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Tellurite Susceptibility and Non-Plasmid-Mediated Resistance in *Escherichia coli*

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Tellurite (TeO_3^{2-}) is highly toxic toward *Escherichia coli* (MIC, ~1 μg ml^{-1}). Mutants (Tel) that were resistant to low levels of TeO_3^{2-} (MIC, ~10 μg ml^{-1}) and collaterally resistant to arsenate were isolated. These Tel mutants were unable to grow on media containing low levels of P_i, which supported growth of the parent strain. When grown at much higher P_i levels they exhibited derepressed levels of the outer membrane *phoE* protein and the periplasmic *phoS* protein, as well as several other proteins indicative of P_i starvation. Tel mutants were markedly defective in $^{32}P_i$ transport, and TeO_3^{2-} was shown to be a potent competitive inhibitor of $^{32}P_i$ transport in the parent strain. The Tel phenotype could be complemented by an F′ plasmid harboring the *phoR*, *phoB*, and *phoA* loci, and curing of the F′ plasmid completely restored TeO_3^{2-} resistance. Of a variety of well-characterized P_i transport mutants, only *phoB* mutants were equally resistant to TeO_3^{2-}, and susceptibility could also be restored in strains carrying an F′ plasmid for the *phoB* region and lost once more after F′ curing. The *tel* and *phoB* loci were equally cotransducible with *lac*. Tel mutants still synthesized alkaline phosphatase, the *phoA* gene product, after P_i starvation, suggesting that the *phoB* locus per se was not involved because *phoB* is a positive regulatory gene for *phoA* expression. The results indicate that TeO_3^{2-} is transported into *E. coli* by a phosphate transport system and that resistance to TeO_3^{2-} specifically selects for as yet uncharacterized mutants in the *phoB-phoA* region of the chromosome.

Tellurium oxyanions, such as TeO_3^{2-}, are highly toxic to both gram-negative and -positive bacteria. Resistance to TeO_3^{2-} has been primarily ascribed to plasmids, especially in various species of *Klebsiella*, *Citrobacter*, and *Pseudomonas* (14, 21, 22). The natural resistance to TeO_3^{2-} has also been used in the identification of *Corynebacterium* species (5). However, the molecular basis of either the acquired or natural resistance is unknown. In some cases the reduction of TeO_3^{2-} to Te and alkylation of Te has been suggested as possible mechanisms (23). TeO_3^{2-} has been recently shown to interact with the *Escherichia coli* and *Klebsiella pneumonia* cell surface to enhance bacteriophage P1 *vir* absorption (J. M. Tomás, M. Regue, R. Pares, and J. Jofre, Can. J. Microbiol., in press).

To try to understand the basis of TeO_3^{2-} resistance, whether chromosomal or plasmid encoded, we felt it was important to first understand the molecular basis of this susceptibility. Most strains of *E. coli* and *Salmonella typhimurium* are highly susceptible to TeO_3^{2-}, and plasmids conferring resistance in these strains have been described (22). Spontaneous mutants resistant to TeO_3^{2-} can be obtained relatively easily; thus, we decided to use this system to examine the molecular basis of susceptibility and resistance in non-plasmid-bearing strains. The results of this study demonstrate that TeO_3^{2-} is transported on the phosphate transport pathway in *E. coli* and also provide an excellent selection method for unusual mutants of this pathway.

MATERIALS AND METHODS

Bacterial strains, bacteriophages, and growth conditions. The strains used in this study, along with their properties and origins, are listed in Table 1. Phages λ, P1, T4, T6, and φ80 as well as colicin V were lab stock. Phages TU1A and TP1 were gifts of M. Schwartz, Paris. These phages were always grown on *E. coli* C600. Luria broth (LB) was used as rich medium, and LB agar was prepared by the addition of 1.5% agar or 0.6% agar for soft agar. The P_i content of P_i-free minimal media (18) was adjusted to between 41 μM (derepressed conditions) and 660 μM (repressed conditions).

Isolation and characterization of TeO_3^{2-}-resistant mutants. Log phase cells (10^8) grown in LB were spread on LB agar containing 5 μg of K_2TeO_3 per ml. After 24 h at 37°C the clearly resistant colonies were purified by restreaking twice on the same media. All TeO_3^{2-}-resistant (Tel) mutants were routinely tested for original amino acid auxotrophic markers, phage and colicin susceptibility by spot testing, and their ability to ferment lactose on MacConkey plates. MICs for heavy metal salts and antibiotics were determined in LB broth after 18 h of incubation at 37°C. Cultures were inoculated at 1% with freshly grown cells.

Sugar fermentation tests were carried out with eosine-methylene blue broth, with sugars sterilized separately as 10% solutions and added at 0.1% final concentration. Overnight cultures of cells grown in LB were used as 1% inocula.

Phosphate growth requirements were determined in minimal medium (19) adjusted to various P_i levels with sterile K_2HPO_4-KH_2PO_4 buffer (pH 7.0). Cells were first grown on minimal medium containing 660 μM P_i to mid-log phase, washed three times with P_i-free medium, and inoculated at 1%. Cultures were incubated at 37°C for 18 h and scored for growth.

Cell surface isolation and analyses. Periplasmic proteins were released by osmotic shock (26). Cell envelopes were prepared by lysis with a French pressure cell at 16,000 lb/in^2, followed by removal of unbroken cells at 10,000 × *g* for 10 min and finally by sedimentation at 100,000 × *g* for 2 h. Cytoplasmic membranes were solubilized twice with sodium *N*-lauryl sarcosinate (7), and the remaining outer membrane

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TABLE 1. *E. coli* strains used in this study

Strains	Description	MIC ($\mu g\ ml^{-1}$) for TeO_3^{2-}	Source
CSH30	F⁻ *trpC*	1	Lab stock
C600	F⁻ *lacY1 leuB6 supE44 thr-1 tonA21* λ⁻	1	Lab stock
KT86	F⁻*lacY1 leuB6 supE44* Tel *thr-1 tonA21* λ⁻	10	This study
KT87	F⁻ *lacY1 leuB6 supE44* Tel *thr-1 tonA21* λ⁻	10	This study
E15	Hrf *fadL701 fep ompF637 ΔphoA8 pit-10 purE relA1 spoT1 tonA22*	1	B. Bachmann
C31	Hfr *fep ompF627 phoS25 pit-10 purE relA1 spoT1 tonA22*	1	B. Bachmann
S26	Hfr *lip ompF627 phoA4 purE relA1 tonA22*	1	B. Bachmann
LEP-1	F⁻ *azi-6 lacI22 lacZ73 mtl-1 phoB23 proC34 purE42 rpsL109 thi-1 trpE38 tsx-67 xyl-5*	10	B. Bachmann
C72	Hfr *fep ompF627 phoS27 pit-10 purE relA1 spoT1 tonA22*	1	B. Bachmann
C90	Hfr *fep ompF627 phoT9 pit-10 purE relA1 spoT1 tonA22*	1	B. Bachmann
10B5	Hfr *fadL701 glpD3 glpR2 lip ompF627 phoA8 pit-1 pst-2 purE relA1 spoT1 tonA22*	2	B. Bachmann
6S5	F⁻ *his-53 lacY1 metB1 nalA12 pit-1 proC24 pst-2 pyrF30 rpsL97 thyA25*	0.25	B. Bachmann
ORF4/KLF251	F'254 F' point of origin: P059 of Hfr OR11 Chromosome markers: *ara-14 azi-6 lacZ36 leu-6 metE-70 mtl-1 proC32 purE-32 recA1 rpsL109 supE44 thi-1 tonA23 trpE-38 tsx-6 xyl-5* λ⁻	1	R. Harkness, Victoria, B.C.
KT201	KT86 + F'254	1	This study
KT202	KT201 cured of F'254	10	This study

fraction was sedimented at 100,000 × *g* for 2 h. Outer membranes were suspended in 10 mM Tris hydrochloride–5 mM $MgCl_2$ (pH 7.3) after brief sonication.

Membrane proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) by a modification of the Laemmli procedure (3) with 12% acrylamide and 0.65% bisacrylamide. Protein gels were routinely stained with Coomassie blue. Protein concentrations were determined by the procedure described by Lowry et al. (12) with bovine serum albumin as a standard.

Genetic techniques and plasmids. Conjugation and F' episome curing were carried out by the method of Miller (15). Transduction experiments were carried out with P1 *cmts* (11) as the high frequency transducing phage. P1 *cmts* was grown in soft agar on a *lac*⁺ *E. coli* strain and transduced into KT86 (Tel^r *lac*⁻). *lac*⁺ transductants were screened for TeO_3^{2-} resistance (10 $\mu g\ ml^{-1}$). Plasmid DNA was isolated from whole cells by alkaline extraction, electrophoresed on 0.7% agarose gels, and stained with ethidium bromide (10).

Phosphate transport and alkaline phosphatase assays. Cells grown on minimal medium plus 330 μM P_i were harvested at mid-log phase and then suspended in P_i-free minimal medium and incubated for 2 h at 37°C (derepressed conditions). The cells were then harvested once more and washed twice with P_i-free minimal medium. These cells (1 ml at 350 μg [dry weight]) were incubated at a concentration chosen to reflect high-affinity transport activity (8×10^{-7} M $^{32}P_i$; 0.10 Ci mol^{-1}) in the presence or absence of different concentrations of P_i, K_2TeO_3 or Na_2HAsO_4. Fractions (0.1 ml) were removed at 30-s intervals for 1 min, filtered (pore size, 0.45 μm; Schleicher & Schull, Inc., Keene, N.H.), washed with 5 ml of P_i-free minimal medium, and assayed for radioactivity by scintillation counting. The rate of $^{32}P_i$ transport was calculated from a linear plot of the rate data. In competition experiments, unlabeled P_i, TeO_3^{2-}, or AsO_4^{3-} was added at different concentrations simultaneously with $^{32}P_i$.

Alkaline phosphatase (EC 3.1.3.1) was measured by the method of Torriani (25).

RESULTS

Isolation and properties of Tel mutants. Spontaneous mutants of *E. coli* C600 occurring at a frequency of 5×10^{-6} were isolated as resistant to 5 μg of TeO_3^{2-} per ml. All Tel mutants were identical to the parent strain with respect to phage susceptibility (T4, T6, φ80, TU1A, and TP1), colicin V susceptibility, and auxotrophic markers (*leu*, *thi*, *thr*) and were Lac⁻ as well. No changes were found in various antibiotic susceptibilities. The ability to ferment each of 10 different sugars was unchanged. Two independent, but typical, Tel mutants (KT86 and KT87) were selected for heavy metal susceptibility studies. Both strains exhibited higher MICs for TeO_3^{2-} (10-fold) and AsO_4^{3-} (5-fold) relative to that of parent strain C600, the MIC of which for TeO_3^{2-} was 1 $\mu g\ ml^{-1}$ and for AsO_4 was 7 mM; but no changes in the MIC toward Na_2AsO_2, $HgCl_2$, $CdSO_4$, $AgNO_3$, $CsCl_2$, or $CuSO_4$ were found. Both Tel mutants showed a minimum growth requirement for P_i of more than three times that of *E. coli* C600 (62 versus 21 μM). These observations suggest that Tel mutants harbor a defect with respect to the transport of P_i.

Outer membranes and periplasmic proteins. After growth under conditions which normally completely repress the production of the periplasmic P_i-binding protein, the *phoS* gene product (38,000 M_r), strain KT86 was found to continue to produce small quantities of a periplasmic protein which was of the approximate molecular weight (MW) of the *phoS* gene product (Fig. 1A). A protein of identical MW was present in P_i-deficient cells of the C600 parent (Fig. 1B).

Synthesis of the PhoS protein is indicative of P_i starvation. After growth under derepressing conditions (40 μM P_i), C600 expressed this protein abundantly (Fig. 1B). As a control, *E. coli* C31 (*phoS*) was specifically defective in the same PhoS protein when grown under derepressed conditions (data not shown). When the outer membrane proteins of C600 and KT86 from cells grown in LB were examined by SDS-PAGE (Fig. 2), a protein of 45,000 M_r was particularly expressed in KT86 but not in C600. However, when C600 was grown under derepressed conditions (41 μM P_i) a protein of this MW was produced. The 46,000 M_r protein corresponds to the well known PhoE porin that is produced in response to P_i starvation (25). An unknown protein of 20,000 M_r present in LB-grown *E. coli* C600 was inexplicably strongly reduced in LB-grown KT86 and practically disappeared in *E. coli* C600 grown under derepressed conditions. The results of these experiments suggest that Tel mutants, even when grown in LB, are starved for P_i.

Transport of $^{32}P_i$. Evidence presented above suggests that Tel mutants may have been defective in P_i transport. When the transport of $^{32}P_i$ was measured directly, after P_i starva-

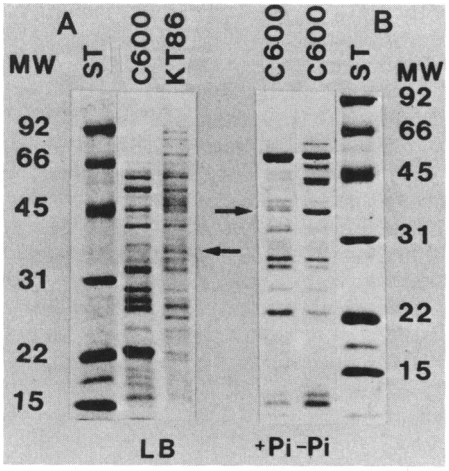

FIG. 1. SDS-PAGE of periplasmic proteins released by osmotic shock (19). (A) MW standards (ST) are indicated in thousands. *E. coli* C600 and KT86 were grown in LB broth. The arrows indicate the 38,000 M_r polypeptide identified as the phosphate periplasmic binding protein (PhoS). (B) *E. coli* C600 was grown under repressed conditions (660 μM P_i [+P_i]) or derepressed conditions (40 μM [−P_i]).

tion for 2 h, KT86 exhibited only 6% of the transport activity of the parent strain (Fig. 3). The incorporation of [U-^{14}C]D-glucose and [^{3}H]proline was not affected in K86 (data not shown), indicating a specific transport defect. This suggests that TeO_3^{2-} enters *E. coli* by one of the two major P_i transport systems (18). This was confirmed in the parent strain C600 by competition assays in which P_i, TeO_3^{2-}, and the known phosphate analog AsO_4^{3-} were compared as unlabeled competitive inhibitors (Fig. 4). TeO_3^{2-} was approximately a 200-fold stronger inhibitor of $^{32}P_i$ uptake than was AsO_4^{3-} and was equally as competitive as P_i (data not shown). Thus, TeO_3^{2-} is a strong competitive inhibitor of P_i transport in *E. coli*, suggesting that it enters the cell via this transport system(s).

Location of the genetic locus for Tel mutants. Evidence

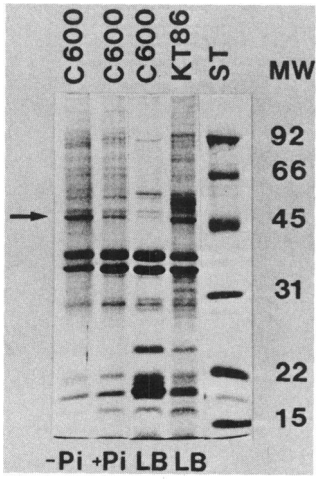

FIG. 2. SDS-PAGE of outer membrane proteins of *E. coli* strains. Outer membranes were prepared by the method of Filip et al. (7). *E. coli* C600 was grown under derepressed conditions (40 μM KH_2PO_4 [P_i]) or repressed conditions (660 μM KH_2PO_4 [+P_i]) or in LB. *E. coli* KT86 was grown in LB broth. MW standards (ST) are indicated in thousands. The arrow indicates the 46,000 M_r band.

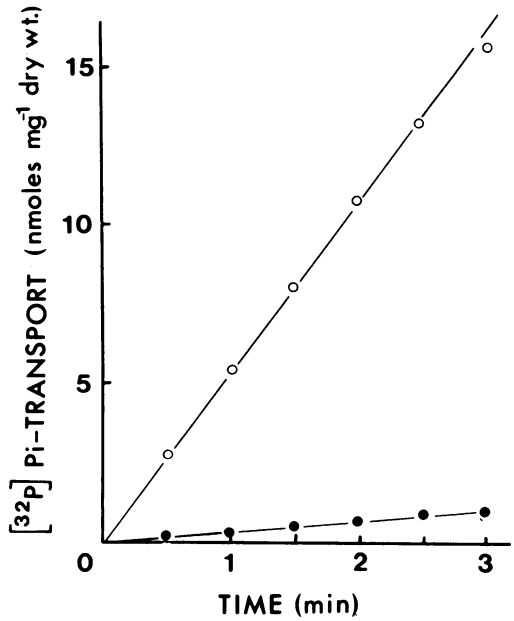

FIG. 3. $^{32}P_i$ transport in wild-type and a Tel mutant of *E. coli*. Cells (0.35 mg [dry weight] ml^{-1}) were starved for P_i for 2 h and then incubated with 0.8 μM $^{32}P_i$ for the times indicated and collected by filtration. Symbols: ○, *E. coli* C600; ●, *E. coli* KT86.

presented above indicates that Tel mutants are possibly defective either in some component or in the synthesis of the P_i transport system of *E. coli*. When a series of well-characterized P_i transport mutants were assayed for TeO_3^{2-} susceptibility (Table 1), only *phoB* mutants showed an MIC

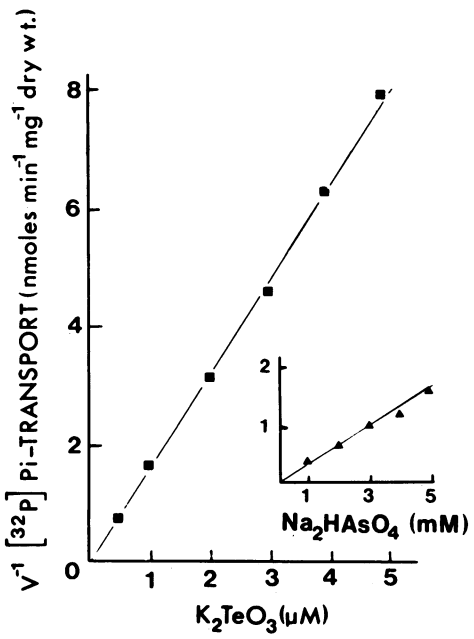

FIG. 4. $^{32}P_i$ transport in wild-type *E. coli*. Symbols: ■, $^{32}P_i$ transport in the presence of different concentrations of K_2TeO_3; ▲, inset, $^{32}P_i$ transport in the presence of different concentrations of Na_2HAsO_4. Note that the rate of $^{32}P_i$ transport is plotted as a reciprocal. Competition for $^{32}P_i$ transport by unlabeled P_i gave a curve that was superimpossible on the TeO_3^{2-} inhibition curve.

TABLE 2. Alkaline phosphatase levels of *E. coli* Tel and mutants

Strain	Relevant phenotype	P_i growth concn (μM)	Sp act (U of enzyme mg of protein^{-1})
		—[a]	
C600		40	0.55
C600		660	<0.002
		—	
KT86	Tel	62	0.47
KT86	Tel	660	0.01
		—	
KT87	Tel	62	0.49
KT87	Tel	660	0.02
LEP-1	PhoB$^-$	62	0.01
LEP-1	PhoB$^-$	660	<0.002

[a] —, limited amount of P_i was required for growth.

for TeO_3^{2-} equivalent to those of strains KT86 and KT87. By conjugating strains ORF4 and KLF251 carrying the episome F'254 (F' *lac*), as a donor for the wild-type *phoB* locus, and strain KT86 (Lac$^-$), as recipient and selecting for Lac$^+$ transconjugants, we obtained strain KT201, a merodiploid for the 6- to 10-min region of the chromosome. The MIC for TeO_3^{2-} of KT201 was found to be reduced to that of *E. coli* C600 (Table 1). After curing the F' from strain KT201 by treatment with acridine orange and selecting for Lac$^-$ colonies, we obtained strain KT202, which was once again resistant to TeO_3^{2-} (Table 1). Thus, both *phoB* and the genetic locus for Tel reside in the *phoR phoB phoA* region covered by F'254. As a confirmation, the transducing phage P1 *cmts* was grown on the donor strain CSH30 (Lac$^+$), and the recipient strain KT86 (Tel^r Lac$^-$) was transduced to Lac$^+$ on lactose minimal plates. Twenty-three percent (46 of 200 colonies) of the Lac$^+$ transductants were also TeO_3^{2-} resistant. Essentially the same frequency of transduction of *phoB* with *lac* was found when the same transduction procedure was used and when strain LEP-1 (*phoB*) was transduced to Lac$^+$ (44 of 200 colonies became susceptible to TeO_3^{2-}). Thus, by the three criteria of equal susceptibility to TeO_3^{2-}, the phosphate transport defect, and a similar genetic location, the *tel* locus was indistinguishable from the *phoB* locus.

Only two known regulatory mutations in this region of the chromosome, either *phoR* or *phoB*, could be candidates for the Tel phenotype. Because the synthesis of alkaline phosphatase is normally constitutive in *phoR* strains and, in contrast, the *phoB* gene product is required for the synthesis of this enzyme (9), we attempted to distinguish these candidates by assaying for the level of alkaline phosphatase in the Tel mutants when grown under both high and low P_i conditions (Table 2). The results indicate that alkaline phosphatase activity is still present in the mutants and can be derepressed to the same degree as that in the parent strain C600. The degree of repression by P_i with the mutants was somewhat less than that with the parent, probably because of the P_i transport defect in these strains. As a control LEP-1, a *phoB* mutant, was largely unable to synthesize alkaline phosphatase even under derepressed growth conditions. Because *phoB* is positively required for *phoA* expression, the Tel mutants could not be simply defective in *phoB*, even though *phoB* mutants were resistant to TeO_3^{2-}.

DISCUSSION

The mechanism of TeO_3^{2-} toxicity and resistance in *E. coli* has not been well understood, yet resistance can be mediated either by a chromosomal mutation or by a plasmid. In either case resistance would presumably be due to the inability to transport TeO_3^{2-}, the acquisition of an efflux mechanism, or the detoxification of the inhibitor (8). Detoxification has been suggested, but not clearly shown, for TeO_3^{2-} resistance plasmids (8, 21).

In the case of the strains studied here, the primary mechanism of resistance in *E. coli* was at the transport level. Tel mutants required higher P_i levels for growth; they exhibited apparent P_i starvation properties (production of *phoS*, *phoE*, and *phoA*, as well as other P_i starvation indicator proteins) under conditions of normally repressible levels of P_i; they were collaterally resistant to both TeO_3^{2-} and AsO_4^{3-}, with the latter being a well-known inhibitor of P_i transport (18); they were unable to transport P_i even under derepressed conditions; and they harbored a defect which mapped in the *phoB-phoR* region at 9 min. Also, because TeO_3^{2-} is an unusually strong competitive inhibitor of the transport of P_i in the parent cells, the conclusion that TeO_3^{2-} enters *E. coli* via a P_i transport system seems inescapable.

The precise locus affected in Tel mutants is not entirely certain; the nature and mechanism of P_i transport and the regulation of these systems are still unclear at present. This lack of clarity stems from an unusual complexity of these systems, especially the *pho* regulon. Results of recent studies (1, 2, 6, 9, 13, 24) have shown that there are several loci involved in the *pho* regulon. Three of these loci (*phoR*, *phoB*, and *phoA*) map in the same general location as the Tel mutants described herein, and they consist of two independent but closely linked loci (*phoB-phoR* and *phoA*). Characterization of the *tel* locus depends on which of two prevalent views of the nature of regulation of the *pho* regulon one embraces. An earlier view (17, 27) presents the *phoB* gene product as a positive regulator of *phoA* (alkaline phosphatase) expression and the *phoR* gene product as an inhibitor of *phoA* expression. From this perspective the *tel* locus cannot be *phoB* because *phoA* was still expressed in Tel mutants; also, the *tel* locus could not be identical to *phoR* because *phoA* expression was not constitutive. Accordingly, the *tel* locus either may be unique or a special class of *phoB* mutations. Another, more recent, view (2, 13, 19, 24) suggests that *phoB* is a positive regulator as well but that *phoR* can act as an autogenous positive or negative regulator of the *phoB-phoR* operon, depending on the intracellular level of P_i. From this perspective the *tel* locus again cannot be simply *phoB*; therefore, it is either a unique *phoR* mutation or perhaps a new locus. We have not sought to further characterize either *phoB* or *phoR* as the locus responsible for the Tel phenotype, primarily because the results would more or less be irrelevant to the major issue here, which is that the P_i transport system is the permeation route for TeO_3^{2-} and that transport deficiency is a means to resistance. It will be of further interest to determine whether plasmid-mediated TeO_3^{2-} resistance has the same molecular basis or whether another mechanism, such as inhibitor efflux, mediates resistance, as recently demonstrated for AsO_4 resistance plasmids (16, 20).

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