

## Identification and Molecular Genetic Analysis of Multiple Loci Contributing to High-Level Tellurite Resistance in *Rhodobacter sphaeroides* 2.4.1

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The ability of the facultative photoheterotroph *Rhodobacter sphaeroides* to tolerate and reduce high levels of tellurite in addition to at least 10 other rare earth metal oxides and oxyanions has considerable potential for detoxification and bioremediation of contaminated environments. We report the identification and characterization of two loci involved in high-level tellurite resistance. The first locus contains four genes, two of which, *trgAB*, confer increased tellurite resistance when introduced into the related bacterium *Paracoccus denitrificans*. The *trgAB*-derived products display no significant homology to known proteins, but both are likely to be membrane-associated proteins. Immediately downstream of *trgB*, the *cysK* (cysteine synthase) and *orf323* genes were identified. Disruption of the *cysK* gene resulted in decreased tellurite resistance in *R. sphaeroides*, confirming earlier observations on the importance of cysteine metabolism for high-level tellurite resistance. The second locus identified is represented by the *tela* gene, which is separated from *trgAB* by 115 kb. The *tela* gene product is 65% similar to the product of the *klaB* (*tela*) gene from the tellurite-resistance-encoding *kilA* operon from plasmid RK2. The genes immediately linked to the *R. sphaeroides tela* gene have no similarity to other components of the *kilA* operon. *R. sphaeroides tela* could not functionally substitute for the plasmid RK2 *tela* gene, indicating substantial functional divergence between the two gene products. However, inactivation of *R. sphaeroides tela* resulted in a significant decrease in tellurite resistance compared to the wild-type strain. Both *cysK* and *tela* null mutations readily gave rise to suppressors, suggesting that the phenomenon of high-level tellurite resistance in *R. sphaeroides* is complex and other, as yet uncharacterized, loci may be involved.

The extraordinary biochemical versatility of *Rhodobacter sphaeroides* 2.4.1 has long been of interest, and much effort has been directed towards understanding the expression and integration of such varied metabolic processes as aerobic and anaerobic respiration, anaerobic photosynthesis, and nitrogen and carbon dioxide fixation in this organism. However, this metabolic flexibility also lends itself to a possible variety of biotechnological and environmental applications. Surprisingly little research has been carried out in this area, particularly in light of the considerable advances in the application of molecular genetics to photosynthetic bacteria (7, 45), with the result that the capacity of *R. sphaeroides* and related photosynthetic bacteria for exploitation in such applications remains largely unexplored.

The resistance of *R. sphaeroides* to an array of toxic heavy metals has much potential in terms of bioremediation and the detoxification of contaminated aquatic environments (21). Furthermore, use of the genetic determinants of resistance and/or detoxification from *R. sphaeroides* in constructing recombinant soil bacteria and plants could greatly increase their bioremediation potential.

We have previously characterized the ability of *R. sphaeroides* 2.4.1 to catalyze the reduction of extremely high con-

centrations (in the millimolar range) of the metalloid oxyanions tellurite and selenite to their ground state (20). A membrane-localized flavin-dependent reductase was suggested to play a crucial role in tellurite reduction; however, it has not yet been identified (20). It is worth noting here that there is a distinction to be made between tellurite reduction and resistance to tellurite. The latter could possibly involve exclusion or efflux of the oxyanion from the cell or chemical modifications different from reduction. Overall, little is known about the mechanism(s) of tellurite resistance in *R. sphaeroides*.

Most gram-negative bacteria are sensitive to very low concentrations of tellurite. However, in addition to *R. sphaeroides*, tellurite resistance in obligately aerobic photosynthetic bacteria and *Thermus* spp. has also been reported (3, 4, 44). So far, five tellurite resistance determinants from gram-negative bacteria, primarily members of the family *Enterobacteriaceae*, have been characterized. Four were isolated from plasmids: two from the IncHI-2 plasmids R478 (40–42) and pMER610 (13–15), both of which were designated *terABCDE*; the *kilA* (*klaA-klaBtelB*) operon from the IncP $\alpha$  plasmid RK2 (37); and the *arsRDABC* locus from the IncF1 plasmid R773 (2, 26, 32). The fifth determinant, *tehAB*, initially believed to have been cloned from the IncHII plasmid (pHH1508a) (38), was subsequently localized to the terminus of the *Escherichia coli* chromosome (31). Interestingly, apart from the similarity between the tellurite resistance loci from the IncHI-2 plasmids, there is no protein sequence homology between the above determinants. Further, apart from the *arsRDABC* locus, which encodes an oxyanion efflux transporter, the mechanism(s) by which the other loci encode resistance to tellurite remains unclear. Three possibilities have been proposed, namely, the direct exclusion, efflux, or reduction of tellurite from or by the cell (18), and two

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recent reports provide indirect evidence which implicates tellurite reduction as the most likely mechanism of resistance encoded by the *terABCDE*, *tehAB*, and *kilA* determinants (17, 34).

Here we report the identification, cloning, sequence, and inactivation of two tellurite resistance determinants from *R. sphaeroides* 2.4.1. The contribution of both loci to high-level tellurite resistance in *R. sphaeroides* is described, and possible mechanisms of tellurite resistance are discussed. The results of this study indicate that multiple loci ensure high-level tellurite resistance in *R. sphaeroides*, some of which may be widespread among different bacterial taxa.

## MATERIALS AND METHODS

**Bacterial strains, plasmids, and growth conditions.** Strains and plasmids used in this work are described in Table 1.

*E. coli* strains were grown at 37°C on LB medium (19) supplemented, when required, with the following antibiotics: tetracycline, 15 µg/ml; ampicillin, 50 µg/ml; trimethoprim, 50 µg/ml; kanamycin, 50 µg/ml; streptomycin and spectinomycin, 50 µg/ml. *R. sphaeroides* and *Paracoccus denitrificans* strains were grown at 30°C on Sistrom's medium A (5) containing succinate as the carbon source supplemented as required with the following antibiotics: tetracycline, 1 µg/ml; kanamycin, 50 µg/ml; trimethoprim, 50 µg/ml; streptomycin and spectinomycin, 50 µg/ml each. Photosynthetic cultures were grown at an incident light intensity of 10 W/m<sup>2</sup> in screw-capped tubes completely filled with liquid media. Strains to be tested for tellurite resistance levels were plated on agar plates containing appropriate levels of tellurite and grown aerobically at 30°C. This approach differs from the method previously described by Moore and Kaplan (20), in which strains being tested for tellurite resistance were inoculated at 5 × 10<sup>7</sup> cells/ml and grown in liquid culture. The liquid culture method may mask the selection of hyperresistant variants among the cells being tested. This could explain the quantitative differences observed between tellurite resistance levels determined by the earlier approach (20) and the approach used in this study. In the present tests, the wild-type strain showed resistance to approximately 150 µg of potassium tellurite per ml.

**Screen for *R. sphaeroides* 2.4.1 genes conferring increased tellurite resistance in *P. denitrificans*.** Previous studies have demonstrated that many *R. sphaeroides* genes are not expressed in *E. coli*, but they are efficiently expressed in the closely related nonphotosynthetic soil bacterium *P. denitrificans* (10, 11). Unlike *R. sphaeroides* 2.4.1, *P. denitrificans* is resistant to only moderate levels of tellurite (approximately 40 µg/ml). Thus, in order to identify cosmids conferring tellurite resistance, an *R. sphaeroides* cosmid library consisting of approximately 800 clones was introduced into *P. denitrificans*. Exconjugants were grown on Sistrom's medium A containing 150 µg of tellurite per ml to select for those cosmids conferring tellurite resistance.

**DNA manipulations, sequence determination, and analysis.** Standard protocols or manufacturer's instructions were followed for DNA isolation and recombinant DNA procedures (19). Pulsed-field gel electrophoresis was performed as previously described (30). DNA sequencing was performed with an ABI 373A automatic DNA sequencer (Applied Biosystems, Inc., Foster City, Calif.) at the DNA Core Facility of the Department of Microbiology and Molecular Genetics, the University of Texas Health Science Center, Houston. Oligonucleotides used for priming standard sequencing reactions were synthesized at the DNA Core Facility or purchased from BRL Life Technologies. DNA and protein sequence analysis was performed with Genetics Computer Group software and tools available from the Baylor College of Medicine Search Launcher (<http://kiwi.imgen.bcm.tmc.edu:8088/search-launcher/launcher.html>).

**Conjugation techniques.** Plasmids were mobilized in biparental matings from *E. coli* S17-1 strains into *R. sphaeroides* or *P. denitrificans* strains essentially as described elsewhere (6).

**Construction of *R. sphaeroides* mutants.** Disruption of the *trgB* gene was achieved by insertion of an ΩSm<sup>r</sup> Sp<sup>r</sup> cassette into the *SacI* site 205 bp from the start of the structural gene in plasmid pUI2816. Deletion of the *trgA* and *trgB* genes involved replacement of the 1,374-bp *SplI-SacII* fragment from pUI2815 with an ΩSm<sup>r</sup> Sp<sup>r</sup> cassette, thereby removing the entire *trgB* gene and approximately 200 bp of the *trgA* gene. To disrupt *cysK*, an ΩSm<sup>r</sup> Sp<sup>r</sup> cassette was introduced into the *NcoI* site 580 bp from the 5' end of the structural gene in plasmid pUI2815. These three constructs were cloned into pSUP202Km or pSUP203 to create pUI2817 (Δ*trgAB*::ΩSm<sup>r</sup> Sp<sup>r</sup>), pUI2820 (*trgB*::ΩSm<sup>r</sup> Sp<sup>r</sup>), and pUI2818 (*cysK*::ΩSm<sup>r</sup> Sp<sup>r</sup>) and mobilized by conjugation into *R. sphaeroides* 2.4.1. Putative double-crossover recombinants (Sm<sup>r</sup> Tc<sup>s</sup>) were selected and analyzed further.

The *R. sphaeroides* *telA* gene was disrupted by introduction of the Tp<sup>r</sup> gene from pSUP5TpMCS into the *HincII* site (p479BS::Tp). A 5.1-kb *PvuII* fragment from pSUP202 containing the *mob* region and the Tc<sup>r</sup> gene was then cloned into p479BS::Tp, and the resulting plasmid p479BS::Tp::mob was mobilized by conjugation into *R. sphaeroides* 2.4.1. Mutant ORFB1 was constructed by inserting an ΩKm<sup>r</sup> cassette into the *Bsu36I* site immediately downstream of the *telA* stop

codon (p479BS::Km<sup>r</sup>). A 4.2-kb *MscI* fragment from pSUP202 containing the *mob* region and the Tc<sup>r</sup> gene was then cloned into p479BS::Km, and the resulting plasmid p479BS::Km::mob was mobilized by conjugation into *R. sphaeroides* 2.4.1. Putative double-crossover recombinants (Tp<sup>r</sup> Tc<sup>s</sup> for *telA* disruption and Km<sup>r</sup> Tc<sup>s</sup> for *orfB* disruption) were selected and analyzed further. The genomic structures of all mutant strains were confirmed by pulsed-field gel electrophoresis and/or Southern hybridization.

**Southern hybridization analysis.** Hybridizations were performed according to the protocol described for Quikhyb rapid hybridization solution (Stratagene). DNA probes were oligolabeled with [α-<sup>32</sup>P]dCTP with random primer (New England Biolabs) and Sequenase enzyme (United States Biochemical) as described elsewhere (27).

**Nucleotide sequence accession numbers.** The nucleotide sequences of the DNA fragments containing *trgAB cysK orf323* and *telA orfB* have been submitted to GenBank under the accession no. AF004296 and AF019377, respectively.

## RESULTS

**Isolation of cosmids from an *R. sphaeroides* 2.4.1 library conferring increased tellurite resistance in *P. denitrificans*.** Two cosmids from an *R. sphaeroides* 2.4.1 library conferring resistance to 150 µg of tellurite per ml in *P. denitrificans* were identified (see Materials and Methods). Restriction analysis of the cosmids, pUI8131 and pUI8381, revealed that as much as 12 kb of insert DNA was common to both cosmids and that, by extension, the same locus conferring resistance to tellurite was likely to be present on both cosmids. Further analysis performed on cosmid pUI8131 demonstrated that the tellurite resistance phenotype was localized on a 4.4-kb *BamHI-PstI* fragment (Fig. 1, pUI2812; Table 2). A 2.0-kb *BamHI-BglII* fragment (Fig. 1, pUI2813) subcloned from pUI2812 retained the ability to confer tellurite resistance in *P. denitrificans*, whereas deletion of an internal 2.4-kb *SacI* fragment from pUI2812 generated a cosmid (Fig. 1, pUI2814) unable to confer increased tellurite resistance to *P. denitrificans* (Table 2).

**Sequence analysis of the tellurite resistance locus from cosmid pUI8131.** The 2.0-kb *BamHI-BglII* fragment from pUI2813 and the larger 4.2-kb *PstI* fragment from pUI2812 (Fig. 1) were subcloned into the cloning vector pBGS8 (Fig. 1, pUI2815; pUI2816), and together with the cosmid pUI8131, the complete DNA sequence for both strands of the 4.4-kb *BamHI-PstI* fragment was determined with specific oligonucleotide primers. Five potential open reading frames (ORFs) were identified (Fig. 1). Two complete ORFs, designated *trgA* and *trgB* (tellurite resistance gene), were identified on the minimum clone (pUI2813) conferring tellurite resistance in *P. denitrificans*. Together, these genes appear to form an operon, with the stop codon of *trgA* overlapping with the start codon for *trgB*. However, in a database search no homologs for the predicted 146- and 361-amino-acid sequences encoded by *trgA* and *trgB*, respectively, were identified. Nevertheless, the codon usage pattern of both genes matches that of functionally characterized *R. sphaeroides* genes. Computer predictions also revealed one potential membrane-spanning domain at the amino terminus of the TrgB protein, a possibility consistent with a role for this protein in tellurite resistance-reduction. Similar analysis predicted the presence of four potential membrane-spanning domains in the protein encoded by *trgA*. Importantly, cosmid pUI2814, containing an internal *SacI* deletion, which removed most of the *trgB* gene but not *trgA*, was incapable of conferring tellurite resistance in *P. denitrificans*, indicating that the *trgA* gene alone does not encode this phenotype.

An incomplete ORF was also identified on the minimum clone conferring tellurite resistance in *P. denitrificans*. This incomplete ORF is predicted to encode 159 amino acids of a protein which displays approximately 26% identity and 36% similarity with the C-terminal portion of the 381-amino-acid *E. coli* cyclopropane fatty acid synthase (*cfa*) gene product (reference 39 and data not shown) and was designated *orfI*. How-

TABLE 1. Strains and plasmids

Strain or plasmid	Relevant characteristic(s)	Source or reference
<i>R. sphaeroides</i>		
2.4.1	Wild type	W. Sistrom
TRGB1	<i>trgB</i> :: $\Omega$ Sp <sup>r</sup> St <sup>r</sup>	This study
TRGAB1	$\Delta$ <i>trgAB</i> :: $\Omega$ Sp <sup>r</sup> St <sup>r</sup>	This study
CYSK1	<i>cysK</i> :: $\Omega$ Sp <sup>r</sup> St <sup>r</sup>	This study
TELA1	<i>telA</i> ::Tp <sup>r</sup>	This study
ORFB1	<i>orfB</i> :: $\Omega$ Km <sup>r</sup>	This study
<i>P. denitrificans</i>		
ATCC 17741	Wild type	ATCC <sup>a</sup>
<i>E. coli</i>		
DH5 $\alpha$ phe	( $\phi$ 80 <i>dlacZ</i> $\Delta$ M15) $\Delta$ <i>lacU169 recA1 endA1 hsdR17 supE44 thi-1 gyrA96 relA1 phe</i> ::Tn10 <i>dCm</i>	9
S17-1	Res <sup>-</sup> Mod <sup>+</sup> <i>recA proA thi</i> ; integrated plasmid RP4-Tc::Mu-Km::Tn7 Tra <sup>+</sup>	28
Plasmids		
pRK415	Mob <sup>+</sup> <i>lacZ</i> $\alpha$ Tc <sup>r</sup> IncP	16
pBGS8	Km <sup>r</sup> cloning vector	29
pUC19	Ap <sup>r</sup> cloning vector	Gibco BRL
pHP45 $\Omega$	Source of $\Omega$ Sp <sup>r</sup> Sm <sup>r</sup> cassette	25
pHP45 $\Omega$ Km	Source of $\Omega$ Km <sup>r</sup> cassette	25
pSUP202/203	pBR325 Mob <sup>+</sup> Ap <sup>r</sup> Cm <sup>r</sup> Tc <sup>r</sup> ColE1; suicide vectors	28
pSUP5TpMCS	pBR325 Mob <sup>+</sup> Ap <sup>r</sup> Cm <sup>r</sup> Tc <sup>r</sup> Tp <sup>r</sup> ColE1; suicide vector	C. Mackenzie
pSUP202Km	Derivative of pSUP202 containing $\Omega$ Km <sup>r</sup> cassette in <i>Hind</i> III site	S. Dryden
pUI8131	Cosmid containing <i>trgA</i> , <i>trgB</i> , <i>cysK</i> , and <i>orf323</i>	8
pUI2812	Derivative of pUI8131 containing 4.4-kb <i>Bam</i> HI- <i>Pst</i> I fragment encompassing <i>trgA</i> , <i>trgB</i> , <i>cysK</i> , and <i>orf323</i>	This study
pUI2813	2.0-kb <i>Bam</i> HI- <i>Bgl</i> II fragment containing <i>trgA</i> and <i>trgB</i> genes in pRK415	This study
pUI2814	pUI2812 derivative with internal 2.4-kb <i>Sac</i> I deletion	This study
pUI2815	4.2-kb <i>Pst</i> I fragment containing <i>trgA</i> , <i>trgB</i> , <i>cysK</i> , and <i>orf323</i> in pBGS8	This study
pUI2816	2.0-kb <i>Bam</i> HI- <i>Bgl</i> II fragment containing <i>trgA</i> and <i>trgB</i> genes in pBGS8	This study
pUI2817	$\Delta$ <i>trgAB</i> :: $\Omega$ Sp <sup>r</sup> Sm <sup>r</sup> in pSUP203	This study
pUI2818	<i>cysK</i> :: $\Omega$ Sp <sup>r</sup> Sm <sup>r</sup> in pSUP203	This study
pUI2819	1.9-kb <i>Pvu</i> II fragment containing <i>cysK</i> gene in pRK415	This study
pUI2820	<i>trgB</i> :: $\Omega$ Sp <sup>r</sup> Sm <sup>r</sup> in pSUP202Km	This study
pUI8479	Cosmid containing <i>R. sphaeroides telA</i>	8
p479-BsS	1.8-kb <i>Sal</i> I- <i>Bsu</i> 36I fragment containing <i>R. sphaeroides telA</i> cloned downstream of P <sub>lac</sub> in pRK415	This study
p479BH	6.8-kb <i>Hind</i> III- <i>Bam</i> HI fragment containing <i>R. sphaeroides telA</i> , <i>orfB</i> , and upstream and downstream genes in pUC19	This study
p479BS	3.7-kb <i>Sal</i> I- <i>Bam</i> HI fragment containing <i>R. sphaeroides telA</i> , <i>orfB</i> , and downstream gene(s) under P <sub>lac</sub> in pUC19	This study
p479BS::Tp <sup>r</sup>	p479BS containing the Tp <sup>r</sup> gene cloned into the <i>Hinc</i> II site of <i>R. sphaeroides telA</i> ; <i>telA</i> ::Tp <sup>r</sup>	This study
p479BS::Tp <sup>r</sup> ::mob	p479BS::Tp <sup>r</sup> containing the 5.1-kb <i>Pvu</i> II fragment including <i>mob</i> region and Tc <sup>r</sup> gene from pSUP202	This study
p479BS::Km <sup>r</sup>	p479BS containing $\Omega$ Km <sup>r</sup> cloned into the <i>Bsu</i> 36I site immediately downstream of <i>R. sphaeroides telA</i> ; <i>orfB</i> :: $\Omega$ Km <sup>r</sup>	This study
p479BS::Km::mob	p479BS::Km containing the 4.2-kb <i>Msc</i> I fragment including <i>mob</i> region and Tc <sup>r</sup> gene from pSUP202	This study
pDT1558	<i>klaA klaB telB</i> operon from tellurite-resistant derivative of plasmid RK2 cloned in pUC19	36
pDT1558 $\Delta$	pDT1558 containing a 0.24-kb <i>Sma</i> I (1462)- <i>Msc</i> I (1701) deletion in <i>klaB</i> (GenBank accession no. M38697 coordinates); <i>klaA klaB' telB</i> . Does not confer tellurite resistance in <i>E. coli</i>	This study

<sup>a</sup> ATCC, American Type Culture Collection.

ever, it is clear that only the 3' end of *orfI* is present on pUI2813 and that it is therefore unlikely to contribute to the tellurite resistance phenotype encoded by this clone.

Approximately 300 bp downstream of *trgB*, another ORF was identified. This ORF displays strong homology with the genes from many other organisms encoding isoenzymes (*O*-acetylserine [thiol] lyase A or B) for the terminal step of cysteine biosynthesis. In *E. coli* and *Salmonella typhimurium*,

the genes encoding these isoenzymes are designated *cysK* and *cysM*. Although the gene identified in this study has the same degree of homology to both *cysK* and *cysM* from *E. coli* (Fig. 2), it has been designated *cysK*.

Immediately downstream of *cysK*, a second ORF was identified, which appeared to form an operon with *cysK*. This ORF had no matches in the database and was designated *orf323*, based on the predicted length of the encoded polypeptide.

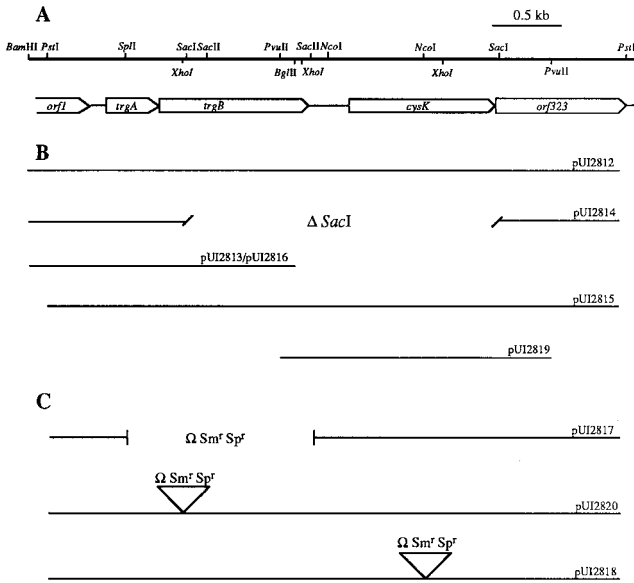


FIG. 1. (A) Physical and restriction maps of the *R. sphaeroides* *trgAB cysK orf323* region. (B) Cosmids and plasmids used for subcloning and complementation. (C) Plasmids used to construct the *trgB*, *trgAB*, and *cysK* chromosomal mutations. The  $\Omega$  cassettes were inserted at the positions indicated.

Further analysis identified two and four potential membrane-spanning domains in the CysK and Orf323 proteins, respectively. It is therefore tempting to speculate that the TrgA, TrgB, CysK, and Orf323 proteins may constitute two membrane-associated complexes or possibly one larger complex.

**Construction and characterization of the *trgA*, *trgB*, and *cysK* null mutations.** The role of the *trgB* gene in conferring increased tellurite resistance when introduced into *P. denitrificans* suggested that it may also be involved in high-level

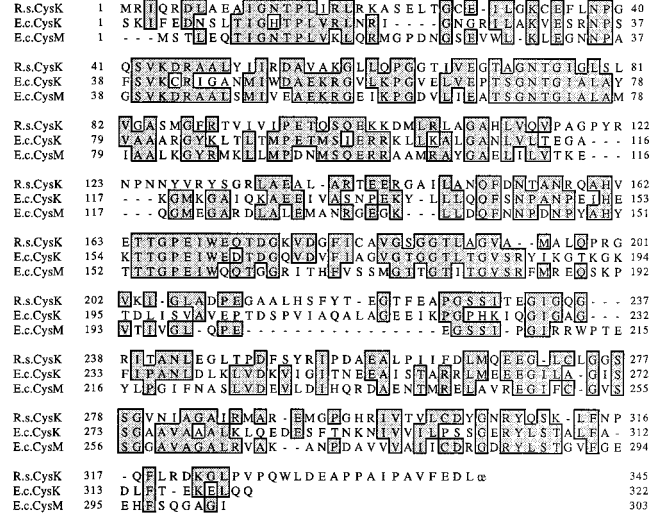


FIG. 2. Alignment of the *R. sphaeroides* 2.4.1 CysK protein (GenBank accession no. AF004296) (*R.s.CysK*) with *E. coli* CysK (GenBank accession no. M21451) (*E.c.CysK*) and *E. coli* CysM (GenBank accession no. M32101) (*E.c.CysM*). Residues identical in at least two sequences are shaded and boxed.

tellurite resistance in *R. sphaeroides* 2.4.1. To investigate this possibility, two mutant strains were constructed. In the first mutant, designated TRGB1, an insertion was introduced into *trgB*. The second mutant, designated TRGAB1, was a deletion mutation lacking the *trgB* gene and the 3' end of the *trgA* gene (see Materials and Methods). Surprisingly, tellurite resistance levels in TRGB1 and TRGAB1 were unaffected compared to those of the wild-type strain, a result somewhat at odds with the ability of the *trg* locus to confer increased tellurite resistance in *P. denitrificans* (Table 2).

Although the *cysK-orf323* operon was not capable of confer-

TABLE 2. Tellurite resistance levels in *P. denitrificans*, *R. sphaeroides*, and *E. coli* strains

Strain	Plasmid	Relevant gene(s) in trans	Resistance to potassium tellurite ( $\mu$ g/ml)	
<i>P. denitrificans</i> ATCC 17741	pRK415	Vector	40	
	pUI8131	<i>trgAB cysK orf323</i>	150	
	pUI8212	<i>trgAB cysK orf323</i>	150	
	pUI8213	<i>trgAB</i>	150	
	pUI8214	<i>trgA</i>	40	
	pUI8479	<i>telA orfB</i>	40	
<i>R. sphaeroides</i> 2.4.1			150	
	TRGB1		150	
	TRGAB1		150	
	CYSK1		75	
	CYSK1	pUI8131	<i>trgAB cysK orf323</i>	150
	CYSK1	pUI2819	<i>cysK</i>	150
	TELA1		50	
TELA1	p479-BsS	<i>telA</i>	150	
ORFB1			150	
<i>E. coli</i> DH5 $\alpha$ phe	p479BH	<i>telA orfB</i>	1	
	p479BS	<i>telA orfB</i>	1	
	p479-BsS	<i>telA</i>	1	
	pDT1558	[ <i>klaA klaB telB</i> ] <sub>RK2</sub>	250	
	pDT1558 $\Delta$ ; p479BsS	[ <i>klaA klaB' telB</i> ] <sub>RK2</sub> <i>telA</i>	1	

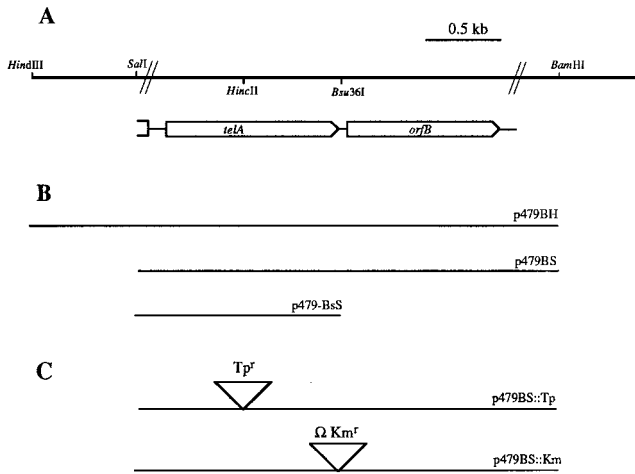


FIG. 3. (A) Physical and restriction maps of the *R. sphaeroides telA orfB* region. (B) Cosmids and plasmids used for cloning and complementation. (C) Plasmids used to construct *telA* and *orfB* mutations. The antibiotic resistance cassettes were inserted at the positions indicated.

ring tellurite resistance in *P. denitrificans*, it had previously been noted that exogenous cysteine added to the growth medium of *R. sphaeroides* 2.4.1 substantially reduces tellurite resistance (20). This observation coupled with the close proximity of the *cysK* and *trgB* genes prompted us to investigate the role of the *cysK-orf323* operon in high-level tellurite resistance. A *cysK* insertion mutation was constructed (see Materials and Methods and Fig. 1). The resulting mutant, CYSK1, remained prototrophic for cysteine, suggesting that, like most other microorganisms, a second isoenzyme for this step in the cysteine biosynthetic pathway also exists in *R. sphaeroides* 2.4.1. However, in contrast to TRGAB1, tellurite resistance in the CYSK1 strain was reduced to approximately half that of the wild-type strain (75 versus 150 µg/ml). This phenotype was complemented by the introduction of cosmid pUI8131 in *trans* (Table 2).

The insertion of an  $\Omega$  cassette in the *cysK* gene terminates transcription originating upstream of the cassette and was likely to have a polar effect on the expression of *orf323*. Therefore, in order to determine if one or the other or both *cysK* and *orf323* were involved in tellurite resistance in *R. sphaeroides*, the *cysK* gene alone was subcloned on a 1.9-kb *PvuII* fragment (Fig. 1, plasmid pUI2819) and introduced into the CYSK1 mutant. Tellurite resistance of strain CYSK1 (pUI2819) was identical to that of the wild-type strain (Table 2). This demonstrated that only the *cysK* gene was required for complementation of the CYSK1 mutant, thereby providing evidence that *orf323* is not required for tellurite resistance, at least with respect to the analysis performed here.

**Cloning and sequence of the homolog of the RK2 plasmid *klaB* (*telA*) gene.** A second, unlinked locus involved in high-level tellurite resistance in *R. sphaeroides* 2.4.1 was identified on cosmid pUI8479 during a search for regulators of photosynthesis gene expression (the results of which will be presented elsewhere). A gene, designated *telA*, which displays significant similarity to the *klaB* (*telA*) gene from IncP $\alpha$  plasmid RK2 (37) was identified, and its role in tellurite resistance was investigated.

Localization of the *telA* gene in pUI8479 is shown in Fig. 3. *R. sphaeroides telA* encodes a protein of 396 amino acids which is 27% identical (65% similar) to the *klaB* (also known as *telA*

R.s.TelA	1	- - - M T S S R R A A E A P R L A E V T A F D L P E P A P A V T P P A	35
RK2.KlaB	1	- -	27
B.s.YaaN	1	M N R D Q S L H I D L L A D P F G G N I E I P G S E A V K A E K E Q V R L V	40
R.s.TelA	36	S A P F E K A Q E I R R R M A E L N V S D S O S T I G F G S K A Q A E L T I	74
RK2.KlaB	28	G L O E S D V P E V H A - V A Q R T I E M G S P O T V A E F G R D V A E H T S R Y	66
B.s.YaaN	41	D V I F E N E K K A T Q L A G Q I D H K N M Q S I V L Y G S Q A Q S K L N F	80
R.s.TelA	75	S Q Q T L A D V K N K Q V G D A Q D S R E R V V S T I R G F S V S E F P V R R	113
RK2.KlaB	67	A D S L I D Q V R N S S L D E A Q E K L T Q V V A K A B S L N V G P D S D N R S	106
B.s.YaaN	81	S H D M T N H V Q K K D Y G E I G E F L G E L M R K L E Q V N P D D E - Q S K K	119
R.s.TelA	114	K A S W W E R L L G R T A P F A R - F V A R Y E D Y V Q Q O I D R R I T Q S L I F H	152
RK2.KlaB	107	R L P E I G P L I D R F R V R S T G F M A R E D T T R E O F E H L V S E V Q I T	146
B.s.YaaN	120	K G F L - A R M F G R V S S S L Q E V L S K Y Q K H S V Q I D R T S L K T E H S	158
R.s.TelA	153	E H R T L K D I K G E D I E Y A R T L D F Y D E E A L V T A A G D E V L A D I D	192
RK2.KlaB	147	Q Q G I A Q R N A S E L E M F A A V R E H R L L G V H L A A G K V R L A E L R	186
B.s.YaaN	159	K N A T I S D N K L L E Q L Y E K N K I Y F A A L N V Y T A A G E L K L F E L K	198
R.s.TelA	193	G R V F P A K E A E V A A T P E Q D R M I K A Q E L R D R A R A R D L E E R V Y	232
RK2.KlaB	187	- - - E Q A E G L R G N V D P G R V Q E A L D A M V A N L D K R I	220
B.s.YaaN	199	T K T T P E L K Q Q A E S S D H - N Q E - A V Q E V N D I Q F A D R L D K K V	236
R.s.TelA	223	H D E K I T R Q V T M O S L P T F R L V Q E N D K A L V T R I N T L V N V Y P	272
RK2.KlaB	231	G D E I A L Q H S A M O S I P T F R M I Q A N N Q M L V D K F H T I R E I T V P	260
B.s.YaaN	237	H D L L S R Q I T I Q S A P Q L R L E Q N T N Q A I A K T I O S I V I T A I P	276
R.s.TelA	273	L W E T O L A Q A V T I Q R S R E A E A E V R G A S D L T N E L I T A N A N L	312
RK2.KlaB	271	A W R R O F M L A E L S I N E O N A V H L A T A I D D T T N D L K R N A L L L	300
B.s.YaaN	277	L W N Q V A I A L T L R Q R N A V D A Q Q V K S D T T E L I K N A E I L	315
R.s.TelA	313	Q Q A N K V L V R K E M E R G V F D T E A V K K A N A T T I A T I N E S N A T I Q	351
RK2.KlaB	301	H R F L S - V E T A K E N Q R L I D V D T L K Q V O T L I L K T V E D V L R I G	339
B.s.YaaN	316	L K N I T E E T A R A N E R G L V D I D T L K K V O E S I T I S T I E E A T T I Q	355
R.s.TelA	352	D E G R A R R A T A E T L Q R M E A L R D D J E A S A R A R R T G T G D T A G	391
RK2.KlaB	340	Q E G V Q K R K D A E K O L A A M R G G D L Q A K L T R Q P V R E L A Q Q E S V	378
B.s.YaaN	356	E E G R I K R R Q A E E T I M M E G Q L K Q K I T I T K E R	386
R.s.TelA	392	T S V R P	396

FIG. 4. Alignment of the *R. sphaeroides* 2.4.1 *TelA* protein (GenBank accession no. AF019377) (*R.s.TelA*) with *B. subtilis* YaaN (GenBank accession no. M96156) (*B.s.YaaN*) and plasmid RK2 *KlaB* (GenBank accession no. M62426) (*RK2.KlaB*). Residues identical in at least two sequences are shaded and boxed.

[37]) gene product from RK2 (Fig. 4). The latter gene belongs to the three-gene *kilA* operon, *klaA-klaB-klaC*. This operon encodes latent high-level tellurite resistance in *E. coli*, which becomes apparent after a point mutation in *klaC*. The mutant form of *klaC* has been designated *telB* (33, 35, 38). To determine if other homologs of the components of the *kilA* operon were also present, we sequenced downstream and upstream of *R. sphaeroides telA*.

Sequencing revealed the existence of a second gene, *orfB*, 12 bp downstream of *telA*. The putative ribosome binding site for *orfB* overlaps the *telA* stop codon, an arrangement which strongly suggests that the genes are transcriptionally and translationally coupled. The putative product of *orfB*, a 321-amino-acid protein, shows no significant homology to the *klaC* (*telB*) gene product from RK2 or to any other protein in the databases. A putative lipid binding site was identified in the possible transmembrane domain in the amino-terminal portion of OrfB. Immediately upstream of *telA*, a third ORF was identified. This gene is divergently transcribed from *telA*, and its predicted amino acid product shows no similarity to that of *klaA* (data not shown). Therefore, the genetic region of the *R. sphaeroides telA* gene is clearly quite distinct from its RK2 counterpart.

**Functional divergence between the *klaB*-*telA* genes.** As previously mentioned, cosmid pUI8479 containing the *telA* gene did not confer increased tellurite resistance in *P. denitrificans*. Further analysis was also performed to determine if the *R. sphaeroides telA* gene in combination with the surrounding genes could increase tellurite resistance in *E. coli*. In addition to cosmid pUI8479, the other plasmids used in this analysis were a pUC19-derived multicopy plasmid, p479BH, containing sequences both upstream and downstream of *telA*, and a derivative, p479BS, containing *telA* and the downstream genes under the control of the strong plasmid *lac* promoter (Table 1; Fig. 3). However, all of the above constructs failed to increase

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tellurite resistance in *E. coli* DH5 $\alpha$ *phe* above background levels (Table 2).

The absence of plasmid RK2 *kilA* operon homologs in the proximity of the *R. sphaeroides telA* gene along with the moderate similarity between the two genes may imply that the *telA* gene products from both sources have diverged significantly and now perform different functions. To test this, we employed plasmid pDT1558 (36), which carries the entire tellurite-resistant variant of the RK2 *kilA* operon, *klaA-klaB-telB*. The *klaB* (*telA*) gene on pDT1558 was inactivated by constructing an internal deletion which should not affect transcription of the downstream *telB* gene. In contrast to pDT1558, the resulting plasmid, designated pDT1558 $\Delta$  (Table 1), conferred no tellurite resistance (<1  $\mu$ g/ml) in *E. coli* DH5 $\alpha$ *phe* (Table 2). The subsequent introduction into DH5 $\alpha$ *phe* (pDT1558 $\Delta$ ) of the *R. sphaeroides telA* gene expressed from the *lac* promoter with or without induction also failed to increase tellurite resistance, thus indicating that the *R. sphaeroides* gene cannot functionally substitute for the RK2 *klaB* (*telA*) gene (Table 2).

#### Construction and characterization of a *telA* null mutation.

To investigate whether, similar to its plasmid RK2 counterpart, the *telA* gene contributes to tellurite resistance in *R. sphaeroides*, we inactivated the chromosomal copy of the gene (see Materials and Methods) (Fig. 3). The strain carrying this mutation, designated TELA1, showed an approximately 67% decrease in tellurite resistance (50  $\mu$ g/ml) compared to wild-type levels (Table 2). Therefore, the *R. sphaeroides telA* gene does contribute to high-level tellurite resistance. No other phenotypes for the TELA1 mutant were detected under the various conditions tested.

The transcriptional-translational coupling of *telA* and *orfB* raised the possibility that the *telA* disruption may have also affected expression of the downstream *orfB*. To test whether *orfB* contributes to the tellurite sensitivity of mutant TELA1, an  $\Omega$ Km<sup>r</sup> cartridge was introduced into the *Bsu*36I site positioned immediately downstream of the *telA* stop codon (Fig. 3). The  $\Omega$  cassette contains efficient transcriptional and translational terminators, and therefore, the existence of an  $\Omega$ Km<sup>r</sup> cassette at the 5' end of the *orfB* gene prevents transcription originating upstream of or within *telA*. The mutant generated, ORFB1, showed the same levels of tellurite resistance as the wild type, thus suggesting that *orfB* is not involved in tellurite resistance (Table 2). Further, mutant TELA1 could be complemented in *trans* by the *telA* gene alone (Table 2).

**Genomic mapping of the tellurite resistance loci.** The genomic locations of the tellurite resistance loci were determined by pulsed-field gel electrophoresis of genomic DNA isolated from mutant strains as well as Southern hybridizations. The  $\Omega$ Sp<sup>r</sup> Sm<sup>r</sup> and  $\Omega$ Km<sup>r</sup> cassettes used to disrupt the *trgB* gene and *orfB* contain *Ase*I and *Sna*BI restriction enzyme sites which were exploited to map the disrupted loci. These sites, as well as sites for *Dra*I and *Spe*I, are rare in *R. sphaeroides* 2.4.1 genomic DNA, and their physical locations have been established (30).

Digestion of genomic DNA from mutant TRGB1 with *Ase*I demonstrated that the 1,105-kb *Ase*I fragment A of chromosome I (Fig. 5) was no longer present and that two new bands of approximately 200 and 905 kb appeared (data not shown). Subsequent digestion of TRGB1 genomic DNA with *Sna*BI mapped the *trgA* locus to a 300-kb chromosome I *Sna*BI fragment E (Fig. 5). This placed the *trg* locus at approximately 1,868 kb clockwise on chromosome I (Fig. 5). Southern hybridizations confirmed the location of the *trg* locus (data not shown).

Digestion of genomic DNA from mutant ORFB1 with *Ase*I resulted in 315- and 790-kb fragments in place of the 1,105-kb

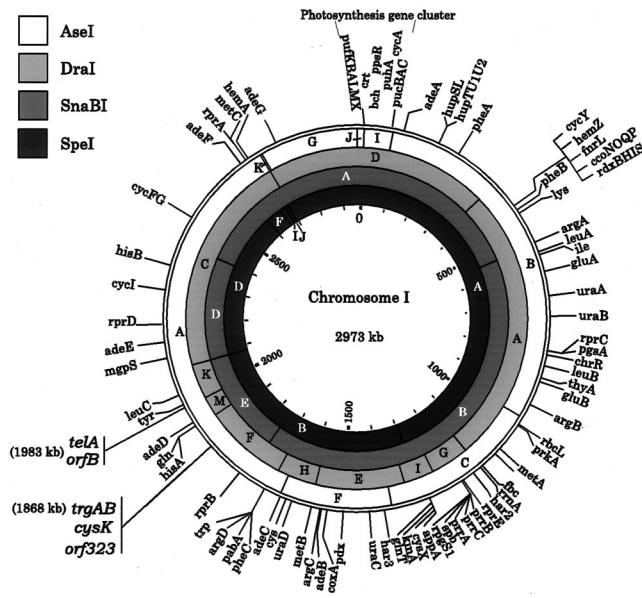


FIG. 5. Map of the *R. sphaeroides* chromosome I. Designations and coordinates of the *trgAB cysK orf323* and *telA orfB* loci are shown enlarged.

fragment A typical of wild-type DNA. Southern hybridization established that *telA* belongs to the 735-kb *Spe*I fragment B, placing it approximately 1,983 kb clockwise on this chromosome (Fig. 5). Therefore, the *trgA* and *telA* loci are separated by approximately 115 kb.

## DISCUSSION

Despite considerable effort, the precise mechanism(s) of tellurite resistance and reduction remains elusive. Recently, it has been demonstrated that the nitrate reductase enzymes of *E. coli* act to reduce tellurite and selenate (1) and that this activity is necessary for the basal (approximately 1  $\mu$ g/ml) resistance of *E. coli* to tellurite. It is interesting to note that *R. sphaeroides* 2.4.1 does not possess nitrate reductase activity, and in this organism, an unidentified membrane-associated flavin-dependent reductase is believed to be responsible for tellurite reduction (20, 21). During anoxygenic photosynthetic growth in the presence of tellurite, the reduction of tellurite plays an important physiological role in the maintenance of cellular redox poise. Therefore, it is not surprising that photosynthetic electron transport and CO<sub>2</sub> fixation pathways are linked to tellurite reduction and that mutations which interfere with these processes drastically decrease tellurite resistance levels (20, 21). In this study, we report the identification and characterization of two loci apparently not involved in respiration, photosynthesis, or CO<sub>2</sub> fixation but which significantly contribute to high-level tellurite resistance in *R. sphaeroides* 2.4.1.

**Tellurite resistance levels may reflect alterations in the cysteine biosynthetic pathway.** Our observation that the disruption of the *R. sphaeroides* 2.4.1 *cysK* gene impaired tellurite resistance levels suggests that there may be a link between cysteine biosynthesis and tellurite resistance. In *E. coli* and *S. typhimurium*, there are two isoenzymes for the terminal step of the cysteine biosynthetic pathway encoded by the *cysK* and *cysM* genes. Mutations in either gene alone do not result in cysteine auxotrophy (43). Here, we also demonstrated that the *R. sphaeroides cysK* null mutation, strain CYSK1, did not result

in cysteine auxotrophy, suggesting that a homolog for the *cysM* gene exists in *R. sphaeroides*. The role of *orf323* is unknown, although its linkage in an operon with *cysK* and the prediction that the proteins encoded by both genes will be membrane localized suggest that Orf323 may have a role in cysteine biosynthesis. The decrease in tellurite resistance levels in CYSK1 is difficult to explain, although it is clear that *orf323* does not contribute to this phenotype.

In *E. coli*, mutations in the *cysK* gene have been shown to interfere with the normal functioning of the sulfate uptake pathway (43). Hence, it may be inferred that by altering sulfate uptake, the sensitivity of *R. sphaeroides* CYSK1 to tellurite has been increased. However, selenite (a chemical analog of sulfite) sensitivity was not affected in the CYSK1 and TELA1 mutants (data not shown). This would seem to argue against the involvement of sulfate uptake in decreased tellurite resistance in mutant CYSK1.

Another possibility is that the *cysK* mutation in *R. sphaeroides* may indirectly affect sulfite reductase activity, thereby lowering tellurite resistance levels. For example, in *E. coli* tellurite resistance conferred by the *tehAB* locus is dependent on a functional sulfite reductase (35). Although the precise mechanism remains unknown, it is clear that an intact cysteine metabolic pathway is important for high-level tellurite resistance in *R. sphaeroides* 2.4.1. This is consistent with the earlier observation that exogenous cysteine in the growth medium of *R. sphaeroides* 2.4.1 is associated with a drastic decrease in tellurite resistance levels (20).

The presence of many duplicate genes, such as *rdxA* and *rdxB* (22, 24) and *hemA* and *hemT* (23) in the genome of *R. sphaeroides* raises the question of whether the *trgA* and/or *trgB* genes also have homologs, a possibility that may explain the absence of a phenotype for the TRGB1 and TRGAB1 strains. The observation that tellurite resistance levels remain unaffected in these strains is somewhat surprising when one considers that the *trgAB* operon alone is sufficient to confer an approximately fourfold increase in tellurite resistance levels in *P. denitrificans*. The absence of known homologs for either *trgA* or *trgB* makes it difficult to speculate on the possible mechanisms by which they may alter tellurite resistance levels in *P. denitrificans*. Both *trgAB* and *cysK-orf323* form separate two-gene operons, and it is possible that the gene products of both operons are functionally independent. Consistent with this, the *trgAB* operon by itself increases tellurite resistance severalfold in *P. denitrificans*. On the other hand, the location of *trgAB* immediately upstream of *cysK orf323* suggests that both operons are involved in the alterations in cysteine biosynthesis which affect tellurite resistance.

**Conservation of the *R. sphaeroides telA* gene among bacteria and plasmids and its involvement in tellurite resistance.** One of the homologs of the *R. sphaeroides telA* gene described here is the *klaB* gene from the *kilA* operon of plasmid RK2. Mutations in both the *R. sphaeroides telA* gene and the RK2 *kilA* operon lower tellurite resistance levels. The mechanism of plasmid RK2 *kilA* operon-encoded tellurite resistance remains unknown. Recent evidence has demonstrated that it is not associated with decreased uptake or increased efflux of tellurite (17, 34). This led to the proposal that the products of the *kilA* operon facilitate tellurite reduction. Nevertheless, the major functions of this operon are believed to be in plasmid maintenance or in contributing to the broad host range of RK2 (12).

Interestingly, the homologs of *klaB (telA)* from plasmid RK2 exist as chromosomal genes in both gram-negative (*telA* in *R. sphaeroides*) and gram-positive (uncharacterized gene *yaaN* in *Bacillus subtilis*) bacteria (Fig. 4). The conservation of this gene

in divergent bacterial groups might signify an important cellular function. In evolutionary terms, one could speculate that the procurement of the chromosomal *klaB (telA)* gene by the predecessor of plasmid RK2 provided the plasmid with a selective advantage. However, it is impossible to predict whether this advantage represented increased tellurite resistance and thus enhanced adaptability or an as-yet-unidentified phenotype for new bacterial hosts of the RK2 plasmid.

It is known that in plasmid RK2 uncontrolled expression of the tightly regulated *kilA* operon is lethal to the host cells, a characteristic which prompted the *kil* designation and which is likely to contribute to stable plasmid maintenance (12). Therefore, apart from involvement in tellurite resistance, the *klaB (telA)* gene products could encode additional properties. However, our analysis of the *R. sphaeroides telA* null mutation revealed no obvious phenotype apart from decreased tellurite resistance.

It is worth mentioning that both *R. sphaeroides telA* and *klaB* from plasmid RK2 form operons with genes encoding membrane-localized proteins. It may therefore be predicted that KLaB (TeLA) function is related to the structure-function of these membrane proteins. However, these proteins are different in plasmid RK2 and *R. sphaeroides*, and no homologs of the RK2 *klaA* or *klaC (telB)* gene have been identified to date.

**Multiple factors contributing to high-level tellurite resistance in *R. sphaeroides*.** In an earlier analysis, we demonstrated that numerous loci involved in respiration, photosynthesis, and CO<sub>2</sub> fixation are important for high-level tellurite resistance in *R. sphaeroides* 2.4.1 (20, 21). These processes involve electron transport and are believed to affect the cellular capacity to reduce tellurite. Here, we have described the identification of two loci, *cysK* and *telA*, which do not appear to be directly involved in electron transport but which significantly contribute to tellurite resistance in this bacterium.

The precise mechanisms underlying CysK- and TeLA-mediated tellurite resistance remain unknown; however, evidence from *E. coli*, in which Kila-mediated tellurite resistance is independent of cysteine metabolism (35), suggests that they are unlikely to overlap. Suppressors with wild-type tellurite resistance levels readily arise in both CysK and TeLA mutant backgrounds (data not shown), suggesting that the phenomenon of high-level tellurite resistance in *R. sphaeroides* 2.4.1 is complex and involves multiple mechanisms, some of which could be universal among bacteria.

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