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The dihydrolipoamide dehydrogenase of *Aeromonas caviae* ST exhibits NADH-dependent tellurite reductase activity

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ABSTRACT

Potassium tellurite (K_2TeO_3) is extremely toxic for most forms of life and only a limited number of organisms are naturally resistant to the toxic effects of this compound. Crude extracts prepared from the environmental isolate *Aeromonas caviae* ST catalize the *in vitro* reduction of TeO_3^{2-} in a NADH-dependent reaction. Upon fractionation by ionic exchange column chromatography three major polypeptides identified as the E1, E2, and E3 components of the pyruvate dehydrogenase (PDH) complex were identified in fractions exhibiting tellurite-reducing activity. Tellurite reductase and pyruvate dehydrogenase activities co-eluted from a Sephadex gel filtration column. To determine which component(s) of the PDH complex has tellurite reductase activity, the *A. caviae* ST structural genes encoding for E1 (*aceE*), E2 (*aceF*), and E3 (*lpdA*) were independently cloned and expressed in *Escherichia coli* and their gene products purified. Results indicated that tellurite reductase activity lies almost exclusively in the E3 component, dihydrolipoamide dehydrogenase. The E3 component of the PDH complex from *E. coli*, *Zymomonas mobilis*, *Streptococcus pneumoniae*, and *Geobacillus stearothermophilus* also showed NADH-dependent tellurite reductase *in vitro* suggesting that this enzymatic activity is widely distributed among microorganisms.

Tellurium is a metalloid belonging to group VIA of the periodic table of elements that shares several chemical characteristics with biologically important elements such as oxygen, sulfur, and selenium. Its elemental form (Te°) is rather scarce in nature and direct experimental evidence concerning tellurium biogeocycling is not available to date. The soluble tellurium oxyanions, tellurites (TeO_3^{2-}) and tellurates (TeO_4^{2-}) are highly toxic for most organisms [1,2]. Tellurite is more toxic than tellurate, especially for Gram-negative bacteria which are susceptible at very low TeO_3^{2-} concentrations (~1 μg/ml or 4 μM) [3].

Some bacteria are naturally resistant to K_2TeO_3 and observations that a number of species exhibit resistance 2000-fold higher than that determined for *E. coli* have been reported [4]. Tellurium salts are routinely used in microbiological procedures to select and isolate specific bacteria such as *Corynebacterium diphtheriae, Streptococcus faecalis*, *Staphylococcus aureus*, *Vibrio cholerae*, and *Shigella spp.* [5].

In general, most tellurite-resistant bacteria develop black colonies when grown in tellurite-amended solid media. The same phenotype is also observed when tellurite-sensitive cells are grown in solid media containing sub lethal tellurite concentrations [3]. Similar observations have been reported in yeast [6]. The chemical nature of the black granules that accumulates inside the cells is elemental tellurium and results from the reduction of Te^{4+} to Te° [7]. In most cases the granules are located near the internal leaflet of the plasma membrane [7–9].

Enzyme activities that reduce tellurite are generically known as tellurite reductases (TR) and have been described in different microorganisms including *Mycobacterium avium* [10], *E. coli* [11], and *Rhodobacter sphaeroides* [12]. Crude extracts derived from these bacteria reduce tellurite using NAD(P)H or $FADH_2$ as electron donors. Partial characterization of a NAD(P)H-dependent tellurite-reducing activity was reported in *Thermus thermophilus* HB8 where it is associated with polypeptides of 53 and 54 kDa [13,14]. Similar results were reported for *Geobacillus stearothermophilus* V where the enzymatic activity was associated with polypeptides of 37.5, 41, and 60 kDa [15].

On the other hand, side activities of some metabolic enzymes such as the *E. coli* nitrate reductases NarG and NarZ are responsible for tellurite reduction, an activity that seems to mediate bacterial basal resistance to K_2TeO_3 [16]. Terminal oxidases of the electron transport chain seem to be involved in tellurite reduction [17]. More recently, it has been shown that catalases from tellurite-resistant *Staphylococcus epidermidis* and bovine pancreas are proficient in reducing tellurite [18].

Searching for new bacterial models for tellurite resistance we recently isolated a naturally tellurite-resistant *A. caviae* ST strain from environmental water samples. The genus *Aeromonas* includes Gram-negative rods that are usually found in aquatic ecosystems

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causing infections to fish. In humans, the bacterium causes diarrhea which is usually due to ingestion of contaminated water. *A. caviae* ST cells grown in tellurite-amended solid media at a minimal inhibitory concentration of 350 μg/ml develop the black colonies that are indicative of tellurite reduction.

Here, we report that the pyruvate dehydrogenase (PDH) complex of *A. caviae* ST, and in particular its E3 component, is involved in the reduction of potassium tellurite. Cells exposed to tellurite exhibit decreased pyruvate dehydrogenase activity suggesting that the complex may represent a novel tellurite intracellular target.

Materials and methods

Bacterial strains and culture conditions. Aeromonas caviae ST and *E. coli* were grown in LB medium at 37 °C with constant shaking. Ampicillin (100 μg/ml) was added to the medium when required.

Purification of tellurite reductase activity. A. caviae ST cells (5 g wet weight) were suspended in 10 ml of 50 mM potassium phosphate buffer pH 6.4 (buffer A) and subjected to sonic disruption. After centrifugation at 10,000*g* for 10 min at 4 °C the supernatant was recovered and streptomycin sulfate at a final concentration of 2% was added. After 30 min incubation on ice, nucleic acids were eliminated by centrifugation at 10,000*g* for 10 min. The supernatant was dialyzed at 4 °C with several changes of the same buffer. This crude extract (10 ml) was loaded onto a phosphocellulose Whatman P11 column (10 × 2.5 cm) that was then washed with several column volumes of buffer A. Bound proteins were eluted with a linear 0–2 M NaCl gradient prepared in the same buffer. Fractions of 1 ml were collected and aliquots of 50 μl were assayed for tellurite reductase at 37 °C for 10 min in 500 μl of a solution containing 20 mM potassium phosphate buffer, pH 7.0, 1 mM K_2TeO_3, 1 mM NADH, and 1 mM 2-mercaptoethanol (TR buffer). Reduction of tellurite was monitored at 500 nm. One unit was defined as the amount of enzyme causing an increase of 0.001 U of OD_{500} min^{-1} ml^{-1}.

Crude extracts were subjected to non-denaturing polyacrylamide (12%) gel electrophoresis (PAGE) to detect tellurite reductase activity *in situ*. The gel containing the fractionated proteins was soaked in TR buffer and incubated at 37 °C for 1 h to identify the black deposits that are indicative of TR activity.

To pinpoint the proteins associated with tellurite reduction, aliquots from active enzymatic fractions were subjected to SDS–PAGE and visualized by staining with Coomassie brilliant blue R250. Protein identification included amino terminal sequencing and amino acid analysis using gel slides. These analysis were performed at Unidad de Espectrometría de Masas, Facultad de Ciencias Químicas y Farmacéuticas, Universidad de Chile, Santiago, Chile.

Pyruvate dehydrogenase activity was determined spectrophotometrically according to the protocol of Brown and Perham [19]. Briefly, reaction mix (500 μl) contained extract and PDH buffer (90 mM Tris–HCl, pH 8.0, 1 mM $MgCl_2$, 2 mM pyruvate, 0.5 mM NAD^+, 0.2 mM coenzymeA, and 0.4 mM thiamine pyrophosphate). NADH generation was monitored at 340 nm. Results were normalized by protein concentration and expressed as % of PDH activity. Specific TR (or PDH) activity determined in crude extracts of cells not exposed to tellurite was considered as 100%. Protein concentration was determined as described by Bradford [20] using bovine serum albumin as standard.

Cloning and expression of A. caviae ST aceE, aceF, and lpdA genes and purification of their gene products. Genes encoding for components E1 (*aceE*), E2 (*aceF*), and E3 (*lpdA*) of the PDH complex were amplified by PCR in 50 μl of a solution containing 10 mM Tris–HCl, pH 8.3, 0.2 mM of each deoxynucleoside triphosphate, 2.5 mM $MgCl_2$, forward and reverse primers (2.5 pmol each), 2–5 ng of *A. caviae* ST genomic DNA and 0.1 U of Taq polymerase (Roche). An initial denaturation step of 3 min at 95 °C was followed by 30 cycles (95 °C for 45 s, 61 °C for 90 s, and 72 °C for 90 s) and a final incubation at 72 °C for 10 min to ensure complete extension of fragments. Primers were designed using the genome sequence published for *Aeromonas hydrophila* ATCC7966 (GenBank Accession No. CP000462) and they are *aceE*(for) CAC CAA TGT CTG ATA TCC TGA AGA ACG A, aceE(rev) CGC GTA CAG CGG GTT GAC, *aceF*(for) CAC CAA TGT CCA AAC AGA TTA TGG TGC, *aceF*(rev) CAG GAC CAG TCG ACG GAT GT, *lpdA*(for) CAC CAA TGA GTA AAG AAA TCA AAA CCC A, and *lpdA*(rev) CTT CTT CTT CTT GGC CTT GG. PCR products were cloned in the expression vector pET101/D TOPO (Invitrogen) and the resulting recombinant plasmids, named pETAE1, pETAE2, and pETAE3, were transformed into *E. coli* BL21(DE3) competent cells.

Escherichia coli cells harboring pETAE1, pETAE2, or pETAE3 were used to purify components E1, E2, and E3, respectively. Cells were grown at 37 C until $OD_{600} \sim 0.6$ and IPTG at 1 mM was added. After 4 h of vigorous shaking, the cells were sedimented, sonicated and the PDH components purified by affinity chromatography on His-Trap™HP (Amersham) columns according to the vendor instructions. A similar experimental approach was followed to purify the E3 component of *G. stearothermophilus* (GenBank Accession No. CAA37631), *Zymomonas mobilis* (GenBank Accession No. CAA63810), and *Streptococcus pneumoniae* (GenBank Accession No. AAK72472) using *E. coli* BL21(DE3) cells carrying plasmids pBSTNAV/E3 [21], pQE709 [22], and pAPH001 [23], respectively. *The* E3 component of *E. coli* (GenBank Accession No. ACA79157) was purified from strain ASKA clone (−) JW0112, a generous gift of the Nara Institute, Japan [24].

Results

Crude extracts of *A. caviae* ST catalyzed the NADH-dependent tellurite reduction as determined by increase of OD_{500} as previously described [14,15]. Tellurite reductase activity was also demonstrated *in situ* after fractionation of *A. caviae* ST crude extracts by native PAGE followed by soaking of the gels in TR buffer (Fig. S1). Tellurite reductase activity was prevented when the extract was heated at 95 °C for 5 min or treated with proteases or SDS, indicating the enzymatic nature of this activity.

Aeromonas caviae ST crude extracts were subjected to purification using a phosphocellulose column and fractions assayed for TR and PDH activity. Fig. 1A shows that both activities co-eluted from the column. Three polypeptides of 43, 58, and 97 kDa were detected when aliquots of active fractions were resolved by SDS–PAGE (Fig. 1B). Amino terminal sequence analysis and bioinformatics were performed to identify the three polypeptides. The best match revealed that they correspond to the E1 (97 kDa), E2 (58 kDa) and E3 (43 kDa) components of the *A. hydrophila* pyruvate dehydrogenase complex.

Aeromonas caviae ST cells were grown with increasing K_2TeO_3 concentrations and cell-free extracts were prepared and assayed for TR and PDH activities. While the TR activity increased with higher tellurite concentration, a ~40% decrease of the PDH activity was observed at the highest tellurite concentration analyzed (Fig. 2).

To unequivocally determine which component(s) of the PDH complex is responsible for the tellurite reductase activity, the *A. caviae* E1, E2, and E3 polypeptides were purified and assayed for TR activity. Only the component 3, dihydrolipoamide dehydrogenase, displayed TR activity *in vitro* (Fig. 3).

To establish whether the E3 component from the PDH complex of other microorganisms is also capable of reducing tellurite, the dihydrolipoamide dehydrogenase genes from *Z. mobilis*, *S. pneumoniae*, *G. stearothermophilus*, and *E. coli* were expressed in *E. coli*

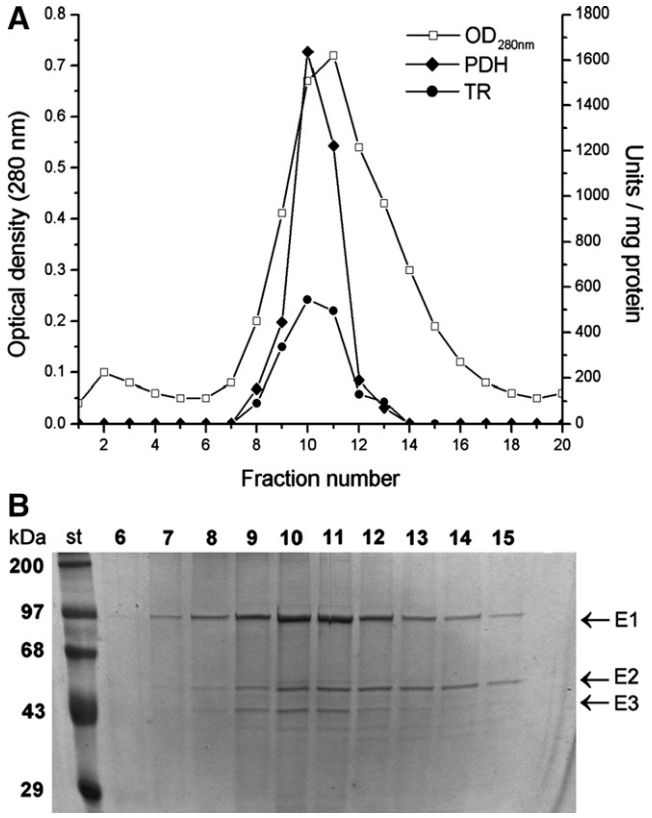

Fig. 1. Purification of tellurite reductase from *A. caviae* ST. The enzymatic activity was purified as described in Materials and methods. (A) Phosphocellulose P11 column chromatography. (B) SDS–PAGE of column fractions indicated at the top. ♦, pyruvate dehydrogenase activity; •, tellurite reductase activity.

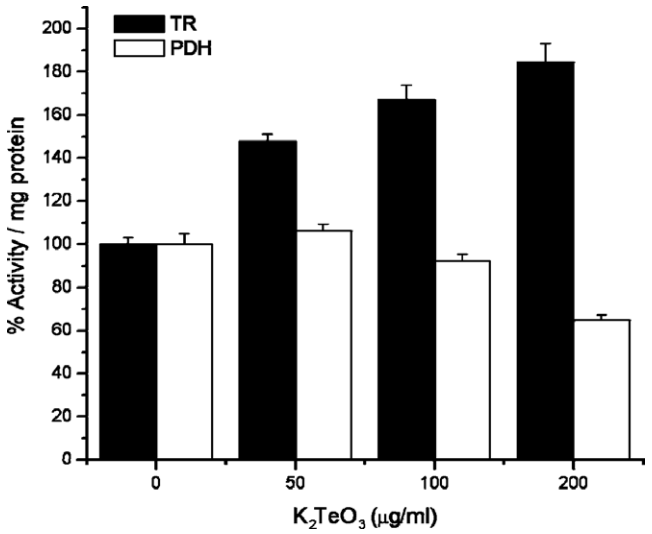

Fig. 2. Tellurite reductase and pyruvate dehydrogenase activities in cell-free extracts of *A. caviae* ST. Cells were grown in LB media with the indicated potassium tellurite concentrations. Cell-free extracts were prepared and assayed for tellurite reductase (TR) and pyruvate dehydrogenase (PDH) as described in Materials and methods. Bars represent standard deviation ($n = 5$).

BL21(DE3). The overexpressed E3 products were purified by His-Trap™HP affinity chromatography and assayed for TR activity (Fig. S2). Results showed (Fig. 4) that the dihydrolipoamide dehydrogenase from the four microorganisms analyzed exhibited an *in vitro* tellurite reductase activity.

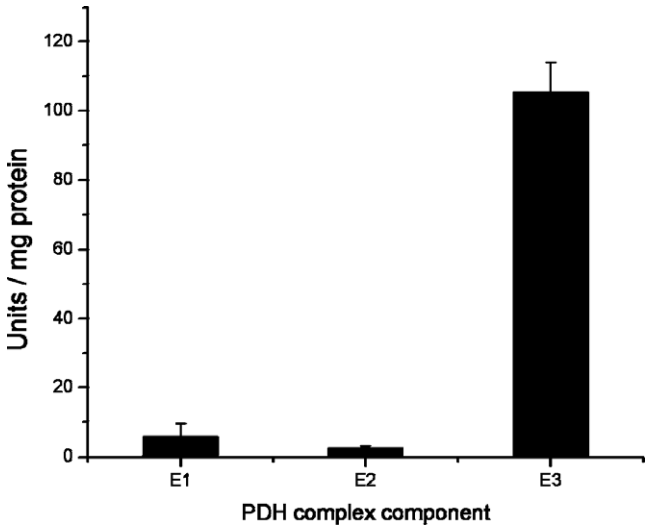

Fig. 3. Tellurite reductase activity present in the E1, E2, and E3 components of the *A. caviae* ST PDH complex. Enzyme activity was determined at 500 nm as described in Materials and methods. Bars represent standard deviation ($n = 5$).

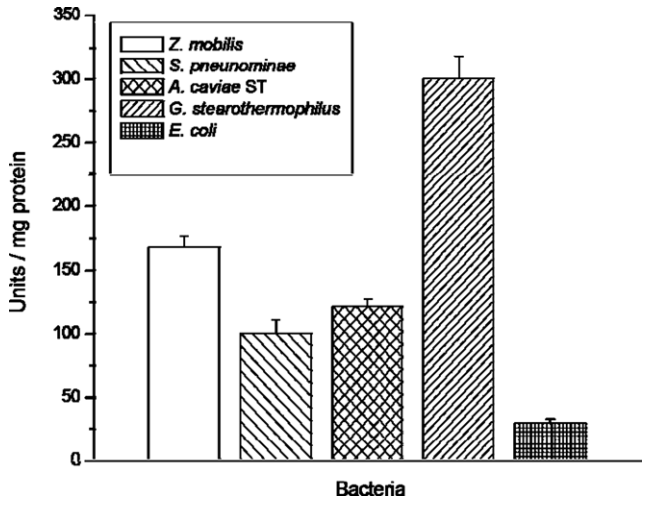

Fig. 4. Tellurite reductase activity of the E3 component of the PDH complex of *Z. mobilis*, *S. pneumoniae*, *A. caviae* ST, *G. stearothermophilus*, and *E. coli*. Activity was determined as described in Materials and methods. Bars represent standard deviation ($n = 5$).

Discussion

The *A. caviae* ST environmental isolate studied here was highly resistant to K_2TeO_3 and crude extracts prepared from this microorganism were capable of reducing tellurite *in vitro*. The cells of most tellurite-resistant microorganisms turn black when grown in tellurite-amended media [7–9,14,25]. *A. caviae* ST displays the same phenotype suggesting that in these cells the reducing activity also occurs *in vivo*.

The NADH-dependent tellurite reductase activity dropped approximately 90% upon heating or proteinase K treatment reflecting the enzymatic nature of this reaction. The TR activity was associated with three main polypeptides that were identified as components of the PDH complex. Fractions containing these polypeptides also exhibited pyruvate dehydrogenase activity (Fig. 1). Cloning, expression and purification of each of the three polypeptides of the PDH complex component showed that only the dihydrolipoamide dehydrogenase protein catalyzed reduction of tellurite *in vitro* (Fig. 3).

The PDH complex catalyzes the irreversible decarboxylation of pyruvate to yield acetyl-CoA thus coupling the glycolytic and

tricarboxylic acid cycle pathways [26]. Consequently, any damage affecting this multienzyme complex would be reflected in vital metabolic processes like ATP synthesis, among others. In this context, *A. caviae* ST extracts prepared from bacterial cells grown in tellurite-amended media had decreased levels of PDH activity suggesting that this complex may represent an intracellular target of the tellurite molecule (Fig. 2). This toxic compound could cause modifications in the normal functioning of the PDH complex that would result in diminished acetyl-CoA production thus limiting the availability of this substrate for Krebs cycle activity. Preliminary qRT-PCR experiments in our laboratory showed that the levels of *ace*E, *ace*F, and *lpd*A transcripts does not significantly change upon tellurite exposure (Vásquez, unpublished data) suggesting that the increased E3-mediated tellurite reduction is probably a function of pre-existing (available) E3 and not due to *de novo* synthesis of this molecule.

The mechanism of E3-mediated tellurite reduction is unknown. It is not clear whether K_2TeO_3 destabilizes the PDH complex as a whole or specifically inhibits a particular component(s) of the complex. The NADH dependence of the tellurite-reducing activity would suggest that this cofactor acts as an electron donor in a fashion similar to the role performed by E3 when catalyzing the reversible reduction of lipoamide.

Because the dihydrolipoamide dehydrogenase is also an enzyme of the α-ketoglutarate dehydrogenase complex [26], tellurite reduction might also affect the normal function of this complex that may represent another intracellular target of the toxic salt.

Escherichia coli cells exposed to K_2TeO_3 show loss of the transmembrane proton gradient and a concomitant decrease of ATP levels without affecting the intracellular concentration of glycolytic intermediates [27] suggesting that component(s) of the electron transport chain could also be targets of tellurite.

Our results suggest that the NADH-dependent tellurite reduction mediated by E3 may decrease acetyl-CoA synthesis which in turn could affect the efficiency of the tricarboxylic acid cycle pathway. The decreased levels of intracellular NADH observed under conditions of telluride exposure would be consistent with the lower ATP levels detected in cells grown in tellurite-amended media [27]. These observations would also help to understand, at least in part, why potassium tellurite is so toxic for most organisms.

The precise molecular mechanisms used by cells to cope with tellurite are not quite well understood. It has been recently demonstrated that tellurite exposure leads to increased concentrations of intracellular oxygen reactive species and hence to the establishment of an oxidative stress status [28,29]. This condition would be concomitant with the dismantling of the PDH complex which is one of the most important complexes of the aerobic metabolism. The toxic effects of tellurite could then be associated with a metabolic stress condition with the Krebs cycle being one of the main targets.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2008.07.119.

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