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Reduction of arsenate to arsenite by the ArsC protein of the arsenic resistance operon of *Staphylococcus aureus* plasmid pI258

(arsenate reduction/oxyanion reductase/plasmid resistances)

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ABSTRACT **The arsenic resistance operon of *Staphylococcus aureus* plasmid pI258 consists of three genes, *arsR* (encoding the repressor regulatory protein), *arsB* (the determinant of the membrane efflux protein that confers resistance by pumping arsenic from the cells), and *arsC* (the small gene whose protein product is required for arsenate resistance only, not for arsenite resistance). ArsC has now been shown to be an arsenate reductase, converting intracellular arsenate [As(V)] to arsenite [As(III)], which is then exported from the cells by an energy-dependent efflux process. The arsenate reductase activity was found in the soluble cytoplasmic fraction in *Escherichia coli* (and not associated with the periplasmic fraction or the sedimentable cell envelope). Purified ArsC protein coupled *in vitro* with thioredoxin plus dithiothreitol (but not 2-mercaptoethanol or reduced glutathione) to reduce arsenate to arsenite.**

The plasmid-determined bacterial resistance system for arsenic and antimony consists of five genes in *Escherichia coli* (1–5) (*arsR, arsD, arsA, arsB*, and *arsC*) but only three genes (*arsR, arsB*, and *arsC*) in *Staphylococcus* species (6, 7). In both bacterial genera, the overall process is very similar. The systems are regulated by the repressor protein ArsR (3, 6–8). A membrane complex consisting of ArsB and ArsA in *E. coli* (9) (but only ArsB has been identified in *Staphylococcus*) carries out the energy-dependent efflux of oxyanions of arsenic, thus resulting in reduced cellular arsenic accumulation and therefore resistance. The ArsA protein was identified as a tightly membrane-associated ATPase whose activity was stimulated by arsenite and antimonite (10, 11). However, the *arsA* gene is missing in the two staphylococcal *ars* operons that have been sequenced (6, 7). The *E. coli* arsenic efflux system is sensitive to osmotic shock (12), leading to the suggestion that a periplasmic or loosely membrane-associated component is required.

The promoter-distal gene, *arsC*, of the staphylococcal and *E. coli ars* operons determines a small hydrophilic polypeptide that has been thought to lack enzymatic activity. ArsC was considered to function as an intracellular substrate-binding protein (2, 4, 5), analogous to the periplasmic substrate-binding proteins for ATP-dependent membrane uptake systems (13). ArsC would then make arsenate accessible to the ArsA/ArsB membrane complex, functioning as an arsenate efflux ATPase. ArsB plus ArsA alone was thought to export arsenite and antimonite (1, 2, 6, 7). We demonstrate that this hypothesis is incorrect. Experimental work in this report establishes that ArsC (in the absence of other *ars* operon gene products) reduces arsenate to arsenite, which is exported by the ArsB protein (G.J. and S. Bröer, unpublished work). Cell-free arsenate reduction assays showed that the reduced thiol compound dithiothreitol (DTT) strongly stimulated ArsC-dependent arsenate reduction. Purified ArsC protein reduced arsenate to arsenite when DTT and purified thioredoxin were added.

Table 1. Bacterial strains and plasmids

Strain or plasmid	Genotype or description	Ref.
E. coli		
AN120	*uncA401 arg rpsL*	14
K38(pGP1-2)	Hfr(λ); T7 RNA polymerase	15
S. aureus		
RN4220	Efficient acceptor of *E. coli* DNA	16
Plasmids		
pGJ101	Intact pI258 *ars* cloned into pSK265	6
pGJ103	Intact pI258 *ars* cloned into pUC19	6
pGJ105	Internal deletion of *arsB* in pGJ101	6
pGJ106	Deletion of 3′ end of *arsC* in pGJ101	6
pGJ107	*arsR*$^+$, partial *arsB* and total *arsC* deletion	6
pGJ109	*arsB* and *arsR* deletion, *arsC*$^+$	*
pGJ401	Intact *ars* cloned into vector pT7-5	6, 15
pUM3	Intact arsABC from R773 in pBR322	1

*This work.

MATERIALS AND METHODS

Bacterial Strains and Plasmids. The bacterial strains and plasmids used are listed in Table 1. Cells were grown in Luria broth (17) with ampicillin (50 μg/ml) or chloramphenicol (5 μg/ml), when required.

Materials. Materials were as described in the previous report (6). Purified *E. coli* thioredoxin (18) was a gift from J. A. Fuchs (University of Minnesota). DNA manipulations were as standard (17). Plasmid pGJ109 was constructed as follows. Plasmid pGJ101 (6) DNA was digested with restriction endonucleases *Nde* I and *Sau*96I. The ends were blunted using DNA polymerase (Klenow fragment) and self-ligated with T4 phage ligase. *S. aureus* transformation with plasmid DNA was carried out according to ref. 6.

Arsenic Uptake and Efflux. The procedure was as described (19). *S. aureus* RN4220 cells carrying plasmids were grown in Luria broth to 50 Klett units turbidity and induced with 50 μM arsenite (for pGJ101 only) at 37°C for 1.5 hr. Cells were harvested by centrifugation, washed, and suspended in triethanolamine (TEA)-buffered minimal medium (19) at 200 mg/ml (dry weight of cells). For arsenic uptake, 100 μl of cell suspension was added to 5 ml of TEA buffer with 0.2% glucose and 5 μM $^{73}AsO_4^{3-}$, and 0.7-ml samples were taken, filtered, and washed with 5 ml of TEA buffer containing 5 mM sodium phosphate (pH 7.5) before scintillation counting. For arsenic efflux, cells were loaded with 5 mM $^{73}AsO_4^{3-}$ at room temperature for 60 min. Loaded cells (50 μl) were diluted into 5 ml of TEA buffer plus 0.2% glucose and 5 mM sodium

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Abbreviations: DTT, dithiothreitol; TEA, triethanolamine.

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phosphate (pH 7.5). Samples (0.7 ml) were filtered and washed for scintillation counting.

Arsenate Reduction. *Whole cells. S. aureus* RN4220 cells containing plasmids were grown in Luria broth to 50 Klett units, induced by addition of 100 μM $NaAsO_2$ for 1.5 hr, harvested by centrifugation, washed, and resuspended at 200 Klett units (≈0.5 mg/ml, dry weight) in TEA medium (19) without phosphate and with 0.2% glucose. $^{73}AsO_4^{3-}$ (1 mM) was added to the cell suspension at 37°C. At indicated times, 15-μl samples were centrifuged for 1 min in a Sorvall microcentrifuge, and supernatants were spotted (5 μl) on thin-layer chromatography (TLC) plates (Kodak Chromagram cellulose, Eastman Kodak) and dried. The plates were developed with 2-propanol/water (9:1, vol/vol), dried, and analyzed by autoradiography or by using an AMBIS β scanner (AMBIS Systems, San Diego) to quantify $^{73}AsO_4^{3-}$ and $^{73}AsO_2^-$.

Cell-free extracts. S. aureus or *E. coli* cells with plasmids were grown in Luria broth, induced, harvested, washed, and suspended in reaction buffer (10 mM Tris·HCl, pH 7.5/1 mM Na_2EDTA/1 mM $MgCl_2$). Cells were disrupted by sonication and cell debris was removed by centrifugation at 100,000 × *g* for 45 min at 4°C. The supernatants were used as crude cell extracts. Arsenate reduction at 37°C was initiated by mixing cell-free preparations with 10 or 100 μM $^{73}AsO_4^{3-}$ (depending upon the activity level present). The reactions were stopped by boiling for 30 sec. Samples were centrifuged in a Sorvall microcentrifuge and the supernatants were spotted (3–5 μl) for TLC. $^{73}AsO_4^{3-}$ and $^{73}AsO_2^-$ were analyzed as above.

Osmotic Shock. A modification of the procedure of Weiner and Heppel (13) was used. *E. coli* cells were grown in Luria broth, induced with 100 μM $NaAsO_2$ for 1.5 hr at 37°C, harvested, and washed with 10 mM Tris·HCl (pH 7.5). Cell pellets were suspended in 33 mM Tris·HCl, pH 7.5/20% sucrose/0.2 mM Na_2EDTA and the mixture was shaken for 5 min at room temperature and centrifuged. Cells were suspended in ice-cold water and shaken for 10 min at 4°C. After centrifugation, the supernatants were concentrated in a Centricon 10 microconcentrator (Amicon) and used as the periplasmic shock fluids. β-Galactosidase was assayed according to Miller (20). Alkaline phosphatase was assayed similarly by hydrolysis of *p*-nitrophenyl phosphate (0.8 mg/ml) in 1 M Tris·HCl (pH 8.0); the reaction was stopped and the pH raised by addition of K_2HPO_4 (26 mg/ml).

The ArsC protein was produced from plasmid pGJ401 (Table 1) in *E. coli* K38(pGP1-2) cells using the T7 RNA polymerase expression system (15). The purification of ArsC protein on two successive columns by FPLC (fast protein liquid chromatography, Pharmacia; a Q Sepharose column followed by a Superose 12 column) based on the method of Rosen *et al.* (21) will be described elsewhere (G.J., unpublished work).

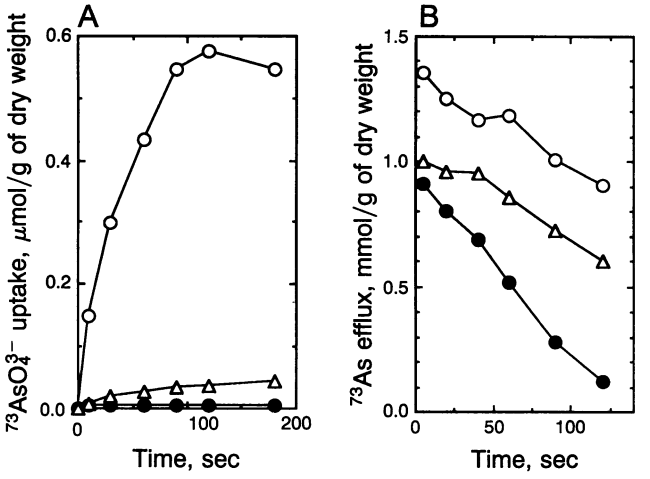

FIG. 1. Arsenic uptake and efflux. *S. aureus* RN4220 cells with the vector plasmid pSK265 (○), with pGJ101 (containing the intact *ars* operon of plasmid pI258) (●), or with pGJ109 (containing only the *arsC* gene) (△) were grown in Luria broth to midlogarithmic phase, induced (for pGJ101 only) with 50 μM $^{73}AsO_2^-$ for 90 min, centrifuged, and suspended in TEA buffer without phosphate. (*A*) Uptake. $^{73}AsO_4^{3-}$ (5 μM) was added and 0.7-ml samples were filtered and washed for scintillation counting. (*B*) Efflux. Suspended cells were loaded with 5 mM $^{73}AsO_4^{3-}$ for 60 min and then diluted 1:100. Samples (0.7 ml) were filtered and washed for scintillation counting.

RESULTS

Decreased Arsenic Uptake and Enhanced Efflux with Cloned *ars* Genes. The cloned *S. aureus* plasmid pI258 is an arsenic resistance determinant that confers lowered uptake of radioactive arsenate into cells (Fig. 1*A*). Quantitatively, the cloned fragment in plasmid pGJ101 showed essentially identical levels of reduced uptake and enhanced efflux as did the intact plasmid (data not shown). Note in the experiment in Fig. 1 that 5 μM $^{73}AsO_4^{3-}$ was added for uptake, but 5 mM $^{73}AsO_4^{3-}$ was used in the efflux experiment.

Surprisingly, the ArsC protein alone (as encoded in plasmid pGJ109) was sufficient to confer reduced arsenic uptake

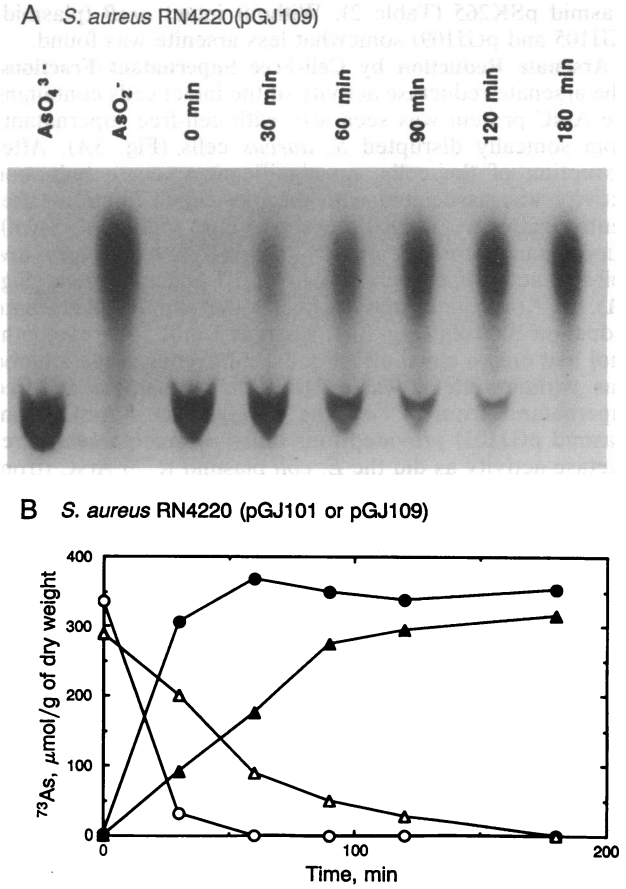

FIG. 2. Arsenate reduction to arsenite by *S. aureus* cells. Cells of strain RN4220 with plasmid pGJ101 or pGJ109 were exposed to 1 mM $^{73}AsO_4^{3-}$, samples were taken at various times, the cells removed by centrifugation, and the supernatants were chromatographed on cellulose TLC plates. (*A*) Autoradiograph (overnight exposure) of TLC plate showing $^{73}AsO_4^{3-}$ control, $^{73}AsO_2^-$ control, and supernatants from RN4220(pGJ109) cells incubated with $^{73}AsO_4^{3-}$ at 37°C for 0, 30, 60, 90, 120, or 180 min. (*B*) Quantitation of the autoradiograph in *A* by the AMBIS β scanner plus similar data for cells of strain RN4220(pGJ101). ○, RN4220(pGJ101) cells, remaining arsenate; ●, RN4220(pGJ101) cells, arsenite; △, RN4220(pGJ109) cells, remaining arsenate; ▲, RN4220(pGJ109) cells, arsenite.

(Fig. 1A) and arsenate resistance (data not shown) but had no measurable effect on the rate of arsenic efflux (Fig. 1B) under standard assay conditions.

Arsenate Reduction to Arsenite by Intact Cells. Staphylococcal cells with ArsC reduced arsenate to arsenite (Fig. 2). With plasmid pGJ101, 93% of the arsenate had been converted to arsenite within 30 min at 37°C and the conversion was complete by 60 min (Fig. 2B). With the *arsC* gene alone in plasmid pGJ109, the reduction of arsenate to arsenite was more gradual, and only about 30% of the added 1 mM $^{73}AsO_4^{3-}$ had been reduced by cells by 30 min of incubation. In addition to the cell-free medium arsenate and arsenite shown in Fig. 2, the *S. aureus* cells retained a small fraction (<5%) of the radioactive arsenate added (data not shown). When cells loaded for 1 hr at 20°C with 5 mM $^{73}AsO_4^{3-}$ were suspended in buffer and the distribution of arsenate and arsenite was measured, essentially all intracellular arsenic was arsenate with strain RN4220(pSK265) but ≈3 times more intracellular arsenite than arsenate was found with cells of strain RN4220(pGJ101) (data not shown).

The reduction of arsenate to arsenite was dependent on the presence of the *arsC* gene and the extent of reduction depended also upon the presence of *arsB* (Table 2). The *arsC*-deletion mutant plasmids pGJ106 and pGJ107 conferred no more arsenate reduction activity than did the vector plasmid pSK265 (Table 2). Without intact *arsB* (plasmids pGJ105 and pGJ109) somewhat less arsenite was found.

Arsenate Reduction by Cell-Free Supernatant Fractions. The arsenate reductase activity of the intact cells containing the ArsC protein was seen also with cell-free supernatants from sonically disrupted *S. aureus* cells (Fig. 3A). After disruption of the cells, no significant arsenate reduction activity was associated with the particulate fraction (after centrifugation at 100,000 × *g* for 45 min) (data not shown). Supernatants from strain RN4220(pSK265) had very low cell-free activity, especially at low DTT concentrations (Fig. 3A). DTT (maximum activity at 300 μM) stimulated arsenate reduction 100-fold (Fig. 3A), whereas 1 mM 2-mercaptoethanol had only a small effect and 1 mM reduced glutathione was without effect (Table 3). With comparable cell-free supernatants from *E. coli*, the staphylococcal ArsC (from plasmid pGJ103) provided four times as much arsenate reductase activity as did the *E. coli* plasmid R773 ArsC (from plasmid pUM3) (Fig. 3B). We do not know whether this difference is due to inherent properties of the two ArsC proteins (which are identical at only 18% of their amino acid positions) or different amounts made from different plasmids and promoters. However, note that the *S. aureus* cell-free preparations (Fig. 3A) showed >10 times the arsenate reductase activity of the most active *E. coli* preparation (Fig. 3B). The sulfhydryl inhibitors *N*-ethylmaleimide and iodoacetate inhibited arsenate reductase activity by ≈80% in crude cell-free preparations (data not shown) and by ≈90% with purified ArsC protein (Table 3). Both *N*-ethylmaleimide and iodoacetate are irreversible inhibitors of enzymes with critical cysteine residues. That the inhibition was incomplete may be due to some of the ArsC protein being present in an oxidized (disulfide) form in the cell-free preparations.

Table 2. Arsenate reduction to arsenite by *S. aureus* cells with various plasmids

Plasmid	*ars* genes	AsO_2^- formed, μmol/g of cells in 1 hr
No cells		<3*
pSK265	Vector, none	31
pGJ101	*arsR arsB arsC*	332
pGJ105	*arsR* (180/429 *arsB*) *arsC*	177
pGJ106	*arsR arsB* (112/131 *arsC*)	30
pGJ107	*arsR* (166/429 *arsB*)	27
pGJ109	(49/104 *arsR*) (56/429 *arsB*) *arsC*	224
pI258	*arsR arsB arsC*	246

Determinations of the distribution of ^{73}As between arsenite and arsenate for each cell type on a single chromatogram. Cells were grown in Luria broth, induced with 100 μM arsenite for 90 min at 37°C, transferred into TEA buffer without phosphate, and exposed to 1 mM $^{73}AsO_4^{3-}$ for 60 min at 37°C before centrifugation and chromatography of the supernatants. For partial deletions of genes, the number of codons remaining divided by the total number of codons in the gene is shown. The added arsenate corresponded to 3.32 mmol/g of cells.
*No detectable activity.

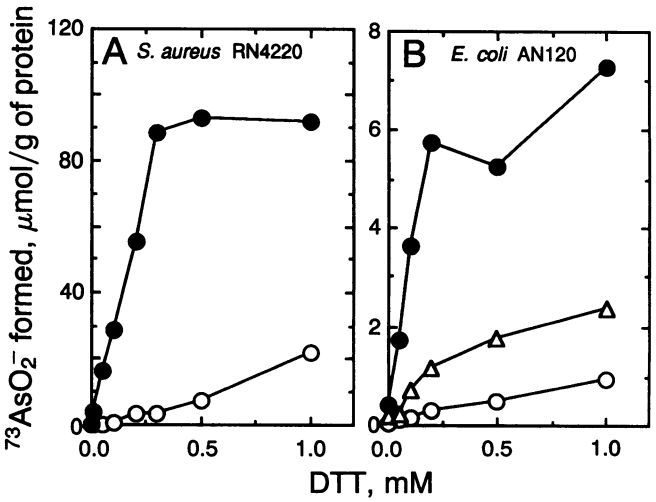

FIG. 3. Arsenate reduction by cell-free preparations requires DTT. Crude 100,000 × *g* supernatant from *S. aureus* cells with plasmid pSK265 (○) or pGJ109 (●) (*A*) or from *E. coli* cells of strain AN120(pUC19) (○), AN120(pGJ103) (●), or AN120(pUM3) (△) was incubated with added 100 μM $^{73}AsO_4^{3-}$ and various concentrations of DTT for 10 min at 37°C. Samples were removed and analyzed by TLC and scanning.

The cell-free arsenate reduction activities seen with crude preparations from *S. aureus* and *E. coli* cells were stimulated by NADPH (but not by NADH) when DTT was absent (Fig. 4). As in Fig. 3, the *S. aureus* pI258 *ars* determinant in *S. aureus* showed 4–8 times greater cell-free activity (Fig. 4A) than the *S. aureus* pI258 *ars* determinant in *E. coli* (Fig. 4B). The *E. coli* pUM3 ArsC gave very low activity in the absence of DTT and was not stimulated by NADPH (Fig. 4B). Although only low-level cell-free activity was seen with

Table 3. Effect of thiol compounds and thiol poisons on arsenate reduction by purified ArsC protein

Thiol added	Thiol inhibitor	AsO_2^- formed, μmol/g of ArsC in 5 min
None	–	<1
DTT	–	340
2-ME	–	4.1
GSH	–	<1
NADH	–	<1
NADPH	–	<1
2-ME + DTT	*N*-Ethylmaleimide	25.1
2-ME + DTT	Iodoacetate	42.9

Purified ArsC protein (70 ng) was incubated with 1 mM *N*-ethylmaleimide or iodoacetate for 5 min prior to addition of 1 mM 2-mercaptoethanol (2-ME) followed by 70 ng of purified *E. coli* thioredoxin and 500 μM DTT. $^{73}AsO_4^{3-}$ (10 μM) was added and after 5 min at 37°C, the radioactive arsenic was analyzed by TLC. For samples without inhibitor, 500 μM DTT, 1 mM 2-ME or reduced glutathione (GSH), or 200 μM NADH or NADPH was added to purified ArsC plus thioredoxin before addition of $^{73}AsO_4^{3-}$.

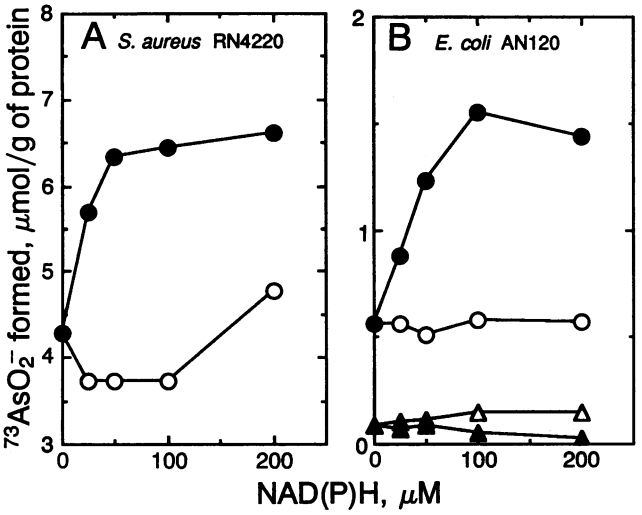

FIG. 4. Arsenate reduction with the staphylococcal or *E. coli* ArsC in *S. aureus* or *E. coli* cells: Effects of pyrimidine nucleotides. Constitutive AN120(pUM3) and RN4220(pGJ109) or induced AN120(pGJ103) cells were disrupted by sonication and high-speed supernatants were used. $^{73}AsO_4^{3-}$ (10 μM) and various amounts of NADH (open symbols) or NADPH (closed symbols) were added and samples were analyzed after 5 min at 37°C. (*A*) *S. aureus* RN4220(pGJ109) supernatant. (*B*) *E. coli* supernatant from strain AN120(pGJ103) (with the staphylococcal *ars* determinant) (circles) or AN120(pUM3) (constitutive *E. coli ars*) (triangles).

preparations from strain AN120(pUM3) (Figs. 3*B* and 4*B*), intact cells of strain AN120(pUM3) reduced arsenate to arsenite more rapidly than cells of strain AN120(pGJ103) (data not shown). Whereas the crude cell-free ArsC preparation responded to NADPH (Fig. 4), purified ArsC protein did not (Table 3).

ArsC Is a Cytoplasmic Protein. Because of the inhibition of arsenic efflux caused by osmotic shock (12) and the previous hypothesis that ArsC functions as a substrate-specificity component of the *ars* transport system (analogous to a periplasmic protein), it was important to localize ArsC in the cell (Table 4). The cytoplasmic (β-galactosidase) and periplasmic (alkaline phosphatase) marker enzymes were distributed as expected (Table 4) and arsenate reductase activity was found primarily in the cytoplasmic fraction, with <1% associated with the membrane fraction and ≈15% released by osmotic shock.

Table 4. Cellular location of the ArsC arsenate reductase activity by osmotic shock

Fraction	β-Galactosidase, units*	Alkaline phosphatase, units†	Arsenate reductase, unit(s)‡
Untreated cells			
Total sonicate	6990	400	16.7
Supernatant	5770	302	27.1
Membrane	450	38	0.6
Shocked cells			
Shock fluid	339	480	3.7
Total sonicate (after shock)	5640	77	27.0
Supernatant	6000	44	31.0
Membrane	86	37	0.2

Cell fractions were from *E. coli* AN120(pGJ103). Osmotic shock fluid, cell sonicate, supernatant, and membrane fraction after 100,000 × *g* centrifugation for 45 min were as described in *Materials and Methods*.

*Rate of *o*-nitrophenol β-D-galactoside hydrolysis (Miller units; ref. 20).

†Rate of *p*-nitrophenyl phosphate hydrolysis: change in A_{420} per hr per OD_{600} unit of cell density.

‡From TLC analysis: nmol of AsO_2^- formed at 37°C per min with various fractions, divided by 100 Klett units turbidity of original cells.

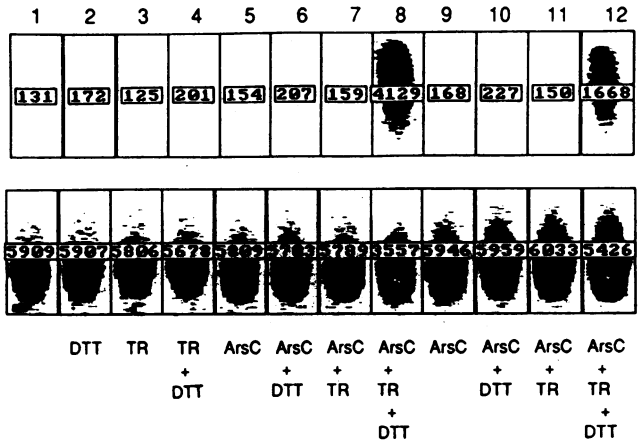

FIG. 5. Thioredoxin and DTT are needed for arsenate reduction. Samples (3.5 μl) of 15-μl reactions for 10 min at 37°C with 10 μM $^{73}AsO_4^{3-}$ were spotted on TLC plates, and the plates were developed for 2.5 hr with 2-propanol/water. $^{73}AsO_2^-$ (*Upper*) and $^{73}AsO_4^{3-}$ (*Lower*) regions of the TLC plate were quantified by scanning (counts in *Insets*) in the AMBIS β-scanner. Lane 1, $^{73}AsO_4^{3-}$ control. Additional components are indicated below each lane: DTT, 500 μM; purified thioredoxin (TR), 1 μg; purified ArsC, 0.2 μg (lanes 5–8) or 0.1 μg (lanes 9–12).

Purified ArsC Couples to Thioredoxin. The staphylococcal ArsC protein was overproduced in *E. coli* K38(pGP1-2) and purified by FPLC. With purified ArsC, strong quantitative conversion of arsenate to arsenite (54% and 23% conversion, with 0.2 μg and 0.1 μg of ArsC, Fig. 5, lanes 8 and 12, respectively) in 10 min was achieved only in the presence of excess (1 μg) purified thioredoxin with 500 μM DTT as reductant. Without thioredoxin, the ArsC protein itself reduced arsenite much more poorly, with 1.0% and 1.3% conversion to arsenite with 0.2 μg and 0.1 μg of ArsC, respectively (lanes 6 and 10). Thioredoxin plus DTT also showed marginal reduction of 0.8% of the added arsenate to arsenite (lane 4). Reaction mixtures containing proteins without DTT (or with DTT but no proteins) did not show arsenate reduction to arsenite. With an assay as in Fig. 5, the ArsC protein appears rather heat-stable, surviving 2 min heating at 80°C. ArsC arsenate reductase activity was decreased >80% at pH 6.3 but was stable in the pH range 7.0–9.5 (data not shown).

DISCUSSION

ArsC protein has an enzymatic function of which we had been previously unaware, the reduction of arsenate to arsenite. Chen *et al.* (1) identified the *arsC* gene of plasmid R773 in *E. coli* and showed by genetic studies that ArsC was needed for arsenate resistance, but not for arsenite or antimonite resistance. A comparable *arsC* gene with the same phenotype was found on the staphylococcal plasmids pI258 (6) and pSX267 (7). The previous hypothesis for the role of ArsC in arsenate resistance (summarized in refs. 2, 4, 5, and 9) was that ArsC was an intracellular substrate-binding protein, analogous to periplasmic substrate-binding proteins that facilitate transport but lack enzymatic activities. The arsenic efflux pathway through the ArsB membrane protein was thought to transport arsenite in the absence of ArsC and to transport both arsenate and arsenite in the presence of ArsC (2, 5, 21). We now have shown that it is arsenite that is exported when arsenate is added to cells containing ArsC.

The ArsC protein of the *E. coli* plasmid R773 was purified and crystallized (21) but its function was not tested. The R773 ArsC also has arsenate reductase activity in our studies (Fig. 3 and data not shown), but under our experimental conditions, the *in vitro* activity of crude preparations of R773 ArsC was much lower than that of pI258 ArsC (Figs. 3 and 4). How this difference results from the 82% nonidentities of aligned amino acid positions (1, 6) remains for subsequent work.

An important unanswered question is the mechanism of enzymatic energy coupling for arsenate reduction. Arsenate reductase activity was cytoplasmic and not membrane-associated or periplasmic (Table 4). The amino acid sequence of ArsC shows no sign of a nucleotide-binding motif. It seems likely that the energy for arsenate reduction is coming from elsewhere in the cytoplasm and being funneled to ArsC. The nonbiological thiol compound DTT strongly stimulated arsenate reduction by ArsC (Fig. 3) and the thiol poisons *N*-ethylmaleimide and iodoacetate inhibited reduction. However, the major intracellular small thiol compound, reduced glutathione, did not reduce arsenate to arsenite in the presence or absence of ArsC (data not shown). For several intracellular protein disulfide exchange reactions, the small protein thioredoxin plays an intermediate role, regenerating reduced cysteine residues on intracellular enzymes (18, 22). The results with cell-free arsenate reductase activity of ArsC were consistent with ArsC reducing arsenate with the involvement of cysteine residues found in the sequence (6, 7): ArsC activity was stimulated by DTT and inhibited by thiol poisons. It appears that *in vitro* the ArsC cysteine residues are maintained in a reduced state by thioredoxin. Thioredoxin in turn can be directly reduced by DTT or by the bacterial enzyme thioredoxin reductase [which requires NADPH for activity (22)]. The experiment with purified thioredoxin and purified ArsC protein *in vitro* suggests that arsenate was reduced with reducing power from DTT → thioredoxin → ArsC → arsenate. We suggest a similar pathway *in vivo* with reducing power from NADPH → thioredoxin reductase → thioredoxin → ArsC → arsenate. However, with the complex interactions of thioredoxin, glutaredoxin, and glutathione in energy coupling for cellular reductase activities (e.g., ref. 23), related alternatives for *in vivo* energy coupling are possible.

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