**Bacterial Toxicity of Potassium Tellurite: Unveiling an Ancient Enigma**

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Biochemical, genetic, enzymatic and molecular approaches were used to demonstrate, for the first time, that tellurite (TeO$_3^{2-}$) toxicity in *E. coli* involves superoxide formation. This radical is derived, at least in part, from enzymatic TeO$_3^{2-}$ reduction. This conclusion is supported by the following observations made in K$_2$TeO$_3$-treated *E. coli* BW25113: i) induction of the *ibpA* gene encoding for the small heat shock protein Ibpa, which has been associated with resistance to superoxide, ii) increase of cytoplasmic reactive oxygen species (ROS) as determined with ROS-specific probe 2′,7′-dichlorodihydrofluorescein diacetate (H$_2$DCFDA), iii) increase of carbonyl content in cellular proteins, iv) increase in the generation of thiobarbituric acid-reactive substances (TBARs), v) inactivation of oxidative stress-sensitive [Fe-S] enzymes such as aconitase, vi) increased levels of superoxide dismutase (SOD) activity, and vii) generation of superoxide radical during *in vitro* enzymatic reduction of potassium tellurite.

**INTRODUCTION**

To date, it is not known if elements like Ag, As, Cd, Cr, Hg, Pb, Te, or some of their derivatives play a defined biological function and they are mainly associated with toxicity [1,2]. Oxidant species such as hydrogen peroxide (H$_2$O$_2$), superoxide anion (O$_2^{-}$), and hydroxyl radical (OH$^.$) are typical byproducts of the aerobic metabolism that can be formed by exposure of cells to free radical-generating molecules like metals and metalloids [8]. Recent indirect evidence suggests a relationship between tellurite toxicity and superoxide generation inside the cell [3,4,5]. Evidence has accumulated in the last few years suggesting that tellurite could exert its toxicity through intracellular generation of reactive oxygen species (ROS).

**RESULTS**

Tellurite triggers expression of the *ibpA* stress response promoter

Aiming to investigate potassium tellurite toxicity in *E. coli* we studied induction of *lacZ* fusions to the well-characterized stress response promoters *ibpA*, *sodA*, *p3SRpoH* and *cspA* in cells exposed to tellurite. Significant promoter activation by K$_2$TeO$_3$ was observed only for the *ibpA* promoter. Fig. 1 shows that *E. coli* cells treated with tellurite exhibited a 10-fold transcription induction as compared with the untreated controls. This finding is interesting because Ibpa protein has been associated with increased resistance to oxidative stress induced by superoxide [12,13]. CspA mRNA is highly transcribed in response to cytoplasmic protein stress [14,15]. Our results showed a 2-fold increase in the *cspA* promoter transcription in response to tellurite. Promoters *sodA* and *p3SRpoH*.

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were used to study transcription induction in cells treated or untreated
moters
ibpA, cspA, p3RpoH
l
The inset clearly shows that the behaviour of
Results are the average of at least 4 determinations.
was calculated dividing the value obtained at 3 h by the value at time 0.
0 and after 3 h with or without tellurite treatment. The fold induction
highest ROS levels in cells treated with 0.5–1.0
This activation was proportional to tellurite concentration, with the
escein diacetate) was used to monitor formation of intracellular ROS
reached the stationary growth phase.
3 h of tellurite treatment may be due to the fact that the cells have
slight increments of
tellurite inside the cell are mediated, at least in part, by an
increase in intracellular ROS concentration which in turn is
related with the generation of metabolic ROS.
These results support previous findings that the oxidative effects
of tellurite inside the cell are mediated, at least in part, by an
increase in intracellular ROS concentration which in turn is
most likely a consequence of tellurite reduction in the bacterial
cytosol [7,16].
Tellurite generates ROS in the cytoplasm of E. coli
The fluorescent probe H$_2$DCFDA (2′, 7′- dichlorodihydrofluor-
escin diacetate) was used to monitor formation of intracellular ROS
tellurite-treated cells (Fig. 2). Cells treated with different sub lethal
cells exhibited a peak of catalase activity during the first 15 min of
treatment. These activities were determined in
Aconitase activity, a ROS-
ROS were determined in cells grown under aerobic conditions
with or without K$_2$TeO$_3$ (Table 1). Aconitase activity, a ROS-
sensitive enzyme containing a [Fe-S] cluster [17,20], decreased
approximately 5-fold in K$_2$TeO$_3$ treated cells. Malate dehydro-
gean, an enzyme reported to be resistant to oxidative stress [17,21],
showed no differences between tellurite-treated and untreated
cells.
To determine the effect of tellurite on ROS-responsive enzym-
behavior, the activities of catalase (CAT) and superoxide
dismutase (SOD) were assayed during the first hour of tellurite
treatment. These activities were determined in
KS400 [18]. K$_2$TeO$_3$ treatment also produced an important increase of
TBARs in E. coli BW25113. This increase was about one half of
that induced by H$_2$O$_2$, an observation that is consistent with the
idea of a secondary toxic effect of tellurite due to superoxide anion
generation during tellurite reduction.

### Determination of ROS-sensitive and ROS-responsive enzymatic activities

A number of enzymatic activities that are known to be affected by
ROS were determined in cells grown under aerobic conditions
with or without K$_2$TeO$_3$ (Table 1). Aconitase activity, a ROS-
sensitive enzyme containing a [Fe-S] cluster [17,20], decreased
approximately 5-fold in K$_2$TeO$_3$ treated cells. Malate dehydrogen-
ase, an enzyme reported to be resistant to oxidative stress [17,21],
showed no differences between tellurite-treated and untreated
cells.

![U Miller Fold Induction](image)

**Figure 1.** Tellurite-induction of β-galactosidase activity in E. coli reporter strains. E. coli reporter strains ADA100 (AB734::ibp: lacZ), ADA310 [AB734:: (cspA: lacZ)], ADA410 [AB734:: (p3RpoH: lacZ)] and ADA510 [AB734:: (suA: lacZ)] containing the stress-responsive promoters ibpA, cspA, p3RpoH and suA fused to the lacZ gene respectively, were used to study transcription induction in cells treated or untreated with 0.5 μg/ml ibpA. β-galactosidase activity was evaluated at time 0 and after 3 h with or without tellurite treatment. The fold induction was calculated dividing the value obtained at 3 h by the value at time 0. Results are the average of at least 4 determinations.

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did not show any detectable activation suggesting that tellurite
does not involve DNA damage or periplasmic stress in E. coli. The
slight increments of β-galactosidase activity seen in controls after
3 h of tellurite treatment may be due to the fact that the cells have
reached the stationary growth phase.

**Tellurite increases oxidation of cytoplasmatic proteins**

The generation of carbonyl groups in proteins, produced by the
modification of side chains of some amino acids, is a suitable
marker to monitor oxidation of the intracellular environment
[17,18]. Spectrophotometric determination of derivatized carbonyl
groups with 2,4 diminotrophenyl hydrazine (DNPH) showed that
crude extracts of K$_2$TeO$_3$-treated E. coli exhibited a 4-fold increase
in the content of oxidized cytoplasmatic proteins compared with the
2-fold increase observed with peroxide (Fig. 3A). Determination of
carbonic groups using specific antibodies against DNPH-
derivatized proteins confirmed the oxidative effect of K$_2$TeO$_3$ on
cytoplasmatic proteins. Although the protein immunoreactive
pattern between untreated and treated cells was not significantly
different, a few high molecular weight proteins were distinctly
observed in tellurite-treated cell extracts (data not shown). Experiments to determine the identity of these proteins are in
progress.

**Tellurite increases cytoplasmic TBARs**

The level of TBARs has been extensively used to assess the
damage of oxidative stress to membrane lipids in many organisms
[18,19]. It has been recently shown that TBARs concentration
significantly increases in E. coli exposed to hydrogen peroxide [18].
We also observed an increase of TBARs in H$_2$O$_2$-treated E. coli
BW25113 (Fig. 3B). However, this increase was only approxi-
mately 30% of that previously observed in E. coli KS400 [18].
K$_2$TeO$_3$ treatment also produced an important increase of
TBARs in E. coli BW25113. This increase was about one half of
that induced by H$_2$O$_2$, an observation that is consistent with the
idea of a secondary toxic effect of tellurite due to superoxide anion
generation during tellurite reduction.

**Table 1.** Effect of potassium tellurite on E. coli malate dehydrogenase and aconitase.

| STRAIN | MIC (μg/ml) |
|--------|-------------|
| wt     | 2.0         |
| ibpA   | 0.06        |
| katG   | 1.5         |
| sodA/sodB | 0.01 |
| ssoS   | 0.75        |

Enzymatic activities were determined in crude extracts obtained from cells
treated or untreated with 0.5 μg/ml K$_2$TeO$_3$ for 30 min as described in Methods.
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treatment followed by a 50% decrease in activity (Fig. 4A). This initial rise in activity could be associated with the generation of peroxide produced along the course of superoxide dismutation by the bacterial superoxide dismutase (SOD).

Crude extracts obtained from tellurite-treated cells showed an important increase in SOD activity as compared to the basal level exhibited by untreated cells (Fig. 4B). The highest rise in SOD activity was observed within the first 15 min and was followed by a lower activity increase. These results show that $O_2^-$ levels increase as a result of tellurite exposure suggesting that the stress conditions generated by $K_2TeO_3$ in $E. coli$ may be associated, at least in part, with intracellular generation of superoxide.

Tellurite induces soxS mRNA synthesis in $E. coli$

To determine whether ROS-response related genes are induced by tellurite exposure, $E. coli$ sodA, sodB, soxS and katG mRNAs were quantitated by PCR using total RNA of tellurite-treated or untreated cells. Fig. 5 shows that $E. coli$ sodA, sodB and katG genes exhibited a rather basal expression in untreated cells. Interestingly, no soxS was detected under the same experimental conditions. After 10 min of tellurite exposure a marginal increase was observed for sodA and sodB mRNAs. Under the same conditions, a strong transcriptional activation was seen for katG and particularly for soxS. Only the housekeeping gapA gene, encoding glyceraldehyde-3-phosphate dehydrogenase, showed a transcription decrease in tellurite-exposed cells (Fig. 5). These results suggest that tellurite causes induction of genes known to be responsive to increases in ROS levels. Presence of soxS mRNA in tellurite-treated cells is also indicative that superoxide radical is being generated.

Absence of $E. coli$ superoxide-responsive genes causes tellurite hypersensitivity

Deletion of genes that are induced upon $K_2TeO_3$ exposure results in an increase of sensitivity to this toxic salt. Interestingly, ibpA-, sodA/sodB- and soxS-deficient strains are the most affected, a result that agrees with observations described above. katG, which is highly induced in the presence of tellurite (Fig. 5) does not seem to be involved in tellurite tolerance (Table 2).
In vitro superoxide generation

Results described above suggest that inside the cell K₂TeO₃ behaves as a ROS generator with superoxide radical being the most likely species generated. To test this assumption an in vitro tellurite-reduction assay was carried out (see Materials and Methods for details). The rationale for these experiments is based on previous observations indicating that superoxide radical is formed during intracellular selenite reduction [22,23]. Because Se and Te share several chemical properties, we reasoned that tellurite reduction may also be associated with superoxide formation.

As expected, in vitro K₂TeO₃ reduction resulted in an increase in WST-formazan absorption (Fig. 6); this increase was proportional to superoxide concentration (not shown). That superoxide was generated during tellurite reduction was demonstrated by performing the assay in the presence of SOD. This enzyme totally inhibited the increase of OD₄₃₈ which did not occur when β-amylase was used. This observation confirms that superoxide radical is formed during tellurite reduction in vitro.

DISCUSSION

Results described in this work represent a step ahead in the understanding of the mechanism underlying potassium tellurite toxicity. We have previously shown that E. coli cells overexpressing G. stearothermophilus V cysteine metabolism-related genes develop a significant increase (~25 fold) in tellurite resistance [9,16,24]. Expression of G. stearothermophilus V cysK and iscS genes prevented the typical decrease in intracellular RSH content caused by tellurite ([16] and unpublished results). Our interpretation was that the cysteine synthase-encoding cysK gene indirectly participates in the generation/regeneration of GSH required to maintain the reductive cytoplasmic environment altered by tellurite.

The IscS desulfurase is probably involved in recovery of tellurite-damaged [Fe-S] clusters that are part of the active site of some ROS-sensitive enzymes [9,10]. Superoxide radical, the ROS that is most probably involved in the oxidative toxic effect of tellurite, would be generated during tellurite reduction in a process similar to that previously described for selenium oxyanions [22,23]. ROS generation by tellurite was suggested by experiments using sodA sodB tellurite-hypersensitive E. coli mutants and by demonstration that the minimal inhibitory concentration of potassium tellurite increased under anaerobicic conditions [9,10]. We hypothesized that tellurite reduction by thiols or cellular reductases generates metallic tellurium (Te⁰) and involves ROS generation as well.

To further study tellurite-mediated toxicity we analyzed activation of stress promoters ibpA, sulA, P53poH and cspA in response to K₂TeO₃. Significant tellurite-mediated transcription activation...
was only observed for the \textit{ibpA} promoter. The \textit{ibp} operon is a member of the \textit{E. coli} \sigma^32 regulon that encodes the small heat shock proteins IbpA and IbpB. This operon undergoes a high level of transcriptional induction in response to a temperature up shift and participates in events associated with oxidative stress. IbpA over expression generates an important increase in resistance to superoxide anions but does not have any effect in resistance of \textit{E. coli} to hydrogen peroxide [12,13]. Results obtained with an \textit{E. coli} \textit{ibpA} mutant (BW25113 \textit{kan::ibpA}) showed a \approx 32 fold increase in tellurite sensitivity as compared to the wild type strain suggesting that IbpA plays a role in \textit{K}_2\textit{TeO}_3 resistance (Table 2).

Our results showed a 2-fold increase in the \textit{cspA} promoter transcription in response to tellurite. CspA mRNA is highly transcribed in response to cytoplasmic protein stress [14,15]. This tellurite-mediated activation is in line with the idea that \textit{TeO}_3^{2−} has an oxidative effect on cytoplasmatic proteins by replacing Se or reacting with catalytic cysteine residues in key cellular proteins.

The \textit{P3\textit{rpoH}} promoter is induced by periplasmic protein misfolding and is transcribed by the \textit{Ecol} holoenzyme [25]. We did not observe tellurite-induced activation of the \textit{P3\textit{rpoH}} promoter suggesting that tellurite-mediated stress mainly affects cytoplasmic proteins. It is well known that \textit{K}_2\textit{TeO}_3 affects proteins and cellular targets by direct interaction and/or its capability to oxidize cellular thiols [7,16]. We hypothesized that ROS produced during \textit{Te}^{4+} generation could account for part of tellurite toxicity. \textit{Te}^{4+} reduction takes place in the cytoplasm and it is probably mediated by thiols and/or by cellular reductases [4,5,26,27]. Results of experiments with the \textit{P3\textit{rpoH}} promoter are consistent with previous findings reporting that a \textit{Rhodobacter capsulatus} mutant in the periplasmic antioxidant gene \textit{senC} does not exhibit sensitivity to \textit{K}_2\textit{TeO}_3, which has been interpreted as evidence that superoxide radical is not generated in the periplasm of this rod [11].

The \textit{sulA} gene is activated as part of the cellular SOS response induced by compounds that generate DNA damage or affect DNA replication [28]. We showed that the \textit{sulA} gene promoter was not activated in response to tellurite suggesting that the toxic effect of tellurite does not involve DNA damage or activation of the SOS response. These results lead to the speculation that \textit{H}_2\textit{O}_2 or hydroxyl radical, that are the ROS directly involved in DNA damage [29], are not generated during tellurite reduction.

To discard a Fenton-like reaction as the one described for chromate [30] we performed in \textit{vitro} incubations of plasmid DNA with \textit{K}_2\textit{TeO}_3 or with \textit{K}_2\textit{TeO}_3 in the presence of \textit{H}_2\textit{O}_2. No DNA

Figure 5. Tellurite induces \textit{katG} and \textit{soxS} mRNA synthesis in \textit{E. coli}. DNA fragments (300 bp) from \textit{E. coli sodA}, \textit{sodB}, \textit{katG}, \textit{soxS} and \textit{gapA} genes were amplified by RT-PCR and fractionated by electrophoresis on agarose gels (1.5%). Total RNA from cells grown with (\textit{K}_2\textit{TeO}_3) or without (control) 0.5 \textmu g/ml potassium tellurite was used as template for the RT-PCR. The estimated DNA (ng) content determined for each band is shown (see Material and Methods for details).

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damage was evidenced as judged by the lack of shift mobility on agarose gels. A similar result was obtained under tellurite reduction conditions confirming that tellurite toxicity does not seem to involve DNA damage (data not shown).

To further assess that the cellular oxidative stress generated by tellurite lies on the intracellular generation of oxygen reactive species a direct measure of cytoplasmic ROS using the intracellular, specific, probe H2DCFDA in E. coli cells treated with different sub lethal K2TeO3 concentrations was made. Tellurite treatment increased cytoplasmic ROS in a fashion that was proportional to K2TeO3 concentration (Fig. 2). These results suggested that in E. coli the tellurite-mediated stress can be associated, at least in part, with an increase in the cytoplasmic ROS concentration.

Protein carbonylation occurs in a number of physiological and pathological processes and it has been suggested that protein carbonylation can be used to determine the ratio between oxidative stress damage and the power of protective systems to avoid it [31]. Tellurite generates an important increase of protein carbonylation can be used to determine the ratio between oxidative stress damage and the power of protective systems to avoid it [31]. Aldehyde-mediated protein oxidation [31]. Aldehyde-mediated protein oxidation can occur in a number of physiological and pathological processes and it has been suggested that protein carbonylation can be used to determine the ratio between oxidative stress damage and the power of protective systems to avoid it [31].

Table 2. Minimal inhibitory concentrations (MIC) of K2TeO3 for E. coli BW25113 strains deficient in ROS-responsive genes.

| Malate dehydrogenase µg/min/mg prot | Aconitase µmol/min/mg prot |
|------------------------------------|----------------------------|
| Control 0.57±0.01                   | 0.380±0.05                 |
| K2TeO3 0.59±0.04                   | 0.085±0.02                 |

Numbers are the mean of 4 independent determinations.

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The increase in TBARS content has been widely used to assess oxidative stress damage to lipids in many microorganisms including E. coli [18,19,31]. Fig. 3B shows that K2TeO3-treated E. coli cells exhibit an increase in lipid peroxidation products (determined as TBARS) suggesting that these tellurite-generated compounds are probably a consequence of ROS generation during the reduction of the tellurium oxanyan. TBARS levels were lower in tellurite-treated cells as compared to cells exposed to hydrogen peroxide (Fig. 3A and 3B). This observation may be related to the multifactor toxic effect of K2TeO3 that would affect different cellular processes through the generation of secondary toxic molecules such as O2-, aldehydes and peroxides. Preliminary results from our laboratory indicate that a putative E. coli aldehyde reductase can be associated with a protection against tellurite-generated lipid peroxidation products (unpublished results).

An alternative approach to monitor the generation of ROS upon tellurite exposure is to estimate induction of some specific ROS-sensitive or ROS-responsive enzymatic activities. The activity of aconitase, a superoxide sensitive enzyme, was evaluated in E. coli cells treated with potassium tellurite. Aconitases possess [4Fe-4S] clusters highly sensitive to superoxide and represent suitable markers to estimate ROS sensitivity. Cells exposed to tellurite exhibited an important decrease in aconitase activity (Table 2). Fumarase A, another ROS-sensitive enzyme, also showed a decreased activity under tellurite stress conditions (unpublished observations). These results confirm that tellurite triggers an enzymatic ROS phenotype, presumably via superoxide generation. The activity level of the ROS-resistant metabolic enzyme malate dehydrogenase was determined as control. No differences in malate dehydrogenase activity of E. coli BW25113 cells treated and untreated with tellurite was detected (Table 1). These results agree with previous observations indicating that tellurite also causes a rapid reduction of ATP levels that does not involve a decrease in the glycolytic pathway or depletion of glucose in E. coli [32]. This effect is most likely due to a shift to anaerobic metabolism similar to that described for other toxic metals [33].

Activation of oxidative stress enzymatic responsive systems estimated by measuring catalase (CAT) and superoxide dismutase (SOD) activities upon K2TeO3 exposure was also analyzed (Fig. 4A and 4B, respectively). Catalase HPI, encoded by the katG gene, is highly induced by an increase in peroxide concentration. Unexpectedly, an increase in CAT activity was observed during the first 10 min of tellurite exposure (Fig. 4A). This result may be a consequence of the hydrogen peroxide produced by superoxide dismutation because an increase in CAT activity was associated with the highest increase of SOD activity (15 min) along the K2TeO3 treatment (Fig. 4B). The fact that CAT activity does not increase at later times suggests that hydrogen peroxide is not the ROS generated in E. coli tellurite-stressed cells (Fig. 4A). In situ catalase activity determinations on native gels showed no correlation between tellurite concentration and catalase activity over 30 min incubation (data not shown). These results also favor the
idea that tellurite does not produce peroxide inside E. coli as does the hydrogen peroxide-hypersensitive katG strain which showed a negligible increase in sensitivity to K$_2$TeO$_3$ (Table 2). E. coli cells over expressing a *Staphylococcus epidermidis* catalase gene showed a slight increase in tellurite resistance suggesting that the role of H$_2$O$_2$ in tellurite resistance is, if any, is not an important feature of K$_2$TeO$_3$ toxicity [34].

It has been shown that SOD activity slightly increases when wt E. coli cells are about to enter the second half of the logarithmic growth phase [35]. We observed similar results in E. coli BW25113 cells that were not treated with tellurite. SOD activity was distinctly induced throughout the course of the experiment confirming that superoxide dismutases play a role in the cellular response of E. coli to potassium tellurite (Fig. 4B).

The slight increment of sodA and sodB mRNA synthesis observed after a brief exposure to K$_2$TeO$_3$ also suggest that superoxide is produced in response to tellurite (Fig. 5). Induction of katG during early stages of tellurite treatment reflects an increase of peroxide concentration that is probably generated by superoxide dismutase as suggested above. High levels of soxS mRNA were also detected under tellurite exposure confirming that tellurite-generated O$_2^-$ activates the most specific and important superoxide response system in E. coli.

An *E. coli* soxS strain (BW25113 kan::soxS) showed a tellurite-sensitive phenotype (Table 2), an observation which is consistent with a role of O$_2^-$-protection systems in response to tellurite exposure. The lower sensitivity to tellurite observed by this mutant when compared with the *ibpA* and *soxAB* strains is presumably due to the fact that the several genes governed by the soxRS regulon are also regulated by other global networks [18,36].

An *in vitro* tellurite reduction assay was used to demonstrate that reduction of Te$_4^+$ to Te$_6^-$ indeed generates O$_2^-$. Production of O$_2^-$ was determined by the use of WST-1, a molecule known to be highly specific and sensitive to reduction by superoxide radicals. Enzymatic reduction of tellurite *in vitro* produced a significant increase in light absorption at 438 nm that is indicative of O$_2^-$ generation (Fig. 6). The superoxide concentration was proportional to tellurite concentration confirming that K$_2$TeO$_3$ reduction involves O$_2^-$ generation (data not shown). The *in vitro* reduction assay performed in the presence of SOD confirmed that superoxide is one of the products of tellurite reduction.

Altogether, our results strongly indicate that the dramatic toxic effect of potassium tellurite in *E. coli* can be associated with the chemical activity of the tellurium oxianion at various levels of bacterial metabolic pathways including, among others, inactivation of [Fe-S] center-containing dehydratases, cytoplasmic thiol oxidation, enzyme and protein carbonylation and membrane peroxidation.

Before the submission of this manuscript Tremaroli et al. [37] inferred that superoxide radicals are generated as a result of the increased superoxide dismutase activity of *Pseudomonas pseudoalcaligenes* cells in response to tellurite exposure.

**MATERIALS AND METHODS**

**Bacterial strains and growth conditions**

*E. coli* BW25113 [38] was the parental strain used in all experiments. *E. coli* mutants deficient in the *katG*, *ibpA* and *soxS* genes were provided by the NARA Institute of Science and Technology, Japan [39]. Cells were routinely grown in LB medium [40] at 37°C with shaking. Antibiotics were added as required. Kan$^R$ insertions into *E. coli* BW25113 chromosomal ROS resistance genes were constructed by the method of Datsenko and Wanner [36].

**Enzyme activity assays**

Cells were disrupted by sonication on ice-cold water and extracts cleared by centrifugation. Aliquots of cell-free extracts were assayed for aconitase [20], malate dehydrogenase [21], catalase and superoxide dismutase [41,42]. Protein concentration was determined by the Bradford method.

**Stress-promoter activation assays**

Promoter regions of the *ibpA*, *cspA*, *spoH* and *sodA* genes have been routinely used as molecular tools to detect and characterize antibacterial agents that induce stress response in *E. coli* due to their strength and specificity [43,44].

*ibpA* gene, encoding for the small bacterial heat shock protein Hpa, undergoes high level of induction following a temperature up shift and has been associated with oxidative stress response in *E. coli* [12,13]. CspA is the major *E. coli* cold shock protein [14]. *E. coli* cells dedicate more than 10% of their synthetic capability to produce CspA shortly after transfer to 10°C [15].

While the P3spoH promoter is activated by unfolded proteins generated during periplasmic stress [25], the *sodA* gene is activated during the course of the SOS response by compounds that damage DNA or affect its replication [28].

*E. coli* strains used in the stress-promoter activation assays are ADA100 [AB734Δ*ibpA*::lacZ], ADA310 [AB734Δ*ibpA*::lacZ], ADA410 [AB734Δ*ibpA*::lacZ] and ADA510 [AB734Δ*ibpA*::lacZ] and were kindly provided by Dr. Francois Banex [43,44].

Flasks (500 ml) containing 100 ml of LB medium were inoculated with 2 ml of overnight cultures and grown at 37°C under aerobic conditions until the optical density at 600 nm (OD$_{600}$) was 0.4. Aliquots of 25 ml were then transferred to preheated 125 ml flasks and the cultures incubated for 3 h in the presence of 0.5 μg/ml K$_2$TeO$_3$. Control cultures contained an equal volume of H$_2$O$_2$. The fold induction was calculated by comparing β-galactosidase activity after 3 h of tellurite treatment divided by the activity at time 0, as described by the group of Banex [43,44]. All experiments were carried out in triplicate or higher.

**β-galactosidase assay**

Following the incubation with tellurite, samples (2 ml) were withdrawn at defined time intervals and OD$_{600}$ determined. Cells were sedimented by centrifugation at 6,500 x g for 8 min, suspended in an equal volume of 50 mM monobasic potassium phosphate (pH 6.5), and disrupted by sonication. After centrifugation at 10,000 x g for 10 min to discard cell debris, aliquots of the cleared lysate were assayed for β-galactosidase activity (triplicate) using the chromogenic substrate O-nitrophenyl-β-D-galactopyranoside [45].

**Tellurite sensitivity assay**

Cells were grown overnight and diluted one hundred-fold with fresh LB medium. Ten μl of these dilutions were transferred into tubes containing 1 ml of LB added with specific amounts of antibiotics and potassium tellurite. Cells were incubated at 37°C for 48 h with shaking and the OD$_{600}$ monitored to determine the minimal inhibitory concentration (MIC).

**Determination of intracellular oxidation levels**

The oxidant-sensitive probe H$_2$DCFDA [17] was used to determine the intracellular levels of ROS in cells treated with 3 different tellurite concentrations (0.2, 0.5, and 1 μM/ml). Cells were grown aerobically in tellurite-amended LB medium until OD$_{600}$ 0.5, washed with 10 mM potassium phosphate buffer (pH 7.0), and
incubated for 30 min in the same buffer containing 10 μM H$_2$DCFDA dissolved in dimethyl sulfoxide. After washing, the cells were suspended in the same buffer and disrupted by sonication. Cell extracts (100 μl) were mixed with 1 ml of phosphate buffer (pH 7.0) and the fluorescence intensity was measured at 2 min intervals over a 30 min period using an Applied Biosystems Cytotoxfluor 4000 Fluorescence multi-well plate reader (excitation, 490 nm; emission, 519 nm). The inset of Fig. 2 represents the emission obtained for all the treatments at a fixed time (28 min). Emission values were normalized by protein concentration.

Determination of protein carbonyl content
The carbonyl content in cellular proteins was determined as described by Semchyshyn et al. [18]. Crude extracts were prepared from E. coli BW25113 cells treated or untreated with K$_2$TeO$_3$ (0.5 μg/ml) or H$_2$O$_2$ (100 μM) for 30 min. Extracts were treated with streptomycin sulfate (2%) and incubated on ice for 15 min. Precipitated nucleic acids were discarded by centrifugation at 14,000 × g for 5 min. After adding four volumes of 10 mM dinitrophenylhydrazine (DNPH, prepared in 2 M HCl) to 100 μl of the nucleic acid-free supernatant, the mixture was incubated at 1 h at room temperature with vortexing every 10–15 min. Proteins were precipitated by adding 500 μl of 20% trichloroacetic acid (TCA) and then sedimented by centrifugation at 14,000 × g for 5 min. The pellet was washed at least three times with an ethanol/ethylacetate mixture (1:1) to remove any unreacted DNPH and redissolved at 37°C with 450 μl guanidine HCl/dithiothreitol. Carbonyl content was determined spectrophotometrically at 370 nm using a molar absorption coefficient of 22,000 M$^{-1}$ cm$^{-1}$ [18].

Determination of thiobarbituric acid-reactive substances (TBARs)
TBARs in cell extracts were determined as described by Rice-Evans et al. [19]. Briefly, 1-ml of cell suspensions were precipitated with 1.0 ml of 20% TCA (w/v) and centrifuged at 10,000 × g for 5 min. Supernatants were mixed with 2.0 ml of a saturated solution containing thiobarbituric acid in 0.1 M HCl and 10 mM butyraldehyde. Samples were then heated for 60 min in a water bath kept at 100°C. Aliquots of 1.5 ml were then removed, chilled, mixed with 1.5 ml of butanol and centrifuged at 4,000 × g for 10 min. The organic fraction was recovered and the OD$_{535}$ was measured spectrophotometrically. TBARs content was determined using a molar extinction coefficient of 156 μM$^{-1}$ cm$^{-1}$ [18,19].

RNA purification and RT-PCR experiments
E. coli BW25113 cells were inoculated in two 200 ml flasks at 1:100 dilution and incubated at 200 rpm at 37°C until the OD$_{600}$ was 0.6. At that moment one culture was amended with K$_2$TeO$_3$ solution (0.5 μg/ml final concentration) and incubated for 10 min, sedimated at 13,000 × g by 3 min and total RNA was isolated using the QIAGEN RNeasy purification kit (Promega) following the vendor recommendations. The ratio OD$_{260/280}$ was determined for the purified RNA using an Agilent 8453 UV-visible spectrophotometer. Independent RT-PCR experiments were performed using 2 μg of total RNA as template. The set of primers used to amplify fragments of approximately 300 bp of E. coli katG, sodA, sodB and ssod genes were skatG 5'-GGTCCGC3CACTAAACCCTTGTTGTC-3', skatG 5'-GAAAGGGCCTGGACTCAGGT3', ssodA 5'-TCCGATAGCCGCTTTAGGCTACC3', ssodB 5'-CG-TGCCATCGCCGTCCGATGCTTA-3', ssodB 5'-TAAACGCGCTTGAATCGGCAA3', ssodS 5'-AAAGATGCTT- GGCCAAGCCCACATTT-3', ssosS 5'-CGAACATATTGAGC- GACGGCGCTTAA-3', and ssoxS 5'-TTACAGGCGGGTCGGC- GATAATGGCT-3'.

RT-PCR conditions included a 30 min incubation at 25°C with RNA-free DNase I (Promega) followed by a DNase-inactivating step of 10 min at 65°C. Synthesis of cDNA was allowed to proceed for 2 h at 42°C using a commercial enzyme and RT-PCR kit (QIAGEN). PCR conditions included an initial denaturation at 95°C for 5 min followed by 20 amplification cycles (95°C for 30 s, 45°C for 30 s, and 72°C for 1 min). A final incubation of 72°C for 10 min was added to ensure full extension of the amplified fragments. PCR products were fractionated on agarose gels (1.5%) and the DNA content was estimated using the Gel Pro 4.0 program (Media Cybernetics).

Determination of in vitro K$_2$TeO$_3$ reduction reaction products
An in vitro K$_2$TeO$_3$ enzymatic reduction assay was developed to demonstrate the generation of superoxide along the course of tellurite reduction. Superoxide radical specifically reduces 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-dihydroxyphenyl)-2H-tetrazolium (WST-1, Cell Technology Lab) to the soluble form WST-formazan that can be easily detected at 438 nm. We have previously established that tellurite can be reduced by different enzymes including catalase [4,26,34]. Reduction mix contained 200 μg/ml of purified bovine liver catalase, 1 mM NADPH, 1 mM potassium tellurite and 50 mM Tris-HCl buffer pH 7.0. Generation of WST-formazan was monitored at 438 nm. Assays in which catalase was replaced by either superoxide dismutase or β-amilase were included as negative controls.

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Author Contributions
Conceived and designed the experiments: CV IC JP DF FA. Performed the experiments: IC JP DF FA GP EF JS MC AE. Analyzed the data: CV IC JP. Contributed reagents/materials/analysis tools: CV. Wrote the paper: CV.

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