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Arsenic Efflux Governed by the Arsenic Resistance Determinant of *Staphylococcus aureus* Plasmid pI258

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The arsenic resistance operon of *Staphylococcus aureus* plasmid pI258 determined lowered net cellular uptake of ^{73}As by an active efflux mechanism. Arsenite was exported from the cells; intracellular arsenate was first reduced to arsenite and then transported out of the cells. Resistant cells showed lower accumulation of ^{73}As originating from both arsenate and arsenite. Active efflux from cells loaded with arsenite required the presence of the plasmid-determined *arsB* gene. Efflux of arsenic originating as arsenate required the presence of the *arsC* gene and occurred more rapidly with the addition of *arsB*. Inhibitor studies with *S. aureus* loaded with arsenite showed that arsenite efflux was energy dependent and appeared to be driven by the membrane potential. With cells loaded with $^{73}AsO_4^{3-}$, a requirement for ATP for energy was observed, leading to the conclusion that ATP was required for arsenate reduction. When the staphylococcal arsenic resistance determinant was cloned into *Escherichia coli*, lowered accumulation of arsenate and arsenite and ^{73}As efflux from cells loaded with arsenate were also found. Cloning of the *E. coli* plasmid R773 *arsA* gene (the determinant of the arsenite-dependent ATPase) in *trans* to the *S. aureus* gene *arsB* resulted in increased resistance to arsenite.

Plasmid-determined bacterial resistance to arsenic and antimony has been described in both gram-positive (4, 16) and gram-negative bacteria (7). Resistant *Escherichia coli* and *Staphylococcus aureus* cells accumulate arsenate to only a small extent (24). Lowered net accumulation of arsenic is the result of an active efflux system (15, 25). Using an *E. coli* mutant which is unable to synthesize ATP from respiratory substrates, Mobley and Rosen (15) showed that efflux determined by the *E. coli* plasmid R773 arsenic resistance operon was ATP dependent.

Arsenic resistance determinants were cloned and sequenced. The *E. coli* plasmid R773 *ars* operon consists of five genes (*arsR*, *arsD*, *arsA*, *arsB*, and *arsC*) (3, 18, 22, 26, 29), but only three genes (*arsR*, *arsB*, and *arsC*) were found on *Staphylococcus* plasmids (9, 20). The *arsR*, *arsB*, and *arsC* gene products of both operons have similarities in sequence and function. The *arsR* gene encodes a repressor protein in both organisms (20, 28). The R773 (*E. coli*) and pI258 (*S. aureus*) ArsR proteins have 30% identical amino acid residues. The *E. coli* ArsB protein is a membrane protein with 12 transmembrane α-helices (18, 23, 30) and has amino acid residues 58% identical to those of the staphylococcal ArsB protein (9). The *arsC* gene encodes an arsenate-to-arsenite reductase (10) in both microorganisms, although the sequence similarity is slight (with only 18% identical residues between the *S. aureus* and *E. coli* versions). The function of the arsenate reductase explains why *arsC* is needed only for arsenate resistance (3, 9, 26). ArsC reduces arsenate to arsenite, which subsequently is transported out of the cells (10). The arsenate oxyanion itself is not transported out of the cell (10). The *E. coli* R773 *ars* operon includes the extra gene *arsA*, which determines an ATPase subunit (3, 8). ArsA has been purified and functions as an arsenite- or antimonite-stimulated soluble ATPase (19), which explains the ATP dependency of transport in the presence of *arsA*. The fifth gene, *arsD*, in the *E. coli* plasmid R773 *ars* operon encodes a secondary down-regulatory protein which functions separately from the repressor *arsR* (29). The staphylococcal plasmid *ars* operons lack an *arsD* gene (9, 20).

Since the *S. aureus ars* system determines energy-dependent arsenic efflux (10, 25) but is missing the ArsA ATPase protein of the *E. coli* arsenic resistance system, two hypotheses were possible for the mechanism of the *S. aureus* arsenic resistance system. First, efflux by ArsB in the absence of ArsA might be driven by a chemiosmotic mechanism in response to the cell membrane potential (18). Alternatively, the staphylococcal *ars* system might function as an ATPase, with the ATPase subunit provided by a previously unanticipated chromosomal equivalent to the plasmid *arsA* gene.

In this study, we have determined the functions of the different genes in arsenic transport and investigated the energetics of arsenite efflux under conditions in which cells were loaded with arsenate or arsenite.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are listed in Table 1.

Materials. Chemicals were of analytical grade. ^{73}As was purchased from Los Alamos National Laboratory (Los Alamos, N. Mex.). Radioactive arsenate was chemically reduced to arsenite and column purified (10, 17).

Arsenic uptake. Arsenic uptake was measured essentially as described previously (24). Cells were grown in Luria-Bertani (LB) broth (supplemented with antibiotics when required) to a cell density of 100 Klett units. Cells were induced with 100 μM arsenite at 37°C for 1 h, harvested, washed twice with TEA phosphate-free buffer (24), and then suspended in the same buffer at a cell density of approximately 70 mg (dry weight) per ml. Uptake assays were initiated by 35-fold dilution of cells into prewarmed buffer

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Genotype or description	Reference or source
Bacteria		
E. coli		
AN120	*argE3 thi-1 rpsL uncA401*	2
JM109	*recA1 supE44 endA1 hsdR17 gyrA96 relA1 thi* Δ(*lac-proAB*)	21
S. aureus RN4220	An efficient acceptor of *E. coli* DNA	14
Plasmids		
pSK265	pC194 derivative with multiple cloning sites from pUC19	11
pArsA	R773 *arsA* gene cloned into pACYC184	6
pGJ101	pI258 *arsRBC* cloned into pSK265	9
pGJ103	pI258 *arsRBC* cloned into pUC19	9
pGJ105	Internal deletion of *arsB* in pGJ101	9
pGJ106	Partial deletion of 3′ *arsC* in pGJ101	9
pGJ107	*arsR*$^+$, partial *arsB*, *arsC*	9
pGJ601	Same as pArsA, but with the *arsA* fragment in the opposite orientation	This study
pUM3	*E. coli* R773 *arsABC* cloned into pBR322	3

containing $^{73}AsO_2^-$ or $^{73}AsO_4^{3-}$. Aliquots (100 μl each) were filtered through nitrocellulose filters, washed, and counted in a liquid scintillation counter (Packard Instrument Co. Inc., Downers Grove, Ill.).

Arsenic efflux. Arsenic efflux was measured after loading cells with radioactive $Na_3{}^{73}AsO_4$ or $Na^{73}AsO_2$ (10, 17, 25). For arsenic efflux from arsenate, two different methods were used. For the experiment illustrated in Fig. 1, cells were grown and induced as described above. Cells were harvested, washed twice with TEA buffer, and suspended at a density of 200 mg (dry weight) per ml in TEA buffer. Cells were loaded with 5 mM $Na_3{}^{73}AsO_4$ for 1 h at 37°C and stored on ice. Efflux was initiated by 100-fold dilution into TEA buffer (pH 7.5) plus 0.2% glucose and 5 mM sodium phosphate. Samples (0.7 ml each) were filtered and washed before scintillation counting. In other experiments, deenergized cells were used. Cells were grown as described above, harvested, washed twice with TEA buffer, suspended at 0.3 mg (dry weight) per ml, and deenergized by incubation with 1 mM 2,4-dinitrophenol (DNP) (for *S. aureus*; 5 mM for *E. coli*) at 37°C for 1 h with vigorous shaking. DNP was removed by washing cells three times with TEA buffer. After being suspended at a density of 70 mg (dry weight) per ml in TEA buffer, the cells were loaded with 5 mM $Na_3{}^{73}AsO_4$ for 1 h at 37°C and stored on ice. Efflux was initiated by 100-fold (if not indicated otherwise) dilution into TEA buffer containing substrates and inhibitors.

For arsenic efflux from arsenite, cells were grown and induced as described above but not deenergized. Cells were incubated with 10 mM cysteine for 30 min at 37°C at a density of 1 mg (dry weight) per ml. Cysteine was removed by washing the cells with TEA buffer. Cells were suspended at a density of 70 mg (dry weight) per ml and loaded with 30 to 50 μM $Na^{73}AsO_2$ for 30 min on ice. Efflux was initiated by 35-fold dilution into prewarmed buffer containing substrates and inhibitors.

Growth inhibition. Growth inhibition was determined as described previously (9, 24).

ATP content. Cells were prepared as described for the transport measurements. Samples (20 μl each) were removed and mixed with 180 μl of dimethyl sulfoxide and diluted with 800 μl of water. These samples were chilled on ice and used for determination of ATP content with a luciferin-luciferase assay (1). Samples were removed at the same time intervals as described for the arsenic efflux measurements.

Membrane potential. The membrane potential was measured on the basis of ^{3}H-labeled tetraphenylphosphonium (bromide) (TPP^+) or $^{86}Rb^+$ distribution as described previously (1). Nonspecific binding of TPP^+ was determined by comparison with the membrane potential measured on the basis of $^{86}Rb^+$ distribution under identical conditions. For calculation, an internal volume of 1.5 μl/mg (dry weight) (12) was used. The membrane potential value in the absence of inhibitors was about 170 mV and is in agreement with previously published values (12).

RESULTS

The cloned pI258 arsenic resistance determinant encodes an arsenic efflux system. The arsenic resistance operon of *S. aureus* plasmid pI258, subcloned to form plasmid pGJ101, determined low accumulation and rapid efflux of ^{73}As radioactivity added as arsenate (Fig. 1A and C) or arsenite (Fig. 1B and D). Two structural genes (*arsB* and *arsC*) are present in the *S. aureus* arsenic resistance system (9). A deletion in the *arsB* gene [in strain RN4220(pGJ105) (Fig. 1B)] resulted in increased arsenite uptake, as if the partially deleted ArsB protein could function backwards in arsenite uptake, and loss of efflux (Fig. 1D). Lowered uptake of arsenate (Fig. 1A) and enhanced efflux of arsenite from arsenate (Fig. 1C) was, however, reproducibly seen with the partial ArsB protein. The possibility of partial functions of the deleted form of ArsB was not further tested.

A deletion of the *arsC* gene in strain RN4220(pGJ106) resulted in increased uptake of arsenate and loss of efflux from added arsenate (Fig. 1A and C). In this strain the ArsC reductase is not functional. Efflux of $^{73}AsO_2^-$ was rapid with strain RN4220(pGJ106) (Fig. 1D).

Accelerated arsenite efflux from arsenite (data not shown) and arsenate (Table 2) was induced by growth on arsenite, which resulted in about a 10-fold increase in the arsenic efflux rate.

Energetics of arsenic efflux. The effects of several energy inhibitors on arsenic efflux were measured with cells loaded with arsenate or with arsenite. To differentiate between energy-driving forces, cellular energetics were poisoned by gradual titration with increasing concentrations of each inhibitor. Efflux of radioactivity from arsenate was strongly inhibited by the F_1F_0 ATPase inhibitor *N,N′*-dicyclohexylcarbodiimide (DCCD), the respiratory poison NaCN, the glycolysis inhibitor NaF, and the uncoupler carbonyl cyanide *m*-chlorophenylhydrazine (CCCP) (Fig. 2A and B). Arsenate-loaded cells showed a direct relationship between arsenic efflux rate and cellular ATP content (Fig. 2A) but not with the membrane potential (Fig. 2B). These experiments did not distinguish between a requirement for ATP for arsenate reduction to arsenite and a requirement for ATP for the subsequent efflux of arsenite. Therefore, we tested the energy requirements in cells loaded with arsenite. Efflux of arsenite from *S. aureus* cells loaded with 30 μM arsenite was not inhibited by NaCN, NaF, or valinomycin (in the presence of up to 10 mM KCl), although these treatments led to a large decrease in the ATP content (Fig. 2C). DCCD also

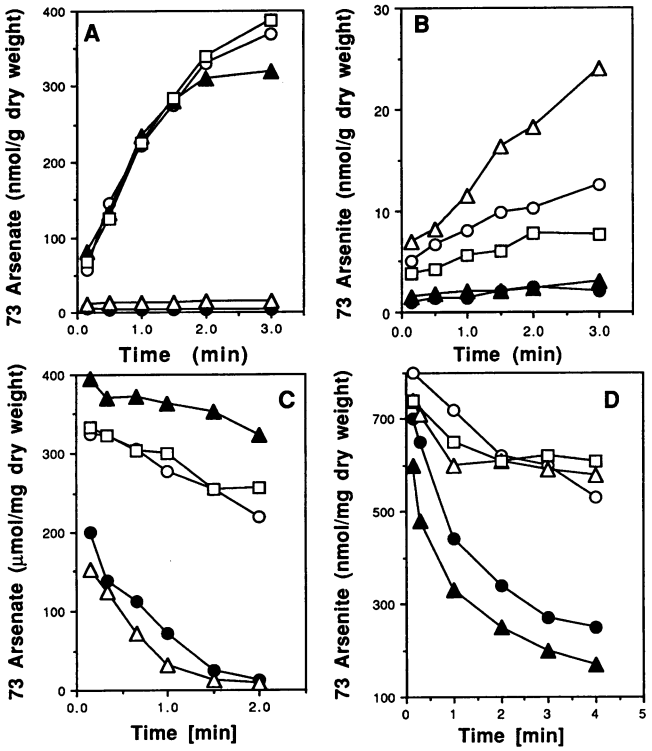

FIG. 1. Uptake and efflux of ^{73}As by *S. aureus* RN4220 cells with intact or partial pI258 *ars* operons. (A) Uptake of $^{73}AsO_4^{3-}$; (B) uptake of $^{73}AsO_2^-$. Cells were grown, induced with 50 μM arsenite, centrifuged, washed, and suspended in TEA buffer with 11 mM glucose at 37°C. At time zero, 5 μM $^{73}AsO_4^{3-}$ or $^{73}AsO_2^-$ was added and uptake was measured by filtering 0.7-ml samples. Symbols: ○, cells with the plasmid vector pSK265, sensitive; ●, cells with plasmid pGJ101, with the intact *ars* operon, resistant; △, cells with plasmid pGJ105 with an internal deletion in *arsB*, sensitive; ▲, cells with plasmid pGJ106, with a partial deletion of *arsC*, arsenate sensitive and arsenite resistant; □, cells with plasmid pGJ107, with a deletion encompassing part of *arsB* and all of *arsC*, sensitive. (C) Efflux of added $^{73}AsO_4^{3-}$; (D) efflux of added $^{73}AsO_2^-$. Symbols are as defined for panels A and B. Cells were grown and induced in LB broth, centrifuged, washed, and suspended in TEA buffer. Cells were loaded with 5 mM $^{73}AsO_4^{3-}$ for 1 h or with 50 μM $^{73}AsO_2^-$ for 30 min. Glucose (11 mM) was added to the efflux solution prior to 100-fold (arsenate) or 35-fold (arsenite) dilution of the cells.

had no effect on arsenite efflux, but the ATP content decreased only slowly with addition of DCCD, whereas the membrane potential increased to up to 210 mV (data not shown), as was expected. Efflux was inhibited under conditions in which cells were completely deenergized by CCCP (data not shown) or valinomycin (in the presence of 300 mM KCl). In careful titration experiments with arsenite-loaded cells, the arsenite efflux rate showed a good correlation to the membrane potential (Fig. 2D) but not to the ATP content of the cells (Fig. 2C).

Increased arsenite resistance of *E. coli* containing the cloned *S. aureus ars* operon plus the *E. coli* R773 *arsA* gene. The staphylococcal pI258 *ars* operon confers resistance to arsenate, arsenite, and antimonite when cloned into *E. coli* (9) (Fig. 3). Complementation to greater arsenite resistance resulted from providing the *E. coli arsA* in *trans* on a second plasmid (Fig. 3). Resistance to arsenate was not increased under these conditions (data not shown). The *E. coli* R773 ArsA protein was synthesized from plasmids pArsA and pGJ601 as measured in Western blot (immunoblot) experiments with anti-ArsA antiserum (data not shown). The resistance level was the same with both *arsA* plasmids in *trans* to the pI258 *ars* operon, although the *arsA* gene alone had little effect on arsenite resistance (Fig. 3). In the *E. coli* plasmid R773 arsenic resistance system the ArsA protein is attached to the ArsB membrane protein (27). However, attempts to measure the physical association of *E. coli* ArsA to the membranes of cells producing pI258 ArsB, by Western blot analysis, were unsuccessful (data not shown).

TABLE 2. Efflux of ^{73}As is energy dependent and inducible[a]

Inducer concn (μM)	Efflux velocity k[b] (1/min)	
	No addition	+ Lactate[c]
0	0.02	0.046
1	0.034	0.075
10	0.035	0.16
100	0.018	0.36

[a] *S. aureus* RN4220(pGJ101) was grown, induced by growth for 1 h with different concentrations of arsenite, and harvested. The cells were energy depleted with DNP and loaded with 5 mM $^{73}AsO_4^{3-}$ for 1 h. After 1,000-fold dilution, samples were filtered at different times, washed twice, and counted.
[b] Efflux rate constant.
[c] Cells were energized by the addition of 10 mM lactate 30 s before the first sample was collected.

Arsenic uptake and efflux from *E. coli* cells with the pI258 *ars* operon. The plasmid pI258 *ars* operon determined low accumulation of radioactive arsenate (Fig. 4A) and arsenite (Fig. 4B) and energy-dependent efflux of radioactivity from arsenate (plasmid pGJ103 [Table 3]) in *E. coli*. The efflux rate was lower, however, than the rate with the *E. coli* plasmid R773 *ars* resistance operon in plasmid pUM3 (Table 3), and net uptake was also lower with plasmid pUM3 than with pGJ103 (Fig. 4A).

Consistent with the resistance data in Fig. 3, lower uptake of arsenate and arsenite was found when the *E. coli arsA* gene was present in *trans* (Fig. 4). However, the cloned *arsA* gene alone also resulted in low accumulation of arsenate or arsenite (data not shown), complicating interpretation of these results. Furthermore, there was no measurable effect of *arsA* present in *trans* on the efflux velocity from *E. coli* cells loaded with radioactive arsenate (Table 3). Conditions for measurement of arsenic efflux from arsenite-loaded *E. coli* cells could not be established.

The energy requirement for arsenic efflux from arsenate-loaded *E. coli* AN120 cells (a mutant strain missing the F_1F_0 membrane coupling ATPase) containing the pI258 *ars* operon was determined. Efflux of ^{73}As added as arsenate was dependent on the addition of glucose and was not inhibited by the respiratory inhibitor cyanide (Table 4), as was expected. Addition of the glycolysis inhibitor fluoride reduced arsenic efflux (Table 4). Efflux was also inhibited by the protonophore CCCP. Since efflux from loaded $^{73}AsO_4^{3-}$ requires both reduction to arsenite and subsequent efflux, the experiment in Table 4 does not establish which process(es) requires ATP.

DISCUSSION

This is the first report on uptake and efflux of radioactive arsenic with *S. aureus* cells containing the plasmid arsenic resistance system since Silver and Keach (25) reported energy-dependent efflux as a basis for arsenate resistance.

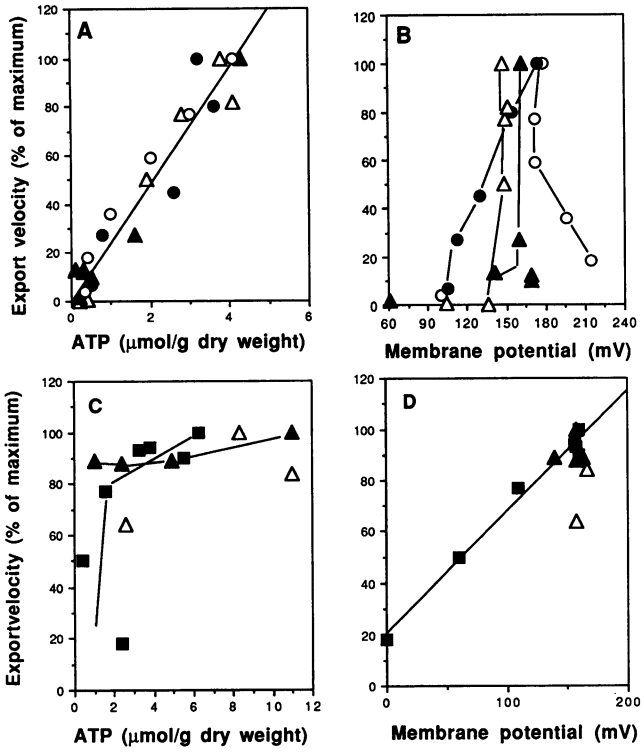

FIG. 2. Relationship of arsenic efflux rate to cellular ATP content and the membrane potential. (A and B) *S. aureus* RN4220 (pGJ101) cells were grown, washed, energy depleted, and loaded with $^{73}AsO_4^{3-}$. Cells were then diluted 1,000-fold into buffer. Increasing concentrations of NaF (1 to 10 mM) (△), NaCN (0.02 to 1 mM) (▲), DCCD (0.5 to 5 μM) (○), and CCCP (0.1 to 3 μM) (●) were added to the cell suspensions 30 s before addition of 25 mM lactate or 10 mM glucose (NaF only). Arsenic efflux was measured as described in Table 2, footnote *a*. The uninhibited control of each titration was set to 100% export velocity. Increased inhibitor concentrations are connected by lines. (C and D) *S. aureus* RN4220 (pGJ101) cells were grown, washed, and loaded with $^{73}AsO_2^-$. Efflux was initiated by 35-fold dilution into buffer with increasing concentrations of NaF (1 to 10 mM) (△), NaCN (1 to 5 mM) (▲), or valinomycin (10 μM in the presence of 0.1 to 300 mM KCl) (■). The uninhibited controls were set to 100% export velocity. In all cases, aliquots of the cells were used to measure ATP content and the membrane potential parallel to the efflux experiments.

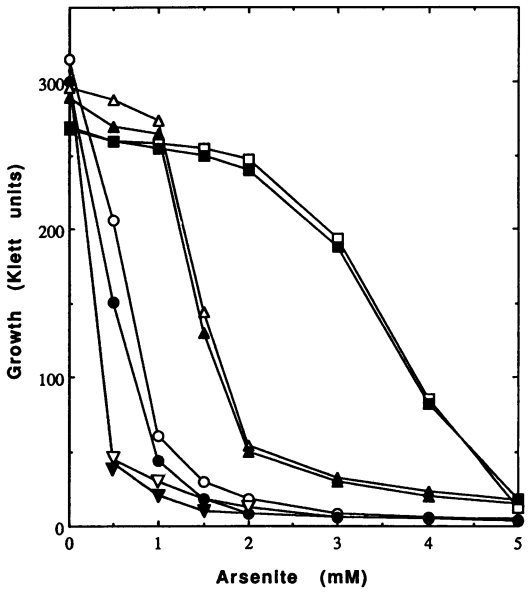

FIG. 3. *E. coli* ArsA increases arsenite resistance for the *S. aureus ars* system cloned into *E. coli*. ▽ and ▼, JM109 cells with the vector plasmid pUC19 and pACYC184, respectively; ○, pArsA containing the R773 *arsA*$^+$ in pACYC184; ●, pGJ601 containing the R773 *arsA*$^+$ in pACYC184 in the opposite orientation of pArsA; △ and ▲, pGJ103 with the intact pI258 *ars* operon in vector pUC19 and with the addition of vector pACYC184, respectively; □ and ■, pGJ103 plus *arsA*$^+$ plasmid pArsA and with pGJ601, respectively, with *arsA*$^+$ in both orientations.

The DNA sequence analysis of the *ars* determinant (9, 20) now enables us to investigate the roles of specific genes. Furthermore, the discovery that the *S. aureus* ArsC protein is an enzyme that reduces AsO_4^{3-} (arsenate) to AsO_2^- (arsenite) requires a reinterpretation of results on efflux of arsenic added as arsenate (10).

We offer a preliminary model which accounts for the data presented. When arsenate is added to the medium, it is taken up by the cellular phosphate transport systems (24, 26). Intracellular arsenate is reduced to arsenite by the ArsC protein, and arsenite is transported out of the cells through the ArsB protein. When arsenite is added to the medium, it is taken up by an unknown pathway (we have not found saturation kinetics for $^{73}AsO_2^-$ uptake [data not shown]) and then rapidly exported through the ArsB protein. The ArsC protein is not involved in arsenite efflux.

Conditions for arsenate loading and efflux (both in *S. aureus* and in *E. coli* cells) were readily established on the basis of previous studies (15, 25). Under conditions similar to those for efflux from arsenate-loaded cells, *ars* operon-dependent arsenite efflux could not be measured from arsenite-loaded cells (data not shown). Therefore, the protocol developed for arsenite efflux studies was substantially different. Although the conditions for uptake and efflux experiments are substantially different (and, of course, are different from those for long-term growth inhibition and resistance), there is in general a consistency between experiments showing reduced uptake and accelerated efflux (Fig. 1 and additional data not shown). It is likely that the need for different protocols for arsenate and arsenite loading reflects

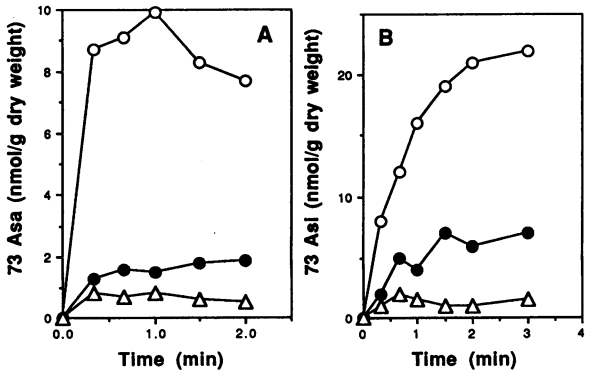

FIG. 4. Uptake of radioactive arsenate (A) and arsenite (B) in *E. coli* AN120 without a plasmid (○), with plasmid pGJ103 (cloned *Staphylococcus* plasmid pI258 *ars* operon) (●), or with pUM3 (cloned *E. coli* plasmid R773 *arsABC*) (△). Conditions are as described in the legend to Fig. 1. Asa, arsenate; Asi, arsenite.

TABLE 3. Efflux of radioactivity from arsenate-loaded energy-depleted *E. coli* cells with the *S. aureus* or *E. coli* arsenic resistance determinants[a]

Plasmid(s)	Conditions	Efflux velocity k (1/min)
pUC19	Added glucose	0.15
pGJ103	No addition	0.15
pGJ103	Added glucose	1.53
pGJ103 and pArsA	Added glucose	1.52
pUM3	Added glucose	6.9

[a] *E. coli* AN120 cells with the indicated plasmids were grown, induced with arsenite, harvested, energy depleted with DNP, and suspended in TEA buffer with added 10 mM glucose (if indicated).

the different chemical reactivities of these arsenic oxyanions. Arsenate competes with phosphate in transport and enzymatic reactions; arsenite is a potent sulfhydryl reagent and an inhibitor of many enzymes with essential thiol groups. Extended loading times with high arsenite concentrations may result in irreversible damage to the cells. For example, whereas most arsenate was pumped from the cells in efflux experiments (Fig. 1C), a significant level of intracellular arsenite remains (Fig. 1D). This remaining radioactivity was not released by permeabilization of the cells with the detergent cetyltrimethylammonium bromide (data not shown). Presumably this arsenite is bound to intracellular thiol compounds.

S. aureus cells with the cloned *ars* operon showed low accumulation and accelerated efflux of arsenite added as arsenate (Fig. 1A and C), as had been shown for plasmid pI258 (24, 25), or of added arsenite (Fig. 1B and D), which had not been tested before. The ArsB membrane protein was needed for arsenite efflux (plasmid pGJ105 [Fig. 1D]), and therefore ArsB is likely to provide the transport channel. When cells with a deletion in the *arsC* gene were loaded with arsenate, no efflux of radioactivity was seen (plasmid pGJ106 [Fig. 1C]). Therefore, ArsB must be specific for arsenite and must not transport arsenate. The presence of the ArsB protein was not essential for efflux of arsenite from arsenate in cells containing the ArsC reductase but only accelerated the rate threefold (10). This result was confirmed in the experimental results shown in Fig. 1A and C (plasmid pGJ105). Two possibilities remain for the efflux of arsenite (generated by arsenate reductase) in the absence of ArsB. Either the arsenite diffuses from the cell without a membrane transport protein or, alternatively, in the absence of ArsB a chromosomally encoded oxyanion carrier functions for efflux, but at a lower rate than ArsB (10). We cannot distinguish between these hypotheses for arsenite efflux under these conditions of slow and continuous production of arsenite from arsenate. We also lack a tested explanation for the increase in arsenite uptake with plasmid pGJ105 (a deletion in *arsB* that would retain only the first 5 of the 12 transmembrane helices shown in the working model for ArsB in reference 18). The extent of stimulation was not always as pronounced as in the experimental results shown, and we tentatively propose that the partial ArsB protein can function in arsenite uptake. The corresponding efflux experiment (Fig. 1D) does not show efflux stimulated by the partial ArsB protein.

TABLE 4. Effect of energy inhibitors on efflux of radioactivity from arsenate-loaded *E. coli* (energy-depleted cells)[a]

Conditions	Efflux velocity k (1/min)
No addition	0.1
Added glucose	1.76
Added glucose + NaF	0.75
Added glucose + NaCN	1.95
Added glucose + CCCP	0.51

[a] AN120(pGJ103) cells were prepared as described in footnote *a* of Table 3; 10 mM NaCN, 10 mM NaF, or 0.1 mM CCCP was added, followed immediately by 11 mM glucose (where indicated), and filtration was started.

Low accumulation and active efflux of arsenic from arsenate was observed only in the presence of ArsC (Fig. 1A and C). These results are now readily explained by the arsenate reductase activity of ArsC, followed by efflux of arsenite (10).

Since the plasmid pI258 *ars* operon lacks the *arsA* gene that determines the ATPase subunit of the *E. coli* R773 efflux pump, it was important to test whether the *S. aureus* system works with ArsB alone through a chemiosmotic mechanism or with an ATP dependency, indicative of an ATPase subunit determined by a gene outside of the plasmid arsenic resistance system. The possibility of a single-polypeptide (ArsB) chemiosmotic transporter evolving into an ATPase by acquisition of a second (ArsA) ATPase subunit was recently discussed (18). Efflux of arsenite from arsenite-loaded cells correlated to the membrane potential and not to the ATP content (Fig. 2C and D). We propose the electrogenic movement of the AsO_2^- anion as the most probable mechanism for ArsB-dependent arsenite efflux. An alternative pathway might be an electrogenic $As(OH)_3$ (neutral)/cation (positively charged) antiport mechanism, and such a pathway might be favored since the pK_a of arsenite is 9.2 and uncharged $As(OH)_3$ is the predominant form at neutral pH. When the membrane potential was varied by energy inhibitors, most inhibitors decreased the potential below the uninhibited value of 170 mV (which is similar to values measured earlier [12]). However, low levels of DCCD increased the membrane potential, presumably because protons that were pumped out by the respiratory chain could not flow into the cells through the F_0 proton channel. Potential values of up to 210 mV were reached, as in previous studies (13). The export of arsenite was inhibited at very low ATP concentrations (Fig. 1C), which always accompanied a greatly reduced membrane potential in these experiments. The arsenite efflux experiments were performed at pH 7.5, at which there is no significant transmembrane pH gradient. Therefore, the inhibitors would not change the internal pH significantly. Nevertheless, a slight decrease of the internal pH in the valinomycin inhibition experiments cannot be excluded.

Arsenite efflux from arsenate-loaded cells seemed to be ATP dependent in *S. aureus* (Fig. 2A and B) and *E. coli* (Table 4). The dependence on ATP in these inhibitor studies with arsenate-loaded cells could equally result from an ATP requirement for arsenate reduction or a subsequent requirement for efflux. For simplicity, and since arsenite efflux appears to be membrane potential dependent (Fig. 1D), we favor a secondary requirement for ATP for arsenate reduction to arsenite. Then, we must assume that reduction is rate limiting in the arsenate resistance process. ATP may be required indirectly through its involvement in thioredoxin-linked cellular reductases (10). Efflux from arsenate-loaded *E. coli* AN120 cells was dependent on added glucose and inhibited by NaF but not by NaCN, results consistent with an ATP requirement. However, CCCP also inhibited efflux of radioactivity from arsenate, a result that is not readily explained for an ATP-dependent process. However, these experiments were performed at pH 6.5, and the addition of

CCCP would then lead to acidification of the cytoplasm, which could in turn be responsible for inhibition of efflux.

The *S. aureus* arsenic resistance system from a gram-positive bacterium functions in the gram-negative bacterium *E. coli* (9). *E. coli* cells with the pI258 *ars* operon show higher resistance to arsenic (9) and low accumulation (Fig. 4) and active efflux of radioactivity from arsenate-loaded cells (Table 3). Accumulation of arsenite was also low, but conditions for energy-dependent arsenite efflux from arsenite-loaded *E. coli* cells could not be established in our experiments. Therefore, the energetics of arsenite efflux could not be tested in *E. coli* with its available mutants. However, the addition of the *E. coli arsA* gene in *trans* to the *S. aureus ars* operon resulted in elevated resistance to arsenite (Fig. 3), suggesting that the pI258 ArsB protein might associate with the R773 ArsA protein (27). The close sequence similarity between *E. coli* plasmid R773 and *S. aureus* plasmid pI258 ArsB proteins (9, 20) favors this hypothesis, and chimeric genes, consisting of part of the *E. coli* and part of the *S. aureus arsB* genes, confer partial arsenite resistance in *E. coli* and higher resistance with added ArsA (5, 6).

In summary, the results described in this report show energy-dependent accelerated arsenic efflux from *S. aureus* cells with the cloned arsenic resistance determinant. There are tentative data supportive of a chemiosmotic mechanism and a lack of ATP dependence for arsenite efflux in *S. aureus* in the absence of an *arsA* gene. Because measurements of energy coupling in wild-type whole cells are not unambiguous, further experiments in subcellular systems are needed to establish the mechanism of energy coupling to arsenic efflux in *S. aureus*.

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