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Escherichia coli 6-phosphogluconate dehydrogenase aids in tellurite resistance by reducing the toxicant in a NADPH-dependent manner

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Exposure to the tellurium oxyanion tellurite ($TeO_3{}^{2-}$) results in the establishment of an oxidative stress status in most microorganisms. Usually, bacteria growing in the presence of the toxicant turn black because of the reduction of tellurite (Te^{4+}) to the less-toxic elemental tellurium (Te^0). *In vitro*, at least part of tellurite reduction occurs enzymatically in a nicotinamide dinucleotide-dependent reaction. In this work, we show that $TeO_3{}^{2-}$ reduction by crude extracts of *Escherichia coli* overexpressing the *zwf* gene (encoding glucose-6-phosphate dehydrogenase) takes place preferentially in the presence of NADPH instead of NADH. The enzyme responsible for toxicant reduction was identified as 6-phosphogluconate dehydrogenase (Gnd). The *gnd* gene showed a subtle induction at short times after toxicant exposure while strains lacking *gnd* were more susceptible to the toxicant. These results suggest that both NADPH-generating enzymes from the pentose phosphate shunt may be involved in tellurite detoxification and resistance in *E. coli*.

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1. Introduction

The tellurium oxyanion tellurite ($TeO_3{}^{2-}$) is harmful mainly for prokaryotic cells. $TeO_3{}^{2-}$ toxicity is mediated in part by the generation of reactive oxygen species (ROS), particularly superoxide (Borsetti et al., 2005; Pérez et al., 2007; Tremaroli et al., 2007; Chasteen et al., 2009; Sandoval et al., 2010). In general, tellurite-exposed *Escherichia coli* show inactivation of oxidative stress-sensitive [Fe-S] cluster-containing enzymes, increased protein carbonylation and lipid membrane oxidation along with activation of superoxide-response genes (Pérez et al., 2007; Calderón et al., 2009). In addition, tellurite causes thiol depletion, especially glutathione (GSH), which, in turn, results in an oxidative stress status (Turner et al., 1999, 2001).

In response to superoxide *E. coli* triggers a coordinated expression of almost 119 genes (*soxRS* regulon) whose biological roles include three different response levels: (i) prevention of oxidative damage, (ii) xenobiotic removal and recycling of damaged macromolecules and (iii) nicotinamide adenine dinucleotide phosphate (NADPH) regeneration (Farr and Kogoma, 1991; Gaudú et al., 1997; Pomposiello et al., 2001; Blanchard et al., 2007).

Intracellular NADPH levels are critical for maintaining a balanced redox status and therefore for survival (Ying, 2008). It has been shown that oxidative stress evokes a metabolic adaptation favoring increased NADPH synthesis, most probably due to augmented activity and expression of glucose-6-phosphate dehydrogenase (G6PDH) (Bériault et al., 2007; Singh et al., 2007; Sandoval et al., 2011).

Previous work from our laboratory has shown that NADPH metabolism may be affected in cells exposed to tellurite. It has been observed that this toxicant can be enzymatically reduced to elemental tellurium (Te^0) in a cofactor-dependent manner in different microorganisms (Chiong et al., 1988; Moscoso et al., 1998; Calderón et al., 2006; Castro et al., 2008; Pugin et al., 2014). NADPH levels can also be affected by non-enzymatic tellurite reduction by glutathione (GSH) or other cell reducing agents (Turner et al., 1999, 2001). On the other hand, NADPH biosynthesis is enhanced as an antioxidant cell response via glucose-6-phosphate dehydrogenase (Sandoval et al., 2011).

Here we report a new NADPH-mediated tellurite reductase (TR) activity that was identified as 6-phosphogluconate dehydrogenase (Gnd). These results suggest that like other metabolic pathways,

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Table 1
Bacterial strains and primers.

E. coli strain	Relevant genotype	Source or reference
BW25113	[a], Δ*(araD-araB)567*, Δ*lacZ4787*(::rrnB-3)	Baba et al. (2006)
pBAD	BW25113 harboring pBAD	Sandoval et al. (2011)
pBAD-*zwf*	BW25113 harboring pBAD-*zwf*	Sandoval et al. (2011)
JW2011	AG1 harboring pCA24N-*gnd* plasmid	Kitagawa et al. (2005)
Δ*gnd*	BW25113 Δ*gnd* (*gnd*::*kan*)	NARA Institute, Japan
AG1	[a], *rec*A1 *end*A1 *gyr*A96 *thi*-1 *hsd*R17(r_K^- m_K^+) *sup*E44 *rel*A1	NARA Institute, Japan

[a] Parental strain.

the pentose phosphate shunt could be involved in tellurite detoxification as well as in the *E. coli* resistance to this toxicant.

2. Materials and methods

2.1. Bacterial strains, plasmids, growth conditions and toxicant treatment

E. coli strains and plasmids used in this study are listed in Table 1. Bacteria were routinely grown in LB medium or glucose-amended M9 minimal medium (Sambrook and Russell, 2001) at 37 °C with vigorous shaking to OD_{600} ~0.5. When required, ampicillin (100 μg ml^{-1}) or chloramphenicol (25 μg ml^{-1}) was added to the medium. Unless otherwise stated, K_2TeO_3 was used at concentrations that ranged 0–2.0 μM. Gene induction in recombinant cells was carried out in the presence of 1 mM IPTG.

Construction of growth curves and minimal inhibitory concentration (MIC) and growth inhibition zones determination were carried out as described previously (Fuentes et al., 2007; Castro et al., 2008, 2009; Pugin et al., 2014).

2.2. Protein purification

E. coli JW2011 carrying plasmid pCA24N-*gnd*-His_6 (NARA Institute, Japan) (Kitagawa et al., 2005) was used to purify Gnd. Cells were grown to OD_{600} ~0.5 and induced with IPTG for 5 h. Cells were suspended in 20 mM sodium phosphate buffer, pH 7.4, 0.5 M NaCl and 20 mM imidazole (buffer A) and disrupted by sonication. After eliminating the cell debris by centrifugation, the crude extract was loaded onto a affinity column (HisTrap HP, GE Healthcare®). After washing exhaustively with buffer A, adsorbed proteins were eluted as recommended by the vendor. Purified Gnd was >95% pure as judged by denaturing polyacrylamide gel electrophoresis (SDS-PAGE).

2.3. Enzyme activity

Cells from 10 ml cultures were disrupted by sonication and extracts cleared by centrifugation. Aliquots of cell-free extracts were assayed for glucose-6-phosphate dehydrogenase (Lushchak et al., 2001), 6-phosphogluconate dehydrogenase (de Silva and Fraenkel, 1979) and tellurite reductase (Chiong et al., 1988; Castro et al., 2008). Protein concentration was determined as described previously (Bradford, 1976) using bovine serum albumin as a standard.

2.4. In situ characterization of TR activity

Crude extracts (100 μg protein) or purified Gnd protein (30–50 μg), were subjected to non-denaturing polyacrylamide (12%) gel electrophoresis to detect *in situ* tellurite reductase activity. After the run, the gel was soaked in reduction buffer (RB) that contained 50 mM potassium phosphate buffer, pH 7.0, 1 mM K_2TeO_3, 1 mM 2-mercaptoethanol and 1 mM NADPH or NADH and incubated at 37 °C for 1 h to identify the black deposits that are indicative of TR activity (Castro et al., 2008).

2.5. Identification of the TR protein

To define the protein(s) associated with tellurite reduction, the black band revealed by native-PAGE was subjected to SDS-PAGE and visualized by Coomassie brilliant blue (R250) staining. Protein identification was carried out by mass spectrometry at Proteomics Resource Center, The Rockefeller University, New York, USA.

2.6. Relative gene expression

The transcriptional level of 6-phosphogluconate dehydrogenase (*gnd*), SoxS DNA-binding transcriptional dual regulator (*soxS*) and hydroperoxidase I (*katG*) genes was assessed as described earlier (Arenas et al., 2010). Their relative expression in *E. coli* BW25113 exposed to tellurite (0.5 μg ml^{-1}) during 5, 10 and 15 min was calculated regarding that of the housekeeping *rpoD* (Sigma 70 factor) and *gapA* (glyceraldehyde 3-phosphate dehydrogenase) genes. Total RNA was prepared using the Favorprep tissue total RNA purification mini kit (Favorgene) and quantified using the Quant-it Ribogreen Kit (Invitrogen). Real time RT-PCR experiments were performed with a LightCycler® RNA Amplification Kit SYBR Green I (Roche Applied Science) using approximately 100 ng of RNA as template. Transcript levels were calculated using the ΔΔCt method. Primers used to amplify the *gnd* gene were 5′-CAGTTCCTGCAGAAAATCACC-3′ (forward) and 5′-ATCCAGCCATTCGGTATGGA-3′ (reverse).

2.7. Data analysis

In general, results were expressed as the mean ± the standard deviation. Differences between experimental groups were analyzed using one-way ANOVA using the GraphPad Prism 5.03 software (GraphPad Software, Inc.). *P*-values less than 0.05 were considered statistically significant.

3. Results

We have previously communicated that an antioxidant response consisting of the activation of enzyme activities involved in tellurite reduction is triggered when bacterial cells are exposed to the toxicant. These activities, that include catalase, dihydrolipoamide dehydrogenase, isocitrate dehydrogenase and other enzymes displaying a branch NAD(P)H-dependent tellurite-reducing activity, are generically known as tellurite reductases (Chiong et al., 1988; Moscoso et al., 1998; Calderón et al., 2006; Castro et al., 2008; Reinoso et al., 2013; Pugin et al., 2014).

3.1. Cofactor preference of TR activity

Since upon *E. coli* exposure to tellurite the NADH/NADPH ratio is modified (Sandoval et al., 2011), it is probable that this situation affects the preference of the reducing activity for the reduced dinucleotide cofactor. Total TR activity was assessed by monitoring the formation of Te^0 at 500 nm (Chiong et al., 1988; Molina et al., 2010). Dialyzed, crude *E. coli* cell-free extracts were used as the enzyme source in the presence of increasing concentrations of either NADH or NADPH (Fig. 1). NADPH clearly proved to be better substrate for TR activity. In fact, 2–3 fold more reductase activity was observed with 0.4–1.0 mM NADPH in regard to NADH (Fig. 1).

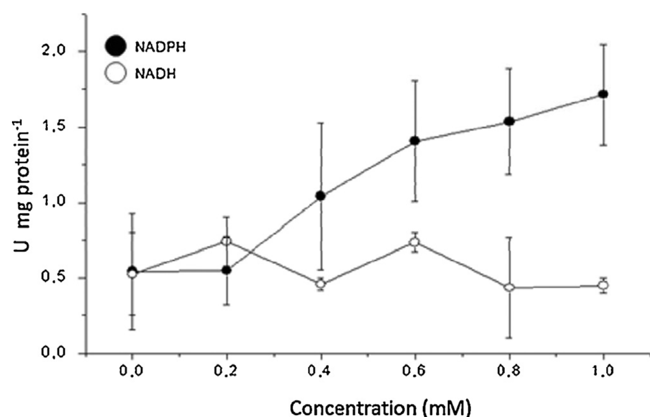


Fig. 1. Assessment of tellurite reductase activity. TR activity (U mg protein^{-1}) was determined in cell-free extracts of wild type *E. coli* BW25113 in the presence of the indicated dinucleotide concentrations as described in Methods. Values represent the average of 3 independent trials ± SD.

Table 2
NADPH-dependent TR activity increases upon *zwf* expression.

Strain Plasmid	Plasmid	TR activity (U mg protein^{-1})	
		NADH	NADPH
BW25113	pBAD	1.1 ± 0.6	2.7 ± 0.6*
	pBAD-*zwf*	1.5 ± 0.2	5.1 ± 0.9*

Enzyme activity was determined as described in Methods. Data represent the mean of 3 independent trials ±SD.
* $P \leq 0.05$.

One of the main sources of NADPH synthesis in tellurite-exposed *E. coli* is glucose-6-phosphate dehydrogenase, whose activity increases in response to the oxidative stress status triggered by the toxicant (Sandoval et al., 2011). When the *zwf* gene (1476 bp) was overexpressed in *E. coli* both TR activity and the NADPH content increased by a factor of 2 and ~35%, respectively. While TR activity in the presence of NADH was almost unchanged, NADPH-dependent reductase increased ~2 fold regarding cells carrying pBAD alone (Table 2). In addition, Fig. 2A shows that TR activity, as revealed after native polyacrylamide gel electrophoresis, is almost observed only with NADPH as the dinucleotide cofactor.

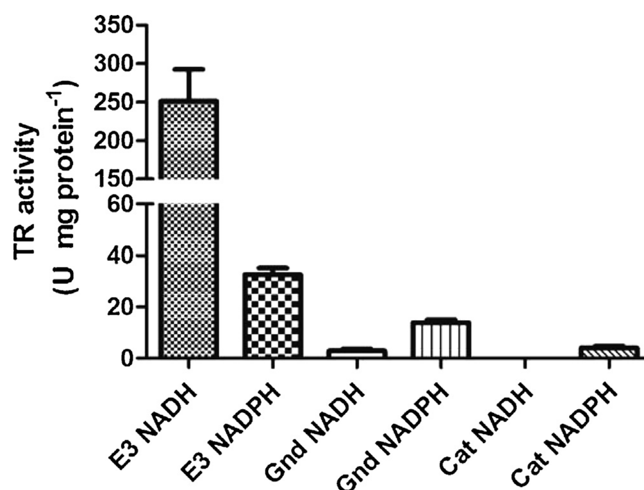


Fig. 3. Tellurite reductase activity. Purified *E. coli* dihydrolipoamide dehydrogenase (E3) and Gnd were assessed for TR activity as determined in Methods. Bovine catalase (Cat) was used as a positive control. Tellurite reduction was determined by monitoring the increase of OD_{500} and TR activity was expressed as U mg^{-1} protein^{-1}. Bars represent the standard deviation ($n = 3$).

3.2. Identification of the reducing activity

The band showing *in situ* TR activity (Fig. 2A, left) was excised and subjected to SDS-PAGE, where a major polypeptide band of ~50–55 kDa was observed (Fig. 2B). This band was excised from the denaturing gel and sent for mass spectrometry sequencing (not shown). The results revealed that two proteins could probably be responsible for TR activity: 6-phosphogluconate dehydrogenase and adenylsuccinate synthetase (PurA). Given that only Gnd exhibits the structural Rossmann-fold NAD(P)H-binding domain (-$GX_{1-2}GXXG$-) usually found in oxidoreductases (Rao and Rossmann, 1973; Pease and Wolf, 1994; Kleiger and Eisenberg, 2002), we decided to proceed further only with this protein. In addition, Gnd activity increased almost five fold in crude extracts of *E. coli* exposed to the superoxide generators tellurite or menadione.

E. coli Gnd was purified almost to homogeneity by affinity chromatography as described in Methods (Fig. 2C). The specific dehydrogenase activity under saturated conditions (20 mM 6-phosphogluconate) was 237,775 ± 29 U/mg protein while TR

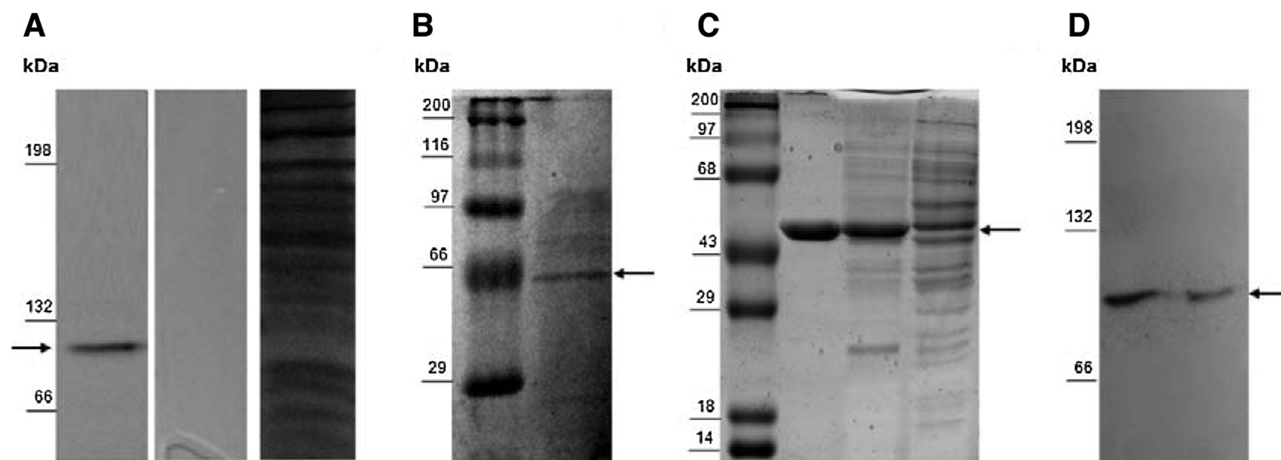


Fig. 2. *In situ* tellurite reductase activity. (A) Crude extracts from untreated *E. coli* BW25113 were fractionated by native PAGE (12%) at 4 °C for 16 h and revealed using either 1 mM NADPH (left) or 1 mM NADH (center) as described in Methods. The right panel represents a gel strip stained with Coomasie blue. (B) NADPH-dependent TR activity band obtained in (A) was excised, fractioned by SDS-PAGE and stained with Coomasie blue. (C) SDS-PAGE and Coomasie blue staining of purified Gnd-His_6 (left), crude extracts of induced and uninduced *E. coli* JW2011 (center and right panel, respectively). (D) NADPH-dependent TR activity visualized after native PAGE as described in (A): *E. coli* BW25113 crude extracts (left) and purified Gnd-His_6 (right). The markers used were high and low range prestained proteins (Gibco BRL #26041-020 and 16040-016, respectively).

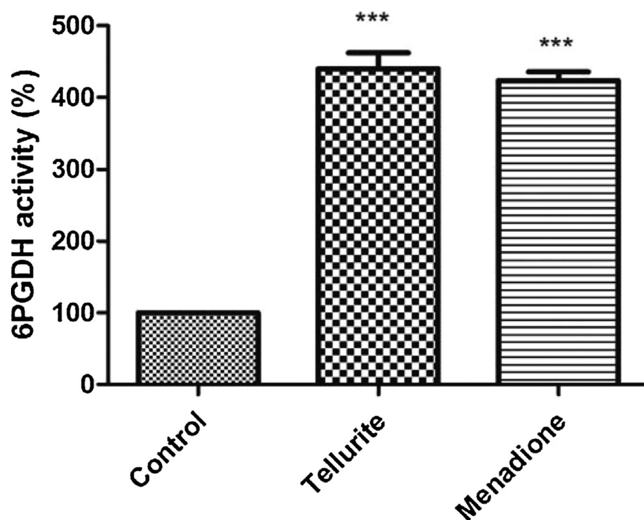


Fig. 4. Determination of Gnd activity. Enzymatic activity was assessed *in vitro* using crude protein extracts from cells previously exposed to tellurite (5 μg ml^{-1}) or menadione (2.5 mM). The reaction mix contained 50 mM Tris-HCl buffer, pH 7.4, 5 mM $MgSO_4$, 0.2 mM $NADP^+$ and the extract (5 μg protein) in a final volume of 1 ml. Incubations were at 20 °C for 2 min and the A_{340} was recorded.

activity was 13.9 ± 1.0 U/mg protein in the presence of 1 mM NADPH. Gnd's TR activity was also assessed by native gel electrophoresis (Fig. 2D, right lane) and compared with that exhibited by other known TR activity-displaying enzymes. Fig. 3 shows that TR activity exhibited by Gnd was higher than that of catalase and much lower than that of dihydrolipoamide dehydrogenase. Interestingly, increased Gnd activity was observed in protein extracts following tellurite- or menadione treatment (Fig. 4).

Table 3
Relative expression of the *gnd* gene in tellurite-exposed *E. coli*.

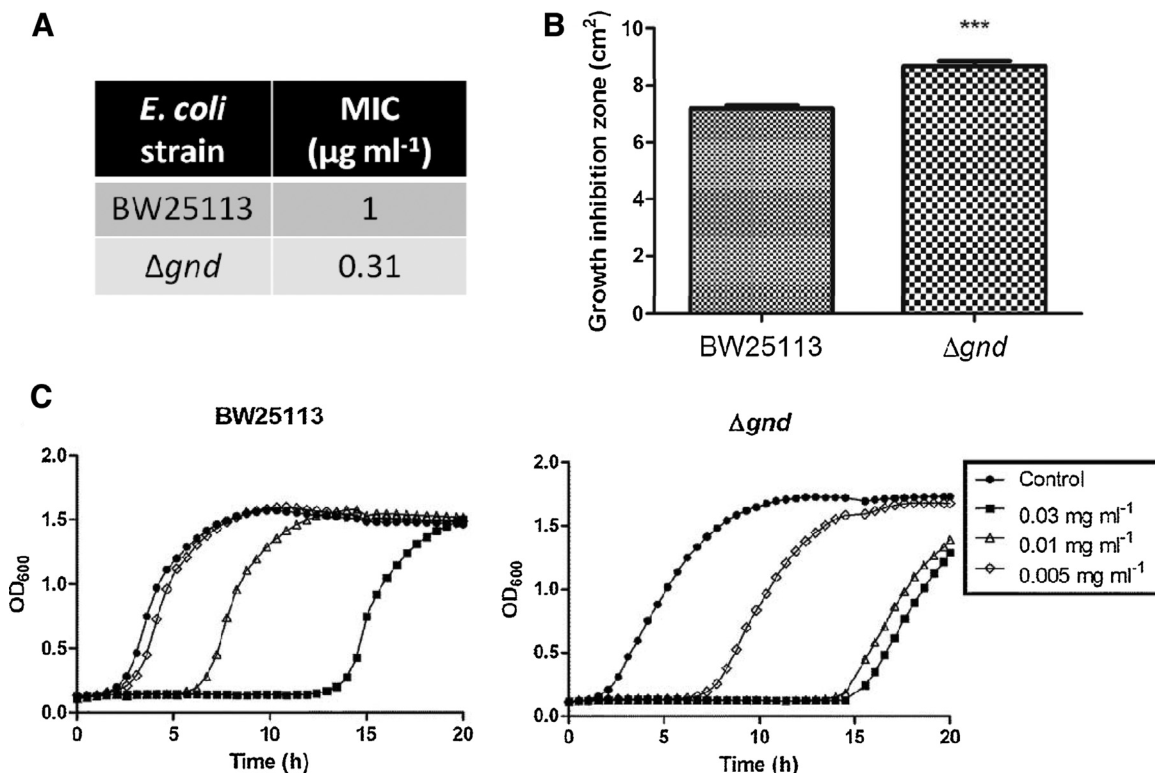
Gene	Relative expression (fold)		
	5 min	10 min	15 min
Gnd	1.5	3.8	1.9
soxS	3.6	>100	6.6
katG	0.8	33.4	51.6

3.3. The Δ*gnd* strain is more sensitive to tellurite than wild type *E. coli*

Next, we assessed the impact of Gnd in tellurite resistance. It was observed that cells lacking the *gnd* gene are more sensitive to the toxicant. Tellurite MIC decreased from 1 μg ml^{-1} (wild type) to 0.31 μg ml^{-1} (Δ*gnd*) (Fig. 5A), a result that correlated well with growth inhibition zones (Fig. 5B). In regard to the wild type control, Δ*gnd* cells showed slower growth at all tellurite concentrations tested (Fig. 5C). Finally, it was observed that at short times of toxicant exposure (i.e., 10 min) *gnd* relative expression showed a subtle induction (3.8 fold) regarding untreated controls (Table 3).

4. Discussion

Despite some progress made to unveil the biochemical mechanism of enzymatic tellurite reduction (Arenas et al., 2014), it is still not completely understood. We previously proposed that the NAD(P)H-dependent tellurite reduction to elemental tellurium by catalase required molecular oxygen with anion superoxide as one of the reaction products (Calderón et al., 2006). Superoxide then triggers an initial signal that enables the cell to induce the expression of several defense mechanisms to counteract the harmful effects of tellurite (Blanchard et al., 2007). In this context, increased expression of *sodA* and *sodB* (Pérez et al., 2007), *yqhD*



E. coli strain	MIC (μg ml^{-1})
BW25113	1
Δ*gnd*	0.31

Fig. 5. Gnd involvement in tellurite resistance. (A) Minimal inhibitory concentrations; (B) growth inhibition zones; (C) growth curves of *E. coli* wild type and Δ*gnd* cells in the presence on the indicated tellurite concentrations (inset).

(encoding a NADP+-dependent aldehyde dehydrogenase) (Pérez et al., 2008) and *btuE* (encoding a glutathione peroxidase) (Arenas et al., 2010) has been observed in tellurite-exposed *E. coli*.

In addition, *E. coli* also responds to tellurite exposure by increasing the G6PDH-dependent synthesis of the powerful antioxidant NADPH (Sandoval et al., 2011). This supplies the redox equivalents required for reducing tellurite to its less toxic, elemental form. Actually, increasing NADPH and decreasing NADH production is a major metabolic bacterial adaptation in response to oxidative stress (Bériault et al. 2007; Singh et al. 2007; Sandoval et al., 2011).

E. coli crude extracts reduced tellurite better with NADPH than with NADH as the electron donor (Fig. 1). The characterization of potential protein(s) responsible for this activity resulted in the identification of Gnd (Fig. 2). This kind of NAD(P)H-dependent tellurite reductase activity has been reported previously in thermophilic bacteria (Chiong et al., 1988; Moscoso et al., 1998), *Staphylococcus epidermidis* and bovine catalases (Calderón et al., 2006) and several purified dihydrolipoamide dehydrogenases (Castro et al., 2008). Interestingly, these last activities are >95% inhibited by tellurite in *E. coli* crude extracts (Calderón et al., 2006; Castro et al., 2008; Reinoso et al., 2012) thus contributing to the previously reported 40% decrease of NADH levels in the presence of the toxicant (Sandoval et al., 2011).

Like G6PDH, Gnd participates in the oxidative pentose phosphate pathway and also catalyzes NADPH synthesis (Nasoff et al., 1984; Pease and Wolf, 1994). In addition to this cofactor-synthesizing activity, no additional function or genetic regulation has been communicated for Gnd (Baker and Wolf, 1984). From a metabolic point of view, the increased carbon flux towards the *E. coli* pentose phosphate shunt resulting from tellurite exposure (Sandoval et al., 2011) becomes a suitable explanation for this pathway being involved in tellurite detoxification. This assumption matches the current pattern of activation of metabolic modules associated with the cell antioxidant response in various organisms (Mailloux et al., 2007, 2009; Singh et al., 2008; Lemire et al., 2010). Although Gnd exhibits a relatively low TR activity regarding dihydrolipoamide dehydrogenase (Fig. 3), the observation that increased Gnd activity in protein extracts following tellurite- or menadione treatment supports the previous statement (Fig. 4). The fact that *gnd* is induced at short times after exposure to the toxicant reinforces this interpretation (Table 3).

The lower growth and increased sensitivity to tellurite observed in *E. coli* Δ*gnd* cells also point out to the involvement of Gnd in tellurite resistance (Fig. 5). Probably Gnd becomes directly involved in toxicant detoxification by reducing it shortly after toxicant exposure.

Upregulation of *katG* and *soxS* genes has been reported previously in tellurite-exposed *E. coli*, which occurs most probably due to the establishment of an oxidative stress status (Pérez et al., 2007). In addition, the expression of other genes shown to be involved in TeO_3^{2-} reduction such as *ndh* (encoding NDH-II dehydrogenase) (Díaz-Vásquez et al., 2014) and *lpdA* (encoding lipoamide dehydrogenase) (Reinoso et al., 2012) is upregulated upon toxicant exposure in *E. coli*. Further work to better characterize the global cell response to tellurite is being carried out in our laboratory.

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