ARSENATE RESISTANT MUTANTS OF ESCHERICHIA COLI AND PHOSPHATE TRANSPORT

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SUMMARY

Arsenate is a substrate for an inorganic phosphate transport system in E.coli U7. Mutants selected for growth in the presence of high concentrations of arsenate lose the capacity to transport arsenate at all concentrations. Most arsenate resistant mutants retain the ability to grow on orthophosphate. Phosphate transport in these mutants is completely abolished in the presence of cyanide, but in the wild type partial activity remains under the same conditions. Strains completely dependent on organic phosphate have also been found. Phosphate transport in the wild type U7 must be mediated by at least two separable systems, one specific for phosphate and the other active for both phosphate and arsenate.

INTRODUCTION

To elucidate the physiology and mechanism(s) of orthophosphate (Pi) transport in Escherichia coli, we have isolated a series of mutants defective in the utilization of Pi. The selection procedure is based on the demonstration that arsenate ion (AsO_4^{-3}, abbreviated here as As) is a substrate for the Pi permeation system in several other micro-organisms (1, 2, 3, 4). Arsenate is a toxic analog of Pi in many intracellular reactions and thus inhibits metabolism and growth (3, 5). Relief from the effects of As on growth could result from a mutation which excludes As from the interior of the cell. Mutants selected for resistance to As might simultaneously become deficient in the transport of As and Pi. L-α-glycerophosphate, which is transported without hydrolysis (6), serves as a source of phosphate in the growth media. A similar toxic analog, phosphite, has been

used to isolate Pi transport mutants in Bacillus cereus (2).

MATERIALS AND METHODS

Strain U7 (ref. 7, Hfr Cavalli, phoA$^-$, thi$^-$) is taken as the wild type for phosphate transport.

Media: A buffered mineral salts mixture containing 15 mM $(NH_4)_2SO_4$, 8 mM $MgSO_4$, 1 mM KCl, 0.2 mM $CaCl_2$, 2 μM $FeSO_4$, 2 μM $ZnCl_2$ and 0.1 M tris-Cl, pH 7.5, is used for all procedures described. To prepare growth medium, 0.6% glycerol and 2 μg/ml thiamine is added after sterilization. Phosphate is provided as sodium orthophosphate or glycerophosphate (α, β isomer mixture, Calbiochem) at the indicated concentrations. Plating medium is prepared as above with the addition of 0.1% sodium citrate and 1.8% agar.

Mutant Selection: A lawn of U7 is spread on plates containing 0.5 mM glycerophosphate and 0.01 M sodium arsenate One drop of ethyl methane sulfonate is placed in the center. Colonies having grown after 3 days incubation at 37° are purified on the same plates.

Uptake Experiments: Cells growing exponentially in glycerol minimal medium supplemented with 1 mM Pi or 5 mM glycerophosphate are washed in 0.02 M tris-Cl (pH 7.5) by centrifugation and resuspended in growth medium lacking Pi. This suspension is warmed at 37° for 3 minutes, then labeled Pi or As is added. Vigorous aeration is maintained throughout by shaking. Samples of 1 ml are collected on membrane filters (Millipore Corp., type HA, 0.45 micron pore size), and washed with chilled buffered salts containing 1 mM Pi. A gas flow counter is used for ^{32}P determinations. ^{74}As activity is measured by liquid scintillation counting using a solution of toluene, 2l, Cellosolve (Fischer Scientific Co.) 1l, PPO, 12 gm, and POPOP, 0.3 gm (Nuclear Chicago Corp.). Concentrated labeled solutions of Pi and As are prepared at least one day in advance and passed through a membrane filter twice before use.

Assays: Pi is determined by the method of Ennor and Stocken (8); As by the method of Mitchell (1). Bacterial density is determined spectrophotometrically at 600 nm.

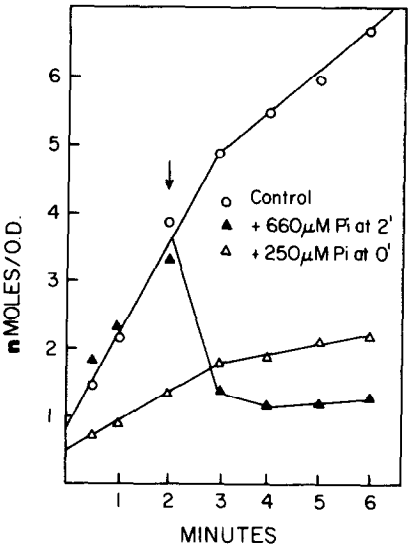

Fig. 1. As Uptake by U7 - The As concentration is 175 μM with a specific activity of 5×10^3 cpm/n mole. Pi is added to 250 μM at 0' or to 660 μM at 2'. The cell suspensions are between 0.19 and 0.23 OD_{600} units.

RESULTS AND DISCUSSION

The wild type U7 rapidly accumulates labeled As when incubated in the absence of Pi (Fig.1). The presence of Pi decreases the initial rate of As uptake. If excess Pi is added to a suspension of cells loaded with As, there is an immediate and rapid efflux of previously accumulated As. In reciprocal experiments, labeled Pi is rapidly accumulated in the absence of As, and As is an inhibitor of Pi uptake (Fig.2). Arsenate does not fully chase previously loaded Pi, indicating esterification of a large fraction of the intracellular phosphate. It should be noted that As and Pi are not equally effective as inhibitors in reciprocal experiments. A 1.4 fold excess of Pi decreases As uptake by 70%, whereas a 2.5 fold excess of As inhibits the initial rate of Pi uptake only by 50%. This series of experiments

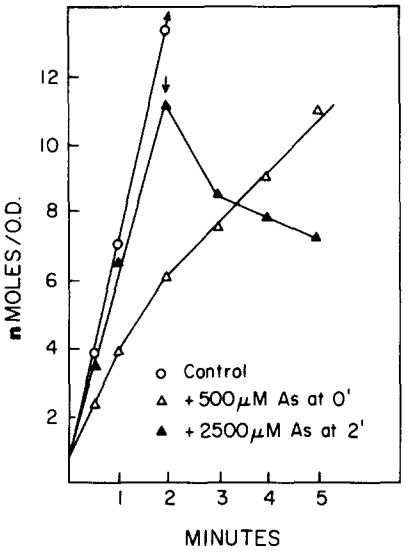

Fig. 2. Pi Uptake by U7 - The Pi concentration is 205 μM with a specific activity of 6×10^3 cpm/n mole. As is added to 500 μM at 0' or to 2500 μM at 2'. The cell suspensions are between 0.18 and 0.23 OD_{600} units.

shows that Pi and As are both substrates for at least one transport system in E. coli and justifies a search for Pi transport mutants among As resistant strains.

TABLE I

PLATING EFFICIENTY OF U7 AND As RESISTANT MUTANTS

STRAIN	Additions to Basic Medium			
	0.5 mM glycerophosphate	1 mM Pi	0.5 mM glycerophosphate + 10 mM As	0.5 mM Pi + 5 mM As
U7	1.0	0.9	1.0×10^{-6}	1.8×10^{-6}
UR13	1.0	1.0	0.9	0.9
UR1	1.0	2.4×10^{-6}	1.0	4.5×10^{-8}

Cultures grown in basic medium with 5 m M glycerophosphate are washed and assayed on plates containing basic medium and the additions shown. Averages of duplicate determinations, taking the number of colonies on 0.5 m M glycerophosphate as 1.0, are shown.

Strains UR1 and UR13 represent two categories of mutants derived from U7 by selection for As resistance. Characterization of these strains is shown in Table I. The wild type grows equally well when Pi or glycerophosphate is the phosphate source, but fails to grow in the presence of As. UR13 has the surprising capacity to grow on Pi in the presence of concentrations of As sufficient to prevent growth of the wild type. UR1 will not grow when provided with Pi as the phosphate source. Some revertants of UR1 selected to grow on Pi have a phenotype similar to UR13. This suggests that the defect(s) in UR13 will also be found in UR1, which should have an additional defect.

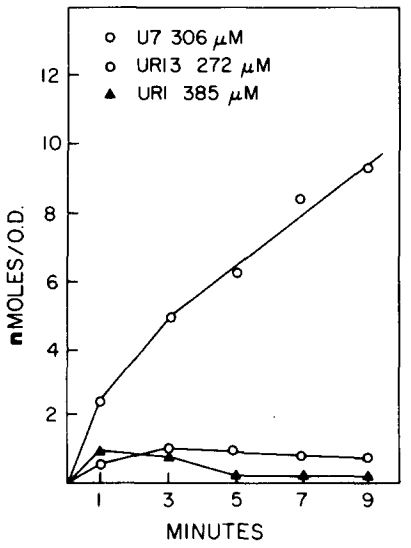

Fig. 3. As Uptake by U7 and As Resistant Mutants - U7 and UR13 were grown with 1 m M Pi as the phosphate source. UR1 was grown with 5 m M glycerophosphate as the phosphate source. As is present at the indicated concentrations with a specific activity of 2.3×10^3 cpm/n mole (U7 and UR13) or 3.3×10^3 cpm/n mole (UR1). Cell suspensions have an $OD_{600} = 0.3$.

Arsenate resistant mutants no longer accumulate As under conditions where the wild type is fully active (Fig.3). UR1 will accumulate Pi but with an initial rate only 10% that of the wild type. UR13 will grow on Pi, but does not accumulate As, suggesting that there is a mechanism of Pi transport for which As is not

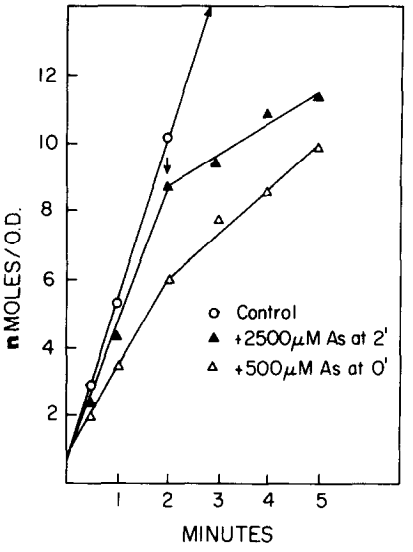

Fig. 4. Pi Uptake by UR13 - All conditions are exactly as in Figure 2.

a substrate. The initial rate of Pi uptake in UR13 is 75% that of the wild type at equal concentrations of Pi (Fig.4). Excess As does not cause net efflux of previously loaded Pi. The 40% inhibition of Pi uptake by a 2.5 fold excess of As in UR13 is slightly less than was found in the wild type. It was noted above that the inhibition of Pi uptake by As was less than would be expected if both substrate and inhibitor had similar affinities for the same transport system. Finding similar inhibition of Pi uptake by As in UR13, which has no As transport system, implies that the Pi transport activity remaining in the wild type despite As competition is primarily via the mechanism that mediates Pi transport in UR13.

We find that accumulation of As in the wild type is not affected by the presence of cyanide (Table II). Because As is transported by the Pi uptake system, it follows that a component of Pi uptake is also insensitive to cyanide. Phosphate uptake in UR13 is more sensitive to cyanide than in the wild type at both concentrations. In the mutant both the initial rate of uptake and the extent of accumulation of Pi are severely inhibited. We therefore conclude that Pi uptake

TABLE II

CYANIDE INHIBITION OF As AND Pi UPTAKE

Substrate	Strain	Rate (nmoles/OD/min) Control	+1 mM KCN	Inhibition (%)
175 μM As	U7	1.6	1.6	-
205 μM Pi	U7	6.4	3.7	42
9 μM Pi	U7	2.8	0.4	86
205 μM Pi	UR13	4.6	0.4	91
9 μM Pi	UR13	2.8	0.04	99

Cells are treated with 1 m M KCN for 3 minutes then labeled substrate is added. Rates are determined for the first minute of uptake.

in UR13 is fully dependent on energy production. Inhibition by cyanide of this Pi specific transport could account for the partial inhibition in the wild type. The increase in inhibition with decreasing concentration suggests that at low external concentrations of Pi, transport is energy dependent.

Characteristics of Pi transport in UR13 can be demonstrated in the wild type by both cyanide poisoning and As competition. Therefore, the Pi specific transport activity in this mutant is the result not of an alteration of an existing non-specific transport mechanism, but of the unmasking of one of at least two mechanisms possessed by the wild type. Strain UR13 has lost the energy independent transport of Pi and As at high substrate concentration. Strain UR1 has lost both permeation pathways.

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