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Energetics of plasmid-mediated arsenate resistance in *Escherichia coli*

(arsenate efflux pump/ATP-coupled transport/heavy metal resistance)

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ABSTRACT **Plasmid R773, which codes for resistances to arsenate, arsenite, and antimony, was introduced into *Escherichia coli* strain AN120, a mutant deficient in the H^+-translocating ATPase of oxidative phosphorylation. Cultures depleted of endogenous energy reserves were loaded with $^{74}AsO_4^{3-}$, and arsenate efflux was measured after dilution into medium containing various energy sources and inhibitors. Rapid extrusion of arsenate occurred when glucose was added. Arsenate was extruded both against and down a concentration gradient. In this strain glucose allows formation of both ATP via substrate-level phosphorylation and an electrochemical proton gradient (or protonmotive force) via oxidation of the products of glycolysis. When oxidation was inhibited by cyanide, glucose metabolism still produced arsenate efflux. Energy sources such as succinate, which supplies a protonmotive force but not ATP, did not result in efflux. Measurement of intracellular ATP concentration under each set of conditions demonstrated a direct correlation between the rate of efflux and ATP levels. Osmotically shocked cells lost the ability to extrude arsenate; however, no arsenate-binding activity was detected in osmotic shock fluid from induced cells. These results suggest that the arsenate efflux system is coupled to cellular ATP rather than an electrochemical proton gradient, possibly by an arsenate-translocating ATPase.**

The plasmid resistance factor R773, described by Hedges and Baumberg in 1973 (1), confers upon its host inducible resistance to arsenate, arsenite, and antimony (2). The plasmid is a member of the F1 compatibility group and is easily transferred among strains of *Escherichia coli* (1) as well as between genera of the Enterobacteriaceae (3). Arsenate resistance is due to an energy-dependent efflux system (4).

Since the advent of antibiotic treatment of microbial infections, the selective pressures on bacterial populations have spread the genes coding for resistance mechanisms. Plasmids often carry these antibiotic and heavy metal compound resistances. Several plasmid-mediated mechanisms have been proposed for protection against antibiotics (5), including alterations of target site, enzymatic degradation, enzymatic alteration, and altered transport. Several examples of transport alterations have been reported. Notably, Levy and McMurry (6) reported a plasmid-mediated tetracycline resistance in which the plasmid-bearing cells demonstrated an apparent block in transport of the antibiotic. Weiss *et al.* (7) reported a plasmid-mediated reduction in uptake of cadmium in *Staphylococcus aureus*. In 1981, Silver *et al.* (2) described inducible resistances to arsenate, arsenite, and antimony in plasmid-bearing strains of *S. aureus* and *E. coli*. In all of these examples, subsequent reports by McMurry *et al.* (8), Tynecka *et al.* (9), and Silver and Keach (4) demonstrated that resistances to tetracycline, cadmium, and arsenate, respectively, were due to energy-dependent efflux systems. The solutes were found to enter the cells but were rapidly expelled.

Generally, transport systems in bacteria have been found to fall into three classes (10): (*i*) group translocation systems, (*ii*) secondary porters linked directly to the protonmotive force, and (*iii*) systems coupled to phosphate bond energy. In the Enterobacteriaceae types *ii* and *iii* can be further classified into those sensitive to the effects of cold osmotic shock and those resistant to the effect (11, 12). By using a mutant defective in the H^+-translocating ATPase, in which the connection between oxidation and phosphorylation is broken, Berger (11) and Berger and Heppel (12) could establish conditions under which the energy resources of the cells consisted solely of phosphate bond energy, solely of a protonmotive force, or a combination of the two. They showed that transport by osmotic shock-sensitive systems is coupled to phosphate bond energy (perhaps ATP, although this has not been conclusively demonstrated), whereas shock-resistant systems are linked to the protonmotive force by cotransport or exchange with an ion (usually H^+) or by electrophoretic movement in response to the membrane potential.

Energy-dependent efflux systems are emerging as a newly recognized class of plasmid-mediated antibiotic and heavy metal resistance in bacteria. The unique nature of these transport systems prompted us to investigate the energetics of the plasmid-mediated arsenate resistance system. On the basis of the following studies of arsenate extrusion in energy-depleted cells of an *E. coli* strain lacking the H^+-translocating ATPase, we conclude that arsenate efflux is coupled to phosphate bond energy and not to a protonmotive force.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions. *E. coli* K-12 strain AN120 (*F⁻ arg rpsL uncA401*) was obtained from F. Gibson (13). *E. coli* K-12 strain J53(R773) (*pro met*) harboring plasmid R773 (with resistances to arsenate, arsenite, antimony, and tetracycline) was kindly provided by S. Silver (2). Plasmid R773 was transferred from strain J53(R773) to strain AN120 by conjugation (14). Recombinants were selected for resistance to streptomycin (100 μg/ml) and tetracycline (20 μg/ml) on LB (14) agar plates. Recombinants were screened for resistance to 2.5 mM arsenite. Cultures were grown either in LB broth or in triethanolamine-buffered minimal medium (2), pH 6.5, supplemented with 5 mM potassium phosphate, glucose (0.5%), arginine (50 μg/ml), and thiamine (2.5 μg/ml). Growth was monitored by measuring optical density at 615 nm. Arsenate resistance was induced by addition of 0.2 mM sodium arsenite to an exponentially growing culture. After 1 hr at 37°C the arsenite concentration was raised to 1 mM, and growth was continued for an additional hour.

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Abbreviations: FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; PMS, phenazine methosulfate.

Transport Assays. *E. coli* AN120(R773) was loaded with radioactive arsenate, and efflux was measured by a modification of the method of Silver and Keach (4). Cells grown in 100 ml of LB broth were induced with sodium arsenite, harvested at late exponential phase, and washed twice with triethanolamine-buffered minimal medium. The cells were depleted of endogenous energy reserves by incubation at 37°C for 2 hr in 100 ml of triethanolamine-buffered minimal medium containing 5 mM 2,4-dinitrophenol and then washed three times with triethanolamine-buffered minimal medium. The cells were suspended in a total of 0.2 ml of triethanolamine-buffered minimal medium containing 3 mM $^{74}AsO_4^{3-}$ (43 mCi/mmol; 1 Ci = 3.7×10^{10} becquerels) and incubated at room temperature for 1 hr. To initiate efflux down a concentration gradient, cells (50 μl) were diluted into 5.2 ml of triethanolamine-buffered minimal medium containing 5 mM potassium phosphate and the various energy sources and inhibitors as described below. Samples (1.0 ml) were taken at intervals by filtration through nitrocellulose filters (0.45-μm pore diameter, Matheson-Higgins Inc., Woburn, MA) and washed with 5 ml of triethanolamine-buffered minimal medium containing 5 mM potassium phosphate. The cells were dried and radioactivity was measured by liquid scintillation counting. Efflux against a concentration gradient was assayed similarly except that the arsenate-loaded cells were diluted into an equal volume of buffer containing 3 mM $^{74}AsO_4^{3-}$ of equal specific activity. The sample size was reduced to 15 μl. Where indicated inhibitors were added to cell suspensions 5 min prior to dilution. Proline transport activity was assayed as described (15).

ATP Determination. Arsenite-induced cells were depleted of endogenous energy reserves and loaded with nonradioactive arsenate (3 mM) for 1 hr as described above. The cell suspensions (25 μl) were added to tubes containing 75 μl of triethanolamine-buffered minimal medium containing 5 mM potassium phosphate and one of the following energy sources: 11 mM glucose, 0.14 mM phenazine methosulfate (PMS) plus 20 mM ascorbate, 10 mM succinate, or 10 mM DL-lactate. Cell suspensions were incubated at room temperature for 2 min. The reaction was stopped with 0.1 ml of ice cold 12% (vol/vol) $HClO_4$, and ATP levels were estimated by the luciferin/luciferase bioluminescence assay of Stanley and Williams (16), using a scintillation counter with the coincidence mode off. ATP solutions of known concentration were used as standards.

Other Methods. The cells were subjected to cold osmotic shock according to a modification (17) of the procedure of Neu and Heppel (18). Shocked and unshocked cells were assayed for proline transport or, after loading with 3 mM $^{74}AsO_4^{3-}$, arsenate efflux. Oxygen consumption was measured in the presence of the various energy sources in triethanolamine-buffered minimal medium by using a Clarke oxygen electrode (Gilson). Protein was determined by the method of Lowry *et al.* (19) with bovine serum albumin as a standard.

Chemicals. ATP and firefly lantern extract were obtained from Sigma. Carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) was a gift from DuPont. $^{74}AsO_4^{3-}$ (41.1 Ci/mmol) was purchased from Amersham. [^{3}H]Proline (1.86 Ci/mmol) was obtained from New England Nuclear. All other salts and chemicals were of reagent grade and were purchased from commercial sources.

RESULTS

Downhill Arsenate Efflux from Energy-Depleted Cells Requires a Source of Energy. Cultures of the H^+-translocating ATPase-deficient mutant AN120 and its R773 plasmid-bearing derivative were depleted of endogenous energy reserves and then loaded with 3 mM $^{74}AsO_4^{3-}$. Arsenate efflux was initiated by dilution into arsenate-free phosphate-containing medium [the presence of phosphate prevents recapture of the arsenate (4)] with or without glucose. Little efflux occurred in the plasmidless strain either with or without glucose (data not shown). The R773 derivative extruded arsenate only in the presence of glucose (Fig. 1).

Downhill Arsenate Efflux Depends on Phosphate Bond Energy. The H^+-translocating ATPase reversibly interconverts the protonmotive force and ATP. During glucose metabolism ATP can be formed directly by substrate level phosphorylation or by oxidative phosphorylation. Conversely, the protonmotive force can be maintained either by the oxidation of reduced substrate by the respiratory chain or from proton translocation coupled to the hydrolysis of ATP by the H^+-translocating ATPase.

Because AN120 is deficient in the H^+-translocating ATPase, there is no interconversion of ATP and protonmotive force. Thus, conditions can be established to provide one energy source but not the other. When dinitrophenol-starved, arsenate-loaded cells were diluted into media containing various oxidizable substrates and inhibitors, the arsenate efflux profiles shown in Fig. 1 were obtained. Glucose produced rapid efflux of arsenate, whereas succinate did not. Inhibition of the respiratory chain by cyanide, which prevents formation of a protonmotive force but not substrate level phosphorylation, did not diminish glucose-driven efflux. However, fluoride, which prevents substrate-level phosphorylation by inhibiting enolase, significantly reduced the rate of arsenate efflux. These results demonstrate that the protonmotive force does not energize arsenate efflux and suggest that the phosphate bond energy of ATP or a derivative is involved in the extrusion process.

To investigate this relationship more closely, the half-time for efflux, the respiratory rate (which provides a measure of the cells' ability to form a protonmotive force), and the intracellular ATP content were measured in arsenite-induced energy-depleted cells presented with various energy sources and inhibitors (Table 1). Efflux occurred to a significant extent only in the presence of glucose and was not inhibited by cyanide. Because cyanide blocks respiration effectively, no protonmotive force can be formed. In concentrations that should have been sufficient to prevent formation of a protonmotive force, the uncoupler FCCP did not prevent efflux. Efflux could be correlated with ATP content but not with the respiratory rate, whether oxidative energy was supplied by physiological substrates such as succinate or by artificial electron donors such as

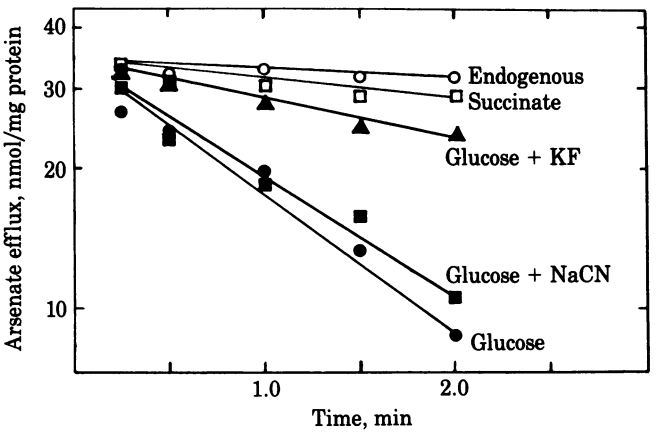

FIG. 1. Source of energy for arsenate efflux. *E. coli* strain AN120(R773) was induced with arsenite, depleted of endogenous energy reserves, and loaded with $^{74}AsO_4^{3-}$. Efflux was initiated by diluting the cell suspension 1:100 into arsenate-free buffer containing the indicated energy sources and inhibitors. ○, Endogenous; ●, 11 mM glucose; ▲, 11 mM glucose and 10 mM KF; ■, 11 mM glucose and 10 mM NaCN; □, 10 mM sodium succinate.

Table 1. Arsenate efflux down a concentration gradient in energy-depleted AN120(R773)

Energy source	Arsenate efflux $t_{1/2}$, min	Oxygen consumption, nmol O/min per mg protein	ATP content,* nmol/mg protein
Endogenous	8.4	30.5	0.1
0.14 mM PMS + 20 mM ascorbate	10.8	–	0.4
10 mM succinate	7.0	116.2	0.2
10 mM lactate	9.8	135.5	0.6
11 mM glucose	1.1	633.5	5.9
11 mM glucose + 10 mM NaCN	1.3	17.2	5.8
11 mM glucose + 10 μM FCCP	1.6	–	4.7
11 mM glucose + 10 mM KF	3.3	–	0.3

* ATP values were determined 2 min after addition of carbon source.

reduced PMS. Glucose-supported efflux was more rapid in the presence of fluoride than might be expected from the ATP levels. This may indicate that other metabolic products of glucose metabolism can support efflux. However, when efflux from energy-depleted cells was measured at early times, there was a lag of 10–20 sec, which correlated well with a lag in ATP synthesis (Fig. 2). This suggests that the immediate donor of energy to the arsenate transport system is ATP itself or another compound that is in rapid equilibrium with ATP.

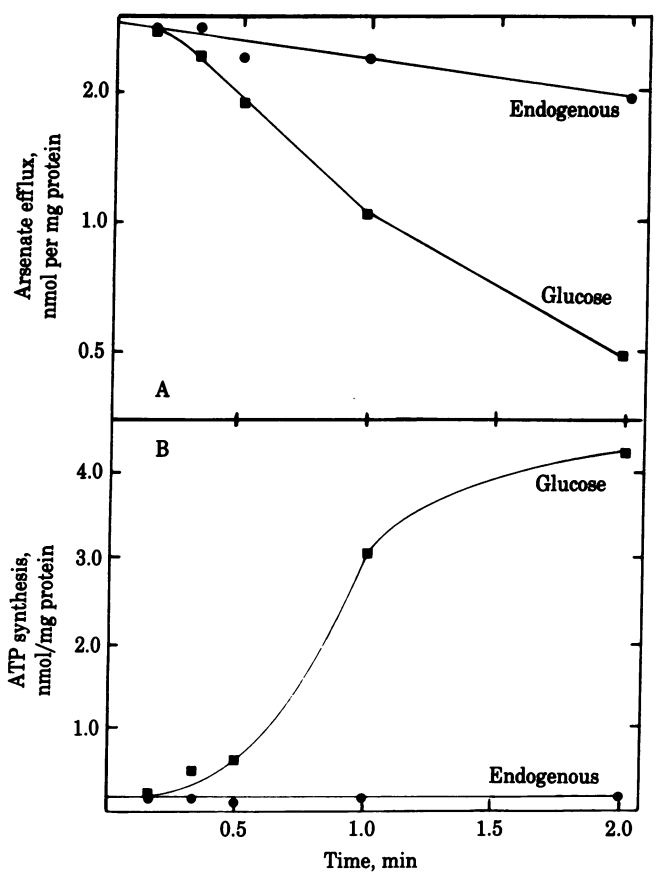

FIG. 2. Correlation between arsenate efflux and ATP synthesis. *E. coli* strain AN120(R773) was induced with arsenite, depleted of endogenous energy reserves, and loaded with $^{74}AsO_4^{3-}$. Arsenate efflux (*A*) and ATP synthesis (*B*) were measured. ●, Endogenous; ■, 11 mM glucose.

Table 2. Arsenate efflux against a concentration gradient in energy-depleted AN120(R773)

Energy source	Arsenate efflux $t_{1/2}$, min
Endogenous	34.1
10 mM succinate	37.0
11 mM glucose	2.3
11 mM glucose + 10 mM NaCN	3.8

Arsenate Efflux Against a Concentration Requires Phosphate Bond Energy. To demonstrate that the requirement for phosphate bond energy was not simply to fulfill a regulatory role, the energy dependence of arsenate efflux against a concentration gradient was examined (Table 2). Arsenate was actively extruded when glucose was supplied, and uphill efflux was insensitive to cyanide. Succinate, which produces only a protonmotive force, was incapable of driving uphill efflux. Thus, phosphate bond energy is necessary and sufficient for transport work.

Arsenate Efflux Is Sensitive to Cold Osmotic Shock Treatment. Because many ATP-linked transport systems rely on periplasmic binding proteins, which are lost during osmotic shock treatment, it was logical to examine the effect of osmotic shock

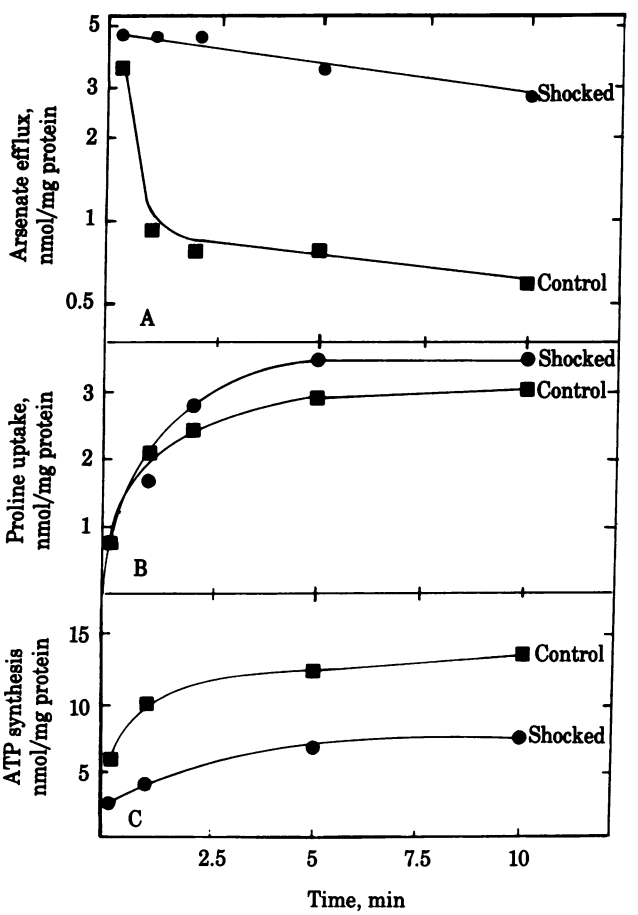

FIG. 3. Effect of osmotic shock on arsenate efflux. *E. coli* strain AN120(R773) was grown in triethanolamine-buffered minimal medium, induced with arsenite, and osmotically shocked. (*A*) Cells were then loaded with $^{74}AsO_4^{3-}$, and arsenate efflux was initiated by 1:100 dilution into buffer containing 11 mM glucose. (*B*) Proline uptake was measured in nonloaded cells by the addition of [^{3}H]proline (0.8 μM final concentration) with 11 mM glucose as energy source. (*C*) ATP levels were measured in osmotically shocked, arsenate-loaded cells. ■, Control cells; ●, osmotically shocked cells.

on the efflux system. We have noted that cells grown in rich medium lose ability to transport most solutes after osmotic shock. For this reason, the cells used for the osmotic shock experiments were grown in triethanolamine-buffered minimal medium with glucose as a carbon source. The cells were osmotically shocked and loaded with arsenate. Osmotic shock drastically reduced the ability of induced plasmid-bearing cells to extrude intracellular arsenate down a concentration gradient (Fig. 3A). Proline transport, a shock-resistant system driven by the protonmotive force (11, 12), was unaffected (Fig. 3B), indicating that loss of the arsenate efflux was not due to nonspecific effects. ATP levels were measured in shocked and unshocked arsenate-loaded cells (Fig. 3C). Osmotic shock treatment resulted in a partial loss of intracellular ATP pools. If the effect of osmotic shock were simply to deplete cellular ATP pools, then a gradual increase in the rate of arsenate extrusion would have been expected as ATP was resynthesized. Within 10 min of incubation with glucose and phosphate, shocked cells resynthesized more than 50% of the ATP found in control cells, a concentration that should have been adequate to drive efflux. Finally, we examined osmotic shock fluid from induced cells for arsenate-binding activity by using equilibrium dialysis against concentrations of $^{74}AsO_4$ between 1 and 100 μM. No binding activity was detected.

DISCUSSION

The results presented here confirm that the arsenate efflux system in plasmid-bearing strains of *E. coli* is energy dependent (4). The data further suggest that arsenate extrusion, both down and against a concentration gradient, is coupled to energy derived from ATP or some closely related phosphorylated compound. These conclusions are supported by the following observations. (*i*) Cells depleted of endogenous energy reserves require a source of energy to initiate arsenate efflux (Fig. 1). (*ii*) A protonmotive force formed by oxidation of respiratory substrates such as succinate and lactate does not drive efflux in a strain deficient in the H^+-translocating ATPase. (*iii*) Arsenate extrusion can be correlated with intracellular ATP concentrations (Table 1). (*iv*) The rate of arsenate extrusion parallels the rate of ATP synthesis (Fig. 2). These observations are consistent with the work of Silver and Keach (4). Although they did not demonstrate directly a dependence on ATP, they showed that arsenate efflux in *S. aureus* was not sensitive to valinomycin, which should dissipate the protonmotive force. They concluded that arsenate efflux in *S. aureus* is not protonmotive force-linked. Because the *S. aureus* and *E. coli* systems have many similarities, they also suggested by analogy that the *E. coli* system might not be linked to a protonmotive force.

Osmotic shock treatment diminished the capability of the transport system to extrude arsenate. Transport systems sensitive to osmotic shock have generally been found to be driven by energy derived from ATP (11, 12). This effect of osmotic shock on such systems is generally attributed to the loss of a periplasmic binding protein (17). To investigate this possibility, we introduced the R773 plasmid into a *phoS* mutant strain, which lacks the phosphate binding protein (20). Induced cells of this strain exhibited normal arsenate efflux, and no arsenate-binding activity was found in osmotic shock fluid from induced cultures. Thus, the phosphate binding protein is not a component of the arsenate efflux system. The data further suggest that there is no plasmid-encoded arsenate binding protein, although a very low affinity binding protein would not be detected by equilibrium dialysis. In his study of the energy requirements for β-methyl galactoside transport in *E. coli*, Wilson (21) showed that both uptake and efflux of β-methyl galactosides utilize phosphate bond energy. Although β-methyl galactoside uptake required the galactose binding protein, efflux did not. The possibility of an alternative galactose binding protein was not explored. However, it is difficult to envision the role of a periplasmic binding protein in an efflux system, in which the solute is transported from cytosol to periplasmic space.

Given the dependence of arsenate extrusion on cytosolic ATP concentration, an arsenate-translocating ATPase would seem likely. Rhoads and Epstein (22) have demonstrated an ATP-coupled ion transport system whose activity is reduced by osmotic shock but that does not utilize a binding protein. Osmotic shock significantly reduces K^+ transport via the Kdp system of *E. coli*, yet the components of this system are localized within the inner membrane and do not include a periplasmic binding protein (23). In this case also protonmotive force-linked transport was unaffected by osmotic shock, suggesting that osmotic shock treatment may have effects other than release of a binding protein.

It is not immediately obvious why a primary pump should be necessary for the maintenance of a low intracellular anion concentration. Because the electrochemical proton gradient is oriented outwards positive and acid, a uniporter could catalyze anion extrusion by simple electrophoretic movement. An ATP-linked pump has one major advantage over a uniporter. The latter is limited to extruding its substrate only to its equilibrium potential—i.e., the ratio of ion inside the cell to outside goes to equilibrium with the proton gradient. This could present problems in the face of high external anion or low protonmotive force. Thus, a uniporter would place the cell at the mercy of its environment. An ATP-linked pump has no such restriction. It can pump out ion far in excess of the proton equilibrium, maintaining a constant and low cytosolic ion concentration more or less independent of the external concentration. The knowledge that extrachromosomal elements can code for primary pumps is of importance for our understanding of the mechanisms of plasmid-mediated drug and heavy metal resistances.

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