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# Effect of tellurite-mediated oxidative stress on the *Escherichia coli* glycolytic pathway

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**Abstract** To unveil the metabolic impact of tellurite in the bacterial cell, the effect of this toxicant on the expression and activity of key enzymes of the *Escherichia coli* glycolytic pathway was analyzed. *E. coli* exposure to tellurite results in: (i) increased glucose consumption, which was paralleled by an increased expression of the glucose transporter-encoding gene *ptsG*, (ii) augmented phosphoglucoisomerase activity and *pgi* transcription, (iii) decreased activity of the enzymatic regulators phosphofructokinase and pyruvate kinase. In spite of these observations, increased intracellular pyruvate, phosphoenol pyruvate and phosphorylated sugars was observed. *E. coli* lacking key glycolytic enzymes was considerably more sensitive to tellurite than the parental, isogenic, wild type strain. Taken together, these results suggest that increasing the availability of key metabolites (pyruvate, phosphoenol pyruvate, NADPH), required to respond to tellurite mediated-stress, *E. coli* shifts the carbon flux towards the pentose phosphate pathway thus facilitating the functioning of the Entner–Doudoroff pathway and/or the glycolytic productive phase.

**Keywords** Tellurite · Glycolysis · Oxidative stress · Pyruvate kinase · Phosphofructokinase

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## Introduction

Glycolysis is a metabolic pathway in which glucose is oxidized to generate ATP, NADH and pyruvate. The route is essential in *Escherichia coli* since six important precursors involved in biosynthesizing macromolecules are generated: D-glucose-6-phosphate (G6P), D-fructose-6-phosphate (F6P), dihydroxyacetone phosphate, 3-phospho-D-glycerate, phosphoenol pyruvate (PEP) and pyruvate (PYR) (Nelson and Cox 2008). Although glucose is the starting molecule of this cytoplasmatic metabolic route in most organisms, *E. coli* and other bacteria have developed a different mechanism by which glucose enters the cytoplasm. The PEP-dependent phosphotransferase system (PTS) allows glucose uptake from the periplasm which is phosphorylated during transport (Bouma et al. 1987; Postma et al. 1993). Given the continuous utilization of glycolytic precursors, most reactions of the pathway are reversible thus allowing the cell to satisfy precursor demands. Exceptions are phosphofructokinase

[PFK, converts F6P into fructose-1,6-bisphosphate (FBP)] and pyruvate kinase (PK, synthesize PYR from PEP) which constitute the pathway check points (Nelson and Cox 2008).

In general, injuries to this pathway can result in severe damage to the cell. Studies carried out with different organisms have shown that glycolysis is affected by several metal ions (Strydom et al. 2006). In this context, while cadmium, zinc and cooper decrease PFK activity (Almeida et al. 2001; Gebhard et al. 2001), zinc inhibits glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Strydom et al. 2006). In addition, PK activity decreases drastically in cells exposed to high manganese concentrations (Malthankar et al. 2004).

On the other hand, while FBP and glyceraldehyde-3-phosphate (G3P) levels drop in the presence of arsenite, those of PEP and PYR increase (Reichl et al. 1988). Other interesting observation refers to the effect of paraquat, a superoxide generator that decreases the expression of several glycolytic genes (Mostertz et al. 2004).

A powerful toxicant for most microorganisms, especially Gram negative bacteria, is the tellurium (Te) oxyanion tellurite ($TeO_3^{2-}$) (Taylor 1999). It is about 100–1,000 fold more toxic than Se, Cr, Cd, Pb or Cu salts (Nies 1999). Te can be incorporated instead of Se in amino acids thus altering the function of proteins (Garberg et al. 1999) while $TeO_3^{2-}$ oxidizes cellular thiols as glutathione (Turner et al. 1999, 2001). Nevertheless, the ultimate mechanisms underlying tellurite toxicity are not fully understood to date. Results from our laboratory and from others have shown that tellurite [Te (IV)] also generate damage as consequence of its reduction to $Te^0$, a process in which reactive oxygen species (ROS), mainly superoxide, are produced (Calderón et al. 2006, 2009; Pérez et al. 2007, 2008; Tremaroli et al. 2007). In turn, superoxide damages proteins (Stadtman 1992; Imlay 2003) and cell membranes (Pérez et al. 2007; Storz and Imlay 1999).

On the other hand, it has been shown that the [4Fe-4S] cluster-containing enzymes aconitase and fumarase are tellurite targets (Calderón et al. 2009). In addition, while the activity of the pyruvate dehydrogenase multienzyme complex decreases in the presence of $TeO_3^{2-}$ (Contreras and Vásquez 2010), that of glucose-6-phosphate dehydrogenase (G6PDH) increases, thus allowing the recovery of NADPH that may have been consumed during tellurite reduction (Sandoval et al. 2011).

Aiming to assess tellurite effects on the bacterial metabolism, we focused in studying the impact of this toxicant on the *E. coli* glycolytic pathway. In particular, tellurite effects on PFK and PK activities, metabolite concentration and changes in gene expression were analyzed.

## Materials and methods

### Bacteria and culture conditions

*E. coli* strains used in this work are listed in Table 1. Bacteria were routinely grown in M9 minimal medium (Sambrook et al. 1989) amended with 0.2 mM glucose at 37°C with shaking. Cultures were started by inoculating 1:100 dilutions of overnight cultures into fresh media. When required, kanamycin (50 μg/ml) was added to the medium.

**Table 1** *Escherichia coli* strains used in this study

| Strain | Relevant characteristics | Source or references |
|---|---|---|
| BW25113 | *lacI*$^q$ *rrnB* Δ*lacZ hsdR*514Δ*ara*BAD Δ*rhaBAD* | Datsenko and Wanner (2000) |
| JW3985 | BW25113 *pgi*::*kan*[a] | |
| JW3887 | BW25113 *pfkA*::*kan* | |
| JW5280 | BW25113 *pfkB*::*kan* | Baba et al. (2006) |
| JW1666 | BW25113 *pykF*::*kan* | |
| JW1843 | BW25113 *pykA*::*kan* | |
| JW1087 | BW25113 *ptsG*::*kan* | |
| GC4468 | wild-type *soxRS*, Δ*lac*U169, *rpsL* | Imlay (2003) |
| DJ901 | GC4468 *soxRS*::*kan* | Privalle et al. (1993) |

[a] *pgi*, *pfkA*, *pfkB*, *pykF*, *pykA* and *ptsG* encode PGI, PFKI, PFKII, PKI, PKII and PtsG, respectively

### Determination of growth inhibition zones and minimal inhibitory concentrations

Cells grown to $OD_{600}$ ~0.3 were diluted 1:2 with fresh medium and plated on M9-glucose plates. After air drying, sterile filter paper disks (6 mm) were placed at the centers of the plates. 10 µl of potassium tellurite ($K_2TeO_3$, 40 mM) or hydrogen peroxide ($H_2O_2$, 10 M) were deposited on the disks and plates were incubated overnight at 37°C.

MICs were determined by inoculating 10 µl of exponentially growing cultures ($OD_{600}$ ~0.3) in 100 µl of M9-glucose medium in 96-well culture plates. Serial 1:2 dilutions of $K_2TeO_3$ or $H_2O_2$ (initial concentrations 80 µM and 10 mM, respectively) were applied to the wells and plates were incubated for 16 h at 37°C.

### Quantifying extracellular glucose

Extracellular glucose was assessed according to Dubois et al. (1956). Briefly, cells were grown to $OD_{600}$ ~0.3, treated for 30 min with 20 µM $K_2TeO_3$ or 1 mM $H_2O_2$ and centrifuged for 5 min at 8,000 × *g*. The sediment was kept to determine protein concentration (Bradford 1976) and the supernatant was diluted 1:10 with glucose-less M9 medium. After mixing with concentrated sulfuric acid and phenol (5%), the mix was heated for 5 min at 80°C. After chilling, absorbance was registered at 490 nm.

### Quantifying PYR, PEP and total phosphorylated sugars (TPS) by HPLC

Cultures grown to $OD_{600}$ ~0.3 were split in two identical aliquots. While the control received no treatment, the other was exposed for 30 min to 20 µM $K_2TeO_3$. Cells were sedimented, suspended in 220 µl of $H_2O$, sonicated and centrifuged for 15 min at 13,000 × *g*. After mixing with 1 volume of 1 M perchloric acid and heating for 10 min at 95°C, the mix was chilled on ice, neutralized with 25 µl of 10 M NaOH and centrifuged as above. After filtering through 0.45 µm sieves, the supernatant was fractionated using an Aminex HPX-87C (BioRad, 250 × 4 mm) column in a Shimadzu apparatus equipped with a model SIL-20A autosampler. Mobile phase was 4 mM $H_2SO_4$ containing 0.1% (v/v) acetonitrile and the flux was set at 0.5 ml/min. PEP and PYR were detected at 220 nm, while TPS were monitored using a refractive index detector.

## Enzyme assays

### PTS transporter

Cells grown to $OD_{600}$ ~0.3 and treated with 20 µM $K_2TeO_3$ or 1 mM $H_2O_2$ for 30 min were used to determine total PTS activity as described by Rungrassamee et al. (2008). The activity of this transporter was determined using an assay coupled to lactate dehydrogenase (LDH), as described by Kornberg and Reeves (1972). One enzyme unit was defined as the amount of enzyme that oxidizes 1 µmol of NADH per min at 37°C.

### PK, PFK, PGI and aldolase

Cells grown to $OD_{600}$ ~0.3 and treated as above were centrifuged for 5 min at 4°C and washed with 0.1 M potassium phosphate pH 7.4 buffer. Cells were suspended in 300 µl of the same buffer containing 1% phenylmethanesulfonyl fluoride (PMSF) and disrupted by sonication. The cell debris was discarded by centrifugation and the supernatant dialyzed for 2 h against 50 mM Tris–HCl pH 7.4 buffer prior to assaying the enzymatic activity. In general, reactions were started with the protein extract.

PGI activity was determined according to Fraenkel and Levisohn (1967). The mix contained 50 mM Tris–HCl pH 7.4 buffer, 10 mM $MgCl_2$, 0.2 mM $NADP^+$, 0.4 mM F6P and 0.5 U of G6PDH. Enzyme activity was registered as the change in $OD_{340}$ at 37°C. One enzyme unit was defined as the amount of enzyme that reduces 1 µmol of $NADP^+$ per min at 37°C.

PFK was assayed as described by Hansen and Schönheit (2000), with the exception that GAPDH was used instead of glycerol phosphate dehydrogenase. The mix contained 25 mM Tris–HCl pH 7.0 buffer, 10 mM $MgCl_2$, 0.2 mM $NAD^+$, 0.5 mM F6P, 0.6 mM ATP, 0.04 U of aldolase and 0.25 U of GADPH. PFK activity was monitored as the change in $OD_{340}$ at 37°C. One enzyme unit was defined as the amount of enzyme that reduces 1 µmol of $NAD^+$ per min at 37°C.

Aldolase activity was measured as described by Jagannathan et al. (1956). The reaction mix contained

3.5 mM hydrazine sulfate and 0.1 mM EDTA. Enzyme activity was registered as the change in $OD_{240}$ at 37°C. One enzyme unit was defined as the amount of enzyme that oxidizes 1 μmol of hydrazine per min at 37°C.

PK activity was determined using an LDH-coupled assay to measure NADH oxidation at 340 nm at 37°C. The reaction mix contained 50 mM imidazole–HCl pH 7.6 buffer, 120 mM KCl, 10 mM $MgSO_4$, 0.2 mM NADH, 5.5 mM PEP, 1.5 mM ADP and 0.5 U of LDH. One enzyme unit was defined as the amount of enzyme consuming 1 μmol of NADH per min at 37°C.

### Flow cytometry

The oxidation-sensitive probe 2′,7′-dihydrodichlorofluorescein diacetate ($H_2$DCFDA, 10 mM final concentration) was added to cells growing in M9-glucose medium. When the cultures reached $OD_{600}$ ~0.3, $K_2TeO_3$ or $H_2O_2$ was added at defined concentrations representing 0.25, 0.5, 1.0 and 2.0 times the respective minimal inhibitory concentrations (10, 20, 40 and 80 μM for tellurite and 1.25, 2.5, 5.0 and 10.0 mM for hydrogen peroxide) and incubation was for another 30 min. Cells were sedimented and suspended with 0.5 ml of 50 mM Tris–HCl pH 7.0 buffer, diluted 1:5 with the same buffer and the fluorescence intensity was determined using a Becton–Dickinson (model FacsCantoII) apparatus equipped with an argon laser.

### Real time PCR

Total RNA from control and toxicant-exposed cells was purified by the RNeasy kit (Qiagen) and quantified using the Quant-iT™ RiboGreen® RNA kit (Invitrogen). 250 ng of purified, DNaseI (Quiagen)-treated RNA were used as template with the LightCycler® RNA Amplification Kit SYBR Green I (Roche Applied Science) kit as recommended. Fluorescence increase was monitored using the LyghtCycler® 2.0 software.

### Statistical analysis

Statistical analysis of the data was carried out using the GraphPad Prism 5.03 (GraphPad Software, Inc) software. A $p < 0.05$ value was used for ANOVA and *t* test analysis.

## Results

### Glucose transport in tellurite-stressed *E. coli*

Given that tellurite generates superoxide (Chasteen et al. 2009) and glucose entrance is increased in *E. coli* exposed to the superoxide generator paraquat (Rungrassamee et al. 2008), it was interesting to assess glucose transport in *E. coli* when grown in the presence of tellurite or hydrogen peroxide (used as positive control of oxidative stress). As expected, decreased levels of extracellular glucose were observed in the presence of both toxicants as compared to controls (Fig. 1a). Also, *E. coli* lacking *ptsG*, the gene encoding the PtsG subunit of the specific glucose transporter, did not show differences in external glucose in the presence of $TeO_3^{2-}$ or $H_2O_2$ as compared to the control condition (Fig. 1b).

### ROS generation in tellurite-exposed *E. coli*

Although it was previously shown that cells grown in tellurite-amended rich media show increased ROS levels (Pérez et al. 2007), it was not known if the same applies for M9 minimal medium, in which *E. coli*

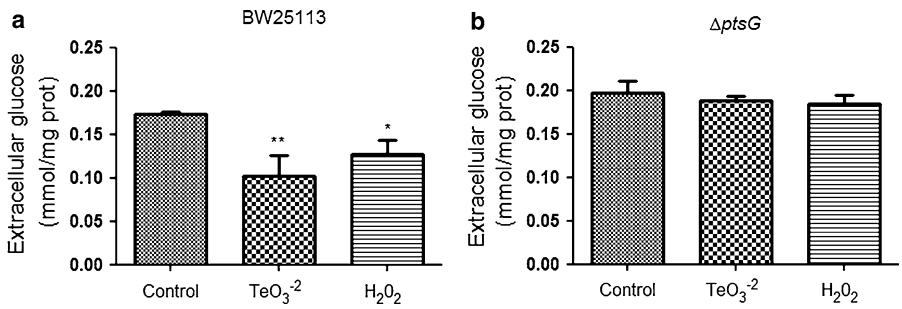


**Fig. 1** Extracellular glucose determination in *E. coli* cultures. Wild type (**a**) and Δ*ptsG* (**b**) strains were exposed to 20 μM tellurite or 1 mM hydrogen peroxide for 30 min at 37°C as described in "Materials and methods". *Bars* represent the average of five independent trials

exhibits ~10-fold more tolerance to tellurite than in LB medium. Tellurite exposure caused the number of fluorescent cells to increase in a concentration-dependent manner, as hydrogen peroxide did (Fig. 2).

## Tellurite effects on *E. coli* glycolytic enzymes and metabolites

Next, enzyme activity as well as the concentration of some key glycolytic metabolites was assessed in tellurite-treated *E. coli*. While phosphoglucose isomerase activity increased ~2.5-fold in treated cells as compared to that observed in untreated controls, PFK activity decreased >80% in the presence of tellurite, suggesting that the pathway could stop at this point. Though aldolase was not affected by ROS elicitors, PK activity decreased by ~40–50% (Table 2). Similar results were observed with hydrogen peroxide in all cases.

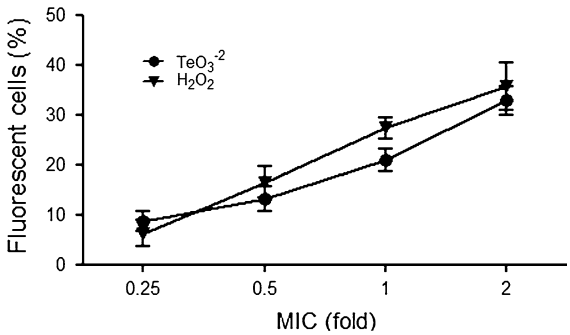


**Fig. 2** Toxicant-mediated intracellular ROS generation in *E. coli*. ROS-producing cells were determined by flow cytometry by measuring the activation of the oxidation-sensitive probe $H_2$DCFDA as described in "Materials and methods". *Bars* represent the average of five independent trials

**Table 2** Enzymatic activity in extracts of toxicant-exposed *E. coli*

| Enzyme | Treatment | | |
|---|---|---|---|
| | Control | $K_2TeO_3$ | $H_2O_2$ |
| PGI | 100 | 241.9 ± 36.6 | 235.9 ± 13.6 |
| PFK | 100 | 17.7 ± 10.0 | 21.0 ± 20.0 |
| Aldolase | 100 | 104.2 ± 12.6 | 105.4 ± 20.3 |
| PK | 100 | 64.9 ± 24.2 | 60.8 ± 13.6 |

Data is presented as % of specific activity and represent the average of four independent trials

When glycolytic intermediate metabolites were analyzed in toxicant-exposed cells, and in spite of the observed decrease in PFK and PK activities, an important increase in PYR, PEP and TPS content (>3-fold) was observed (Fig. S1).

## Expression of genes encoding glycolytic enzymes in tellurite-exposed *E. coli*

Since ROS-mediated stress, particularly by superoxide, results in decreased expression of bacterial glycolysis-involved genes (Mostertz et al. 2004), the *E. coli* transcriptional response under tellurite treatment was assessed. To accomplish this, the expression of *pgi*, *pfkA*, *pfkB*, *pykF* and *pykA* (encoding phosphoglucose isomerase, phosphofructokinase I, phosphofructokinase II, pyruvate kinase I and pyruvate kinase II, respectively) as well as that of the PTS-encoding *ptsG* gene was evaluated. Since previous results from our laboratory stated that the expression of the glycolytic *gapA* gene was not affected by tellurite (Pérez et al. 2008), induction studies were carried out using *gapA* as the housekeeping gene. According to the results shown in Fig. 1 and Table 2, increased transcription of *pgi* and *ptsG* was expected. Figure 3 shows that this was the case, since a 3- and a 2-fold increase was observed for *ptsG* and *pgi* expression, respectively. Curiously, and given the enzyme activity results shown in Table 2, increased *pfkA* and *pykA* and *pykF* transcription was observed in tellurite-exposed cells (Fig. 3).

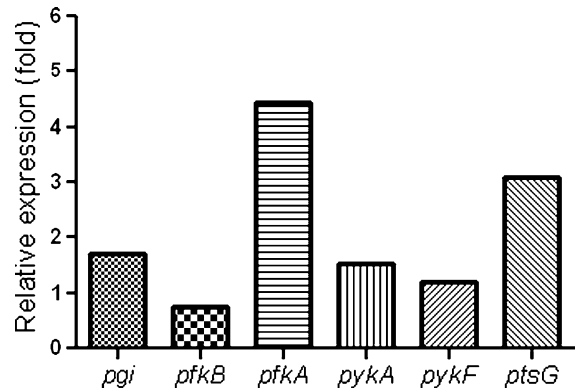


**Fig. 3** Relative expression of glycolytic genes in tellurite-stressed *E. coli*. Transcriptional induction of *pgi*, *pfkB*, *pfkA*, *pykA*, *pykF* and *ptsG* was analyzed after exposing exponentially growing *E. coli* ($OD_{600}$ ~0.3) for 15 min to 20 μM tellurite. Data represent the average of duplicated experiments

### Tellurite tolerance of *E. coli* strains lacking glycolytic genes

To confirm the involvement of glycolysis in the bacterial response to tellurite, the tolerance of *E. coli* lacking glycolytic genes to this toxicant was determined. Table 3 shows that Δ*pfkA*, Δ*pykF*, Δ*pykA* and Δ*ptsG* strains are more sensitive to the tellurite than the isogenic, parental, wild type strain. While similar results were observed with $H_2O_2$ for Δ*pgi*, Δ*pfkA* and Δ*ptsG* strains, interestingly cells lacking *pykA* or *pykF* were not affected by peroxide. These results were confirmed by MIC determination, Δ*pfkA*, Δ*ptsG*, Δ*pykA* and Δ*pykF* cells showing lower tellurite or $H_2O_2$ MICs than control, wild type cells (Table 3).

No difference in tellurite tolerance was observed between cells lacking PGI, aldolase B, phosphoglycerate kinase, phosphoglycerate mutase, PEP synthetase or fructose-1,6-bisphosphatase when compared to the wild type control (not shown).

## Discussion

It is well known that glucose transport is a limiting step of the glycolytic pathway. Results shown in Fig. 1 suggest that glucose incorporation is enhanced in the presence of oxidative stress elicitors, supporting the idea that it depends, at least in part, on *ptsG* expression. Since *ptsG* is positively regulated by *soxS* (Rungrassamee et al. 2008) and given that this regulatory gene is induced by tellurite (Pérez et al. 2007; Chasteen et al. 2009), it was expected to some extent that tellurite would favor PTS-mediated glucose incorporation.

As *ptsG*, the *pgi* gene is also positively regulated by *soxS* (Rungrassamee et al. 2008). In this context, it was of interest to analyze PGI activity in cells exposed to the toxicant. To assess if the observed effect (Table 2) was dependent on tellurite-induced *soxS* activation, PGI activity was determined in Δ*soxRS* cells. While in the parental, GC4468 cells a threefold increase was observed, no differences in PGI activity were detected among control and tellurite-exposed *soxS*-lacking cells (Fig. S2). In addition, tellurite exposure caused the number of fluorescent cells to increase in a concentration-dependent manner (Fig. 2), strengthening the idea that tellurite-enhanced glucose incorporation is related to ROS generation. As expected, *ptsG* and *pgi* transcription was increased in the presence of tellurite (Fig. 3).

Toxicant treatment resulted in decreased activity of the key regulatory enzymes PFK and PK (Table 2). Since PFKI represents >90% of the *E. coli* PFK activity in vivo (Kotlarz et al. 1975), the tellurite sensitivity observed for *pfkA*-lacking cells (Table 3) could be related to low levels of PFKII activity in this genetic background. As expected, Δ*pfkB* cells behave similarly to the wild type control (Kotlarz et al. 1975). In turn, tellurite sensitivity of Δ*ptsG* cells probably reflects the impaired glucose entry to the cytoplasm.

Conversely to the observed decrease in PFK and PK activities, the expression of their respective encoding genes was enhanced in the presence of tellurite, probably to avoid a strong depletion of glycolytic intermediates. Recently, a similar phenotype has been described for *E. coli* cells lacking defined genes of the electron transport chain (Kumar and Shimizu 2011). In spite of these results, increased amounts of phosphorylated intermediates were observed (Fig. S1), which could be generated through the pentose phosphate shunt (PPP) or other pathways. In this context, a recent report shows that the metabolic flux is redirected as response to paraquat, where most of G6P is shifted to the PPP resulting in increased NADPH/NADH ratios and Krebs cycle malfunctioning (Rui et al. 2010). Results from our laboratory indicate that in addition to G6PDH, the last enzyme of the oxidative phase of the PPP, 6-phosphogluconate dehydrogenase, also exhibits increased activity upon tellurite exposure (unpublished data), which could represent an alternative PEP supply. Interestingly, it has been shown that exposure

**Table 3** Growth inhibition zones and minimal inhibitory concentrations of tellurite and $H_2O_2$ for the indicated *E. coli* strains

| *E.coli* strain | MIC | | Inhibition zone ($cm^2$) | |
|---|---|---|---|---|
| | $K_2TeO_3$ (μM) | $H_2O_2$ (mM) | $K_2TeO_3$ | $H_2O_2$ |
| Wild type | 40 | 5 | 3.2 ± 0.1 | 9.5 ± 0.2 |
| Δ*pgi* | 20 | 2.5 | 5.3 ± 1.7 | 13.0 ± 1.0 |
| Δ*ptsG* | 10 | 2.5 | 10.6 ± 1.4 | 18.6 ± 0.4 |
| Δ*pfkA* | 5 | 1.25 | 7.4 ± 0.1 | 15.4 ± 0.5 |
| Δ*pfkB* | 40 | 5 | 3.3 ± 1.0 | 9.4 ± 0.5 |
| Δ*pykA* | 10 | 5 | 7.1 ± 1.2 | 10.0 ± 0.7 |
| Δ*pykf* | 10 | 5 | 6.9 ± 0.4 | 8.7 ± 0.6 |

Data represent the average of four independent trials

to the arsenic oxyanion arsenite (structurally similar to tellurite) also results in increased PEP and PYR levels (Reichl et al. 1988).

Summarizing, this work shows the involvement of a previously not investigated metabolic pathway, glycolysis, in bacterial tellurite resistance. Upon toxicant exposure, while glucose transport and PGI activity increase, the activity of the regulatory PFK and PK enzymes is decreased. This ends in F6P accumulation which can then be directed to the pentose phosphate shunt. This would increase reducing power and generate PEP—which is required to augment glucose entrance by PTS—resulting in enhanced pyruvate levels. Finally, this re-directioning of the carbon metabolic flux helps the bactium to tolerate in a better form tellurite-mediated oxidative stress. Figure S3 depicts the current picture regarding glucose metabolism in tellurite-exposed *E. coli*.

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