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Antimonite Is Accumulated by the Glycerol Facilitator GlpF in *Escherichia coli*

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In a search for genes responsible for the accumulation of antimonite in *Escherichia coli*, Tn*phoA* was used to create a pool of random insertional mutants, from which one antimonite-resistant mutant was isolated. Sequence analysis showed that the Tn*phoA* insertion was located in the *glpF* gene, coding for the glycerol facilitator GlpF. The mutant was shown to be defective in polyol transport by GlpF. These results suggest that in solution Sb(III) is recognized as a polyol by the glycerol facilitator.

Resistance to antimonite [Sb(III)], arsenite [As(III)], and arsenate [As(V)] is encoded by both plasmid-borne and chromosomal arsenical resistance (*ars*) operons (2, 3, 10). These operons encode transport systems that extrude the toxic metalloids, thus lowering the intracellular concentration and producing resistance (2, 8, 13). Arsenate is accumulated by both the Pit and Pst phosphate transport systems, although the Pit system may be responsible for the majority of uptake (13). The pathways for antimonite and arsenite accumulation have not been defined. In an attempt to identify the cellular transporters for the metalloid salts, *Escherichia coli* AW3110 (2) was subjected to random Tn*phoA* mutagenesis (7).

Random Tn*phoA*-mediated mutagenesis to obtain Sb(III)-resistant mutant. Strains, plasmids, and phage used in this study are given in Table 1. *E. coli* AW3110, which lacks the chromosomal *ars* operon (2), was infected with λb221 *rex*::Tn*phoA* cI857 (7). Cells were plated on Luria-Bertani (LB) agar containing 1 mM either potassium antimonyl tartrate or sodium arsenite plus 35 μg of kanamycin/ml. One mutant was obtained on antimonite-containing agar. No arsenite-resistant mutants were isolated. Colonies of the antimonite-resistant strain, OSBR1, were white on plates containing 20 μg of 5-bromo-4-chloro-3-indolyl phosphate (XP)/ml. This could indicate an intracellular localization of the alkaline phosphatase portion of the moiety, an out-of-frame fusion, or fusion with the reading frames in the opposite orientation.

Antimonite resistance is due to a single Tn*phoA* insertion. To determine whether the mutant strain carried the Tn*phoA* insertion in a single locus, the kanamycin resistance phenotype was transduced back into strain AW3110 by generalized transduction with P1 phage. All transductants were Sb(III) resistant. Southern blot hybridization was performed with *Bam*HI-digested genomic DNA of OSBR1, with DNA from AW3110 as a control, by using a 485-bp Tn*phoA*-specific probe. The result of the Southern blot confirmed the existence of only a single Tn*phoA* insertion (data not shown).

The Tn*phoA* insertion is located in the *glpF* gene. Since there is no *Bam*HI site between the site of fusion in Tn*phoA* and the kanamycin phosphotransferase gene, and there is a *Bam*HI site immediately following the 3′ end of the kanamycin phosphotransferase gene (5), chromosomal DNA of OSBR1 was digested with *Bam*HI. The portion of DNA proximal to the fusion junction was cloned into the unique *Bam*HI site of pUC18; the transformed colonies were screened for Km^r. The resulting plasmid, pOSBR1, contained a 5.7-kb insert composed of 0.8 kb of *E. coli* chromosomal DNA and 4.9 kb from Tn*phoA*. Sequence analysis confirmed that the Tn*phoA* insertion was located at 88 min on the *E. coli* chromosome in the *glpF* gene (9, 14, 15). Double-stranded plasmid DNA was prepared with the Qiagen DNA purification system. Sequencing was performed by using the Pharmacia Cy5 labeled autosequence kit (Pharmacia Biotech Inc.) and ALF*express* apparatus according to the method of Sanger et al. (12). The sequence of the insert was determined by using two primers, one complementary to the end of the *phoA* gene and the other complementary to the vector at the *Bam*HI site. The *Bam*HI site of the insert was found within the *glpK* gene (9, 14, 15). The *phoA* gene was fused near the 170th codon of *glpF* but in the opposite orientation to the *glpF* reading frame, which explains the white color of colonies on XP.

Growth properties and metal resistance of *E. coli* OSBR1 (*glpF*::Tn*phoA*). To confirm that the Tn*phoA* insertion inactivated GlpF, cells of strains AW3110 and OSBR1 were streaked onto agar plates containing M9 medium (11) with either 0.2% glucose or 0.5% glycerol as the sole carbon source. The parental strain grew on both media; the mutant grew on glucose but not on glycerol (data not shown). The lack of growth on glyc-

TABLE 1. Strains, phage, and plasmids

Strain, phage, or plasmid	Genotype or description	Reference or source
E. coli strains		
JM109	*recA1 supE44 endA1 hsdR17 gyrA96 relA1 thi* Δ(*lac-proAB*) F′[*traD36 proAB*$^+$ *lacI*^{q} *lacZ*ΔM15]	11
AW3110	Δ*ars*::*cam* F$^-$ IN(*rrnD-rrnE*)	2
OSBR1	AW3110 *glpF*::Tn*phoA*; Km^r	This study
Plasmids		
pUC18	Cloning vector; Ap^r	16
pOSBR1	5.7-kb *Bam*HI fragment in pUC18; Km^r	This study
Phage λTn*phoA*	Tn*5* IS*50*L::*phoA* (Km^r)	7

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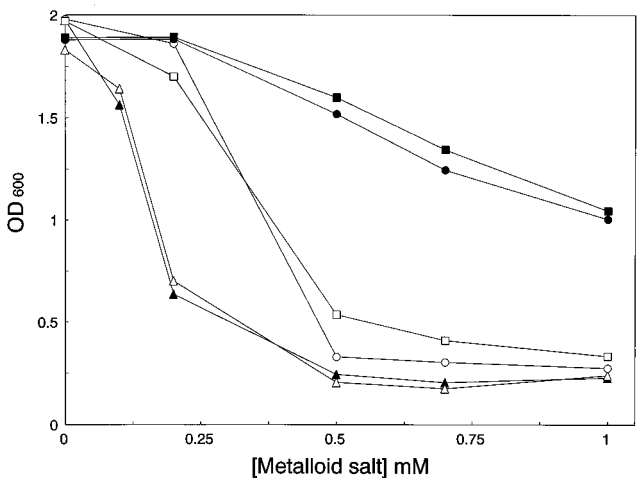

FIG. 1. Antimonite and arsenite resistance. Metalloid resistance was assayed in cells of *E. coli* AW3110 (Δ*ars*) (open symbols) or OSBR1 (Δ*ars glpF*::Tn*phoA*) (solid symbols). Cells were grown in LB media with the indicated concentrations of potassium antimonyl tartrate (squares), antimony trichloride (circles), or sodium arsenite (triangles) for 24 h at 37°C with shaking, following which turbidity at 600 nm was measured.

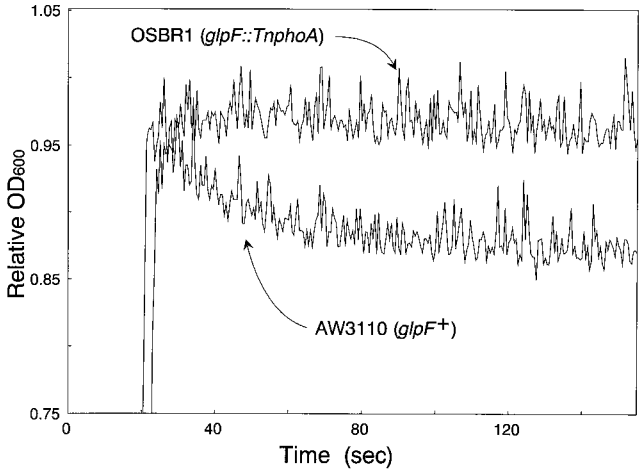

FIG. 2. GlpF activity in wild-type and antimonite-resistant cells. Cells were grown overnight in M9 medium containing 1% tryptone at 37°C with shaking. In the morning the culture was diluted 10-fold in 50 ml of the same medium supplemented with 0.5% glycerol and allowed to grow at 37°C with shaking to mid-exponential phase. The cells were harvested, washed twice with 30 ml of a buffer consisting of 50 mM MOPS (morpholinepropanesulfonic acid)-KOH, pH 7.0, and suspended in 1 ml of the same buffer, all at room temperature. Cells (0.2 ml) were rapidly mixed with 0.8 ml of 0.25 M xylitol in the same buffer at room temperature. The optical changes resulting from plasmolysis and deplasmolysis of the cells were recorded at 600 nm.

erol is most likely due to a polar effect on expression of the *glpK* gene rather than a direct result of the transport defect. The mutation conferred resistance to antimonite (Fig. 1). The fact that the cells were equally resistant to potassium antimonyl tartrate and $SbCl_3$ demonstrated that the form of Sb(III) is not important for the phenotype. Thus, it is unlikely that the complex of tartate and Sb(III) is not the substrate of GlpF. Of interest is the fact that no resistance to sodium arsenite was observed. There are two possibilities to explain this result. First, arsenite may not be transported by GlpF. Second, there may be alternate routes of arsenite uptake into *E. coli*. The effects of other metals on the growth of *E. coli* OSBR1 were examined. When cells were streaked onto LB agar containing the salts of various metals, no increase in resistance to any was found (Table 2).

GlpF activity in wild-type and antimonite-resistant cells. Following plasmolysis of cells by the osmotic shock of dilution in xylitol, a GlpF substrate, cells with the GlpF polyol facilitator exhibit a rapid swelling that is absent in GlpF-defective strains (4). This swelling is accompanied by a decrease in light scattering at 600 nm. Strain AW3110 exhibited a half time of swelling of approximately 50 s (Fig. 2), consistent with the reported value of 0.7 min (4). Strain OSBR1 showed no optical changes following dilution in xylitol, indicating a lack of GlpF activity. It was not possible to test for Sb(III) transport directly, since no radioactive isotope of antimony is commercially available, and the antimony salts are not soluble at the concentrations required for the optical measurement of GlpF activity.

In summary, these results indicate that the major route of

TABLE 2. MICs of metal salts for *E. coli* strains[a]

Metal salt	MIC (mM) for strain:	
	AW3110[b]	OSBR1[c]
Cadmium acetate	1.6	1.6
$ZnSO_4$	2	2
$CuSO_4$	6	6
$NiCl_2$	5	5
$NaCrO_4$	6	5

[a] Cells were grown overnight and streaked out on LB plates containing varying concentrations of metal salts. Growth was monitored after 24 h at 37°C. The MIC is the concentration at which no growth was detected.
[b] Genotype, Δ*ars*.
[c] Genotype, Δ*ars glpF*::Tn*phoA*.

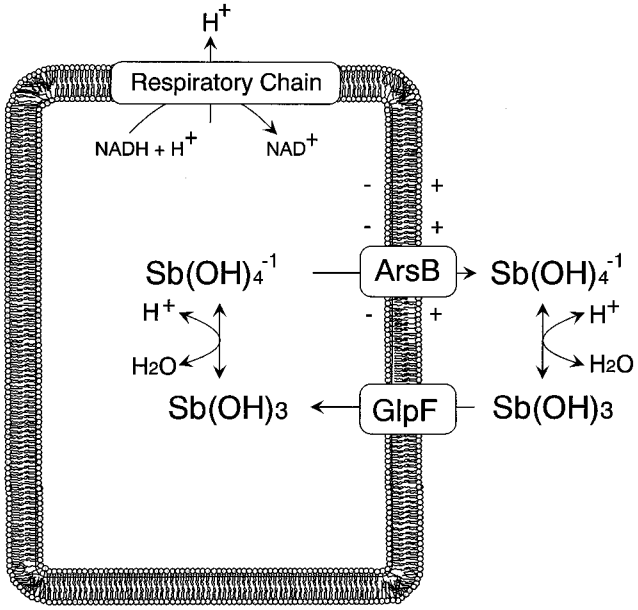

FIG. 3. Routes of Sb(III) entry and efflux in *Escherichia coli*. In solution the major species of Sb(III) would be the neutral $Sb(OH)_3$. This inorganic triol is transported into the cell by GlpF, which catalyzes the facilitated diffusion of a wide range of polyols (4). ArsB, which catalyzes electrophoretic extrusion of anionic forms of Sb(III) (6), would extrude the toxic metalloid from the cell. This cycle provides an endogenous resistance to moderate levels of environmental antimonite. Cells become hypersensitive to Sb(III) when ArsB is absent. Resistance is increased either in the absence of GlpF or by expression of a plasmid-borne *ars* operon.

entry of antimonite into cells of *E. coli* is via the GlpF polyol facilitator. At a neutral pH the major form derived from antimonous acid is the neutral monomeric species $Sb(OH)_3$ (1). This suggests that $Sb(OH)_3$ may be recognized by GlpF as the inorganic equivalent of a polyol. In contrast, it appears that ArsB, which catalyzes the extrusion of arsenite and antimonite, transports an anion (6). Although $Sb(OH)_3$ is in equilibrium with the anionic species $Sb(OH)_4^-$ with a pK_a value of 11.8, it is not clear whether that anion is the true substrate of ArsB. However, since most strains of *E. coli* have chromosomal *glpF* (14, 15) and *arsB* genes (2), cells exposed to antimonite would have a cycle of Sb(III) uptake by GlpF and extrusion by ArsB, producing a moderate level of resistance (Fig. 3). In the absence of ArsB, cells become hypersensitive to Sb(III) (2), and plasmid-borne *ars* operons produce high-level resistance (10, 13). In this study we demonstrate that, even in the absence of ArsB, resistance can also result from inactivation of GlpF, which therefore must be the major route of entry of Sb(III) into cells.

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