Sulfur is an essential element that is required for the biosynthesis of proteins, enzyme cofactors, and other important biomolecules. In bacteria, this element can be assimilated into sulfur-containing amino acids through enzymatic fixation from inorganic sources, such as sulfate and/or thiosulfate (15, 38). Although tellurium shares several chemical properties with sulfur, no biological function for Te is known to date. Conversely, some tellurium compounds, like the oxoanion tellurite (TeO$_3^{2-}$), are extremely toxic for most forms of life, especially microorganisms (34).

It has been proposed that K$_2$TeO$_3$ toxicity could be due to the oxidation of cellular thiols such as glutathione (37) or the generation of superoxide radical during tellurite reduction, which would cause a redox imbalance resulting in intracellular oxidative stress (5, 23, 26, 33, 34, 36).

Maintenance of cell redox balance is one of the most important processes involving molecules synthesized from reduced sulfur taken from the environment. Glutathione (GSH) is one of the major nonprotein thiols in living organisms, including humans, yeast, and bacteria (6, 10). GSH has been involved in resistance to osmotic and oxidative stress as well as in Escherichia coli resistance to the toxic effects of electrophiles like methylglyoxal (6, 11, 31). A protective effect of GSH against oxidative stress has also been described for Lactococcus lactis and Rhodobacter capsulatus (17, 18).

Three genes involved in tellurite resistance have been described previously for the thermotolerant gram-positive rod Geobacillus stearothermophilus V (27, 33, 41). The genes that are involved in the metabolism of cysteine are cysK, iscS, and cobA, and they encode a cysteine synthase (CysK), a cysteine desulfurase (IscS), and a uroporphyrinogen-III C-methyltransferase (SUMT), respectively. CysK catalyzes the last step of inorganic sulfur fixation into l-cysteine, while SUMT is involved in the biosynthesis of siroheme, an essential sulfate reductase cofactor that participates in the inorganic assimilation of sulfur (15). We recently demonstrated that cobA and ubiE genes from G. stearothermophilus V confer increased tolerance to oxyanions of selenium and tellurium when expressed in E. coli (1, 32). Finally, IscS, which yields sulfur and l-alanine from l-cysteine, has been shown to be involved, along with IscA and IscU, in the recovery of [Fe-S] clusters (9, 29).

The purpose of this study was to evaluate the responsiveness of cysK, iscS, and cobA from G. stearothermophilus and some genes of the E. coli Cys regulon in medium containing potassium tellurite. Results indicate that bacterial tolerance to tellurite involves, at least in part, several components of the cysteine metabolic pathway.

**MATERIALS AND METHODS**

**Bacterial strains, culture conditions, and stress induction.** E. coli strains and plasmids used in this work are listed in Table 1. Cells were grown routinely in LB medium (26) at 37°C with shaking. Experiments were initiated with the addition of 1:100 dilutions of overnight cultures to the medium. Cell cultures reaching an optical density at 600 nm (OD$_{600}$) of $\approx$0.6 were amended with K$_2$TeO$_3$ (0.5 µg/ml), H$_2$O$_2$ (125 µg/ml), diamide (850 µg/ml), or paraquat (250 µg/ml), as required. Controls received equal volumes of sterile water.

**Antimicrobial disk assay and determination of MICs.** Overnight cultures of E. coli or its derivatives were diluted 100-fold with LB medium. Ten microliters of K$_2$TeO$_3$ (10 µg/ml), H$_2$O$_2$ (1.25 mg), or paraquat (3.0 mg) was then spotted independently onto sterile filter paper disks that were placed in the center of the plates. Growth inhibition zones were determined after incubation at 37°C for 24 h. To determine MICs, cells were grown with shaking at 37°C in LB medium supplemented with appropriate concentrations of the compounds under study (7).

**RNA extraction, plasmid construction, and cell transformation.** RNA purification and DNA sequencing was performed using the RNeasy kit (QIAGEN). Briefly, cultures of E. coli K-12 or its derivatives (OD$_{600}$ of $\approx$0.6) were split in two and one was amended with 0.5 µg/ml K$_2$TeO$_3$ and incubated for 10 min. Cells were sedimented at 13,000 × g for...
TABLE 1. Bacterial strains and plasmids

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant characteristic(s)</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>E. coli</em> TOP10</td>
<td>F^− mcrA Δ(mrr-hsdS-MCR-mcrBC) d80lacZΔM15 ΔlacX74 deoR rupG recA1 araD139 Δ(ara-leu)7697 gntU galK rpsL(Str^r) endA1 λ^−</td>
<td>Invitrogen</td>
</tr>
<tr>
<td><em>E. coli</em> BW25113</td>
<td>lac^− rmB^−, ΔlacZ99, hisΔR514 ΔaraBAD^−, ΔgalRAΔΔ303 DmcrAB^−</td>
<td>Datsenko and Wanner (8)</td>
</tr>
<tr>
<td>JW2415</td>
<td><em>E. coli</em> BW25113 ΔcysA</td>
<td>Baba et al. (3)</td>
</tr>
<tr>
<td>JW2720</td>
<td><em>E. coli</em> BW25113 ΔcysC</td>
<td>Baba et al. (3)</td>
</tr>
<tr>
<td>JW2722</td>
<td><em>E. coli</em> BW25113 ΔcysD</td>
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<tr>
<td>JW3582</td>
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<td>JW2414</td>
<td><em>E. coli</em> BW25113 ΔcysM</td>
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</tr>
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<td>E. coli AB734</td>
<td>F^− lac− (del)</td>
<td>Shapiro and Baneyx (30)</td>
</tr>
<tr>
<td>E. coli ADA110</td>
<td>ABT734 ΔαβδG::lacZ</td>
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</tr>
<tr>
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<tr>
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<td>ABT734 ΔαβδG::lacZ</td>
<td>C. Vásquez (39)</td>
</tr>
<tr>
<td><em>G. stearothermophilus</em> V</td>
<td>Wild-type Tel^−</td>
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</tr>
<tr>
<td><strong>Plasmids</strong></td>
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<td>Cloning vector, Ap^r Tet^−</td>
<td>Bolivar et al. (4)</td>
</tr>
<tr>
<td>pBluescript-SK</td>
<td>Cloning vector, Ap^r</td>
<td>Stratagene</td>
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<td><em>G. stearothermophilus</em> iscS gene cloned in pBluescript-SK, Ap^r</td>
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<td><em>G. stearothermophilus</em> iscS gene cloned in pBluescript-SK, Ap^r</td>
<td>This study</td>
</tr>
</tbody>
</table>

a Tel^−, tellurite resistance; Tet^−, tetracycline resistance; Ap^r, ampicillin resistance.

3 min and used for RNA extraction. The OD_{260nm}/OD_{280nm} ratio for the purified RNAs was determined with an Agilent 2100 Bioanalyzer spectrophotometer.

*G. stearothermophilus* V, *cobA*, *cysK*, and *iscS* genes containing 300 bp upstream of their ATG initiation codons were amplified by PCR using primers listed in Table 2. PCR conditions included an initial denaturation at 95°C for 5 min, followed by 30 amplification cycles (95°C for 30 s, 55°C for 30 s, and 72°C for 30 s). A final incubation at 72°C for 10 min was included to ensure complete extension of the amplified fragments. PCR products were cloned in pGEMT Easy (Invitrogen). Recombinant plasmids were digested with HindIII, and the released fragments were purified and cloned independently into the medium-copy-number vector pBR322 or the high-copy-number vector pBluescript-SK (Table 1).

*E. coli* TOP10 cells were used in all transformations. Cells were made competent by electroporation (MicroPulse, Bio-Rad) using 0.2-cm cuvettes and a 2.5-kV pulse.

**β-Galactosidase assay.** *E. coli* cultures (1.5 ml) harvested at different time intervals were subjected to centrifugation at 13,000 × g for 3 min. Cells were permeabilized by the addition of 1.5 ml ice-cold buffer Z (40 mM NaH_2PO_4, 60 mM NaHPO_4, 10 mM KCl, 1 mM MgSO_4, and 50 mM β-mercaptoethanol, pH 7.5) and assayed for β-galactosidase activity. The o-nitrophenyl-β-D-galactopyranoside (ONPG) substrate was used as described previously by Miller (20). Protein concentration was estimated using the Bradford reagent (Sigma).

**Bioinformatics and computation analysis.** Sequence analysis and primer design were performed using Vector NTI 8.0 (Informax, Inc.). Nucleotide sequences of the *G. stearothermophilus* V *cobA, cysK*, and *iscS* genes and those of the *E. coli* cys regulon (*cysD, cysB, cysC, cysE, cysG, cysL, cysK, cysM, and cbl*) were obtained from GenBank (accession numbers Y427647, AF533655, AF196621, and NC_009013). Analysis of variance statistical analyses were performed with a 0.05 level of confidence.

**Real-time RT-PCR.** The induction of gene expression was calculated based on the difference between the crossing points of each quantitative reverse transcription-PCR (RT-PCR) determination (Cp − Cpt, where Cp and Cpt are the crossing points in the absence and presence of tellurite, respectively). Crossing points define the cycle at which the amplification becomes exponential, and they are inversely proportional to the amount of the specific RNA template present in the sample.

Approximately 2 μg of RNA from control or tellurite-treated cells was used for real-time RT-PCR experiments using a LightCycler RNA amplification kit (Roche Applied Science) according to instructions of the vendor. The specific oligonucleotide primers used are listed in Table 2. PCR products were visualized and analyzed using a LightCycler 2.0 instrument.

**RESULTS**

Behavior of *E. coli* strains carrying *G. stearothermophilus* genes in tellurite-containing medium. *G. stearothermophilus* V *cobA, cysK*, and *iscS* genes containing their own promoters were separately cloned in the medium- and high-copy-number plasmids pBR322 and pBluescript-SK, respectively. The resulting recombinant plasmids were used to transform *Escherichia coli*, and bacterial tolerance to K_2TeO_3 and other oxidative stress inducers was evaluated by measuring growth inhibition zones in tellurite-amended solid medium. Figure 1 shows that pBRcoba, pBRcysK, and pBRiscS increased *E. coli* resistance approximately 20% for tellurite, 25% for paraquat, 25% for diamide, and 20% for hydroperoxide. In turn, *E. coli* cells carrying pSiscK, pSiscK, and pSiscS exhibited an additional 20% tolerance to K_2TeO_3 and hydroperoxide (see Fig. S1S in the supplemental material), suggesting a gene dosage effect. This behavior was not observed for the thiol reducer diamide (data not shown).
To circumvent the repression of cysteine biosynthesis in rich medium, the same experiments were conducted in M9 minimal medium, the same experiments were conducted in M9 minimal medium. The parental isogenic G. stearothermophilus strain AB734 was used as a control. Sublethal concentrations of K₂TeO₃ had very little effect on P̂3rpoH and sulA activation, while E. coli ADA110 and ADA310 showed increased β-galactosidase activities, suggesting that tellurite affects cytoplasmic proteins and has no evident effect on DNA (not shown).

Real-time RT-PCR was used to monitor the functional capacity of the G. stearothermophilus V cobA, cysK, and iscS genes promoters by K₂TeO₃. Alignment of nucleotide sequences upstream of the ATG initiation codon of cobA, cysK, and iscS showed similarities of 40 to 44%. No binding motifs to E. coli or Bacillus subtilis sigma factors such as σ₇₀ and σ₄₃ were found.

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cysK, 2.54 for iscS, 5.02 for ubiE, and −0.95 for gapA. Thus, while expression of the *G. stearothermophilus* V genes increased in the presence of potassium tellurite, that of the gapA housekeeping gene decreased by approximately 10% (Fig. 3).

Expression of *E. coli* cysteine metabolism-related genes in cells exposed to potassium tellurite. An experimental approach similar to that described in the previous section was followed to evaluate the effect of potassium tellurite on the expression of *E. coli* cysteine metabolism genes. The participation of Cys regulon genes in tellurite tolerance was investigated using *E. coli* BW25113 and its isogenic derivatives cysA, cysC, cysE, cysG, cysI, cysK, cysM, and cbl. The loss of cysteine metabolism-related genes is paralleled with an increase of growth inhibition areas for all toxic compounds included in this study. In general, strains ΔcysA, ΔcysK, ΔcysM, Δcbl, and ΔcysC exhibited the highest sensitivity to the reactive oxygen species (ROS) generators paraquat and hydrogen peroxide as well as to potassium tellurite (Fig. 4).

Total RNA from untreated cells and cells treated with potassium tellurite was used, along with specific primers for each gene of the regulon, as templates for performing real-time RT-PCR (Fig. 5). Most of the *E. coli* cysteine metabolism-related genes showed increased expression when cells were grown in the presence of potassium tellurite. The ΔCp values were 0.92 for cysA, 0.32 for cysB, 0.19 for cysC, 1.06 for cysE, 0.35 for cysG, 1.06 for cysI, 1.17 for cysM, 2.04 for cysK, 2.94 for cbl, and −1.3 for gapA.

**DISCUSSION**

Living organisms require sulfur for the biosynthesis of proteins and other essential enzymatic cofactors and reducing
agents. After incorporation into the cell, sulfur is reduced to sulfide that then reacts with O-acetyl-L-serine to form L-cysteine (15). Cysteine has several vital functions in the catalytic cycle of many enzymes, is part of important reducing agents like glutathione, and is required for the biosynthesis and repair of [Fe-S] centers of several essential proteins, including cytochromes, fumarases, and aconitases.

Cysteine-containing molecules like glutathione and thioredoxin play a major role in maintaining an intracellular reducing environment and protecting the cell from oxidative damage (6, 12, 22). It has been reported that GSH is ubiquitous in most gram-negative bacteria and is absent in most gram-positive organisms examined so far (10, 18). These observations suggest that glutathione is not unique in protecting against oxidative stress or maintaining the redox balance of cells. It has been shown that thioredoxin 1 (TrxA), thioredoxin 2 (TrxB), and thioredoxin reductase (TrxC) are not essential for survival in E. coli. trxA and trxB cells exhibit increased sensitivity to H2O2, a phenotype that is not observed in trxC cells (25). E. coli strains with mutations in the GSH system are also viable. For example, gshA and gshB mutants and gorA mutants, encoding the two enzymes of glutathione biosynthesis and glutathione reductase, respectively, exhibit increased sensitivity to the GSH-oxidizing agent diamide (2). Prinz et al. (24) determined that E. coli requires the thioredoxin or the GSH/Grx system to grow aerobically and that mutants in these systems were incapable of reducing cytoplasmic disulfide bonds.

We have previously identified three G. stearothermophilus V genes (cobA, cysK, and iscS) that encode enzymes involved in cysteine metabolism and whose expression mediates tellurite resistance in E. coli (33, 40, 41). The participation of cysK, cysM, and iscS genes in tellurite resistance as well as in oxidative stress has been also documented with other microorganisms (19, 21, 26).

Tellurite toxicity is thought to result from the oxidizing character of this molecule. More recently, the idea that tellurite would damage the cell through the establishment of oxidative stress has emerged. This stress condition could be a consequence of the drastic decrease of the concentration of cellular antioxidants and/or could be associated with the presence of some kind of ROS. The observation that superoxide generation results from enzymatic tellurite reduction supports this assumption (5, 23).

The above considerations allow the speculation that an increase in cellular antioxidants would result in higher tolerance to ROS elicitors. Expression of G. stearothermophilus V genes containing their own promoters in medium- or high-copy-number plasmids supports this idea (Fig. 1; see Fig S1S in the supplemental material).

To date, most studies concerning bacterial response to oxidative stress have been focused on hydrogen peroxide-, alkylhydroperoxide-, or superoxide-induced stress. Little is known about disulfide stress, a subcategory of oxidative stress that causes the accumulation of nonnative disulfide bonds in the
cytoplasm. E. coli cells were also exposed to the thiol-specific oxidant diamide. E. coli cells harboring the _G. stearothermophilus_ V _cobA_, _cysK_, and _iscS_ genes exhibited smaller growth inhibition zones than controls that did not express these genes, suggesting that they have a protective effect against the toxic effect of diamide. Leichert et al. (16) reported that _B. subtilis_ isolates independently exposed to hydrogen peroxide or diamide showed similar gene expression profiles, suggesting that they share the same response mechanism. These results agree with our observation that the expression of _G. stearothermophilus_ V genes protects _E. coli_ against the toxic effects of hydrogen peroxide and diamide.

This protective effect was further observed in transformed _E. coli_ reporter strains ADA110 and ADA310 (30), which exhibited important reductions of β-galactosidase activity when exposed to K₂TeO₃. The levels of β-galactosidase in these _E. coli_ ADA strains expressing _Geobacillus_ genes were indistinguishable from those of controls grown in the absence of tellurite (Fig. 2).

It is well known that living organisms have evolved defense mechanisms to maintain the cell's homeostasis under adverse conditions. In _Escherichia coli_, for example, temperature up-
shifts and other kinds of stress induce the synthesis of heat shock proteins when misfolded proteins accumulate in the cytoplasm (13). In this context, gene induction by tellurite poisoning has been reported for E. coli and Proteus mirabilis. The E. coli gusB gene and P. mirabilis terZABCDE operon are positively regulated by tellurite (14, 35). The presence of sequences similar to OxyR binding motifs in the ter operon of P. mirabilis suggested that such induction would be dependent on this transcriptional regulator. However, no OxyR-like binding motifs were found within regulatory regions of the G. stearothermophilus V genes (not shown), suggesting that the K2TeO3-induced positive regulation observed in Fig. 3 is not dependent on this regulator.

We further studied the involvement of the E. coli Cys regulon genes in tellurite-amended medium. Results showed that strains defective in cysteine metabolism-related genes exhibited higher sensitivity to K2TeO3 than did the isogenic, parental, wild-type strain (Fig. 4), suggesting that most of the elements of the cysteine biosynthetic pathway are required to manage the tellurite-induced stress. In addition, all the Cys regulon genes studied here were turned on in the presence of tellurite even in rich medium like LB, where transcription of the cysB and cysE regulatory genes is repressed (Fig. 5).

In conclusion, we have shown that genes of cysteine biosynthesis are induced in the presence of potassium tellurite. The behavior of the Cys regulon elements could reflect a tellurite-mediated derepression, which in turn could be explained by the depilation of cellular thiols or sulfur-containing molecules such as glutathione.

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