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Expression of *Aeromonas caviae* ST pyruvate dehydrogenase complex components mediate tellurite resistance in *Escherichia coli*

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ABSTRACT

Potassium tellurite (K_2TeO_3) is harmful to most organisms and specific mechanisms explaining its toxicity are not well known to date. We previously reported that the *lpdA* gene product of the tellurite-resistant environmental isolate *Aeromonas caviae* ST is involved in the reduction of tellurite to elemental tellurium. In this work, we show that expression of *A. caviae* ST *aceE, aceF*, and *lpdA* genes, encoding pyruvate dehydrogenase, dihydrolipoamide transacetylase, and dihydrolipoamide dehydrogenase, respectively, results in tellurite resistance and decreased levels of tellurite-induced superoxide in *Escherichia coli*. In addition to oxidative damage resulting from tellurite exposure, a metabolic disorder would be simultaneously established in which the pyruvate dehydrogenase complex would represent an intracellular tellurite target. These results allow us to widen our vision regarding the molecular mechanisms involved in bacterial tellurite resistance by correlating tellurite toxicity and key enzymes of aerobic metabolism.

The low-abundance chalcogen element, tellurium (Te), belongs to the 16 (VIA) group of the periodic table and exhibits no toxicity in its elemental state (Te^0). However, its soluble oxyanions tellurite [TeO_3^{2-}, Te(IV)] and tellurate [TeO_4^{2-}, Te(VI)] are highly toxic both for prokaryotes and eukaryotes [1]. Tellurite is by far more toxic than tellurate, especially for Gram negative bacteria, even at concentrations as low as 4 μM [2]. Bacterial sensitivity to tellurium compounds is well documented. In fact, Alexander Fleming wrote about the antibacterial properties of potassium tellurite (K_2TeO_3) as early as 1932 and since then, tellurite has been used routinely as a selective agent in microbiological culture media [3].

TeO_3^{2-} resistance (Tel^R) determinants have been found in plasmids or the chromosome in different bacterial species which exhibit great variability of aminoacid/nucleotide sequences thus hampering the proposition of a general mechanism to explain the tellurite resistance/toxicity phenomenon. Some of the determinants identified so far play a role in diverse metabolic pathways such as *Escherichia coli* nitrate reductases NarG and NarZ, which are able of tellurite reduction and seem to be responsible for the basal resistance of this bacterium to tellurite [4]. Other enzymes exhibiting the ability to reduce tellurite are *Staphylococcus epidermidis* catalase [5] and dihydrolipoamide dehydrogenase from *Aeromonas caviae* and other bacteria [6]. In addition, *Geobacillus stearothermophilus* V *cysK* and *iscS* genes, encoding cysteine synthase and cysteine desulfurase, respectively, have been also shown to mediate tellurite resistance in *E. coli* [7,8]. Similar results were reported for the *Staphylococcus aureus cysM* gene [9].

Tellurite entry to the cell would occur most probably through phosphate carriers [10], although monocarboxylate [11] and iron [12] transporters could also be involved in TeO_3^{2-} entrance into the cell. Once inside the cell, tellurite would be partially reduced by nitrate reductase or other enzymes [4–6]. When tellurite concentration reaches levels high enough to overcome this barrier, important biomolecules are affected. One of the first intracellular tellurite targets to be identified was glutathione (GSH), whose levels significantly decrease upon tellurite exposure, affecting seriously the redox balance of the cell [13]. Later, Lohmeier-Vogel et al. [14] communicated that tellurite exposure compromises the cell metabolism by causing the loss of the transmembrane pH gradient and decreased cytoplasmic ATP levels in *E. coli*. In addition, it has recently been shown that *E. coli* [15] and *Pseudomonas pseudoalcaligenes* KF707 [16] exhibit a noticeable increase of intracellular reactive oxygen species (ROS) when exposed to potassium tellurite, indicating, at least in part, that tellurite exerts its toxic effects through the establishment of an oxidative stress status in the cell.

Regardless of the above considerations, the ultimate mechanism(s) underlying tellurite toxicity is(are) not known to date and it has been repeatedly argued that tellurite toxicity results from its strong oxidant character [2].

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We recently reported that chromatographic fractions containing the pyruvate dehydrogenase (PDH) complex of the tellurite-resistant environmental isolate *A. caviae* ST (hereafter *A. caviae*), in addition to PDH activity, exhibited tellurite reductase (TR) activity. Molecular cloning, expression studies, and purification of each PDH complex component allowed tracing TR activity to the E3 component, dihydrolipoamide dehydrogenase [6]. In this work we show that expressing *A. caviae aceE, aceF*, and *lpdA*, encoding pyruvate dehydrogenase, dihydrolipoamide transacetylase, and dihydrolipoamide dehydrogenase, respectively, mediate tellurite resistance in *E. coli*.

Materials and methods

Bacterial strains and culture conditions. Aeromonas caviae, *E. coli*, and their derivative strains used in this study are shown in Table 1. Cells were grown routinely at 37 °C in LB medium with constant shaking. When required, ampicillin (100 μg/ml), kanamycin (50 μg/ml) or chloramphenicol (25 μg/ml) was added to the medium.

Cloning and expression of A. caviae aceE, aceF, and lpdA genes. Genes encoding *A. caviae* component E1 (*aceE*), E2 (*aceF*), and E3 (*lpdA*) were amplified by PCR as previously described [6]. PCR products were cloned into pBAD TOPO® generating pBAE1, pBAE2, and pBAE3 recombinant plasmids which were subsequently introduced by electroporation into competent *E. coli* cells. Cells were grown in arabinose-amended LB medium to induce the expression of the respective proteins.

Detection of intracellular ROS. (a) H_2DCFDA (dihydro-dichlorofluorescein diacetate): *E. coli* was grown aerobically in arabinose-amended LB medium ($OD_{600} \sim 0.5$), washed with 10 mM phosphate buffer, pH 7.0 (buffer A), and exposed to sublethal tellurite concentrations (0.2 μg/ml) for 15 min. Cells were then incubated for additional 30 min in the presence of 20 μM H_2DCFDA (prepared in dimethyl sulfoxide). After washing twice with buffer A, cells were disrupted by sonication. Cell debris was eliminated by centrifugation for 10 min at 13,000*g* and fluorescence intensity was determined in an Applied Biosystems Citofluor 4000 Fluorescence Multi-well Plate Reader (excitation 410 nm, emission 519 nm) as described previously [15]. Results were normalized per mg of protein and expressed as percent fluorescence. (b) DHE (dihydroethidine): *E. coli* cells were grown as above, washed with 50 mM phosphate buffer, pH 7.4, centrifuged and suspended in the same buffer containing 0.2% glucose. Cells were incubated for 30 min at room temperature in the presence of potassium tellurite (0.2 μg/ml) and 127 μM DHE. Cell-free extracts were prepared and fluorescence intensity was determined (excitation 490 nm, emission 590 nm) as described earlier [17]. Results were normalized per mg of protein and expressed as percent fluorescence.

Determination of β-galactosidase activity. Cultures of *E. coli* carrying pBAD, pBAE1, pBAE2 or pBAE3 were grown in the presence of the inducer arabinose to an $OD_{600} \sim 0.3$ and then exposed to potassium tellurite (0.5 μg/ml) for 3 h at 37 °C with shaking as described previously [25]. OD_{600} was measured and cultures were chilled on ice. Then 100 μl of cell suspension were mixed with 800 μl of 60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, and 50 mM 2-mercaptoethanol, pH 7.0 and cells were permeabilized by adding chloroform and 0.1% SDS. The mix was preincubated for 10 min at 30 °C and after adding the substrate ONPG (*O*-nitrophenyl-galactoside, 0.73 mg/ml final concentration) it was incubated at 25 °C for 20 min. Reactions were halted placing the tubes for 10 min at 110 °C. Activity was expressed as Miller units per mg protein according to the procedure described by Miller [18].

Results

Clones of *E. coli* BW25113 carrying *A. caviae aceE, aceF* or *lpdA* genes cloned into pBAD expression vector were assessed for tellurite resistance by measuring growth inhibition zones in arabinose-amended agar plates. Expression of each *A. caviae* PDH complex component generated a Tel^R phenotype in *E. coli* BW25113. Indeed, each clone individually expressing the structural genes of *A. caviae* PDH complex exhibited reduced growth inhibition zones (~30%) as compared to the parental, isogenic strain, carrying the cloning vector alone (Fig. 1A). Similar results were obtained by determining tellurite minimal inhibitory concentrations (not shown). In turn, *E. coli* lacking *aceE*, *aceF* or *lpdA* genes were more sensitive to tellurite and showed growth inhibition zones ⩾85% than that of the parental, wild type strain. Genetic complementation of these mutants with the respective heterologous genes restored tellurite resistance levels to those of the control strain, again showing that the three genes of the PDH complex participate in K_2TeO_3 resistance (Fig. 1B).

Table 1
Bacterial strains used in this work.

E. coli strain/plasmid	Relevant genotype	Source or reference
BW25113	*lacI*^{q} *rrnB* Δ*lacZ hsdR*514 Δ*araBAD* Δ*rhaBAD*	Nara Institute, Japan
BW25113/pBAD	*E. coli* BW25113 carrying pBAD expression vector Ap^R	This work
BW25113/pBAE1	*E. coli* BW25113 carrying *aceE* from *A. caviae* ST cloned in pBAD (pBAE1) Ap^R	This work
BW25113/pBAE2	*E. coli* BW25113 carrying *aceF* from *A. caviae* ST cloned in pBAD (pBAE2) Ap^R	This work
BW25113/pBAE3	*E. coli* BW25113 carrying *lpdA* from *A. caviae* ST cloned in pBAD (pBAE3) Ap^R	This work
ADA110	*E. coli* AB734 λφ(*ibp::lacZ*) carrying pBAD Ap^R	Shapiro and Baneyx (2002)
ADA110/pBAD	Ap^R	This work
ADA110/pBAE1	Ap^R	This work
ADA110/pBAE2	Ap^R	This work
ADA110/pBAE3	Ap^R	This work
JW0110	*E. coli* BW25113 *aceE::kan*	Baba et al. (2006)
JW0111	*E. coli* BW25113 *aceF::kan*	Baba et al. (2006)
JW0112	*E. coli* BW25113 *lpd::kan*	Baba et al. (2006)
JW0110/pBAE1	Ap^RKan^R	This work
JW0111/pBAE2	Ap^RKan^R	This work
JW0112/pBAE3	Ap^RKan^R	This work
QC774	*E. coli* DE(*lac*)4169 *rpsL* DE(*sodA-lacZ*)49 DE(*sodB-kan*)1-DE(2) Cm^RKan^R	Nara Institute, Japan
QC774/pBAD	$Cm^RKan^RAp^R$	This work
QC774/pBAE1	$Cm^RKan^RAp^R$	This work
QC774/pBAE2	$Cm^RKan^RAp^R$	This work
QC774/pBAE3	$Cm^RKan^RAp^R$	This work

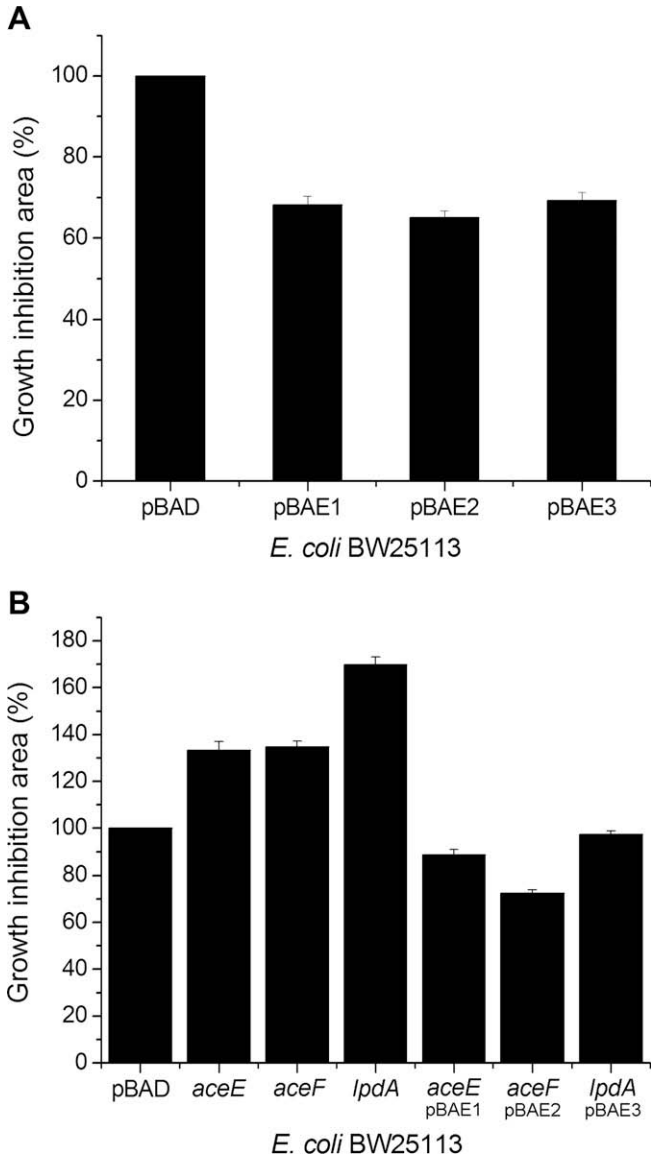

Fig. 1. Genes from the PDH complex of *A. caviae* mediate tellurite resistance in *E. coli*. (A) Growth inhibition of *E. coli* carrying cloned *A. caviae aceE* (pBAE1), *aceF* (pBAE2), and *lpdA* (pBAE3) genes in the presence of potassium tellurite. (B) Growth inhibition of *E. coli* lacking the indicated genes before and after being complemented with the heterologous *A. caviae* genes. Sterile filter disks containing 10 μg of K_2TeO_3 were placed in the centers of LB-ampicillin plates and cells were grown overnight at 37 °C. In both cases, the growth inhibition area of the control, parental strain carrying the expression vector pBAD alone (7.1 cm^2) was taken as 100%. Bars represent the standard error ($n = 5$).

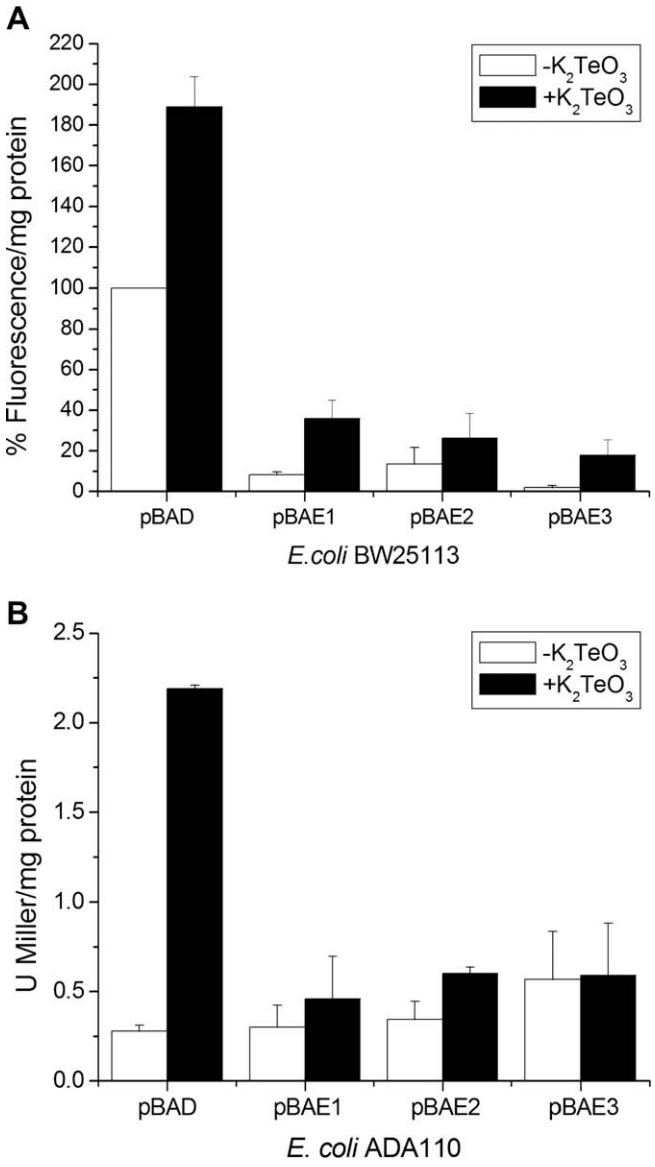

Fig. 2. Expression of *A. caviae aceE*, *aceF*, and *lpdA* results in decreased ROS generation in *E. coli*. (A) Levels of tellurite-induced ROS were monitored by assessing the activation of the probe H_2DCFDA in *E. coli* strains carrying the referred *A. caviae* genes after exposure to tellurite (0.2 μg/ml) for 30 min at 37 °C. Fluorescence was determined and normalized per mg of protein (100% was the fluorescence intensity of H_2DCFDA observed in extracts from the unexposed, parental, isogenic strain). Bars represent the standard error ($n = 5$). (B) β-galactosidase activity in *E. coli* ADA110 expressing pBAE1, pBAE2, and pBAE3 in the presence and absence of K_2TeO_3 (0.5 μg/ml) was determined as described in Methods. Bars represent the mean of four independent trials ±SE.

On the other hand, *E. coli* cells expressing *A. caviae aceE*, *aceF*, and *lpdA* genes did not exhibit increased intracellular ROS upon tellurite exposure. Conversely, a strong decrease in fluorescence emission was observed in all cases as compared with untreated controls, suggesting that expression of *A. caviae* genes results in a protective effect against these oxidant oxygen derivatives (Fig. 2A). This protective effect against ROS was observed even in untreated cells. In fact, cells expressing *aceE*, *aceF*, and *lpdA* but not exposed to tellurite showed fluorescence levels representing less than 15% of those exhibited by untreated controls (Fig. 2A, white bars).

Further evidence that expression of *A. caviae aceE*, *aceF*, and *lpdA* in *E. coli* results in decreased ROS levels was obtained using *E. coli* ADA110 [19], a reporter strain containing a transcriptional fusion of the *lacZ* gene to the *ibpA* gene promoter. IbpA is a chaperon that responds to cytoplasmic stress [20] and to superoxide-induced oxidative stress [21]. Fig. 2B shows that *E. coli* ADA110 carrying pBAE1, pBAE2 or pBAE3 exhibit decreased β-galactosidase activity upon tellurite exposure as compared to control cells carrying pBAD alone, confirming that tellurite treatment generates lower ROS levels in cells expressing the *A. caviae* genes.

Since superoxide is the leading ROS generated upon tellurite exposure in *E. coli* [5,15], we decided to look at the expression of *A. caviae aceE*, *aceF*, and *lpdA* in the oxidative stress-sensitive, SOD-deficient, *E. coli sodAB* in the presence of both tellurite and the superoxide-specific probe dihydroethidine. Fig. 3 shows that intracellular superoxide levels are notoriously lower in cells expressing the *A. caviae* genes as compared to the control strain, even in the absence of tellurite (Fig. 3, white bars). These results

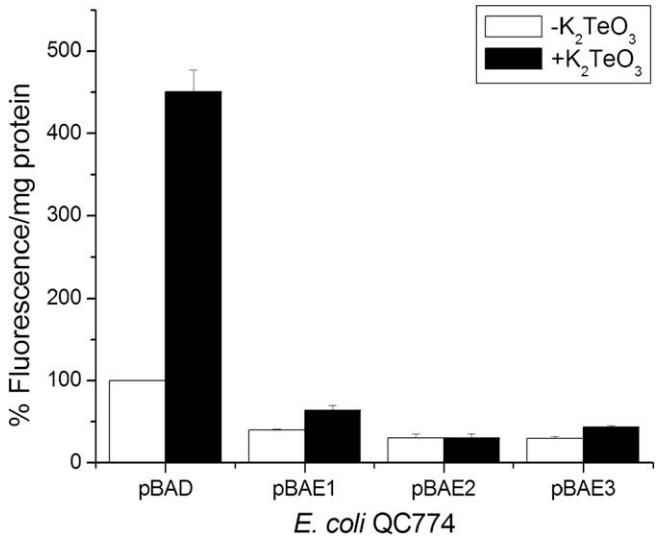

Fig. 3. Expression of *A. caviae aceE*, *aceF*, and *lpdA* results in decreased tellurite-induced superoxide in *E. coli sodAB*. Superoxide levels were assessed following the activation of dihydroethidine in tellurite-stressed *E. coli sodAB* expressing the indicated genes after being exposed to potassium tellurite (0.2 μg/ml) for 30 min. Fluorescence was determined and normalized per mg of protein (100% was the fluorescence intensity of dihydroethidine observed in extracts from the unexposed, parental, isogenic QC774/pBAD strain). Bars represent the mean of four independent experiments ±SE.

reinforce the protective effect of the *A. caviae* gene products when *E. coli* grows in the presence of the toxic tellurium oxyanion.

Discussion

Several studies on bacterial tellurite resistance have suggested that it would consist of multifactor phenomena in which different metabolic pathways and/or their substrates or products would play a role as resistance-like determinants [4,7,9,15,22–25].

In this work we have shown that expression of the structural genes of the *A. caviae* PDH complex (*aceE*, *aceF*, *lpdA*) resulted in a tellurite resistance phenotype in *E. coli*, as evidenced by lower growth inhibition zones (Fig. 1). To determine if the observed tellurite resistance was related to cytoplasmic ROS content, the superoxide-sensitive fluorescent probe dihydroethidine was used. Observed fluorescence levels in *E. coli* strains carrying pBAE1, pBAE2, and pBAE3 were substantially lower than those of the control strain, even in the absence of tellurite, suggesting that *aceE, aceF*, and *lpdA* expression effectively protects *E. coli* from tellurite-induced oxidative stress (Fig. 2A). Decreased intracellular ROS levels as a consequence of *A. caviae aceE*, *aceF*, and *lpdA* expression were also observed in tellurite-exposed *E. coli* ADA110 (Fig. 2B). In addition, decreased superoxide levels observed in *A. caviae aceE*-, *aceF*-, and *lpdA*-expressing *E. coli sodAB* confirmed the above observations (Fig. 3). Expression of the referred *A. caviae* genes also protects *E. coli* from hydrogen peroxide stress (our unpublished observations).

In general terms, during aerobic metabolism, the PDH complex catalyzes the formation of acetyl-CoA which allows the functioning of the Krebs cycle. In turn, most of the NADH required to make ATP is made in the cycle. Tellurite resistance observed in *E. coli* as consequence of heterologous gene expression could be explained considering some studies demonstrating that *E. coli* exposure to oxidative elicitors results in selective damage to proteins that include pyruvate dehydrogenase (AceE) and dihydrolipoamide transacetylase (AceF) [26]. Similar results have been observed in yeast, along with damaged Krebs cycle enzymes such as aconitase and the α-ketoglutarate dehydrogenase complex [27]. In this context, increased E1 and E2 content resulting from the expression of *A. caviae aceE* and *aceF* in *E. coli* would increase ROS targets that could avoid damage to other macromolecules as proteins and/or membrane lipids. Since lipoic acid, forming part of dihydrolipoamide transacetylase, can scavenge free radicals [28], AceF acting as antioxidant *per se* may represent an alternative explanation for increased tellurite resistance in *aceF*-expressing cells.

On the other hand, no pyruvate dehydrogenase activity has been detected in *E. coli lpd* mutants, causing cytoplasmic pyruvate to accumulate. At the same time, acetyl-CoA levels were barely detectable and low levels of Krebs cycle activity were observed [29]. Then *lpd* cells would be unable to generate appropriate NADH and/or ATP levels to face tellurite stress. In this context, resistance augments observed in *lpdA*-expressing cells could be due to tellurite reduction by E3, which would diminish intracellular tellurite concentration thus decreasing tellurite-generated superoxide. Dihydrolipoamide dehydrogenase shows tellurite reductase activity as part of the PDH complex itself or as free protein [6], requiring in both situations NADH as the electron donor. If tellurite concentrations overcome certain levels, this enzyme and other proteins having TR activity would be sidetracked to tellurite reduction diverting them from their main physiological roles which would have important consequences for the cell's metabolism. For instance, the PDH complex would synthesize lower amounts of acetyl-CoA which would in turn restrict functioning of the Krebs cycle. This would avoid the generation of enough NADH required for both tellurite reduction and the electron transport chain. In turn this would result in a decreased proton transmembrane gradient that would explain the decreased ATP synthesis observed in *E. coli* by Lithgow et al. [9].

Summarizing, the tellurite resistance phenotype observed upon expression of *A. caviae aceE*, *aceF*, and *lpdA* in *E. coli* would be a consequence of the three proteins being tellurite targets which might help in protecting the cell machinery from being excessively exposed to tellurite. Experiments to gain further insight on tellurite toxicity are under way in our laboratory.

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