected to delicate control mechanisms, commonly acting at the transcriptional level [7; reviewed in 8]. These regulatory systems will be mentioned in this review but, for space reasons, will not be detailed.

9.2 Micronutrient Cations

As most living organisms, pseudomonads require the essential micronutrient cations derived from copper, cobalt, nickel and zinc, used mainly as enzyme cofactors and regulatory effectors. For these purposes, divalent cations form complexes with diverse ligands within the cells. Higher concentrations of these transition metals, however, may exert toxic effects on most cells as harmful complexes may be formed with varied biomolecules. This dual behavior has made it necessary for bacteria to develop strict homeostasis mechanisms in order to avoid metal toxicity, while allowing intracellular basal levels of the essential ions. Homeostasis commonly includes transmembrane uptake and efflux systems that carefully regulate intracellular cation levels. This review emphasizes on those pseudomonad systems devoted to tolerate the noxious effects of toxic divalent cations, not considering the physiological mechanisms for micronutrient acquisition and use. Also, systems for the homeostasis of essential but almost not toxic cations (i.e. iron and manganese) will not be considered.

9.2.1 Copper

Copper is an essential metal, mainly required by aerobic cells as a cofactor for electron transport and redox enzyme systems [9]. Copper exists in the cytoplasm in the Cu(I) reduced state, being its ability to undergo redox Cu(II) to Cu(I) transformations partly responsible of its toxic properties. Additional toxicity effects derive

from the ability of copper to displace other metals (i.e. Ni^{2+}, Co^{2+}, Mn^{2+}, Zn^{2+}) from essential complexes as well as to unspecifically bind to biomolecules [10]. Bacterial copper transport and homeostasis has been widely studied in *Escherichia coli* and in Gram-positive *Enterococcus hirae* (reviewed in [9, 11–15]) and will not be treated here. Some copper resistance systems related to pseudomonads will be next described.

The *copABCD* operon from *Pseudomonas syringae* plasmid pPT23D was one of the first bacterial copper-resistance systems analyzed [16]. pPT23D was found in a copper-resistant *P. syringae* pv. *tomato* strain isolated from copper-treated tomato fields [17]. Unlike other cation resistance mechanisms, the *cop* operon encodes a copper-sequestering system that prevents copper ions from entering the cytoplasm (Fig. 9.1A). CopA and CopC are periplasmic copper-binding proteins able to capture 11 and 1 copper atoms per polypeptide, respectively [18]. CopA also displays

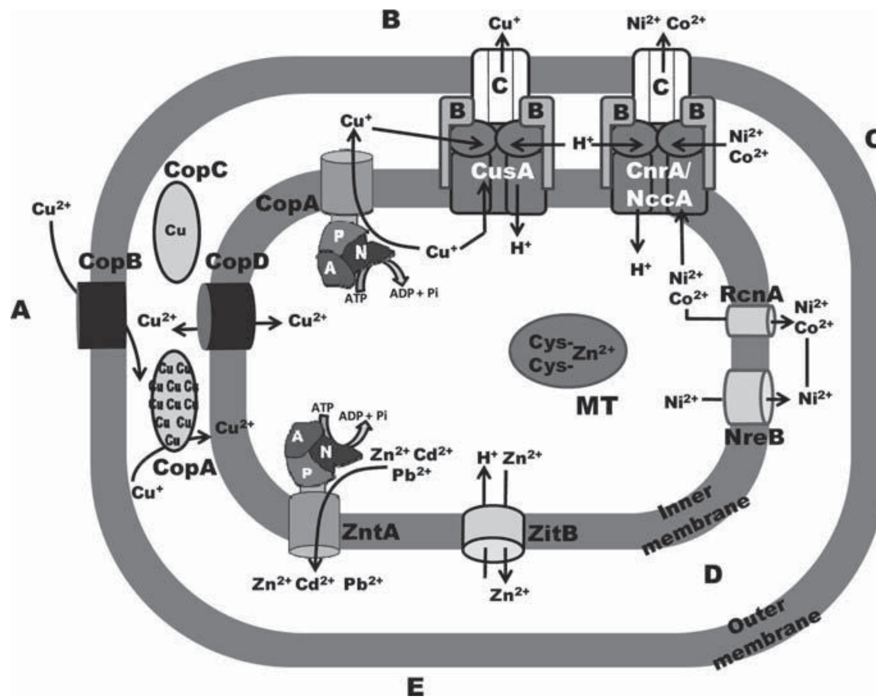

Fig. 9.1 Mechanisms of resistance to essential cations in pseudomonads. *A*, Cop copper binding system. *B*, P-type ATPase CopA and RND complex CusCBA. *C*, nickel/cobalt resistance systems. RND complexes CnrCBA and NccCBA; MFS transporters RcnA and NreB. *D*, zinc metallothioneins (MT). *E*, zinc resistance systems. P-type ATPase ZntA and CDF transporter ZitB. P-type ATPases domains shown are: P, phosphorylation domain; N, nucleotide-binding domain; A, activator and phosphatase domain. The functions of the resistance systems are described in the text. Note that CopA in *A* is a periplasmic multi-copper oxidase of the COG2132 protein family whereas CopA in *B* is a P-type ATPase of the COG2217 protein family. These two non-homologous proteins received the same name

a multi-copper oxidase activity, transforming Cu(I) to Cu(II), similar to that of CueO from *E. coli* [19] and may protect periplasmic enzymes from copper damage. CopC is probably a chaperone which delivers copper to CopD, an inner membrane protein with eight transmembrane segments. Outer membrane protein CopB also binds Cu^{2+} and is proposed to function in concert with CopD in Cu^{2+} uptake [20]. Copper-inducible expression of the *cop* operon is regulated by a chromosomally-encoded repressor and by a plasmid-borne two-component CopR/S system [21]. Chromosomal homologs of CopA and CopB have been identified in many pseudomonads [22], whereas CopC and CopD are less common and seem to be auxiliary determinants for optimal copper resistance. An additional gene, transcribed from a different promoter, encodes the small periplasmic CopE protein, which is related to PcoE from *E. coli* and to SilE from the *Salmonella* silver-resistance operon; as PcoE, CopE seems to bind Cu(I) and may function as a copper chaperone [9]. The other pseudomonad *cop* genes also show sequence similarity with the corresponding genes from the plasmid-mediated *E. coli* copper-resistance *pco* operon, although the latter system catalyzes the efflux of copper rather than its binding [23].

Other potential copper-resistant determinants studied in enterobacteria have been identified from the sequenced genomes of pseudomonads. For example, the genomes of *P. aeruginosa* [3] and *P. putida* [6] possess homologs of the CusCBA system, a proton-driven RND transporter which effluxes Cu^+ (and Ag^+) from the cytoplasm [24] (Fig. 9.1B), and of SilP, a P-type ATPase which extrudes Ag^+ (and probably Cu^+) [25]. Also, a homolog of the widespread P-type ATPase CopA [26], able to efflux copper from the cytoplasm, has been located in the *P. aeruginosa* genome [27] (Fig. 9.1B). A transcriptomics analysis of *P. aeruginosa* PAO1 showed that a P-type ATPase (ORF PA3920), three RND transporters (PA1436, PA2520, and PA3522), and two CDF family members (PA0397 and PA1297) were up-regulated in response to copper exposure [28]. One of the RND determinants encodes the *czrCBA* system [29], mentioned below because it confers Cd^{2+}, Zn^{2+} and Co^{2+} resistance by an efflux mechanism. The CDF systems encode homologs of the CzcD and RzcB transporters which confer resistance to divalent cations in other bacteria [30]. It is possible that some of these systems also efflux copper. These findings confirm that efflux systems constitute a major strategy for copper homeostasis as well as a main protection barrier for pseudomonads against copper toxicity.

9.2.2 Cobalt and Nickel

Cobalt and nickel are similar transition metals of oxidation state II. They play essential roles for microorganisms as cofactors for many diverse metalloenzymes. Thus, uptake and homeostasis systems for these micronutrient divalent cations must exist in all bacteria [31–33]. Bacterial $Co^{2+}$and Ni^{2+} homeostasis systems have been studied with great detail in *E. coli* and to a lesser extent in other microorganisms [33]. As with copper, high levels of Co^{2+} and Ni^{2+} may exert toxic effects on microorganisms [23, 34]. The main mechanism of Co^{2+} and Ni^{2+} toxicity probably relates to their potential interference with iron (and possibly manganese) homeostasis. As

for most divalent metal cations, the main tolerance bacterial strategies to cope with excess Co^{2+} and Ni^{2+} are usually associated with membrane efflux systems.

Cobalt and nickel resistance systems have not been studied directly in pseudomonads. However, the identification of homologous genes for metal cation resistance in the genomes of species of *Pseudomonas* indicates that these bacteria have the potential to display tolerance mechanisms against Co^{2+} and Ni^{2+}. Co^{2+} resistance is usually accompanied by resistance either to Ni^{2+}, Cd^{2+}, or Zn^{2+}.

Two RND systems from megaplasmids of *Cupriavidus metallidurans* (previously named *Alcaligenes eutrophus* and *Ralstonia metallidurans*), the *cnrCBA* and *nccCBA* operons, confer resistance to both Co^{2+} and Ni^{2+} [4, 35, 36]. The CnrCBA system from plasmid pMOL28 is formed by the three typical RND polypeptides: CnrA, an inner membrane transporter, CnrC, located in the outer membrane, and CnrB, a membrane fusion protein bridging the periplasmic space [37]. This tripartite complex functions as a chemiosmotic pump driven by the proton-motive force that effluxes the cations probably from the cytoplasm to the periplasm and then to the outside (Fig. 9.1C). Additional *cnrYXH* genes regulate the expression of the efflux pump [38, 39]. The NccCBA complex is structurally and functionally similar to CnrCBA and is also regulated by corresponding *nccYXH* genes [40] (Fig. 9.1C). Unlike Cnr, the Ncc system, besides Co^{2+} and Ni^{2+} resistances, also confers resistance to Cd^{2+}.

The CzcCBA complex from plasmid pMOL30 of *C. metallidurans*, the first characterized RND system related to heavy metals, confers resistance to Cd^{2+}, Co^{2+} and Zn^{2+} [4, 41, 42] and will be described below in the cadmium section. A variant of this system, the Czn complex from *Helicobacter pylori*, has a distinct substrate specificity, exporting Cd^{2+}, Zn^{2+} and Ni^{2+} [43]. Also located in the Czc determinant is CzcD, a member of the CDF family, originally reported as a regulatory protein [44] but later found to confer low resistance to Co^{2+}, Cd^{2+} and Zn^{2+} [30]. Similar CDF transporters related to cation efflux, DmeF and FieF, have been identified in the *C. metallidurans* chromosome [45]. An interesting interplay between the Czc/Cnr RND systems and CDF proteins has been reported. CDFs seem to first export the cations from the cytoplasm to the periplasm and then RNDs pump them from the periplasm to the outside [45– 47].

Transporters of the MFS group have been also assigned functions in Co^{2+} or Ni^{2+} efflux. This includes the first MFS protein found to be involved in metal transport, NreB of *Achromobacter xylosoxidans*, only transporting Ni^{2+} [48], and RcnA from *E. coli* which effluxes Co^{2+} and Ni^{2+} [49] (Fig. 9.1C). NreB and RcnA are histidine-rich polypeptides displaying a distinct topology of 12 and six transmembrane segments, respectively. The RcnR repressor regulates the expression of RcnA [50]. RcnA has been found to be also controlled by the global regulator Fur and was proposed to function as a connector of cobalt, nickel and iron homeostasis [51]. The P-type ATPase ZntA from *E. coli*, which confers cation resistance by the efflux of Zn^{2+}, Cd^{2+} and Pb^{2+} (see the sections of these metals below), is stimulated by Co^{2+} and Ni^{2+} [52] and may also efflux these ions although with little efficiency.

The genomes of *P. aeruginosa* [3] and *P. putida* [6] contain structural and regulatory *czc* genes (two copies in *P. putida*) which are probably involved in the efflux of

Co^{2+} and other divalent cations. Accumulation of Ni^{2+} as a resistance strategy has been reported in strains of *P. aeruginosa* [53] and *P. putida* [54], but the mechanisms involved have not yet been studied.

9.2.3 Zinc

A widely distributed enzyme cofactor, zinc displays affinity for ligands possessing oxygen, nitrogen or sulfur. As mentioned for copper, toxicity of zinc is associated with its ability to replace other metals (i.e. Ni^{2+}, Co^{2+}, Mn^{2+}) from enzymes or by forming complexes with other biomolecules. Zinc homeostasis has been studied with detail in several bacterial species [55]. Zinc occurs naturally as the divalent cation Zn^{2+} and the level of the metal is regulated by processes of Zn^{2+} uptake, sequestration by metallothioneins (MT), and efflux from the cytoplasm [56].

The pumps of Zn^{2+} efflux are usually not restricted to Zn^{2+} as a substrate, and may also catalyze transport of other divalent cations [56]. Zn^{2+} is exported across the cytoplasmic membrane by the P-type ATPase ZntA, described in *E. coli* [57], and by its closest homologue, CadA, first described in *Staphylococcus aureus* [58]. ZntA was the first example described of a specific Zn^{2+} transporting protein in *E. coli* [57], but now is known to transport a broad range of soft metal ions, including Cd^{2+}, Pb^{2+}, Ni^{2+}, Co^{2+} and Cu^{+} [59] (Fig. 9.1E). ZntA is a protein of 732 amino-acid residues with all the characteristics of a soft metal ion-translocating P-type ATPase, which include a cysteine-rich hydrophilic amino-terminal region that contains a single metal-binding motif $GMDC_{AA}C$ [56]. ZntA is regulated by ZntR (the *zntR* gene is located in another region of the chromosome in *E. coli*), that belongs to the MerR family of regulators. The expression of ZntA is induced by Zn^{2+}, Cd^{2+} and Pb^{2+}, being Cd^{2+} the more effective inducer [60, 61]. ZntR functions as a dimeric protein and tightly binds to its cognate promoter, P_{zntA}, located upstream of the *zntA* start codon [56]. A well-characterized system of Zn^{2+} transport is the CzcCBA complex of *C. metallidurans* [4], described below in the cadmium section.

Another protein that has been associated with zinc resistance is ZitB, a CDF transporter that mediates efflux of Zn^{2+} in *E. coli* [62] (Fig. 9.1E). ZitB is closely related to CzcD that transports Cd^{2+}, Zn^{2+} and Co^{2+} [30] (described in the cadmium section). The expression of the *zitB* gene leads to a significant increase in Zn^{2+} resistance and to reduced Zn^{2+} accumulation in *zntA*-disrupted *E. coli* cells [62]. It has been proposed that ZitB contributes to Zn^{2+} homeostasis at low concentrations of zinc, while ZntA is required for growth at higher concentrations [62].

Zinc-regulated genes have been analyzed in *Pseudomonas fluorescens* employing mutagenesis [63]. One of the genes identified was a *zntA*-like gene that was inducible by the presence of Zn^{2+}, Cd^{2+}, Pb^{2+}, Ni^{2+}, Hg^{2+}, and Ag^{+} ions. A mutant in this gene exhibited only hypersensitivity to Zn^{2+}, Cd^{2+} and Pb^{2+}, suggesting that it encodes a transporter for these cations. The *P. putida* strain S4 employs a dual strategy for zinc resistance [64]. One strategy is mediated by an inducible ATPase that effluxes the ion during the exponential phase of growth. The second mechanism is the accumulation of Zn^{2+} that can be stored by proteins in the outer membrane and the periplasm.

In addition to membrane transport pumps, some bacteria produce metallothioneins (MT) [15]. MTs are small poly-thiol proteins that bind metal cations, lowering their free concentrations within the cytoplasm (Fig. 9.1D). The best characterized prokaryotic MT is SmtA from *Synechococcus* PCC 7942, which protects against elevated levels of Zn^{2+} [65, 66]. For a long time SmtA was the only prokaryotic MT identified [67], but currently other related bacterial MTs, called BmtA, have been described [68]. MTs were purified from *P. putida* and *P. aeruginosa* strains and found that they were associated with three to four Zn^{2+} atoms [68]. Additional BmtA-like proteins were identified in *P. fluorescens* strains pf01 and SBW25. Most bacterial MTs identified to date have been found in cyanobacteria and pseudomonads [68].

9.3 Toxic Cations

This group of elements includes heavy metals with no known biological function and clear toxic effects over living cells: cadmium, lead and mercury. Potentially toxic metals which are irrelevant in biological terms, mainly by their presence at very low levels or in non available forms in most environments (i.e. gold, thallium, aluminum), will not be considered in this review. As with the essential cations, transmembrane efflux systems are also used by bacteria as key resistance mechanisms against toxic metal cations. Mercury represents a unique case for which intracellular sequestering followed by detoxification has evolved as a best suited bacterial tolerance strategy.

9.3.1 Cadmium and Lead

Cadmium chemistry is closely related to that of essential zinc. Cadmium and lead commonly form cations of oxidation states II, although lead may also exist in the IV valence. Lead differs from cadmium and zinc in their chemical coordination properties. In contrast to zinc, cadmium and lead bind preferentially sulfur (soft) ligands. Due to their similarity, zinc homeostasis and cadmium and lead resistance mechanisms often overlap, as reflected by their sharing of uptake and efflux transporters and metal-responsive regulatory proteins [59]. Intracellular Cd^{2+} is maintained at low levels through the control of sequestration or efflux of the ion. Cd^{2+} can be effluxed from bacterial cells by at least three systems: the P-type ATPase CadA, a large single polypeptide, the CzcCBA system, a three-polypeptide chemiosmotic RND complex that functions as an ion/proton exchanger, and CzcD, a single CDF membrane protein acting as a chemiosmotic efflux pump [15].

The P-type ATPase CadA from pI258 plasmid of *S. aureus* is the most studied Cd^{2+} resistance system [58]. CadA homologs have been found in several bacterial species, including pseudomonads. The system is localized in the *cadAC* operon. CadA catalyzes the active efflux of Cd^{2+}, Zn^{2+}, or Pb^{2+} [58, 69], and contains all the characteristic domains of a P-type ATPase [70, 71] (Fig. 9.2A). CadC is a transcriptional regulator needed for full Cd^{2+} and Zn^{2+} resistance in *S. aureus* [72].

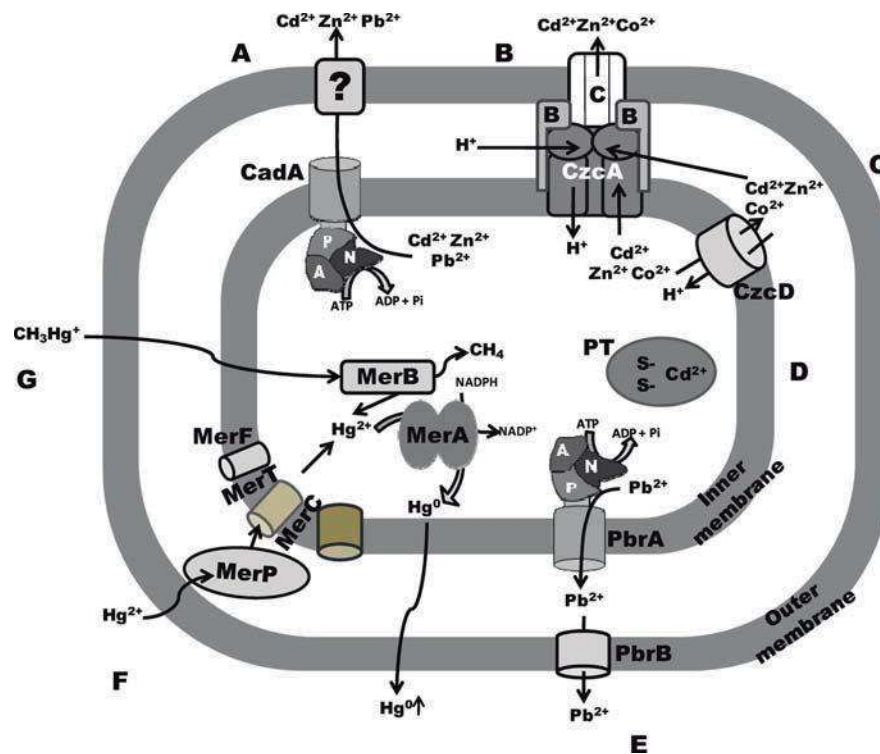

Fig. 9.2 Mechanisms of resistance to toxic cations in pseudomonads. *A*, cadmium P-type ATPase CadA. *B*, multication RND complex CzcCBA. *C*, CDF transporter CzcD. *D*, cadmium pseudothioneins (PT). *E*, Pbr lead resistance system. *F*, mercury resistance Mer system. *G*, organomercurial resistance MerB enzyme. P-type ATPases domains shown are: P, phosphorylation domain; N, nucleotide-binding domain; *A*, activator and phosphatase domain. The functions of the resistance systems are described in the text

The best studied homologue of CadA, the above mentioned ZntA from *E. coli,* is 30% identical to CadA.

The level of resistance to Cd^{2+} varies widely within species of *Pseudomonas* [73, 74]. *P. putida* 06909 possesses the *cadA* and *cadR* genes that are homologs to *zntA* and *zntR* from *E. coli*, respectively. CadA from *P. putida* 06909 confers a high level of resistance to Cd^{2+}, partial resistance to Zn^{2+}, and, unlike ZntA, does not confer Pb^{2+} resistance [75]. The level of Cd^{2+} resistance conferred by the *cadA* gene in *P. putida* 06909 is 17-fold higher than that conferred by *zntA* in *E. coli*. Homologous ORF's PA3690 (*cadA*) and PA3689 (*cadR*) are also present in the genome of *P. aeruginosa* PAO1, but their function has not been elucidated yet. CadA sequences from *P. putida* and *P. aeruginosa* both have a histidine-rich N-terminal extension that is missing in other CadA sequences; this region is probably responsible for the higher level of resistance to Cd^{2+} of these strains [75]. CadR is a transcriptional regulator of the MerR family [76]. CadR represses its own

expression in the absence of Cd^{2+}, it is induced in the presence of Cd^{2+}, and is necessary for full resistance to Cd^{2+} and Zn^{2+} [75]. *P. putida* KT2440 possesses the CadA1 and CadA2 transporters. CadA2 confers resistance to Cd^{2+} and Pb^{2+} in *P. putida* whereas CadA1 does not seem to confer metal tolerance in *P. putida*, but confers Zn^{2+} resistance when overexpressed in *E. coli*. CadA1 expression is inducible by Zn^{2+}. CadA2 is considered a housekeeping resistance mechanism against Cd^{2+} and Pb^{2+} [77]. CadA2 is constantly expressed at a high level even when Cd^{2+} is absent, but its expression increases in the presence of metals.

The second Cd^{2+} resistance determinant, the CzcCBA system, actively transports Cd^{2+}, Zn^{2+} and Co^{2+} out of the bacterial cell [15] (Fig. 9.2B). One of the best characterized systems is the *czc* determinant from *C. metallidurans* [36]. The system is organized like other three-component RND transporter complexes. CzcA is a cation/proton antiporter located in the cytoplasmic membrane that effluxes toxic cations to the periplasm [78]. CzcA is essential for cation transport and is considered the core of the complex. CzcB is a membrane fusion protein which spans the periplasmic space, bringing the outer and inner membranes in close position. The third component, CzcC, is an outer membrane protein that effluxes cations from the periplasm to the outside [59]. The CzcCBA system catalyzes the efflux of both toxic and essentials cations and, for that reason, is tightly regulated by downstream and upstream regulatory regions.

Czc homologues have been detected by Southern hybridization in several *Pseudomonas* strains, including *P*. *aeruginosa* PAO1 [29]. The system is annotated as *czcCBA*-like in the PAO1 strain genome and probably confers resistance to Zn^{2+}, Cd^{2+}, and Co^{2+} [3]. In the environmental isolate *P. aeruginosa* CMG103, the *czrSRCBA* gene cluster confers a high level of resistance to Cd^{2+} and Zn^{2+} [29]. In *P*. *aeruginosa* PT5 (a PAO1 derivative) cross-resistance between heavy metal and antibiotic pumps has been reported [74]. The two-component system CzcS-CzcR controls the expression of the Czc efflux pump and also regulates negatively the expression of the OprD porin, leading to carbapenem resistance. A *czcCBA* system is also functional in *P. putida* KT2440, which possesses two copies of the transporter [77]. CzcCBA1 confers Zn^{2+} resistance and its expression is induced by the metal; Cd^{2+}, and possibly Pb^{2+}, are also transported by CzcCBA1, but is less efficient when it acts as a Cd^{2+} or Pb^{2+} transporter. CzcCBA2 also confers Zn^{2+} resistance, but its expression is not induced by any metal.

The third Cd^{2+} resistance determinant, CzcD, is an efflux pump that belongs to the CDF protein family [30] (Fig. 9.2C). The function of CzcD has been analyzed only in *C. metallidurans* but there are homologs in the genomes of *P. aeruginosa* PAO1 [3] and *P. putida* KT2440 [6]. CzcD is located in the cytoplasmic membrane and possesses at least six transmembrane helices. The level of resistance to Cd^{2+}, Zn^{2+} and Co^{2+} conferred by CzcD is lower as compared to that conferred by CzcA. CzcD is also involved in the regulation of the expression of the CzcCBA efflux system.

A distinct cadmium resistance mechanism reported in *Pseudomonas* involves cadmium-binding proteins called pseudothioneins (PT) [79] (Fig. 9.2D). PTs have been identified in a *P. putida* strain adapted to grow in 3 mM cadmium.

Pseudothioneins CdPT1, CdPT2 and CdPT3 are synthesized in different growth phases, being CdPT1 the major protein produced during the exponential phase. As metallothioneins, PTs are small cysteine-rich proteins (3.5–7 kDa). PTs have a lower cysteine content than mammalian metallothioneins (12–23% compared to 33%), but have in common that bind Cd^{2+}, Zn^{2+} and Cu^{2+} [79].

ZntA of *E. coli* and CadA of *S. aureus* also confer Pb^{2+} resistance in *E. coli* cells expressing the corresponding genes. A mutant strain with a disruption of the *zntA* gene showed hypersensitivity to Pb^{2+}; expression of *cadA* from pI258 plasmid complemented the phenotype, indicating that *cadA* also confers Pb^{2+} resistance [69].

The only specific mechanism of Pb^{2+} resistance described so far is the *pbr* system, reported in *C. metallidurans* CH34 [80] (Fig. 9.2E). In contrast to the *cad* and *znt* systems, which only comprise the ATPase with a regulatory gene, the *pbr* system is constituted by several genes arranged in the divergent operons *pbrUTR* and *pbrABCD*. These operons encode proteins involved in three different processes: uptake, efflux and accumulation of lead, which together confer maximal Pb^{2+} resistance. The role of PbrU on lead resistance has not been elucidated. PbrT is a permease that takes up Pb^{2+}; expression of *pbrT* alone in the absence of the *pbrABCD* genes results in Pb^{2+} hypersensitivity, due to an increase in Pb^{2+} uptake. The *pbrR* gene encodes the PbrR repressor that belongs to the ArsR/SmtB family of regulators. PbrR controls transcription of the *pbr* structural genes. PbrA is an inner membrane P-type ATPase, closely related to the CadA and ZntA ATPases, that effluxes Pb^{2+} to the periplasm. PbrB is an outer membrane lipoprotein that probably functions in removing Pb^{2+} from the periplasmic compartment, assisting PbrA for lead resistance [80, 81] (Fig. 9.2E). PbrC is probably an aspartic peptidase that removes the signal peptide from PbrB before it is transported to the periplasmic space; PbrC is required with PbrB for full resistance [80, 81]. PbrD is an intracellular protein that may bind Pb^{2+} with a cysteine-rich metal-binding motif but is not essential for Pb^{2+} resistance [80]. The Pbr system has not yet been identified in *Pseudomonas* strains. In the *P. putida* KT2440 genome, of a total of 61 open reading frames with a putative role in metal homeostasis and detoxification, there seems not to be homologues to *pbr* genes [6].

9.3.2 Mercury

Mercuric ions (Hg^{2+}) display a rather strong affinity for sulfur-containing ligands, thus its toxicity relates mostly to protein sulfhydryl poisoning and to binding to other relevant thiol compounds. A main ubiquitous resistance mechanism is used by bacterial cells to tolerate mercury: the reduction of Hg^{2+} to Hg^0. This biotransformation converts highly toxic cationic mercury into the metallic species, an almost innocuous volatile form (Fig. 9.2F). Methylation of mercury will not be included in this chapter as does not seem to represent a resistance mechanism; this microbial modification often yields toxic organomercurial derivatives.

Mercury resistance has been reported since many years in *Pseudomonas* species [82, 83], including *P. cepacia*, *P. fluorescens*, *P. putida*, *P. putrefaciens*, and *P. stutzeri* [84]. The reduction of Hg^{2+} to Hg^{0} is mediated by the mercuric reductase encoded by the *merA* gene, which is located in the mercury resistance *mer* operon [85, 86]. The *mer* operon is often encoded on mobile genetic elements [84]. The simplest and most studied Gram negative *mer* determinant is that from transposon Tn*501*, originally identified in *P. aeruginosa* [82]. The *mer* operon shows several genetic arrangements depending on its origin. The *mer* operon of Tn*501* consists of *merRTPADE* genes which encode polypeptides with regulatory, transport and enzymatic functions; other *mer* genes, such as *merB, C, F, E* and *G,* are localized on different *mer* operons [84, 86]. The *mer* genes are widely distributed among pseudomonads as shown in Fig. 9.3.

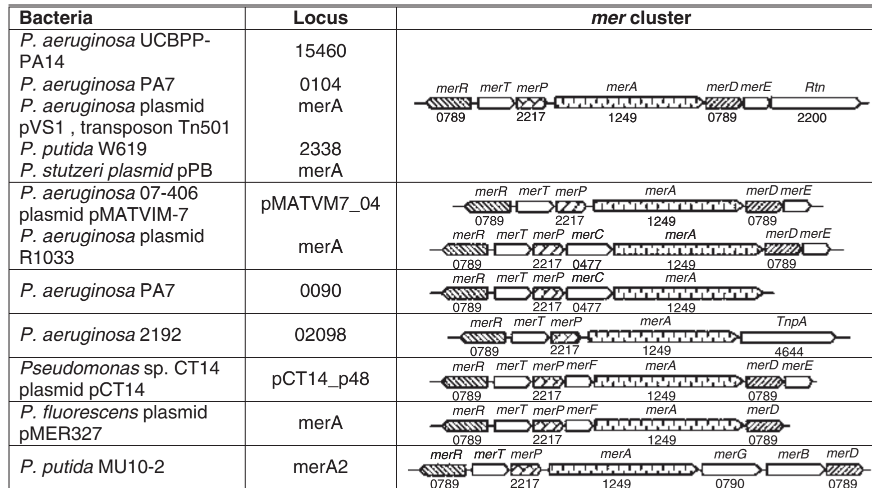

Fig. 9.3 Schematic representation of the arrangement of the *mer* genes located in the genomes of pseudomonads. The columns indicate the *Pseudomonas* strain and the microbial locus for each *merA* gene. *Boxed arrows* indicate genes and the direction of transcription. Numbers below genes indicate the COG family to which each gene belongs [87]

The resistance mechanism consists of the initial binding of Hg^{2+} ions by a pair of cysteine residues on MerP, a mercury binding protein located in the periplasm of Gram-negative bacteria [88]. Mercuric ions are then transferred via a redox exchange mechanism to a pair of cysteines on MerT, an inner membrane transport protein (Fig. 9.2F). MerT is present in most *mer* operons from Gram negative bacteria. MerT is essential for mercury resistance and is the only Mer protein that interacts directly with MerP. Other Hg^{2+} membrane transporters are encoded by *merC* and *merF* genes. Deletion of merC and merF had no effect on the mercury resistance level [86]. Hg^{2+} is finally transferred via cysteine residues to the N-terminal domain of MerA, the homodimeric flavoprotein mercuric reductase,

the key component of the mercury detoxification system. The enzyme catalyzes the two-electron reduction of Hg^{2+} to volatile elemental mercury (Hg^0), which is nonenzymatically removed from the growth medium (Fig. 9.2F). The MerA enzyme utilizes NADPH as an electron donor and requires an excess of exogenous thiols for activity. MerA is located in the cytoplasm, where NADPH is abundant [85, 86].

In some bacteria, resistance to organomercurial compounds is also conferred by *mer* operons encoding the additional organomercurial lyase enzyme MerB (Fig. 9.2G). MerB is a monomeric cytosolic enzyme that cleaves the Hg–C covalent bond of both alkyl and aryl mercurials, releasing Hg^{2+}, which is then transformed by the MerA mercuric reductase. The activity of the MerB enzyme was first determined in *Pseudomonas* K-62 [86, 89]. Bacteria possessing MerB are tolerant to both inorganic and organic mercurials (broad-spectrum resistance); in contrast, the narrow-spectrum resistance determinants, where the *mer* systems lack *merB*, only confer resistance to Hg^{2+} [90]. Organomercurials are highly lipid-soluble as to enter the cell efficiently without a specific uptake system. The product of the *merG* gene lies in the periplasm and probably reduces permeability to organomercurials in soil pseudomonads [86]. Deletion of *merG* in *Pseudomonas* K-62 resulted in a decrease in phenylmercury resistance [91]. The regulatory genes for the *mer* system are *merR* and *merD* [86]. MerR belongs to the large MerR family that, as mentioned above, includes transcriptional regulators for Cd^{2+}, Zn^{2+}, Cu^{2+} and Pb^{2+}. MerR is an activator of the *mer* cluster and in Gram negative bacteria is divergently transcribed from the major *mer* promoter [76].

9.4 Toxic Oxyanions

Arsenic and chromium are toxic nonessential metalloids that may be present as environmental pollutants. The main tolerance mechanism developed by bacteria for arsenic and chromium oxyanions is their efflux from the cytoplasm by specific orthologous membrane transporters. Oxyanions derived from essential elements that generally lack toxicity (i.e. molybden and tungsten) or those that, while showing toxicity commonly occur at very low levels in the environment (uranium and vanadium) are not described here.

9.4.1 Arsenic

Arsenic is a metalloid present in numerous disturbed and natural ecosystems. It can exist in multiple oxidation states, with the most common being arsenite [As(III)] and arsenate [As(V)]. Although some microorganisms can utilize As(V) for anaerobic respiration [92] or oxidize As(III) as a sole energy source, arsenic is generally toxic to most microbes [93]. Arsenate (AsO_4^{3-}) is a toxic analog of phosphate (PO_4^{3-}), and most organisms take up arsenate via phosphate transporters [94]. As(V) toxicity is due to the uncoupling of ATP phosphorylation, that would directly impact energy flow, as well as to the inhibition of nucleic acid and phospholipid syntheses

[95]. In bacteria, the pathway for uptake of trivalent metalloids as As(III) is through the polyol transporter GlpF, which belongs to the family of aquaglyceroporins [96]. As(III) toxicity is predominantly due to its ability to covalently bind protein sulfhydryl groups [93]. Arsenate and arsenite (AsO_2^-) oxyanions are detoxified by an interplay of redox, transport, sequestration, and covalent modification reactions [94] (Fig. 9.4A).

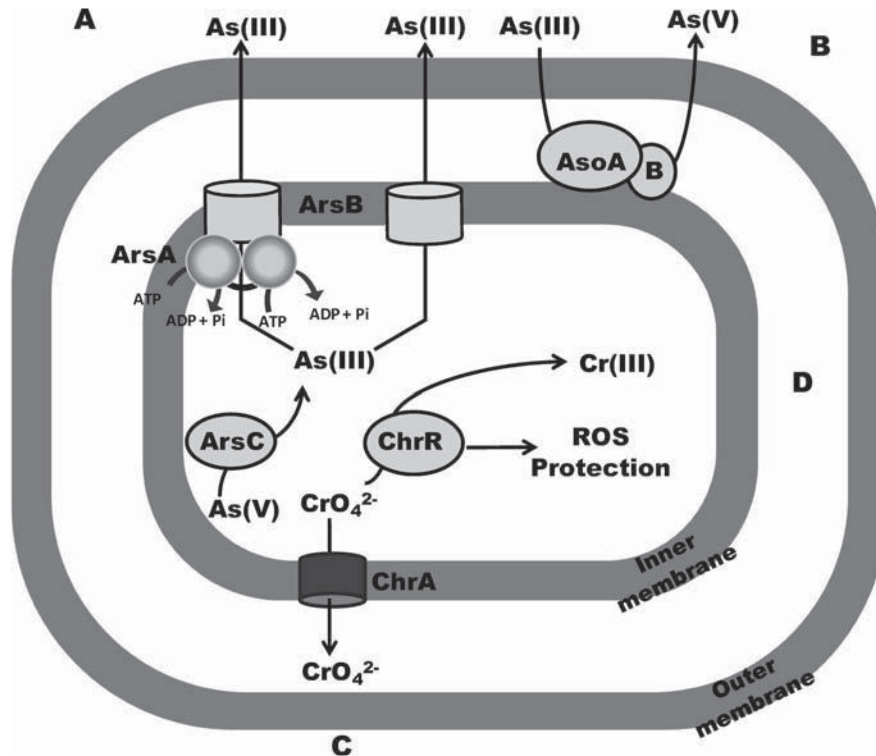

Fig. 9.4 Mechanisms of resistance to toxic oxyanions in pseudomonads. *A*, arsenic resistance ArsABC and ArsBC systems. *B*, AsoAB arsenite oxidase complex. *C*, chromate resistance ChrA transporter. *D*, ChrR chromate reductase. The functions of the resistance systems are described in the text

Bacteria adapt to arsenic toxicity mainly by the development of resistance mechanisms conferred by chromosomal or plasmid-encoded arsenical resistance (*ars)* operons [96]. Once the trivalent form of the metalloid accumulates in the cell, resistance is produced by their removal from the cytosol [96]. The *ars* clusters are widely distributed among pseudomonads, as shown in Fig. 9.5.

The mechanism of resistance to arsenic conferred by *ars* genes has been best characterized from *E. coli* plasmid R733 [97]. The *ars* operon consists of genes *arsRDABC*. The *arsA* gene encodes the ATPase enzyme subunit of a protein complex composed of an ArsA dimer bound to ArsB, an inner membrane polypeptide

Bacteria	Locus
arsR 40640, *arsB* 1055, *arsC2* 0394, *arsH* 0431	
P. putida KT2440	1927 – 1930 2715 – 2718
P. putida F1	3034 – 3037
P. putida GB-1	3077 – 3080
P. aeruginosa PAO1	2277 – 2280
P. aeruginosa PACS2	01002767 – 01002770
P. aeruginosa C3719	01187 – 01190
P. aeruginosa 2192	01380 – 01383
P. aeruginosa UCBPP-PA14	35130, 35110, 35100, 35080
P. fluorescens Pf0-1	2400 – 2397
P. fluorescens Pf-5	2186 – 2183
P. syringae pv. syringae B728a	1504 – 1502
arsC1 1393, *wrbA* 0655	
P. syringae pv. syringae B728a	3702 – 3703
P. syringae pv. tomato T1	4747 – 4748
P. syringae pv. tomato str. DC3000	1687 – 1686
P. syringae pv. phaseolicola 1448A	3723 – 3724
arsR 0640, *arsC2* 0394, *PTPc* 2453, *ArsD*, *arsA* 0003, *arsC2* 0394, *ACR3* 0798 *trkA* 2072, *arsR* 0640, *arsH* 0431, *PTPc* 2453	
P. stutzeri A1501	0241 – 0234 2095 – 2098
arsR 0640, *arsC2* 0394, *ACR3* 0798, *arsH* 0431	
P.putida W619	5146 – 5149
arsR 0640, *arsB* 1055, *arsC2* 0394	
P. entomophila L48	3306 – 3308

Bacteria	Locus
arsC1 1393, *wrbA* 0655, *DUF2069* 3308	
P. putida KT2440	1645 – 1643
P. putida F1	4072 – 4074
P. putida GB-1	1247 – 1245
P. putida W619	1207 – 1205
P. aeruginosa PAO1	0950 – 0948
P. aeruginosa PACS2	01001431 – 01001429
P. aeruginosa C3719	05495 – 05493
P. aeruginosa 2192	06125 – 06123
P. aeruginosa PA7	4559 – 4561
P. aeruginosa UCBPP-PA14	51980, 51990, 52000
P. fluorescens Pf-5	4456 – 4458
P. fluorescens Pf0-1	4228 – 4230
P. mendocina ymp	2989 – 2991
P. entomophila L48	1354 – 1352
P. stutzeri A1501	2824, 2826, 2827
ProP 0477, *arsB* 1055	
P. syringae pv. syringae B728a	1681 – 1682
P. syringae pv. tomato str. DC3000	3799 – 3798
P. syringae pv. tomato T1	0009 – 0010
P. syringae pv. phaseolicola 1448A	3604 – 3603
arsC1 1393, *trkA* 2072, *ProP* 0477, *DUF619* 1247 *arsR* 0640, *arsB* 1055, *arsC2* 0394, *arsH* 0431, *arsC1* 1393	
P. syringae pv. tomato T1	3165 – 3168 3161 – 3165
asoB 0723, *asoA* 0243	
Pseudomonas sp. TS44	aoxA, aoxB

Fig. 9.5 Schematic representation of the local genomic context of *ars* and *aso* genes located in the genomes of pseudomonads. All *ars* and *aso* genes shown are located in chromosomes. The *columns* indicate the *Pseudomonas* strain and the microbial locus for each gene. Preliminary information on *aso* genes is also included. *Boxed arrows* indicate genes and direction of transcription. Numbers below genes indicate the COG family to which each gene belongs. [87]

(Fig. 9.4A). As(III) is the substrate of the ArsAB efflux pump, which is an As(III)-translocating ATPase [97]. ArsB alone is sufficient for As(III) resistance and proton motive force-dependent As(III) efflux; bacteria lacking ArsA are still resistant to arsenic [96] (Fig. 9.4A). *arsC1* belongs to the COG1393 family [87] and encodes an enzyme that reduces As(V) to As(III), which is subsequently extruded from the cell; arsenate reductase activity is required for optimal resistance to As(V) [93] (Fig. 9.4A). In vitro, the reductase activity requires both reduced glutathione (GSH) and any of the three *E. coli* glutaredoxins, Grx1, Grx2 or Grx3 [96]. The *arsD* gene is constitutively expressed and encodes a regulatory protein that controls the maximal expression of the *ars* operon [93]. Finally, *arsR* encodes a repressor that controls the expression of the *ars* operon and can be induced by As(III), Sb(III) or even bismuth.

ars homologous sequences have been identified in chromosomal DNA from *P. aeruginosa* [98]. Phylogenetic analysis showed that *P. aeruginosa* and *P. putida* possess *ars*C chromosomal homologs [99]; in the case of *P. aeruginosa*, its genome contains separate genes for glutaredoxin- and thioredoxin-coupled ArsC reductases [96]. The chromosome of *P. fluorescens* MSP3 possesses a less complex arsenic operon (*arsRBC*) which confers resistance to arsenate and arsenite [100].

Analysis of the *P. putida* KT2440 genome revealed two very similar systems, *arsRBCH*, for arsenic resistance [6]. The distinct gene *arsH* is located downstream of *arsC* and is transcribed in the same direction. The *arsH* gene product of *Yersinia*

enterocolitica was reported to confer resistance to both arsenite and arsenate and was assigned a possible role as a transcriptional regulator [101]. However, *P. putida arsH* genes, denominated ArsH1 and ArsH2, showed a significant similarity to plant NADH oxidoreductases and to *Bacillus subtilis* azoreductase [6]. ArsH is widely distributed in bacteria and sparsely in fungi, plants, and archaea [96], but its role in arsenic resistance is still unclear.

Bacterial oxidation of As(III) to less toxic As(V) may be considered as a resistance mechanism. Arsenite oxidase (Aso) from *Alcaligenes faecalis* is the best understood example of this detoxification activity [94]. The enzyme is formed by a molybdopterin-containing subunit and a Fe-S Rieske subunit encoded by the *asoA* and *asoB* genes, respectively [102] (Fig. 9.4B). The *aso* genes form part of the so called "arsenic gene island" encoding proteins related to arsenic resistance and homeostasis; these include putative periplasmic oxyanion binding proteins, probably associated with ABC membrane transporters, as well as an arsenite efflux pump (ArsAB) ATPase. An ortholog of the AsoA Mo-pterin subunit of arsenite oxidase was identified in the genome of *Pseudomonas* sp. TS44 (Fig. 9.5), suggesting that some pseudomonads also possess the ability to oxidize arsenite [102].

As(III) toxicity via a mechanism involving peroxidation of unsaturated fatty acids was found in *P. putida* [103, 104]. It was proposed that this process leads to the generation of organic hydroperoxides and oxygen radicals, which induce components of the oxidative stress response such as superoxide dismutase (SOD) and catalase [93]. Catalase activity increased in response to the presence and oxidation of As(III) [104]. These studies also showed that the levels of glutathione reductase (Gor) increased upon exposure of *P. putida* to As(III). One function of Gor in *E. coli* is to recycle oxidized glutathione back to reduced glutathione, which is the reductant for the As(V) reductase that converts As(V) to As(III). The latter is then actively removed from the cell by the ArsB effflux pump [93].

Mutants of *P. aeruginosa* PAO1 affected in the *arsB*, *crc* (the catabolite repressor control protein) and *gor* genes are more sensitive to As(III) than wild-type strain [93]. The *crc* mutant was more sensitive to H_2O_2 in the presence of As(III); the sensitivity to As(III) was assumed to be due to an abnormal regulation of genes under Crc control. Double *sodA*/*sodB* mutants also exhibited increased sensitivity to As(III), suggesting that the oxidative stress response is involved in As(III) resistance [93].

9.4.2 Chromium

The biological effects of chromium depend on its oxidation state. At the extracellular level, Cr(VI) (usually in the form of chromate, CrO_4^{2-}) is highly toxic to most bacteria, whereas Cr(III) is relatively innocuous by its inability to traverse cell membranes. In the cytoplasm, chromium toxicity is mainly related to the process of reduction of Cr(VI) to lower oxidation states [i.e. Cr(III) and Cr(V)] in which free radicals may form [105, 106]. Bacterial resistance determinants may be encoded either by chromosomal genes or by plasmids [107, 108]. Usually genes located

on plasmids encode membrane transporters which directly mediate efflux of chromate ions from the cytoplasm. On the other hand, resistance systems encoded within bacterial chromosomes are generally related to specific or unspecific Cr(VI) reduction, free-radical detoxifying activities, repairing of DNA damage, and processes associated with sulfur or iron homeostasis [106].

The best understood mechanism of resistance to chromate is the efflux of $C_rO_4^{2-}$ conferred by the ChrA protein encoded by the *P. aeruginosa* plasmid pUM505 [109]. ChrA is a membrane protein of 416 amino acid residues which displays a topology of 13 transmembrane segments (TMS) [110]. ChrA functions as a chemiosmotic pump that effluxes CrO_4^{2-} from the cytoplasm using the proton motive force [111, 112] (Fig. 9.4C). Plasmid pMOL28 from *C. metallidurans* [113], plasmid 1 from *Shewanella* sp. ANA-3 [114], and transposon Tn*OtChr* from *Ochrobactrum tritici* 5bv11 [115] all encode ChrA homologs which confer resistance to chromate. The resistance mechanism seems to be the same for these homologs from proteobacteria, as all caused reduced accumulation of CrO_4^{2-}.

Structure-function analyses have been conducted with the *P. aeruginosa* ChrA protein. Random mutagenesis of the *chrA* gene showed that most essential amino acids are located in the amino terminal end of ChrA [116]. In agreement with this finding, phylogenetic analysis of ChrA homologs revealed that the amino terminal halves are more conserved than the carboxyl halves [117], suggesting that the two halves of ChrA carry out different functions in the transport of CrO_4^{2-}.

The ChrA proteins belong to the CHR superfamily of transporters, first described by Nies et al. [107] as a small group of prokaryotic proteins involved in CrO_4^{2-} and sulfate (SO_4^{2-}) transport. This superfamiliy was classified as TC no. 2.A.51 [118] and includes proteins encoded in chromosomes and plasmids. The protein databases currently contain several dozens of homologs, including proteins from eukaryotes, and has been named as the CHR superfamily of chromate ion transporters [117]. The CHR superfamily contains two families, large proteins (comprising seven LCHR subfamilies) and short proteins (comprising three SCHR subfamilies). All pseudomonads with genomes sequenced have ChrA homologs from subfamilies LCHR1 or LCHR5, as well as other *chr* related genes (Fig. 9.6).

Plasmids pB4 [119], from a *Pseudomonas* sp. strain, and pUM505, from *P. aeruginosa* [106], possess *chrBAC* gene clusters that share high sequence similarity with the resistance determinant from plasmid pMOL28. A function of the ChrB protein in the inducibility of the *chrA* gene by CrO_4^{2-} had been previously demonstrated in *C. metallidurans* [113]. The presence of *chrC*, a gene encoding a probable SOD enzyme, is another variable feature of the *chr* gene clusters [120] (Fig. 9.6).

A second mechanism of resistance to CrO_4^{2-} is the transformation of hexavalent chromium to the trivalent form [105, 108]. Microbial reduction of Cr(VI) to Cr(III) is not a plasmid-associated trait. Chromate reduction has been demonstrated in diverse pseudomonad species, including *P. ambigua* [121], *P. fluorescens* [122] and *P. putida* [123], although only a few enzymes have been characterized to date [105]. Initial studies suggest that chromate reductases may have a different primary role other than chromate reduction; this secondary function for chromate reductases

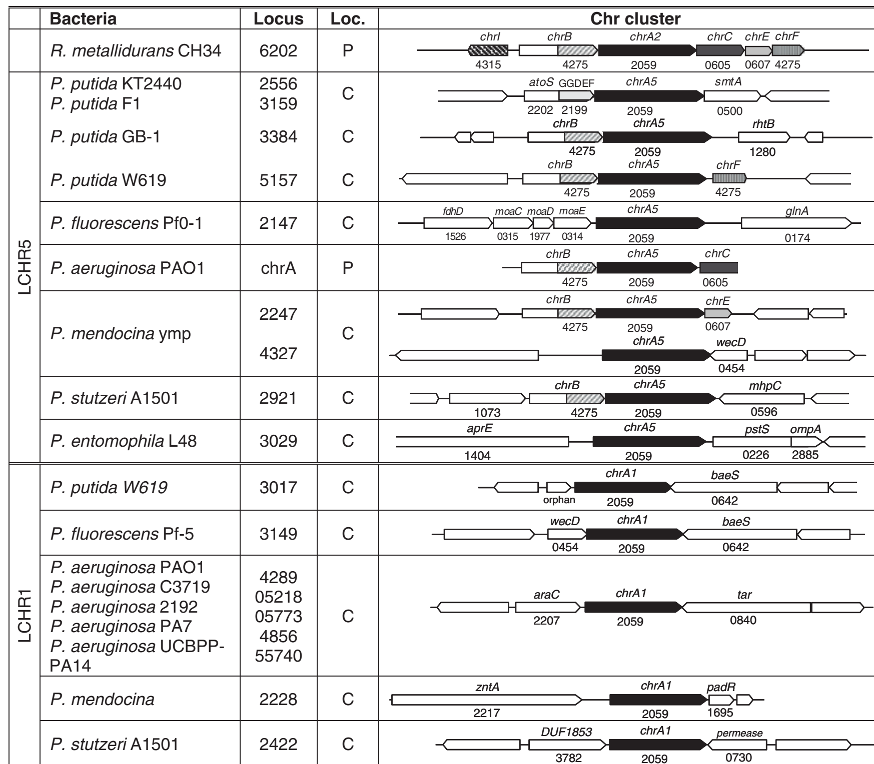

Fig. 9.6 Schematic representation of the local genomic context of *chr* genes located in the genomes of pseudomonads. All *chrA* genes belong to the LCHR1 or LCHR5 subfamilies of the CHR superfamily [117]. The columns indicate the *Pseudomonas* strain, the microbial loci and the location (Loc.) for each *chrA* gene (P, plasmid; C, chromosome). *Boxed arrows* indicate genes and direction of transcription. Identified genes are indicated according to the characterized *chr* determinant from *C. metallidurans* plasmid pMOL28. Numbers below genes indicate the COG family to which each gene belongs [87]

may be related to the recent introduction of Cr(VI) to the environment by industrial pollution.

The currently best studied chromate reductase is the ChrR enzyme from *P. putida,* a soluble flavin mononucleotide-binding enzyme which reduces Cr(VI) to Cr(III) [124] (Fig. 9.4D). Purified ChrR revealed that a quinone reductase activity produced a flavin semiquinone during CrO_4^{2-} reduction; this activity transferred >25% of the NADH electrons to reactive oxygen species (ROS) and generated the Cr(V) species transiently. This property of ChrR provides an antioxidant defense mechanism to *P. putida* by shielding cells against H_2O_2 toxicity [125] (Fig. 9.4D). ChrR in one pathway reduces Cr(VI) to Cr(III) generating intermediary Cr(V) and ROS, and, by an additional mechanism, reduces quinones to protect against ROS. ChrR from *P. putida* belongs to the NADP(H)-dependent FMN reductase (FMN_red) protein family, currently comprising about 250 homologs [106].

Besides chromate efflux and reduction, several other resistance mechanisms to deal with chromium are displayed by bacteria. Since oxidative stress is responsible for most toxic efects of chromate, protection and detoxification systems against this process is an important part of the defensive barrier. Protection of bacterial cells from DNA damage caused by CrO_4^{2-} is another defensive shield. Cr(VI) has long been known to induce the *E. coli* SOS repair system that protects DNA from oxidative damage [126]. DNA helicases RecG and RuvB, and the FtsK protein, components of DNA repair and chromosome segregation processes, have ben shown to participate in the response to DNA damage caused by CrO_4^{2-} exposure in *P. aeruginosa* [127, 128]. Additional protective strategies may be related with sulfur or iron metabolism, but these systems have not been analyzed in pseudomonads.

9.4.3 Tellurium

Tellurium is a rare-earth metalloid with a low abundance in the Earth's crust but which derivatives may be pollutants in industrial waste discharges. The tellurite oxyanion (TeO_3^{2-}) is highly toxic for most microorganisms, particularly Gram-negative bacteria [129]. Tellurite toxicity in *E. coli* is several orders of magnitude higher than that of heavy metals such as cobalt, zinc and chromium [23, 130]. Studies on TeO_3^{2-} metabolism and toxicity in *E. coli* showed that the oxyanion interacts with reduced thiols and that glutathione is the initial target of tellurite reactivity [131]. Tellurite, as chromate, is reduced intracellularly producing toxic intermediates which may cause DNA damage [132].

Despite several genetic determinants related to TeO_3^{2-} resistance have been analyzed to the molecular detail, a general mechanism to explain this phenotype is not available [15, 133]. Instead, a variety of possible biochemical strategies used by bacteria to defend themselves from tellurite toxicity have been reported.

Antibiotic-resistant clinical isolates of enterobacteria and *P. aeruginosa* commonly possess plasmids conferring TeO_3^{2-} resistance [134, 135]. A TeO_3^{2-} resistance determinant from the chromosome of *P. syringae* pv. *pisi* encodes a methyl transferase enzyme that may detoxify tellurium by methylation [136]. Similarly, the *P. putida* genome contains genes that may encode tellurium (and selenium) methylation activities [6]. *P. aeruginosa* PAO1 mutants affected in the genes encoding DNA helicases RuvB, RecG and the DNA translocase FtsK, that function in DNA repair and chromosome segregation, respectively, showed an increased susceptibility to tellurite [127, 128]. Protection from DNA damage caused by TeO_3^{2-} exposure was proposed as the role of those enzymatic activities in conferring TeO_3^{2-} resistance. The precipitation of TeO_3^{2-} by a siderophore, pyridine-2, 6-bis(thiocarboxylic acid), produced by *P. stutzeri* KC has been proposed as another mechanism for TeO_3^{2-} detoxification [137]. A detailed metabolomics study of the tellurite hyper-resistant *Pseudomonas pseudoalcaligenes* KF707 strain revealed that the resistance phenotype involves a variety of complex cell modifications, including the induction of the oxidative stress response, resistance to membrane alterations, and a rearrangement of cellular metabolism [138].

The *tehAB* operon from the *E. coli* chromosome encodes TehA, an integral membrane protein, and TehB, a weakly membrane-associated protein [139]. A possible role for this operon in the efflux of TeO_3^{2-} has been discarded [140]. In contrast, the *E. coli* ArsAB ATPase, which effluxes arsenite, is also able to transport TeO_3^{2-} [141]. The TehAB system confers TeO_3^{2-} resistance by a distinct strategy involving thiol redox enzymes, such as glutathione reductase and thioredoxin reductase, as a mechanism of oxidative protection [139]. The TehB protein was found to bind S-adenosyl methionine as a methylation cofactor that detoxifies TeO_3^{2-}, as mentioned above for the *P. syringae* tellurium resistance determinant [142]. Other *E. coli* tellurite resistance systems, the *kilA* and *ter* determinants, seem to function by protecting glutathione from being reduced by TeO_3^{2-} [131].

9.5 Other Toxic Ions

To close the listing of heavy metals displaying deleterious effects on bacteria, a brief outline will be given next for the environmentally important elements silver, tin and selenium, for which resistance mechanisms have been only barely analyzed in pseudomonads.

Silver is a highly toxic metal with several biomedical uses, mainly as an antiseptic. Even though numerous examples of silver resistance have been reported in pseudomonads [143–145], no studies on resistance mechanisms have yet appeared. As mentioned in the section of copper, the genomes of *P. aeruginosa* [3] and *P. putida* [6] possess homologs for the CusCBA system, a proton-driven RND transporter which may efflux Ag^+ from the cytoplasm [24], and for SilP, a P-type ATPase which extrudes Ag^+ (see below) [25]. A rather complex Ag^+ resistance system, first described from a *Salmonella* plasmid [25, 146], was later found widely spread in other enterobacterial plasmids [147]. It consists of three operons. s*ilE* encodes the Ag^+-inducible periplasmic SilE protein, which binds Ag^+ ions and prevents their entry to the cytoplasm. Next, the *silCFBAP* operon encodes the P-type ATPase SilP, which transports Ag^+ from the cytoplasm to the periplasm, the RND complex SilCBA, an efflux pump able to extrude periplasmic Ag^+ to the outside, and SilF, a periplasmic chaperone that escorts Ag^+ from SilP to the SilCBA pump. A third operon, *silRS*, encodes a typical two-component regulatory system that controls the expression of the Sil system.

Tin is a nonessential metal whose inorganic forms have little toxicity. In contrast, organotins, widely used organometallic compounds, are highly toxic for microorganisms [148]. Organotin-resistant bacteria have been isolated from polluted ecosystems [148, 149]. Several pseudomonad strains resist organotins by breaking Sn-C bonds [150, 151]. A distinct resistance mechanism is displayed by a *P. stutzeri* strain possessing the *tbtABM* operon [152]. TbtABM is a RND system which effluxes tributyltin from the cytoplasm. TbtABM also confers resistance to antibiotics and aromatic compounds and shows homology with *P. putida* multidrug resistance systems. No further details on this organotin resistance mechanism have been reported.

Selenium, a metalloid related to sulfur and tellurium, is required as a micronutrient by most microorganisms. However, toxic selenium oxyanions selenate (SeO_4^{2-}) and selenite (SeO_3^{2-}) may be generated by industrial activities. The reduction of selenite (and less frequently of selenate) to elemental Se^0, considered as a detoxification mechanism, may be carried out by varied bacterial species [153–155], including pseudomonads [156]. Heavy metal-resistant *C. metallidurans* has the ability to reduce selenite to Se^0, which accumulates as granules in the cytoplasm [155]. The *P. putida* genome contains genes that may encode selenium methylation activities [6]. No genes or biochemical mechanisms are available for bacterial selenium resistance.

9.6 Concluding Remarks

Pseudomonads have evolved diverse resistance mechanisms to cope with heavy metal toxicity. Due to the distinct chemical properties of each toxic metal(loid), and to the different levels to which the microorganisms are exposed, bacteria with varied defense systems have been selected. Strategies involving the exclusion of cytoplasmic ions by membrane efflux pumps seem to be a preferred mechanism. Except for mercury, and probably for tellurite, all the toxic ions treated here may be the substrates of efflux pumps. These transporters belong to a variety of membrane protein families (RND, CDF, MFS, P-type ATPases) frequently widespread among all life domains. A second common resistance mechanism involves the use of redox enzymes. For most chemical elements susceptible to generate different valence species with a lower toxicity (i.e. mercury, arsenic, chromium, selenium), redox detoxification systems are usually deployed by pseudomonads. As may be inferred from the resistance systems described above, understanding the interactions of pseudomonads with toxic heavy metals largely benefited from biochemical and genomic information generated in other bacteria. A conclusion that may be drawn is that the relatively large genomes of pseudomonads are plenty of genetic determinants for heavy metal resistance, which is in tune with the diverse environments that these bacteria use to inhabit.

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ANEXO 2

Transporte de sulfato y oxianiones relacionados en bacterias.

Como parte de las actividades académicas del programa de Doctorado se me asignó la redacción de una revisión bibliográfica crítica y exhaustiva sobre el transporte de sulfato y oxianiones relacionados en bacterias. En esta revisión participaron el M.C. César Díaz-Pérez, alumno del Doctorado, el Dr. Héctor Riveros-Rosas y el Dr. Carlos Cervantes, asesor de la tesis.

A continuación se presenta el manuscrito generado de esta revisión el cual está en proceso de ser enviado para su publicación en una revista científica internacional.

Bacterial Transport of Sulfate and Related Oxyanions

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Abstract

Sulfur is an essential element for microorganisms which can obtain it from varied compounds, being sulfate the preferred source. The first step for sulfate assimilation, sulfate uptake, has been studied in several bacterial species. This article reviews the different bacterial transporters for sulfate and related oxyanions. The uptake of sulfate is carried out by sulfate permeases that belong to SulT (CysPTWA), SulP, PiT (CysP), and CysZ families. Sulfate is structurally related to the oxyanions molybdate, tungstate,

selenate and chromate. Molybdate is transported mainly by the high-affinity ModABC system and tungstate by the TupABC and WtpABC systems. CysPTWA, ModABC, TupABC, and WtpABC are analogous ATP-binding cassette (ABC)-type transporters with similar organization and properties. The uptake of selenate and chromate oxyanions occurs mainly through sulfate permeases. The toxic oxyanion chromate can also be extruded out of the cell by CHR chromate transporters.

1.- Introduction

Sulfur (S) is an essential element widely required by living organisms because it plays several important roles in cells. Sulfur is a component of the amino acids cysteine and methionine as well as of cellular cofactors including biotin, coenzyme A, S-adenosylmethionine, thiamine, glutathione, lipoic acid, and of iron-sulfur clusters (Scott *et al.*, 2007). Sulfate is the preferred sulfur source for most organisms (Marzluf, 1997; Leustek *et al.*, 2000; Kertesz and Wietek, 2001; Markovich, 2001; Stec *et al.*, 2006), and is the second most abundant soluble oxyanion, after phosphate, in the bacterial cell (Silver and Walderhaug, 1992). Sulfate belongs to the group VI of oxyanions, which includes other structurally similar members such as selenate, molybdate, tungstate, and chromate (Markovich, 2001). Sulfate is taken up by membrane transporters called sulfate permeases. The term sulfate permease is used to designate the active transport system that ensures the entrance of inorganic sulfate to the cytoplasm of bacterial cells (Hryniewicz *et al.*, 1989). Bacterial sulfate permeases belong to several protein families such as ATP-binding cassette (ABC)-type and SulP. Due to their similar structural characteristics, related oxyanions can be transported inside the cell by the same type of carriers.

The molybdate (MoO_4^{2-}) and tungstate (WO_4^{2-}) oxyanions also play important roles in the bacterial cell because they are the main source of essential metals molybdenum (Mo) and tungsten (W) (Hille, 2002). Molybdenum is an essential trace element for most living systems. Molybdenum-containing enzymes, or molybdoenzymes, are ubiquitous in nature and play important roles in the global cycles of nitrogen, carbon, and sulfur (Kisker *et al.*, 1997). Tungsten has been also reported as an essential trace element for some bacteria

and archaea (Johnson *et al.*, 1996). Molybdate and tungstate are taken up by high-affinity transporters of the ABC-type (Schwarz *et al.*, 2007).

Selenium is an essential element for microorganisms and its common biological forms are selenocysteine (the so-called twenty-first amino acid) and selenomethionine (Stolz *et al.*, 2006). Selenate (SeO_4^{2-}) and selenite (SeO_3^{2-}) are the predominant inorganic forms of selenium in aerobic environments. High concentrations of selenium oxyanions are highly toxic and mutagenic for bacteria and mammals. Selenate is taken up by the sulfate permeases and by an alternative system that also transports selenite (Lindblow-Kull *et al.*, 1985; Turner *et al.*, 1998).

In contrast with essential sulfur, molybdenum, tungsten, and selenium, chromium is a controversial element (Stearns, 2000). Chromium (III) is considered an essential nutrient required by humans and animals (Vincent, 2004). However, chromium essentiality is challenged because no specific enzyme or cofactor containing chromium has been identified (Stearns, 2000). Chromium is considered as not essential for microorganisms. Chromate (CrO_4^{2-}) and dichromate ($Cr_2O_7^{2-}$) are Cr(VI) derivatives higly toxic for most cells. Chromate is transported inside bacterial cells mainly by sulfate transporters (Ramírez-Díaz *et al.*, 2008). Due to the toxicity of chromate some bacteria possess the CHR transport system that extrudes chromate out of the cell (Cervantes *et al.*, 2001).

Since sulfate, molybdate, tungstate, and selenate are the principal sources of the essential elements sulfur, molybdenum, tungsten, and selenium, respectively, it is important to understand with detail how those oxyanions are transported inside the cell. This review is focused on bacterial sulfate transporters, some of which have not been considered in other reviews (Kertesz, 2001). A detailed description of the sulfate transporters Sbp/CysPTWA, SulP, CysP and CysZ is given in the first part of the review. A comparative description of the bacterial transporters for related oxyanions, molybdate, tungstate, selenate and chromate is presented in the second part.

2.- Sulfate transport

Sulfate is transported inside bacterial cells by carriers belonging to the SulT family, the SulP family (Kertesz, 2001), CysP, that pertains to the phosphate inorganic transporter (Pit) family (Mansilla and de Mendoza, 2000), and the putative sulfate transporter (CysZ)

family (Rückert *et al.*, 2005) (**Table 1**)(**Figure 1**). In addition, sulfate can also be transported by the ModABC molybdate transport system (**Table 2**).

Once inside the cell, sulfate is first reduced to sulfide which is then used to synthesize cysteine (**Figure 2**). Internal sulfate is first activated via an ATP-dependent reaction by the ATP sulfurylase or sulfate adenylyl transferase (EC 2.7.7.4), leading to the formation of adenosine 5'-phosphosulfate (APS) and pyrophosphate. In *Escherichia coli*, and many other proteobacteria, this activity requires two proteins, the catalytic subunit (COG0175 protein family) encoded by the *cysD* gene and the GTP regulatory subunit (COG2895 protein family) encoded by the *cysN* gene (Sekowska *et al.*, 2000; Guédon and Martin-Verstraete, 2006). In eukaryotes, archaea, firmicutes, cyanobacteria and ε-proteobacteria this activity requires only one protein (COG2046) encoded by the *sat* gene (Bradley *et al.*, 2009). APS formed is then the substrate of APS kinase (EC 2.7.1.25), encoded by the *cysC* gene, in a reaction that utilizes a second ATP molecule to phosphorylate the 3'-OH position of APS, which is transformed to 3'-phosphoadenosine phosphosulfate (PAPS) (Sekowska *et al.*, 2000)

In

. In *E. coli* the genes encoding for these enzymes are organized in the *cysDNC* operon. In the next step of the reduction pathway, PAPS is reduced to sulfite (SO_3^{2-}) by PAPS reductase (EC 1.8.4.8), encoded by the *cysH* gene, yielding adenosine 3'-5' diphosphate (PAP) as a by-product. CysH from *E. coli* uses reduced thioredoxin or glutathione as electron donors (Guédon and Martin-Verstraete, 2006). The next step is the reduction of sulfite into sulfide by NADPH-sulfite reductase (EC 1.8.1.2), an enzyme comprising two subunits (Sekowska *et al.*, 2000). The α subunit, encoded by the *cysJ* gene, contains FAD, whereas the β subunit, encoded by the *cysI* gene, contains an iron-sulfur center and a siroheme prosthetic group (analogous to that in siroheme-dependent nitrite reductases). The genes involved in the reduction of PAPS to sulfide form the *cysJIH* operon.

Interestingly, in the *Mycobacterium tuberculosis* genome, the *cysJ* gene is absent, and the *nirA* gene (homologous to *cysI*) encodes a protein that posseses activity as

ferredoxin-sulfite reductase (EC 1.8.7.1); NirA uses six ferredoxins as electron donors instead of NADPH (Schnell *et al.*, 2005) .

The synthesis of cysteine is achieved by the reaction of sulfide with *O*-acetylserine, which is previously formed by the reaction of serine and acetyl-coenzyme A, catalyzed by serine acetyltransferase (EC 2.3.1.30), encoded by the *cysE* gene. Next, two homologous enzymes (COG0031) *O*-acetylserine sulfhydrylase A (under aerobic conditions) or B (under anaerobic conditions), also called *O*-acetylserine (thiol)-lyases A and B (EC 2.5.1.47), encoded by the *cysK* and *cysM* genes, respectively, convert *O*-acetylserine and sulfide into cysteine and acetate (Sekowska *et al.*, 2000; Guédon and Martin-Verstraete, 2006; Zhao *et al.*, 2006) (**Figure 2**) (see section 2.1.4).

In addition, *O*-acetylserine sulfhydrylase B (CysM) can also use thiosulfate ($S_2O_3^{2-}$) as a substrate, yielding S-sulfocysteine, which is subsequently reduced to cysteine by a still uncharacterized mechanism (Sekowska *et al.*, 2000; Guédon and Martin-Verstraete, 2006) (**Figure 2**). Thiosulfate may also be transported inside bacterial cells by the sulfate permease CysPTWA.

2.1.- Sulfate permeases of the SulT family

Sulfate uptake through the SulT permease was initially described in mutant strains of the enterobacterium *Salmonella typhimurium* that were unable to use either sulfate or thiosulfate as a sulfur source but that were able to utilize sulfite, sulfide, and cysteine (Dreyfuss, 1964). Sulfate transport is an energy-dependent process with an apparent *Km* for sulfate of 36 μM that is strongly inhibited by lower concentrations (5μM) of thiosulfate (Dreyfuss, 1964). The thiosulfate ion can also be used as a sulfur source by some bacteria (Sekowska *et al.*, 2000). The transport of both sulfate and thiosulfate is repressed when the *S. typhimurium* cells are grown in the presence of cysteine and is derepressed in the presence of the cysteine analog n-djenkolic acid (Dreyfuss, 1964). These data showed that sulfate and thiosulfate use the same transport system, which, for this reason, is also named as the sulfate-thiosulfate permease (Dreyfuss, 1964; Sirko *et al.*, 1990). Sulfate transport is inhibited in several bacterial species by sulfite (Pardee, 1966) and by chromate (Pardee, 1966; Ohtake *et al.*, 1987; Nies and Silver, 1989).

The sulfate-thiosulfate permease belongs to the sulfate/tungstate uptake transporter (SulT) family (TC# 3.A.1.6) of the ABC superfamily of transporters and is constituted by sulfate and tungstate porters, according to the transporter classification database (TCDB) (Saier, 2000; Kertesz, 2001; Saier *et al.*, 2006), a system that classifies transporters according to mode of action and phylogeny (Saier, 2000).

The SulT permease is constituted by the typical components of ABC-type transporters (Sirko *et al.*, 1990) (**Figure 1**). ABC importers are composed of a substrate-binding protein and a core of four structural domains: two transmembrane domains (TMD) and two hydrophilic cytoplasmic domains containing the ATP-binding cassette, also named as nucleotide-binding domains (NBD) (Higgins, 2001; Bouige *et al.*, 2002). The substrate-binding protein, which binds the substrate for the first time, confers high affinity and specificity to the transporter and transfers the substrate to the membrane domains (Tam and Saier, 1993; Higgins, 2001). The substrate-binding protein is located in the periplasmic space in Gram-negative bacteria, whereas in Gram-positive bacteria and archea is inserted into the membrane via a lipid anchor (Oswald *et al.*, 2006). The TMDs are constituted by two transmembrane proteins, each with five or six predicted membrane-spanning α-helices, which provide the translocation pathway. The NBDs, which hydrolyze ATP and energetically drive the transport, are considered as the motor domains of ABC transporters. The NBDs of ABC transporters display high conservation in sequence and conformation, suggesting that the mechanism of ATP hydrolysis is similar in all ABC transporters (Higgins, 2001, Oswald*, et al.*, 2006).

The SulT sulfate-thiosulfate permeases of *S. typhimurium* and *E. coli* are encoded by the *cysPTWAM* operon and the *sbp* gene (**Table 1**) (Sirko *et al.*, 1990). The first gene in the operon, *cysP*, encodes the periplasmic thiosulfate-binding protein CysP; the *cysTW* genes encode membrane proteins CysT and CysW, respectively; *cysA* encodes the ATP-binding protein CysA; and *cysM* encodes the above mentioned *O*-acetylserine sulfhydrylase B, involved in the biosynthesis of cysteine. Finally, located in another chromosomal region, the *sbp* gene encodes the periplasmic sulfate-binding protein Sbp (Sirko *et al.*, 1990), which serves as an initial high-affinity receptor for sulfate (**Figure 1**).

2.1.1.- Periplasmic sulfate (Sbp)- and thiosulfate (CysP)-binding proteins

The SulT permease consists of two periplasmic substrate-binding proteins, Sbp and CysP (Hryniewicz *et al.*, 1990; Sirko *et al.*, 1995) (**Figure 1**). The Sbp sulfate-binding protein of *S. typhimurium* was the first periplasmic substrate-binding protein identified (Pardee, 1966; Pardee and Watanabe, 1968) and crystallized (Pflugrath and Quiocho, 1988). The Sbp protein of *S. typhimurium,* of 329 amino acids (accession number P02906), consists of two similar globular domains that adopt an ellipsoidal shape, with the ligand-binding site located deep in the cleft between the two domains and where the sulfate substrate is bound completely desolvated (Pflugrath and Quiocho, 1985). Sulfate is tightly held in place by seven hydrogen bonds, five of which are donated by main-chain peptide NH groups, another by a serine hydroxyl, and the last by the indole NH moiety of a tryptophan side-chain; there are no positively-charged residues nor cations or water molecules within van der Waals distance to the sulfate dianion (Pflugrath and Quiocho, 1985). In order to liberate sulfate, the protein must undergo a conformational change (Pflugrath and Quiocho, 1985), a behavior common to other substrate-binding proteins, like the maltose-binding protein (Ehrmann *et al.*, 1998). Sbp of *S. typhimurium* binds sulfate with a *Km* of 0.1 μM and chromate with almost the same affinity; it is able to discriminate by a factor of about 10^5 in favor of SO_4^{2-}, SeO_4^{2-}, and CrO_4^{2-} against HPO_4^{2-} or $H_2PO_4^-$, indicating that Sbp only binds tetrahedral, fully ionized oxyanions (Jacobson and Quiocho, 1988). Sbp of *E. coli* is very similar in size, sequence and antigenicity to the *S. typhimurium* protein and probably binds sulfate by a similar mechanism (Jacobson *et al.*, 1991).

The second periplasmic sulfate-binding protein of the SulT permease is CysP. In *E. coli*, CysP is a 313 amino acids protein (accession number P16700) whose major activity is thiosulfate binding (Hryniewicz *et al.*, 1990). A mutation in *cysP* decreases about 60 times the affinity for thiosulfate binding (Hryniewicz *et al.*, 1990).

CysP shows aproximately 44% sequence similarity with Sbp, from both *E. coli* and *S. typhimurium*; they belong to two different but related protein families (COG4150 and COG1613, respectively). Interestingly, the amino acid residues essential for sulfate binding are conserved in both proteins (Hryniewicz *et al.*, 1990). Sbp and CysP have partially overlapping activities, since a mutation inactivating one of them does not result in lacking of sulfate or thiosulfate binding or uptake (Sirko *et al.*, 1995). In contrast, mutations in both genes result in the inability to use sulfate or thiosulfate as sulfur source

(Sirko *et al.*, 1995). It has been suggested that both CysP and Sbp interact with membrane proteins CysT and CysW of the SulT permease, which explains the failure to isolate transport-defective mutants affected in either *sbp* or *cysP* (Hryniewicz *et al.*, 1990). The presence of two substrate-binding proteins in the SulT permease, whose function overlap may be an adaptive advantage for the cell under certain environmental conditions.

2.1.2.- CysT and CysW membrane proteins

The second and third genes of the *cysPTWAM* operon, *cysTW*, encode the membrane proteins CysT (also named CysU, accession number P16701)) and CysW, which constitute the transport channel of the SulT permease (accession number P0EAB0) (Sirko *et al.*, 1990) (**Figure 1**). In *E. coli*, CysT is a 277-amino-acids protein and CysW a 291-amino-acids protein, which share 30% identity (53% similarity) with each other; each protein displays six putative transmembrane segments (TMSs) (Sirko *et al.*, 1990). A mutant affected in *cysT* removes 11 and 6.5 times less sulfate and thiosulfate_from the incubation mixture, respectively, than the wild type, which results in the inability of cells to grow with sulfate and thiosulfate as sulfur source (Hryniewicz *et al.*, 1990). In *E. coli* and several other bacteria, mutations in the *cysTWA* gene cluster confer a requirement for cysteine or for a sulfur source different than sulfate, but in *Burkholderia cenocepacia* transposon insertions in the *cysW* gene do not result in cysteine auxotrophy (Farmer and Thomas, 2004). The *cysW* mutants of *B. cenocepacia* has similar growth properties than the wild-type strain in the presence of ≥0.5mM of sulfate, even when sulfate uptake by the mutants was 50-fold less efficient (Farmer and Thomas, 2004). Since the genome of *B. cenocepacia* apparently does not possess additional copies of *cysW* genes, it was proposed that CysT may partially substitute CysW in sulfate uptake by this bacterium (Farmer and Thomas, 2004).

2.1.3.- CysA, the ATPase subunit

In the *cysPTWAM* operon, the *cysA* gene encodes CysA, the ATPase subunit of the transporter (**Figure 1**). CysA couples the hydrolysis of ATP to the translocation of sulfate (Sirko *et al.*, 1990). The crystal structure of CysA from the Gram positive

thermoacidophilic bacterium *Alicyclobacillus acidocaldarius* has been solved to a resolution of 2.0 Å (Scheffel *et al.*, 2005); this is the only CysA protein structurally analyzed to date.

CysA displays two different dimers in the asymmetric unit, the well defined CysA-1 and the CysA-2 that is fairly flexible (Scheffel *et al.*, 2005). CysA from *A. acidocaldarius* is a functional dimer, with each monomer composed of a regulatory domain, which is present only in some ABC-ATPases, and the nucleotide-binding domain, which has a fold essentially similar to other ABC-ATPases. The regulatory domain has a similar fold in all the ABC-ATPases where it is present, and it has been suggested that it might function as a signal-transduction module (Scheffel *et al.*, 2005). The NBD of CysA is subdivided into a catalytic subdomain and a helical subdomain. The NBDs of the ABC-ATPases possess the highly conserved motifs, the 'Walker' A motif, with consensus sequence GXXGXGKS/T, where X can be any residue, the 'Walker' motif B, with sequence hhhhD, where h stands for hydrophobic amino acids, and the ABC signature motif LSGGQ (also called C-loop) that is unique to the ABC transporter superfamily. The NBDs also possess several motifs with only one highly-conserved residue, such as the Q-, Pro-, D- and H-loops (Oswald *et al.*, 2006). All these motifs are highly conserved on CysA proteins.

2.1.4.- CysM, the *O*-acetylserine sulfhydrylase B

In *E. coli* and *S. typhimurium* the last gene of the *cysPTWAM* operon, *cysM*, encodes the already mentioned *O*-acetylserine sulfhydrylase B (*O*-acetylserine (thiol)-lyase) (Sirko *et al.*, 1990). This enzyme participates in the central anabolic pathway of bacteria to produce cysteine (**Figure 2**). In *E. coli* and *S. typhimurium* two isoenzymes have been characterized, the *O*-acetylserine sulfhydrylases A (CysK) and B (CysM), which are produced under aerobic and anaerobic growth conditions, respectively (Kredich and Tomkins, 1966). Both are homodimeric enzymes, have about 43% of amino acids in common, and catalyze the same step in cysteine biosynthesis. The proposed chemical catalytical mechanism of both isoenzymes is similar, but they have different substrate specificities. CysM seems to be less substrate selective than CysK (Tai *et al.*, 1993). Both enzymes can use sulfide but, despite their high similarity, only CysM is able to use thiosulfate in lieu of sulfide to synthesize S-sulfocysteine (**Figure 2**) (Kredich, 1992). The

three-dimensional structures of CysM and CysK show that they exhibit an overall fold very similar to each other, the main difference being a more hydrophilic active site of CysM due to the presence of two ionizable residues that replace two neutral residues in CysK. The turnover number of CysM is higher than CysK, and the *Km* for their substrate *O*-acetylserine is lower (Burkhard *et al.*, 1998; Claus *et al.*, 2005; Chattopadhyay *et al.*, 2007). CysM plays an important role in thiosulfate utilization, since it allows the incorporation of sulfur from thiosulfate without the need of sulfate reduction, a step which consumes two ATP molecules (Kredich and Tomkins, 1966). In spite of the importance of *cysM*, this gene is found as part of the *cysPTWAM* operon mainly in γ-proteobacteria, and it is absent in the operons of other bacteria such as *M. tuberculosis* (Woof, 2002)*,* and the cyanobacterium *Synechococcus elongatus* PCC7942 (Laudenbach and Grossman, 1991) (**Figure 3**).

2.1.5.- Distribution of the *cysPTWAM* operon

The *cysPTWAM* operon is widely distributed among bacteria and displays two different alternative forms depending of the substrate-binding protein present, the *sbp*-containing operons and the *cysP*-containing operons (**Figure 3**). The *sbp*-containing operon is unique in some members of the α-, β-, and γ–proteobacteria, and is the only operon in all δ-, ε-proteobacteria, actinobacteria, firmicutes and cyanobacteria (**Figure 3**). A few members of α-, β-, and γ–proteobacteria have only the *cysP*-containing operon, like *Silicibacter sp.*, *Herminiimonas arsenicoxydans*, and *Yersinia pestis* (**Figure 3**). The two alternative operons are present in some members of the α-, β-, and γ–proteobacteria such as *Agrobacterium tumefaciens*, *Janthinobacterium sp.*, *and Pseudomonas mendocina* (**Figure 3)**.

The *sbp*-containing and the *cysP*-containing operon show distinct gene arrangements among the different taxonomic groups. Both operons have a minimum of four genes: *sbp*, *cysT*, *cysW* and *cysA* (**Figure 3**). The exception is like *Magnetospirillum magneticum* that shows a minimum of three genes: *cysP*, *cysT* and *cysW* (**Figure 3**). The operons of some bacteria also contain genes involved in sulfur metabolism (**Figure 3).** In various bacteria, like *E. coli*, the *sbp* gene is located in another region of the chromosome and does not form part of the operon.

The presence of the *sbp*-containing operon or the *sbp* gene alone in all taxa analyzed probably is due to the preference of sulfate against thiosulfate as a sulfur source. The presence of two operon forms in several bacterial taxa indicates the importance of the redundancy of the uptake system, and a posible advantage for the organisms. In *E. coli,* a mutant that does not produce the Sbp protein, can grow on sulfate since CysP acts as a functional backup for the Sbp protein (van der Ploeg *et al.*, 1997; Han and Lee, 2006).

Sulfate permeases with similar characteristics to the SulT system have been described in actinobacteria. Such is the case for *M. tuberculosis*, where the sulfate permease components are encoded by a cluster formed by the *subI* and *cysTWA* genes. *subI* encoded a sulfate-binding protein that is similar to Sbp. The *Km* for sulfate uptake of the *M. tuberculosis* permease is 36 μM (Wooff *et al.*, 2002), a value identical to the *Km* of the *S. typhimurium* SulT permease (Dreyfuss, 1964).

In *B. cenocepacia*, the sulfate transporter is encoded by the *sbp cysTWA* gene cluster (Farmer and Thomas, 2004), which forms part of an operon that is probably expressed from a promoter located upstream of the *sbp* gene (Iwanicka-Nowicka *et al.*, 2007).

A sulfate-thiosulfate SulT permease has been also described in the cyanobacterium *Synechococcus elongatus* PCC 7942 (previously known as *Anacystis nidulans* R2) (Green and Grossman, 1988; Laudenbach and Grossman, 1991). Sulfate transport in *S. elongatus* is light- and energy-dependent (Green and Grossman, 1988), and, as it occurs in *S*. *typhimurium*, is inhibited by thiosulfate, selenate and chromate (Green and Grossman, 1988). The genes that encode *S. elongatus* sulfate permease (cysA *sbpA*-*cysT*-*orf81*-*cysR*-*cysW)* have a different arrangement as compared with those of the *E*. *coli* and *S*. *typhimurium* operons **(Figure 3)**, but mutations on the corresponding genes cause the same effects on sulfate transport (Laudenbach and Grossman, 1991). *cysA* is the first gene and is transcribed in the opposite direction to the rest of the genes; insertional inactivation of *cysA* results in cysteine auxotrophy (Green *et al.*, 1989). *cysT* and *cysW* gene products are essential for sulfate transport since mutations on each of these genes decrease the V_{max} for sulfate transport about 25 times compared to the wild-type strain (Laudenbach and Grossman, 1991). The genome of *S. elongatus* possess three copies of the *sbp* gene, the *sbpA* gene already described, other located in an adyacent operon, and the third *sbp* gene is located in another region of the chromosome

(**Figure 3**). Unlike the *E. coli* and *S. typhimurium* operons, the *cysT* and *cysW* genes from *S. elongatus* are separated by the ORF81 and the *cysR* gene (Laudenbach and Grossman, 1991) (**Figure 3**). Neither the *cysR* gene nor the Orf8l products are require for growth on sulfate or thiosulfate, but mutants in these genes are incapable of growth in thiocyanate as a sulfur source (Laudenbach and Grossman, 1991). The *cysR* product has homology to regulatory proteins which possess the 'helix-turn-helix' motif involved in DNA binding, but is not related to the CysB protein that regulates sulfate transport in *E. coli* and *S. typhimurium* (Laudenbach and Grossman, 1991).

The genome of another cyanobacterium, *Synechocystis* sp. PCC 6803, contains an operon encoding a sulfate permease similar to that of *S. elongatus* (Kohn and Schumann, 1993). The order of the genes is *sbpA*-*orf97*-*cysT*, where *sbpA* encodes Sbp, a sulfate-binding protein, the o*rf97* encodes a protein with 61.5% of sequence similarity to ORF81 from *S. elongatus*; and *cysT* encodes a channel-forming protein. These proteins probably have similar functions to the proteins of the homologous operons already described (Kohn and Schumann, 1993).

2.1.6.- Regulation of the sulfate transport

Sulfate and thiosulfate uptake varies according to the availability of cysteine or other sulfur sources. Sulfate transport is repressed when cells are grown in the presence of cysteine and is derepressed in the absence of sulfate (Dreyfuss, 1964). Gene products required for transport of sulfate and thiosulfate are regulated as part of the cysteine regulon (Hryniewicz and Kredich, 1991), which includes genes involved in the biosynthesis of cysteine from sulfate (Kredich, 1992).

The maximum expression of the cysteine regulon, that includes the *cysPTWAM* operon, requires both sulfur limitation and the presence of the inducers, *O*-acetylserine, that is a precursor in the biosynthesis of cysteine (**Figure 2**), or *N*-acetylserine, that is derived nonenzymatically from *O*-acetylserine by an intramolecular *O*-to-*N*-acetyl shift. The repression of the cysteine regulon by growth on cysteine, or other readily utilizable sulfur sources, is due to the inhibition of serine transacetylase by cysteine that results in a decrease in the synthesis of the inducer *O*-acetylserine (Kredich, 1992) (**Figure 2**). The transcriptional activator CysB is also a requeriment for high expression levels of the

regulon (Hryniewicz and Kredich, 1991). In *E. coli,* excess of sulfate in the growth medium reduces the expression of the genes regulated by CysB and by the inducer *N*-acetylserine, by 40 to 50% of fully derepressed values, whereas in the presence of cysteine gene expression is completely repressed (van der Ploeg *et al.*, 1996). A similar regulation occurs in *Pseudomonas aeruginosa*, where the *sbp* and *cysTWA* genes are overexpressed when cells are grown in the absence of sulfate (Hummerjohann *et al.*, 1998; Tralau *et al.*, 2007).

CysB belongs to the LysR family of regulators (Kredich, 1992). Like other LysR-type transcriptional regulators, CysB acts with a dual function capable of either activation or repression of transcription from different target promoters (Lochowska *et al.*, 2001). The inducers *O*-acetylserine or *N*-acetylserine stimulates binding of CysB to the promoters of the *cysJIH* operon (which encodes enzymes involved in the reduction of sulfate and sulfite; **Figure 2**), and of the *cysK*, *cysP*, and *cysB* genes (Monroe *et al.*, 1990; Hryniewicz and Kredich, 1991; Ostrowski and Kredich, 1991).

In *S. typhimurium* several different types of CysB-binding sites (CBS) have been identified which are categorized by function and by their responses to the inducer (Hryniewicz and Kredich, 1995). The *cysB* gene is negatively autoregulated by the binding of the CysB protein to the positions -10 to +36 relative to the major transcription *cysB* start site (Ostrowski and Kredich, 1991). In this case, the inducer *N*-acetylserine inhibits the binding of CysB to the *cysB* promoter and partially reverses the inhibition of transcription initiation caused by CysB; this means that CysB has the ability to respond to acetylserine with either an increase or a decrease in the affinity for different DNA sequences (Ostrowski and Kredich, 1991).

In *S. typhimurium* and *E. coli* the CysB proteins contain 324 amino acids and their sequences are 95% identical (Ostrowski *et al.*, 1987). CysB is a homotetramer of 36-kDa subunits (Miller and Kredich, 1987) that possesses several functional domains, including a DNA-binding “helix-turn-helix” (HTH) motif (Ostrowski *et al.*, 1987). The CysB regulator is widely analyzed and already are described the regions and amino acids involved in each step of the mechanism of regulation. Mutational analysis of CysB from *E. coli* identified essential regions for DNA binding, inducer response, and oligomerization (Lochowska *et al.*, 2001). Mutations within the predicted HTH motif and its close vicinity,

localized in the N-terminal region, impaired the ability of CysB to negatively autoregulate and also to activate the *cysP* promoter, which indicates that these regions are crucial for the DNA-binding function of CysB (Lochowska *et al.*, 2001). In the central region of CysB were located mutations that alter the response to the inducer. Changes in the C-terminal region affected DNA binding, oligomerization and stability of CysB (Lochowska *et al.*, 2001).

2.2.- Sulfate permeases of the SulP family

The sulfate permease SulP family (TC# 2.A.53) is a large and ubiquitous protein family with hundreds of sequenced members derived from the three domains of life (**Table 1**); however, only a few proteins belonging to this family have been functionally characterized (Saier, 2000; Kertesz, 2001; Saier *et al.*, 2006). The SulP proteins possess from 430 to 900 amino acids (Saier, 2000), but the bacterial members are of 434 to 573 residues, with some exceptions. In contrast to the SulT-type sulfate permeases, which transport sulfate and related oxyanions, SulP family members are inorganic anion uptake carriers or anion:anion exchange transporters (Felce and Saier, 2004). Some proteins of the SulP family may be fused to different catalytic or ligand-binding domains or may be associated with enzymes. The fused domain can determine substrate specificity for many of these proteins. If the carrier is fused to enzymes related to sulfur metabolism, for example, this suggests that it may function in sulfate uptake (Felce and Saier, 2004).

The genome of the γ-proteobacterium *Acidithiobacillus ferrooxidans* contains the *sulP* gene that encodes a SulP-type sulfate permease which exhibits 11 predicted TMSs (Váldes *et al.*, 2003). The s*ulP* gene is separated by 10 base pairs from the upstream gene *cab1* encoding a carbonic anhydrase. The juxtaposition of *cab1* and *sulP* in *A. ferrooxidans* suggests that SulP functions as a SO_4^{2-}:$HCO3^-$ antiporter (Váldes *et al.*, 2003).

SLC26 anion exchangers are a gene family that is a part of the SulP family; they transport a wide number of monovalent and divalent anions such as sulfate, chloride, iodide, formate, oxalate, the hydroxyl ion, and bicarbonate (Mount and Romero, 2004). SLC26 proteins are predicted to have 10-14 TMSs and a C-terminal extension, designated as the STAS (from sulfate transporters and anti-sigma antagonist) domain, that appears to play

a role in the function/regulation of the transport activity (Shibagaki and Grossman, 2006). The SLC26 proteins appear to be assembled as dimers composed of two identical subunits with the amino and carboxyl termini at the intracellular membrane side (Detro-Dassen *et al.*, 2008), which suggest that the SulP family members are dimers. The genome of *M. tuberculosis* contains the SulP-type gene Rv1739c, which is related to SLC26 (Zolotarev *et al.*, 2008). The expression of Rv1739c in *E. coli* increases the uptake of sulfate but not of chloride, formate, or oxalate; sulfate uptake was inhibited by sulfite, selenate and thiosulfate (Zolotarev *et al.*, 2008). The expression of Rv1739c in *Mycobacterium bovis* BCG with the *cysA* gene deleted did not complement sulfate auxotrophy, suggesting that Rv1739c requires the *cysA* gene for sulfate transport (Zolotarev *et al.*, 2008).

Several proteins annotated as probable sulfate transporters of the SulP family have not been functionally characterized and they may be carriers of other ions. For example, the BicA transporter of the marine cyanobacterium *Synechococcus* sp. PCC700 is a HCO_3^- ion transporter that was initially reported as a sulfate transporter (Price *et al.*, 2004).

2.3- The CysP sulfate permease

Contrary to the extensive knowledge available for sulfate transport in Gram negative bacteria, mainly through the studies in *E. coli* and *S. typhimurium*, there is few detailed information reported in Gram-positive bacteria. One of the few sulfate transporters identified and biochemistry characterized in Gram-positive bacteria is the sulfate permese CysP, encoded by the *cysP* gene (named before as *ylnA*), identified in *Bacillus subtilis* (Mansilla and de Mendoza, 2000). Although this protein shares the same name, it is unrelated to the thiosulfate-binding protein, also named as CysP, from *E. coli* and *S. typhimurium* (already described in section 2.1).

Expression of the *cysP* gene from *B. subtilis* in *E. coli* mutants affected in *cysA97*, *cysT* or *cysP-sbp* genes restored the ability of the strains to grow in minimal medium with sulfate as a sole sulfur source; moreover, the *E. coli* mutant strain carrying a plasmid with *B. subtilis-cysP* gene accumulated sulfate, whereas the *E. coli* strain without the *cysP* gene did not show accumulation of sulfate, indicating that *B. subtilis* CysP is a sulfate transporter (Mansilla and de Mendoza, 2000).

The *B. subtilis* CysP permease is predicted as a membrane protein with 11 TMS, and two homologous domains that might have arisen by a tandem internal gene duplication event (Mansilla and de Mendoza, 2000; Salaun *et al.*, 2001). The amino and carboxyl termini of CysP are located at the extracellular membrane side (Salaun *et al.*, 2001; Virkki *et al.*, 2007).

B. subtilis CysP has not sequence homology with SulT- or SulP-type sulfate permeases, but shows similarity with phosphate permeases of the inorganic phosphate transporter (PiT) family (TC# 2.A.20) (**Table 1).** CysP possesses the Pho4 domain (COG0306), present in all members of the PiT family, confirming the relationship between the *B. subtilis* CysP permease and the PiT family (Mansilla and de Mendoza, 2000). However, it is unknown if, in addition to functioning as a sulfate permease, CysP is able to transport inorganic phosphate. To obtain additional insights to this last possibility, we constructed a phylogenetic tree with 786 protein sequences that belong to the PiT family (including the CysP permease). Genes encoding transporters of the PiT family are widely spread through the three domains of life, suggesting an ancient origin for this protein family. The archaeal and bacterial proteins are of a 300-550 aa length, whereas the eukaryotic proteins range from 500-1100 aa length. The larger eukaryotic mermbers have an intracellular domain only present in these proteins.

Three major clusters are observed in the phylogenetic tree for the PiT family (**Figure 4A**). Cluster I, on the right-hand side of the tree, is the most numerous group (374 sequences) and is represented mainly by a mixture of archaeal and bacterial clades, either of Gram-positive and Gram-negative. The inorganic phosphate transporters reported in *E. coli*, PitA and PitB, are included in this cluster (Harris *et al.*, 2001). Cluster II (bottom, left-hand side of the tree) is almost as numerous as Cluster I (340 sequences), and comprise members of the three domains of life. This group includes characterized eukaryotic phosphate transporters: mPit-2 from mouse (Bai *et al.*, 2000), Pht2;1 from potato (Rausch *et al.*, 2004), Pht2;1 from Arabidopsis (Daram *et al.*, 1999), and Pit-1 and Pit-3 from human (Ravera *et al.*, 2007). The Cluster III (top, left-hand side of the tree) comprise 72 proteins (41 from archaea and 31 from bacteria). The sulfate permease CysP from *B. subtilis* is included in this group, but no additional characterized sequences belong to this group.

Figure 4B shows a detailed view of Cluster III. This group comprises two branches: subgroup III-A is formed mainly by members of the bacterial clade Firmicutes (including *B. subtilis* CysP) and the archaeal clade Euryarchaeota; subgroup III-B is formed by members of the bacterial clades Thermotogae, Cyanobacteria and proteobacteria, and the archaeal clades Euryarchaeota and Crenarchaeota. Additionally, there is one divergent archaeal protein (YP_001040684 from *Staphylothermus marinus*) in cluster III.

CysP from *B. subtilis* belongs to an operon that comprises four ORFs encoding enzymes involved in sulfur metabolism (Dam *et al.*, 2007). This genetic arrangement is present also in *B. licheniformis*; however, several other species from the subgroup III-A possess orthologous *cysP* associated with sulfur metabolism genes. This last suggests that CysP from *B. subtilis* is not the only PiT family member involved in sulfate transport.

Bøttger and Pedersen (2005) identified inside PiT family sequences two related motifs containing highly conserved aspartate residues critical for phosphate transport. Indeed, the replacement of these aspartates with asparagines severely impaired the transport function, but did not perturb the overall protein architecture (Bøttger and Pedersen, 2005). However, the aspartate residues critical for phosphate transport are not strictly conserved. Interestingly, CysP from *B. subtilis* possesses the two conserved motifs, but the aspartate residues are replaced by glycine and asparagine (Figure 4B). This finding agrees with the fact that CysP is a sulfate permease and suggests that this protein is not able to transport inorganic phosphate.

It is important to mention that the two motifs reported by Bøttger and Pedersen (2005) are conserved with no exception in all 786 sequences analyzed in the Figure 4A as members of the PiT family.

To compare the motifs differences among the protein sequences of PiT family members, we performed a sequence logos (**Figure 5**).

The motif logos of Clusters I and II (that comprise 714 sequences, including the experimentally characterized phosphate transport proteins), illustrate that both critical aspartate residues are strictly conserved, with none exception. This suggests that all members of cluster I and II are phosphate transporters. In contrast, the logos analysis of Cluster III-A (comprising 30 sequences, including CysP from *B. subtilis*) shows that no acidic residues are conserved inside N and C motifs. This suggests that the protein

members of this cluster are not phosphate transporters, and that probably they function as sulfate permeases. The logos of Cluster III-B (41 sequences with no characterized protein) shows that the acidic residues are present, but not strictly conserved, and therefore their transport function is uncertain. Additional experimental data are needed to prove the above mentioned hypotheses.

2.4.-The CysZ sulfate permease

Sulfate permeases similar to SulT, SulP or CysP were not identified in the genome of the Gram-positive soil bacterium *Corynebacterium glutamicum*; instead, *C. glutamicum* has the CysZ sulfate permease (TC# 9.B.7) (**Figure 1**), which is proposed as the principal sulfate permease in this microorganism (Rückert *et al.*, 2005). CysZ, predicted to possess six TMSs, is encoded by the *cysZ* gene of in the *cysIXHDNYZ* operon. The enzymes encoded by *cysIHDN* genes catalyze similar reactions to the known orthologous in *E. coli* (Rückert *et al.*, 2005) (**Figure 1**). Furthermore, strains of *C. glutamicum* with deletions in each of the *cysIHDN* genes were unable to grow in a medium containing sulfate or sulfite as a sulfur sources, demonstrating that they are involved in the reduction of inorganic sulfur compounds. In contrast, the *cysXYZ* genes from *C. glutamicum* are described for the first time. CysZ belongs to the putative sulfate transporter family (**Table 1**) (Rückert *et al.*, 2005). CysZ is a high-affinity sulfate transporter because a *C. glutamicum* mutant affected in *cysZ* was unable to grow with less than 5 mM sulfate as a sole sulfur source. Growth of the mutant was restored by increasing sulfate, and above 30 mM sulfate the growth was similar to wild-type strain, suggesting the presence of a low-affinity sulfate transporter that functions at high sulfate concentrations. The CysX and CysY proteins are probably involved in electron transfer and biosynthesis of the siroheme cofactor of sulfite reductase, respectively. It has been suggested that they participate in the sulfite into sulfide reduction (Rückert *et al.*, 2005).

The genome of *E. coli* possesses a gene that encodes the CysZ protein, reported as a sulfate transporter in the TCDB database; however it is only 13% identical to CysZ from *C. glutamicum*. A BLAST search of CysZ from *C. glutamicum* aligned mostly with homologs located in related bactea, *like C. efficiens*, *Mycobacterium* sp. and *Bacillus cereus*. In contrast, CysZ from *E. coli* aligned with homologs located in enterobacteria like *Shigella*

dysenteriae, S. typhimurium and *Vibrio cholerae*. Even when CysZ from *E. coli* is reported as a sulfate permease its function has not been experimentally tested.

3.-Molybdate and tungstate transport

Molybdenum is a transition trace metal that is required by almost all living organisms including microorganisms, plants, and animals. The few species that do not require molybdenum use tungsten. Molybdenum-containing enzymes, or molybdoenzymes, are found in all aerobic organisms, whereas tungsten-containing enzymes are only found in obligate thermophilic anaerobes (Hille, 2002). Molybdenum and tungsten are transported in the form of the oxyanions molybdate (MoO_4^{2-}) and tungstate (WO_4^{2-}) which are taken up through the membrane by high affinity ABC-type transporters. These transporters allow bacteria to scavenge the less abundant molybdate or tungstate ions in the presence of sulfate, whose concentration in sea water is about 10^5 times higher than that of molybdate (Schwarz *et al.*, 2007). Due to the similarity between molybdate and tungstate oxyanions they can be transported by the same carrier (Grunden and Shanmugam, 1997; Bevers *et al.*, 2006).

In *E. coli*, molybdate may be taken up through three transport systems: 1) the ModABC system (Maupin-Furlow *et al.*, 1995), 2) the CysPTWA (SulT) sulfate-thiosulfate permease in *mod* mutants (Rosentel *et al.*, 1995), and 3) a nonspecific low-efficiency anion transport system that requires high molybdate concentrations (Lindblow-Kull *et al.*, 1985) (**Table 2**).

3.1-The ModABC molybdate transporter

The high-affinity ModABC molybdate system, which also transports tungstate and sulfate (Grunden and Shanmugam, 1997; Self *et al.*, 2001), belongs to the molybdate uptake transporter (MolT) family (TC# 3.A.1.8) (**Table 2**). In *E.* coli, the ModABC transporter is encoded by the *modABC* operon (Maupin-Furlow *et al.*, 1995). The *modA* gene encodes the periplasmic molybdate-binding protein ModA; *modB* encodes the integral membrane protein ModB; and *modC* encodes the ATPase subunit ModC (Maupin-Furlow *et al.*, 1995). (**Figure 6**)

The molybdate-binding protein ModA binds specifically molybdate and tungstate and does not bind sulfate, phosphate and selenate (Rech *et al.*, 1996). *E. coli* ModA binds

molybdate and tungstate with a *Kd* of about 20 nM (Imperial *et al.*, 1998), but a lower affinity (*Kd* of 0.29 μM for molybdate and 0.58 μM for tungstate) is shown by ModA from the phytopathogen bacterium *Xanthomonas axonopodis* pv. *citri* (Balan *et al.*, 2006). The crystal structure of the ModA proteins from *E. coli*, *Azotobacter vinelandii* and *X. axonopodis* has been solved to high resolution (Lawson *et al.*, 1998; Balan *et al.*, 2008). It was found that they possess an ellipsoidal conformation and that the oxyanion is bound through seven hydrogen bonds, similar to the Sbp sulfate-binding protein. Since ModA proteins have a low affinity for sulfate (*Kd*>2mM), they probably discriminate between molybdate and sulfate due to small differences in the sizes of the two oxyanions (Lawson *et al.*, 1998).

The crystal structures of ModA/WtpA molybdate- or tungstate-binding proteins (see section 3.3 for tungstate transport) of the archaea *Archaeoglobus fulgidus*, *Methanosarcina acetivorans*, *Methanocaldococcus jannaschii*, *Pyrococcus horikoshii* and *Pyrococcus furiosus* have a fold highly similar to that of the bacterial ModA proteins from *E. coli*, *A. vinelandii* and *X. axonopodis*, but their transport properties have not been elucidated (Hollenstein *et al.*, 2009). The main structural differences of archaeal proteins include an additional β-sheet located on the protein surface and, most notably, an oxyanion-binding site with an octahedral coordination for the molybdenum and tungsten atoms (Hollenstein *et al.*, 2009), in contrast with bacterial ModA proteins which possess a tetrahedral coordination (Hu *et al.*, 1997; Lawson *et al.*, 1998; Balan *et al.*, 2008). These differences suggest distinct oxyanion-binding modes of the ModA/WtpA proteins from archaea and bacteria. This change in increasing the coordination chemistry may be an effective way to distinguish molybdate and tungstate from highly-abundant sulfate, but it is not a requirement for high-affinity binding of the oxyanion (Hollenstein *et al.*, 2009).

The membrane channel of the ModABC transporter is constituted by the ModB protein (**Figure 6**). The recent crystallization of ModB proteins from the archaea *A. fulgidus* and *M. acetivorans*, has shown that ModB possesses six TMSs (Hollenstein *et al.*, 2007; Gerber *et al.*, 2008). ModB functions as a homodimer to form the channel for molybdate transport (**Figure 6**) (Grunden and Shanmugam, 1997), distinct to the SulT sulfate permease where two proteins, CysT and CysW, constitute the channel for sulfate transport (**Figure 1**) (Sirko *et al.*, 1990). ModB proteins have the conserved motifs

LPLVLPP(V/T/S)VhG(F/Y)XL, where h is a hydrophobic amino acid and X stands for any amino acid, and FAR(S/T)LGEFG(A/V)(T/V), which are also conserved in CysT, CysW, and in the phosphate permease proteins PstA and PstC (Self *et al.*, 2001). These motifs constitute the gate of the ModB protein that separates the translocation pathway from the extracytoplasmic space (Hollenstein *et al.*, 2007).

ModC is the ATPase of the ModABC complex that energizes the transport of molybdate. ModC possesses all the characteristic ATPase motifs described above, such as the 'Walker' A and B motifs, and the signature sequence of ABC transporters, but does not have additional unique sequences. The crystal structures of ModC proteins from *A. fulgidus* and *M. acetivorans* have shown that they have similar folds and contain the highly-conserved motifs (Hollenstein *et al.*, 2007; Gerber *et al.*, 2008). The two ModC subunits exhibit a "head to tail" arrangement, with the conserved phosphate-binding loop (P-loops) juxtaposed to the ABC signature motifs (LSGGQ) of the opposite subunit (Hollenstein *et al.*, 2007; Gerber *et al.*, 2008). Even when the ModC proteins have a similar fold, only ModC from *M. acetivorans* has a regulatory domain; this is a region of approximately 120 amino acid residues appended to the C-terminus, which is also present in the CysA protein (Gerber *et al.*, 2008).

Similar *modABC* operons encoding molybdate transport proteins have been identified in the genomes of several bacterial species, but only a few have been characterized (Self *et al.*, 2001). These include those of *A. vinelandii* (Mouncey et al. 1995), *Staphylococcus carnosus* (Neubauer *et al.*, 1999), *Rhodobacter capsulatus* (Wang *et al.*, 1993), *Klebsiella pneumoniae* (Grunden and Shanmugam, 1997), *Anabaena variabilis* (Zahalak *et al.*, 2004), *Bradyrhizobium japonicum* (Delgado *et al.*, 2006), and *X. axonopodis* pv. *citri* (Balan *et al.*, 2006).

The crystal structures of putative ModABC transporters of *A. fulgidus*, with ModA bound to molybdate or tungstate (Hollenstein *et al.*, 2007) (**Figure 7**), and *M. acetivorans*, were recently solved (Gerber *et al.*, 2008). The *A. fulgidus* ModABC transporter is constituted by a single ModA protein with the molybdate oxyanion bound to the external side of a $ModB_2C_2$ complex. Molybdate or tungstate oxyanions are bound in a cleft between the two lobes; both lobes interact with ModB protein and there are several charged amino acid residues localized in the interface. ModA aligns the substrate-binding cleft with the

entrance to the translocation pathway on ModB, which is constituted by two monomers (Hollenstein *et al.*, 2007) (**Figure 7**). The ModB subunits form a large cavity (the translocation pathway), constituted by TMSs 3 and 5, that is only accessible from the cytoplasm, and is separated from the extracytoplasmic space by a closed gate, adopting an inward-facing conformation (Hollenstein *et al.*, 2007). ModC was purified in a nucleotide-free state, adopting an open conformation. The ModB–ModC interface transmits critical conformational changes, thus coupling ATP binding and hydrolysis to transport. A stoichiometry of two ATP per imported molybdate has been proposed, based on the structure of the $ModB_2C_2A$ complex (Hollenstein *et al.*, 2007).

The ModBC transporter of *M. acetivorans* was crystallized with tungstate bound to ModC (Gerber *et al.*, 2008). As mentioned above, ModC subunits contain regulatory domains which provide two oxyanion-binding sites located at the dimer interface, with both subunits contributing to both tungstate-binding pockets. The role of oxyanion binding in this region is to decrease transporter function by preventing the dimerization of ModC subunits (Gerber *et al.*, 2008). The rate of ATP hydrolysis of the transporter was inhibited at low micromolar concentrations of molybdate or tungstate added, with an apparent inhibitory constant of ~5 μM; in *A. fulgidus* the ATPase activity was insensitive even to high concentrations (1 mM) of molybdate or tungstate (Gerber *et al.*, 2008). This mechanism of regulation, known as trans-inhibition, occurs when substrates exert a concentration-dependent inhibitory effect on the transporter after translocation has occurred (Gerber *et al.*, 2008). In *M. acetivorans*, it has been suggested that the trans-inhibition mechanism can substitute the genetic regulation of the molybdate transporter due to the absence of ModE, the ModABC transporter regulator, in the genome (Gerber *et al.*, 2008) (see next section).

3.1.1-Regulation of the ModABC transporter

Transcription of the *modABC* operon is negatively controlled by the molybdate-responsive ModE protein (Mouncey *et al.*, 1995; Walkenhorst *et al.*, 1995; Grunden *et al.*, 1996). In *E. coli*, ModE is encoded by the *modEF* operon that is transcribed in the opposite direction with respect to *modABC* (Mouncey *et al.*, 1995). The ModF protein has two ATP/GTP-

binding motifs, but its function in molybdate transport or metabolism is unknown (Grunden *et al.*, 1996; Grunden and Shanmugam, 1997).

Repression of the transcription of *modABC* operon by ModE is enhanced by molybdate, and mutations in *modE* gene derepressed the expression of the operon even in the presence of molybdate (Grunden *et al.*, 1996). The *modE* gene is constitutively transcribed at low levels, which is consistent with its proposed role as a repressor, since higher levels of *modE* gene expression would keep completely repressed the transporter. The molybdate-binding domain in ModE contains the consensus sequence TSARNQXXG (amino acids 125 to 133) that is essential for the interaction of the protein with molybdate (Grunden and Shanmugam, 1997). ModE has been also reported as an activator of the *moaABCDE* operon, which encodes the enzymes involved in molybdopterin cofactor biosynthesis (McNicholas *et al.*, 1997).

The active form of ModE that binds to the modA operator is the ModE-molybdate complex (Anderson *et al.*, 1997). *E. coli* ModE functions as a homodimer and each dimer binds two molecules of molybdate with a high affinity (Kd = 0.8 μM); ModE also binds tungstate (Anderson *et al.*, 1997). Molybdate or tungstate binds directly to ModE without a requirement for any other component; the binding of the oxyanion induces a conformation change of ModE (Anderson *et al.*, 1997; Gourley *et al.*, 2001; Schüttelkopf *et al.*, 2003). In *E. coli* the apparent K_d for the interaction between the *modA* operator and the ModE-molybdate complex is of 0.3 nM, and this value increased to 8 nM in the absence of molybdate (Self *et al.*, 2001).

The ModE monomer consists of two domains, the N-terminal domain, which possesses the HTH motif for DNA binding and is primarily responsible for ModE dimerization, and the C-terminal domain, which is the putative molybdate-binding component (Hall *et al.*, 1999). *E. coli* ModE discriminates between oxyanions based on their size and charge, binding molybdate or tungstate but not other tetrahedral oxyanions such as phosphate, sulfate, or vanadate (Gourley *et al.*, 2001).

3.2- The nonspecific anion transport system

The growth of *E. coli* mutants affected in the *mod* genes in rich LB medium, where the SulT sulfate-thiosulfate permease is repressed, indicates that another pathways, is also

involved in molybdate transport (Rosentel *et al.*, 1995). These mutants required higher molybdate concentrations, which indicate that this distinct system is a low-affinity transporter. This pathway has been named as the nonspecific anion transport system in *E. coli* because it can also transport sulfate, selenate, selenite (Lindblow-Kull *et al.*, 1985; Lee *et al.*, 1990; Self *et al.*, 2001) (**Figure 6**). To our knowledge there is not a recent analysis of this transporter.

3.3- The TupABC and WtpABC tungstate transporters

The tungstate oxyanion (WO_4^{2-}) is taken up by bacterial cells through the ABC-type transporters TupABC, which is highly specific for tungstate (Makdessi *et al.*, 2001), WtpABC, that transports both tungstate and molybdate (Bevers *et al.*, 2006), and by the already described molybdate ModABC transporter (Grunden and Shanmugam, 1997) (**Table 2**, **Figure 6**). TupABC, the first tungstate-specific transporter described, is encoded by the *tupABC* operon identified in the Gram negative bacterium *Eubacterium acidaminophilum* (Makdessi *et al.*, 2001). TupA, a periplasmic tungstate-binding protein of 286 amino acids, is highly specific for tungstate and does not bind molybdate, sulfate, chromate, selenate, phosphate or chlorate (Makdessi *et al.*, 2001). TupB is an inner membrane protein of 228 amino acids which possesses five TMS and has low similarity (<20%) to the ModB protein. TupC, of 214 amino acids, is the ATPase subunit of the complex (Makdessi *et al.*, 2001) (**Figure 6**). Homologs to this system have been identified in the archaea *Methanobacterium thermoautotrophicum* and *Haloferax volcanii,* and the Gram negative bacteria *Vibrio cholerae* and *Campylobacter jejuni* (Makdessi *et al.*, 2001).
The WtpABC transporter, identified in the archaeon *Pyrococcus furiosus*, is able to take up both tungstate and molybdate **(Table 2)** (Bevers *et al.*, 2006). WtpA binds tungstate with a higher affinity (Kd = 0.017 nM) than TupA (Kd = 500 nM) and ModA (Kd = 20 nM). The affinity for molybdate (Kd = 20 nM) is similar to that of ModA (Kd = 11 nM) (Bevers *et al.*, 2006). WtpA has a weak identity/similarity with ModA (18%/30%) and TupA (16%/31%), indicating that this is a new class of tungstate- and molybdate-binding proteins. Sequence similarities of WtpB with ModB and TupB are 53% and 50%, respectively, and those of WtpC with ModC and TupC are 51% and 56%, respectively (Bevers *et al.*, 2006). WtpA seems to be an archaeal tungstate transporter, whereas TupA

and ModA occur predominantly in bacteria. The WtpABC system is present in some archaea that do not express homologues of TupA or ModA, such as *P. furiosus* and *M. jannaschii* (Bevers *et al.*, 2006). Some archaea and bacteria have homologous genes for more than one type of tungstate transporter systems, such as *M. acetivorans* that possesses ModA and WtpA transporters, and *Desulfovibrio vulgaris* that has TupA and ModA homologs (Bevers *et al.*, 2006).

4.-Selenate transport

Selenium is an analog of sulfur and may substitute for sulfur in certain thiols. Although selenium is an essential element for microorganisms, is toxic at elevated concentrations. The common biological forms of selenium are the selenocysteine and selenomethionine amino acid analogs (Stolz *et al.*, 2006). In *E. coli* it has been described a single carrier that can transport selenate (SeO_4^{2-}), selenite (SeO_3^{2-}), and sulfate (Lindblow-Kull *et al.*, 1985). Sulfate is the preferred substrate, with an affinity (Km = 2.1 μM) about eight times higher than that for selenate (Km = 17.1 μM) and about 50 times higher for selenite (Km = 102 μM). All three oxyanions compete with each other for their transport (Lindblow-Kull *et al.*, 1985).

The transport of selenate also occurs through the SulT sulfate permease system (Turner *et al.*, 1998). An *E. coli* mutant strain affected in the *cysA* gene showed impaired selenate reduction due to its inability to take up selenate (Bébien *et al.*, 2002). Selenate uptake into *E. coli* cells also requires the transport protein YbaT (a probable amino acid or metabolite transport protein) and the outer-membrane porin NmpC, because a mutation of the corresponding genes inhibited selenate reduction without any change in growth rate (Bébien *et al.*, 2002).

Selenate transport through the sulfate permease has been also analyzed in the β–proteobacterium *Cupriavidus metallidurans* CH34 (previously *Alcaligenes eutrophus* or *Ralstonia metallidurans*) (Avoscan *et al.*, 2009). Cells grown under sulfate-limited conditions (0.3 mM sulfate) accumulated up to six times more selenate than cells grown in sulfate-rich (3 mM) medium, probably due to the repression of the sulfate permease (Avoscan *et al.*, 2009). These findings indicate that *C. metallidurans* takes up selenate using the sulfate permease. Sulfate transport by the already mentioned SulP-type

Rv1739c permease of *M. tuberculosis* expressed in *E. coli* was inhibited by selenate and selenite (Zolotarev *et al.*, 2008), which suggests that both selenium oxyanions can be also taken up through this permease.

5.-Chromate transport

Chromium is a non-essential metal for microorganisms and plants (Cervantes *et al.*, 2001). Chromium exists in nature as two main species, the trivalent form, Cr(III), which is relatively innocuous because is less soluble at physiological pH, and the hexavalent form, Cr(VI), considered a more toxic species (Ramírez-Díaz *et al.*, 2008). Inside the cell, Cr(VI) is readily reduced to Cr(III) by the action of various enzymatic and nonenzymatic activities; the Cr(III) generated inside the cells may then exert diverse toxic effects in the cytoplasm (Ramírez-Díaz *et al.*, 2008).

The toxic oxyanion chromate (CrO_4^{2-}) is taken up by bacterial cells through sulfate permeases (Ohta *et al.*, 1971; Ohtake *et al.*, 1987; Nies and Silver, 1989; Mansilla and de Mendoza, 2000). Once inside the cell before being reduce and exert the toxic effects, chromate can be extrude of the cell through CHR proteins that represent a mechanism of chromate resistance (Ramírez-Díaz *et al.*, 2008) (**Figure 6**).

5.1.- Chromate uptake

Chromate an analogous of sulfate, is transported into the cell through the sulfate transport systems **(Figure 1**) and it has been identified as a competitive inhibitor of sulfate transport (Ohta *et al.*, 1971; Ohtake *et al.*, 1987; Nies and Silver, 1989; Mansilla and de Mendoza, 2000). Transport of both chromate and sulfate oxyanions by the same carrier has been used as a tool to isolate chromate-resistant strains which are usually sulfate transport-negative mutants (Ohta *et al.*, 1971).

In *Pseudomonas fluorescens* LB300 and *C. metallidurans* the level of chromate resistance depends on the sulfur source; when cells are grown under sulfur starvation, sulfate transport is derepressed, chromate uptake is enhanced, and cells are more sensitive to chromate that when cells are grown in a medium with high sulfur or cysteine concentrations that repress sulfate transport (Ohtake *et al.*, 1987; Nies and Silver, 1989). In *P. fluorescens*, the affinity of the sulfate transporter for both oxyanions is similar, with a

Km of 6.4 μM for sulfate and of 19 μM for chromate; the V_{max} was almost five times higher for sulfate (0.84 μmol of SO_4^{2-} min^{-1} g^{-1}) than for chromate (0.17 μmol of SO_4^{2-} min^{-1} g^{-1}) (Ohtake *et al.*, 1987), demonstrating that sulfate is the preferred substrate. The *Ki* for chromate inhibiting sulfate transport was 12.7 μM whereas a higher *Ki* for sulfate (302 μM) was determined (Ohtake *et al.*, 1987).

The previously described sulfate permease CysP from *B. subtilis* also transports chromate inside the cell (Mansilla and de Mendoza, 2000) **(Figure 1**). *E. coli* cysteine-auxotroph mutants with the *cysT*, *cysP*, *sbp* or *cysA97* genes altered are chromate-resistant, probably due to their inability to transport the toxic ion inside the cell. The expression of CysP in these chromate-resistant strains conferred sensitivity to chromate, confirming that this oxyanion can be transported inside *B. subtilis* cells by the CysP permease (Mansilla and de Mendoza, 2000).

5.2.-Chromate efflux

The most studied chromate transport system is the ChrA protein which is encoded by the *P. aeruginosa* pUM505 plasmid (Cervantes *et al.*, 1990). ChrA functions as a chemiosmotic pump that extrudes chromate from the cytoplasm using the proton-motive force (Alvarez *et al.*, 1999; Pimentel *et al.*, 2002). ChrA, a 416-amino-acid residues protein, displays a topology of 13 TMSs (Jiménez-Mejía *et al.*, 2006). Efflux of chromate by *P. aeruginosa* cells showed a typical saturation kinetics with an apparent *Km* of 82 μM chromate and a V_{max} of 0.133 nmol chromate min^{-1} mg $protein^{-1}$. Sulfate and molybdate inhibit chromate efflux in a concentration-dependent fashion, which suggests that ChrA protein binds these anions (Pimentel *et al.*, 2002). Mutations in basic residues Arg98, Arg201, Arg340 and Lys394, located in cytoplasmic loops, and Arg154 and Lys248, located in TMSs, abolished chromate resistance, suggesting that these positively-charged amino acids interact directly with chromate during its transport (G. Moreno-Contreras, unpublished results).

In *C. metallidurans* two genes that encode ChrA proteins have been identified, $ChrA_1$ encoded by pMOL28 plasmid, and $ChrA_2$ encoded by a chromosomal gene; these proteins confer chromate resistance by a similar efflux mechanism as in *P. aeruginosa* (Juhnke *et al.*, 2002). A similar ChrA transporter has also been identified in the facultative

anaerobic Gram-negative bacteria *Shewanella* sp. ANA-3 (Aguilar-Barajas *et al.*, 2008), but in contrast with the ChrA proteins from *P. aeruginosa and C. metallidurans*, that are functional only in their respective hosts (Cervantes *et al.*, 1990; Nies *et al.*, 1990), the ChrA protein from *Shewanella* conferred chromate resistance in both *E. coli* and *P. aeruginosa* (Aguilar-Barajas *et al.*, 2008). A ChrA transporter with a similar function in chromate efflux was recently identified in a chromosomally-located transposon of the α-proteobacterium *Ochrobactrum tritici* strain 5bvl1, isolated from a chromate-polluted environment (Branco *et al.*, 2008).

The ChrA proteins belong to the CHR superfamily of chromate ion transporters that currently comprises hundreds of homologs from all three domains of life (Nies *et al.*, 1998; Nies, 2003; Díaz-Pérez *et al.*, 2007). The CHR superfamily is constituted by members of two sizes: short chain proteins (the SCHR family) of about 200 amino-acid residues, with only one domain and that are encoded by adjacent gene pairs, and the large chain proteins (the LCHR family) of about 400 amino-acid residues, with homologous N-terminal and C-terminal halves. LCHR proteins are proposed to have derived from gene duplication/fusion events (Díaz-Pérez *et al.*, 2007). The genomes of some bacteria, such as *C. metallidurans* and several *Burkholderia* species, encode multiple SCHR and LCHR homologs. Dramatic examples of the possession of multiple genes encoding CHR homologs are *B. vietnamiensis* (five LCHRs and two pairs of SCHRs) and *B. xenovorans* (four LCHRs and two pairs of SCHRs) (Díaz-Pérez *et al.*, 2007), the function of these homologs have not been yet tested.

The ChrA proteins previously described from *P. aeruginosa, C. metallidurans, Shewanella sp. ANA-3* and *O. tritici* belong to the LCHR family.

The function of the paired-genes encoded SCHR proteins Chr3N (the amino domain) and Chr3C (the carboxyl domain) from *B. subtilis* was recently demonstrated (Díaz-Magaña *et al.*, 2009). These short-chain proteins are encoded by adjacent paired genes *chr3N* and *chr3C* which are transcribed as a part of a bicistronic mRNA. The expression of paired *chr3N*-*chr3C* genes in *E. coli* conferred chromate resistance, but the expression of single *chr3N* or *chr3C* genes did not confer chromate resistance, indicating that both genes are required for function. Thus, the SCHR proteins seem to form heterodimers in the

membrane, which effluxes chromate ions from the cytoplasm by a similar mechanism as the long-chain LCHR proteins (Díaz-Magaña *et al.*, 2009).

6.-Conclusions

Sulfate is preferentially used as a sulfur source and probably due to this reason the bacterial sulfate permeases comprise very different transporters belonging to several families, being the sulfate-thiosulfate SulT permease of the ABC superfamily located only in prokaryotes, the wider distributed and studied, nevertheless the number of sulfate permeases biochemically tested is still low. Therefore there are many unknown characteristics of the less studied sulfate permeases of the SulP, Pit and CysZ families. As the sulfate permease CysP from *B. subtillis* that is located in the PiT family of phosphate transporters, however, the phylogenetic analysis suggest that more members of the PiT family are involved in sulfate transport, being good objective of analysis.

Even when the related oxyanions molybdate and tungstate are also essentials their transporters belong basically to the ABC-type transporters, and, in contrast with sulfate permeases the molybdate and tugstate transportes are more specific.

For selenate and chromate there are not specific uptake systems, they are taken up by membrane transporters of structurally related oxyanions such as sulfate and molybdate. On the other hand, chomate can be detoxified throught the CHR efflux systems, that are widely distributed in all three domains of life.

Most of the oxyanions analyzed are transported through ABC-type transporters which form one of the largest of all protein families with a diversity of physiological functions.

Table 1. Bacterial transporters of sulfate.

Transporter	Family	TC number [1]	Organisms	References
Sulfate-thiosulfate permease (CysPTWAM)	Sulfate/tungstate uptake transporter SulT	3.A.1.6	*Salmonella typhimurium* *Escherichia coli* *Mycobacterium tuberculosis* *Synechococcus elongatus* *Burkholderia cenocepacia*	(Ohta *et al.*, 1971) (Sirko *et al.*, 1990; Wooff *et al.*, 2002) (Laudenbach and Grossman, 1991; Farmer and Thomas, 2004)
SulP	Sulfate permease SulP	2.A.53	*Acidithiobacillus ferrooxidans* *Mycobacterium tuberculosis*	(Váldes *et al.*, 2003) (Zolotarev *et al.*, 2008)
CysP	Inorganic phosphate transporter PiT family	2.A.20	*Bacillus subtilis*	(Mansilla and de Mendoza, 2000)
CysZ	Putative sulfate transporter	9.B.7	*Corynebacterium glutamicum*	(Rückert *et al.*, 2005)

[1] According to the Transport Classification Database (TCBD).

Table 2. Bacterial transporters of molybdate and tungstate

Transporter	Family	TC number[1]	Substrates	Organisms	References
ModABC	Molybdate uptake transporters MolT family	3.A.1.8	Molybdate Tungstate Sulfate	*Escherichia coli*	(Maupin-Furlow *et al.*, 1995)
Sulfate/thiosulfate permease (CysPTWA)	Sulfate/tungstate uptake transporter SulT	3.A.1.6	Sulfate Chromate Molybdate Selenate	*Escherichia coli*	(Rosentel *et al.*, 1995) (Turner *et al.*, 1998)
Nonspecific anion transport system	N.R.[2]	N.R.	Molybdate Sulfate Selenate Selenite	*Escherichia coli*	(Rosentel *et al.*, 1995)
TupABC	Sulfate/tungstate uptake transporter SulT	3.A.1.6	Tungstate	*Eubacterium acidaminophilum*	(Makdessi *et al.*, 2001)
WtpABC	Sulfate/tungstate uptake transporter SulT	3.A.1.6	Tungstate Molybdate	*Pyrococcus furiosus*	(Bevers *et al.*, 2006)

[1] According to the Transport Classification Database (TCBD).
[2]N.R. Non reported on the database TCBD

Figure 1. Bacterial sulfate transporters. A, Sulfate-thiosulfate SulT permease constituted by the sulfate- or thiosulfate-binding proteins, Sbp or CysP, respectively, the membrane proteins CysT and CysW, and the CysA ATPase. **B**, The SulP transporter that contains the STAS domain. **C,** The CysP transporter. **D**, The CysZ transporter. Sulfate (SO_4^{2-}), thiosulfate ($S_2O_3^{2-}$), molybdate (MoO_4^{2-}), selenate (SeO_4^{2-}) and chromate (CrO_4^{2-}). The oxyanions transported by each system are indicated. Details for each system are given in the text.

Figure 2. Sulfate and thiosulfate assimilation in *S. typhimurium* and *E. coli*. Sulfate is first taken up by the sulfate permease and then is reduced to sulfide which reacts with *O*-acetylserine to produce cysteine. *O*-acetylserine is formed by reaction of serine with acetyl-CoA. The thiosulfate assimilation pathway is shown in the right part of the figure. Thiosulfate is taken up by the sulfate permease and then reacts with *O*-acetylserine to produce s-sulfocysteine that is subsequently reduced to cysteine. The enzymes and genes encoding the involved enzymes are indicated for each reaction. Details of each step are given in the text.

Figure 3. Distibution and organization of the *cysPTWA* operon and *cys*-related genes. The clades of the different analyzed species are shown to the left. The name of representative species and the distribution of the *sbp*- and *cysP*-containing operons for each one are indicated. Arrows indicate the genes and direction of the transcription. The genes with similar shading encode proteins with similar function. The name for each gene is given in the bottom. The distributions of the different operons were constructed with the STRING database (von Mering *et al.*, 2007), using *cysP* and *sbp* genes sequences.

Figure 4. Phylogenetic analysis of the Pit/CysP protein superfamily. **A,** Phylogenetic tree showing the distribution of the three main clusters of the superfamily. **B,** CysP family group tree showing the two subgroups of the family. Bacteria, Archaea and Eukarya domains are indicated with dash, grey and black lines, respectively. The arrow indicates the location of *Bacillus subtilis* CysP protein. Asterisks indicate the horizontal gene transfer events. Scale bar represents 0.1 amino acid substitutions per site. Tree

constructed with the minimum evolution (ME) method using 786 protein sequences that belong to the three protein subfamilies containing at least one characterized protein member. Similar topologies were obtained using the neighbor-joining (NJ) and maximum parsimony (MP) methods. Entries include sequence access number and species name. Trees were calculated using MEGA 4.0, using the TJJ (Tamura *et al.*, 2007).

Figure 5. Sequence logos of conserved N- and C-terminal motifs of PiT family members. The sequences of PiT family were sorted in clusters according to the results of phylogenetic analysis. Numbering is according to the amino acid sequence of CysP from *B. subtilis* (accession number: o34734). The sequence logos were made using WebLogo (http://weblogo.threeplusone.com/). Each logo consists of stacks of amino acid letters, one stack for each position in the sequence. The overall height of the stack indicates the degree of sequence conservation at that position, while the height of letters within the stack indicates the relative frequency of each amino acid at that position (Crooks *et al.*, 2004). Alignments used to elaborate the logos are provided as supplementary data.

Figure 6. Bacterial oxyanion transporters. A, Molybdate transporters, the ModABC transporter are constituted by ModA, the molybdate-binding protein; ModB, the membrane protein; and ModC, the ATPase. MoO_4^{2-} may also be taken up by a nonspecific transporter. **B**, Tungstate transporters, TupABC and WtpABC systems are constituted by TupA and WtpA, the substrate-binding proteins; TupB and WtpB, the membrane proteins; and TupC and WtpC, the ATPases. **C**, Chromate efflux systems, ChrA and Chr3N-Chr3C transporters. Molybdate (MoO_4^{2-}), tungstate (WO_4^{2-}), sulfate (SO_4^{2-}), selenate (SeO_4^{2-}), selenite (SeO_3^{2-}) and chromate (CrO_4^{2-}). The oxyanions transported for each system are indicated. Details for each system are given in the text.

Figure 7. Structure of the ModABC transporter from *Archaeoglobus fulgidus.* Front view of the $ModB_2C_2$ complex. The complex in ribbon representation shows the ModB subunits colored yellow and green, the ModC subunits colored red and magenta, the binding protein ModA colored blue, and with bound tungstate (green spheres). The lipid

bilayer is also shown. The figure was contructed with VMD and PyMol V 0.99, using the datas published by (Hollenstein *et al.*, 2007).

Figure 1

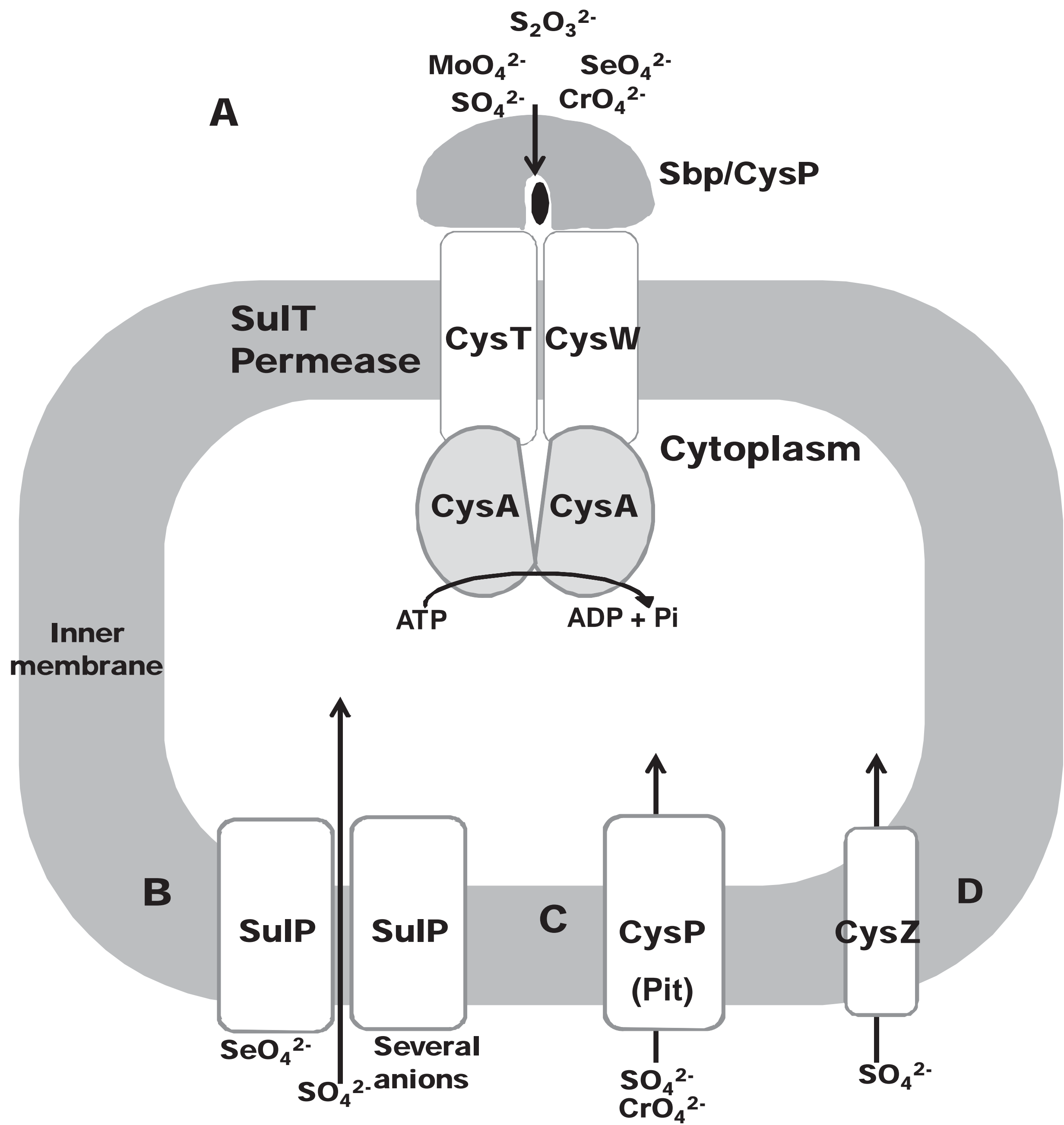


Figure 2

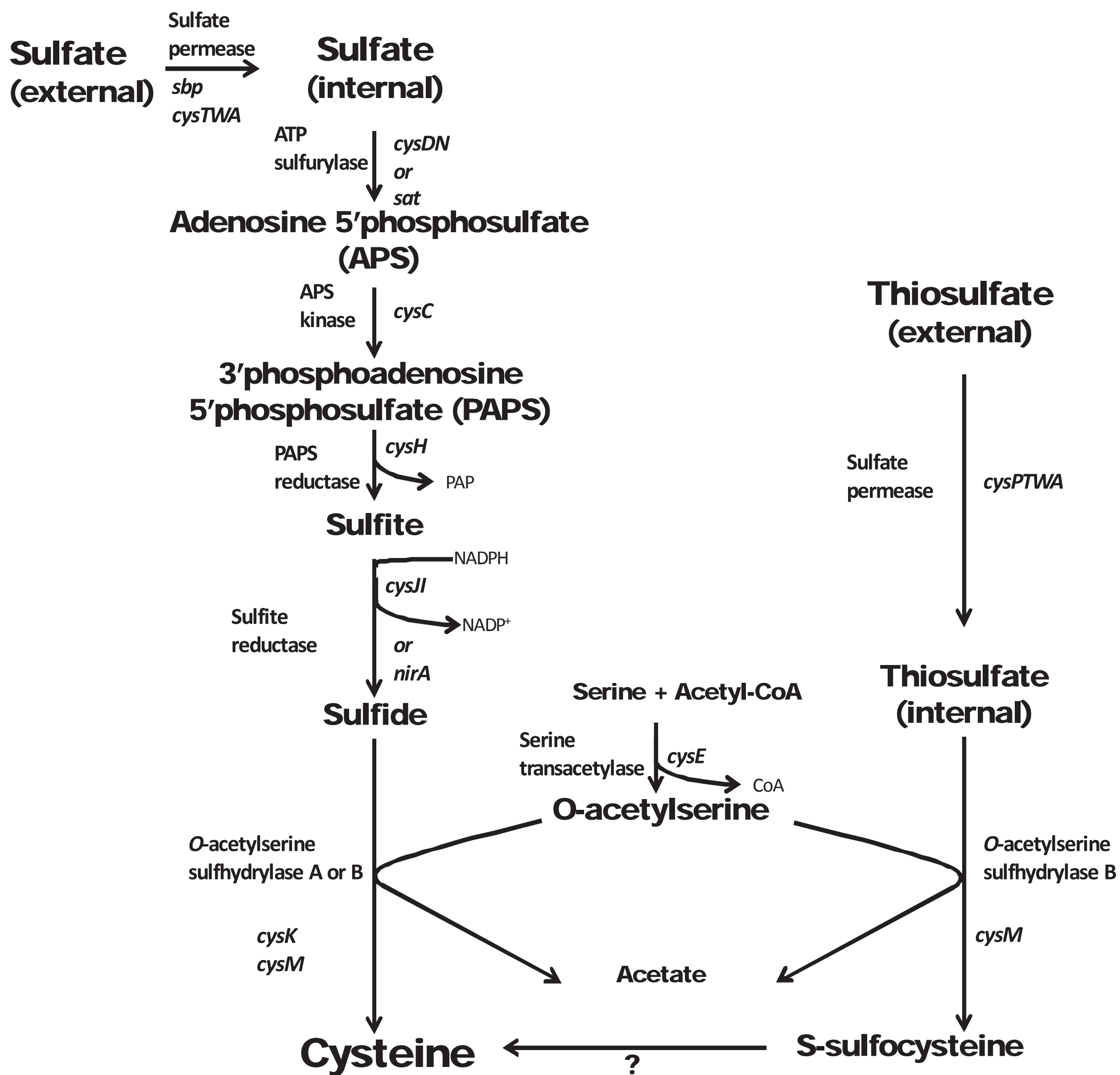


Figure 3

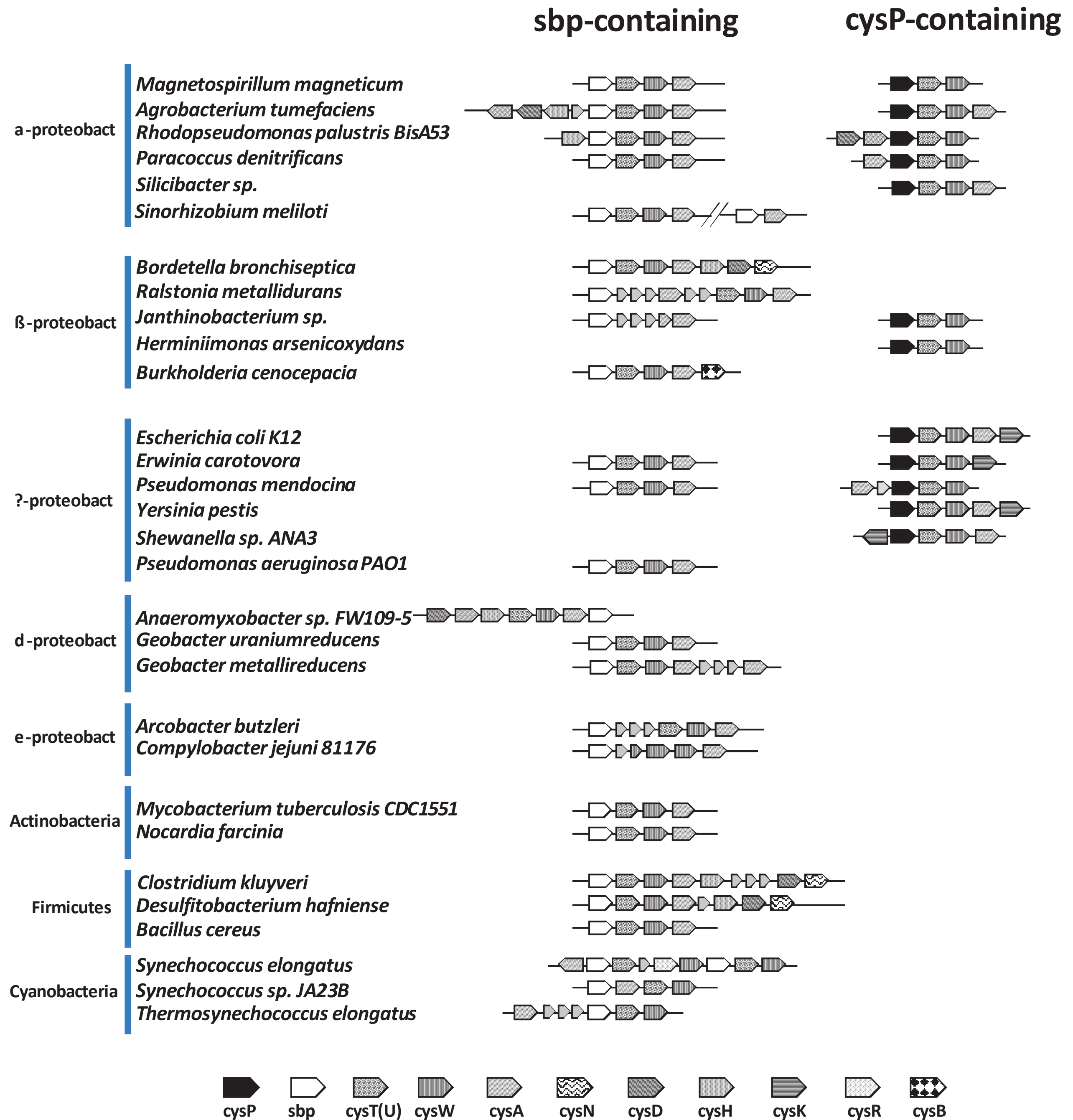
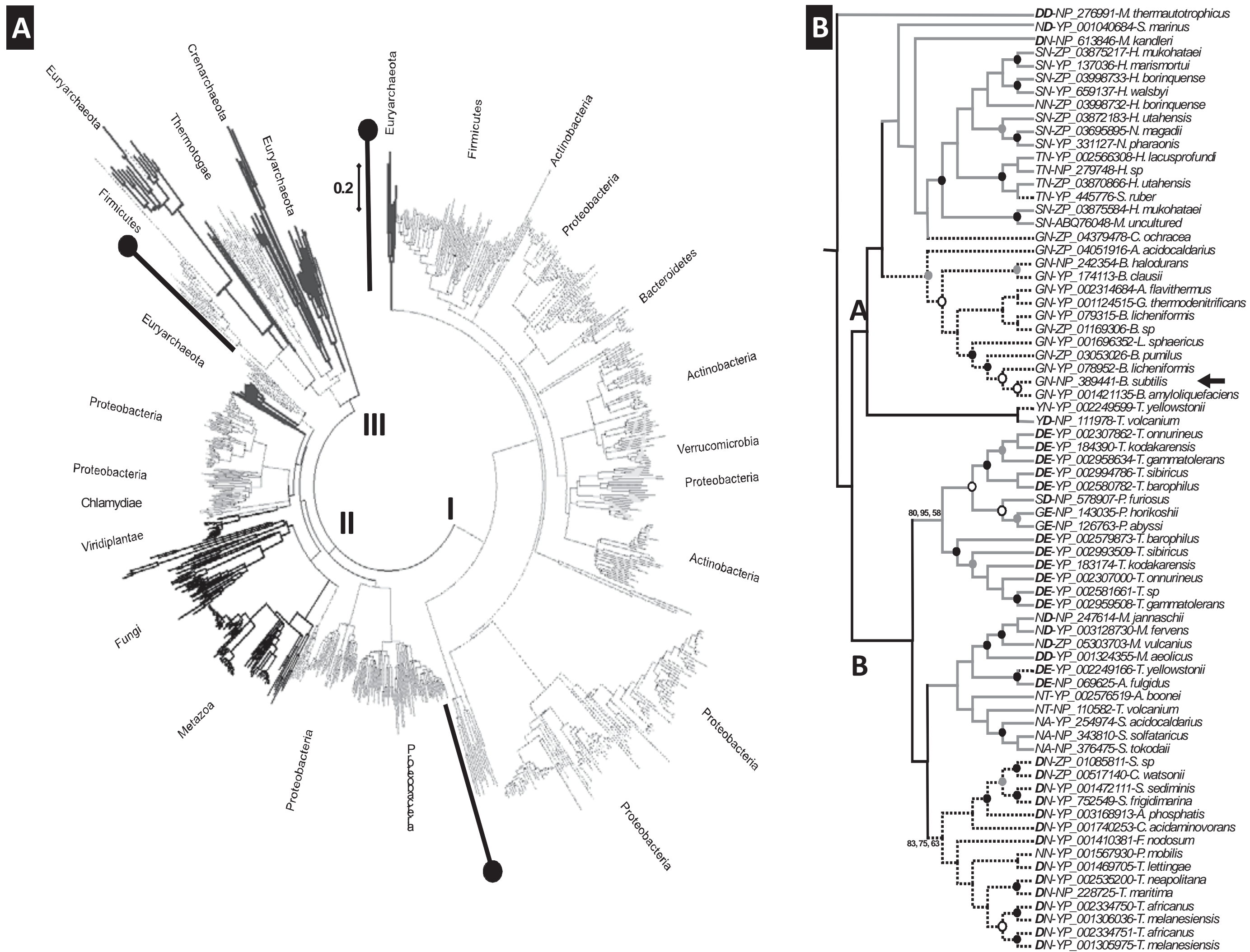
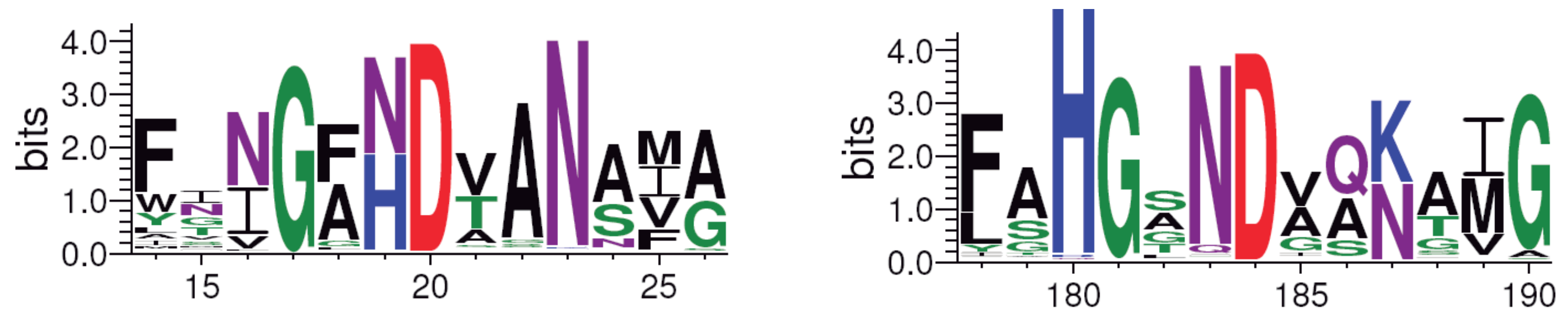


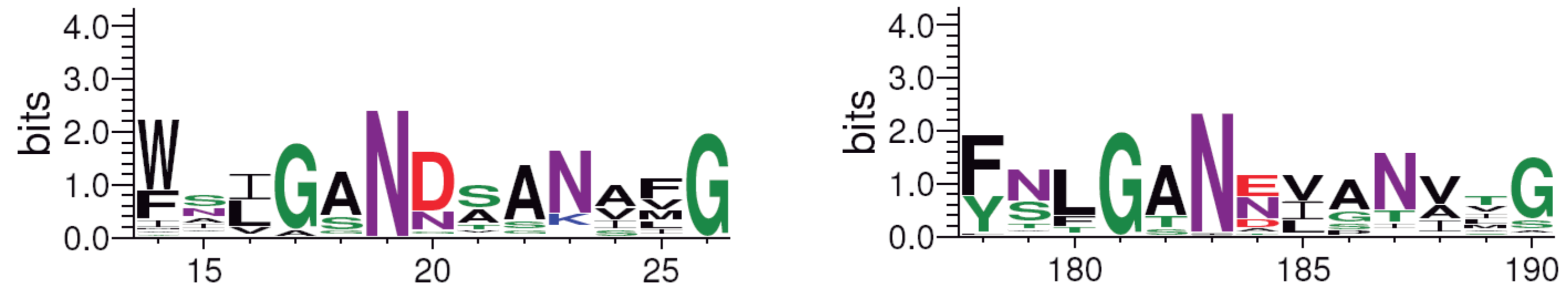
Figure 4



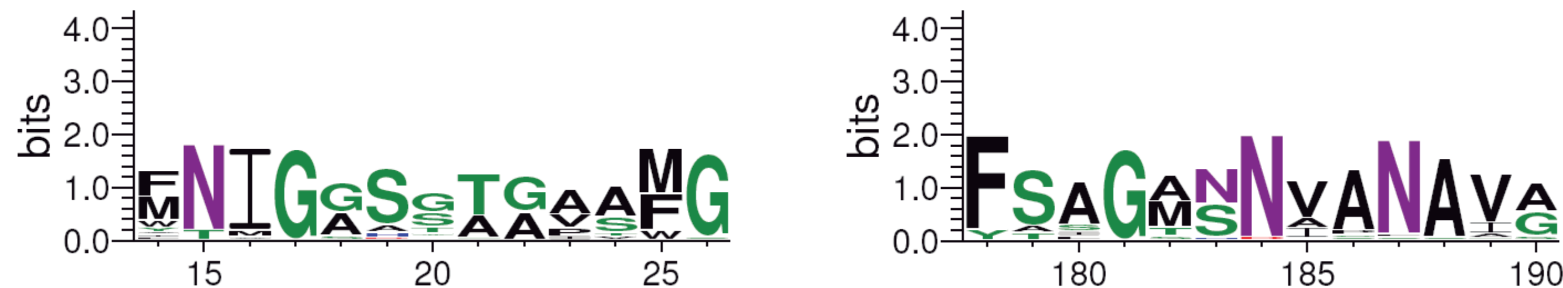
Cluster I and II



Cluster III-B



Cluster III-A



N- terminal motif **C- terminal motif**

Figure 6

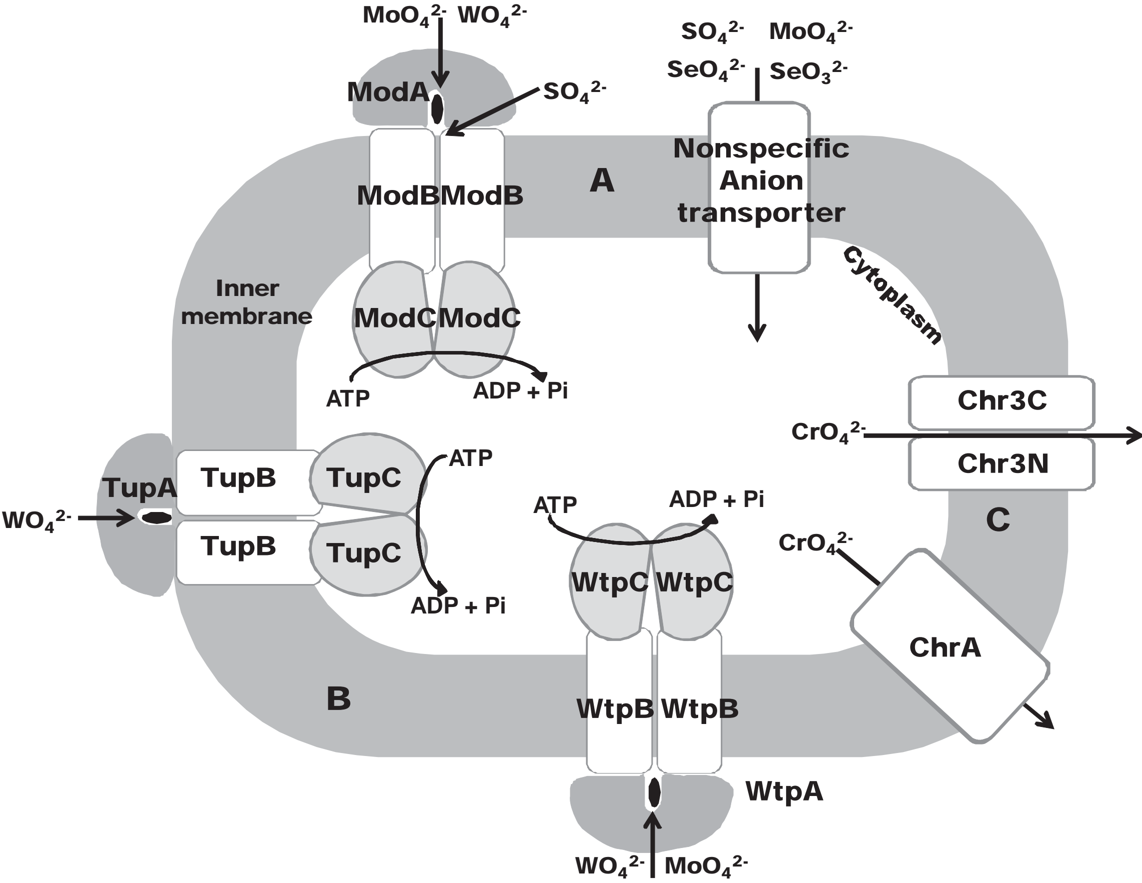
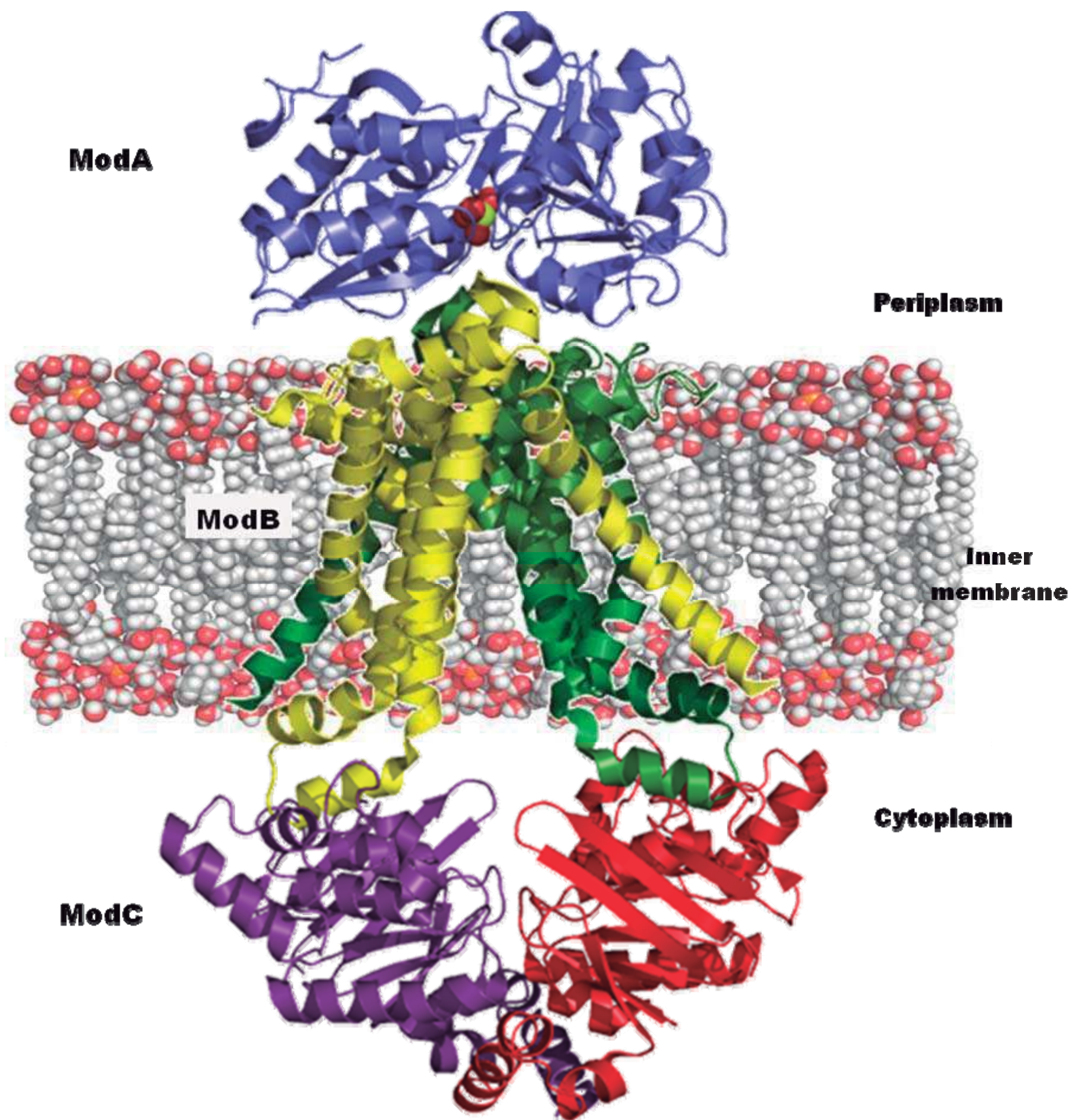


Figure 7



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