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Tellurite resistance gene *trgB* confers copper tolerance to *Rhodobacter capsulatus*

Corinna Rademacher · Marie-Christine Hoffmann · Jan-Wilm Lackmann · Roman Moser · Yvonne Pfänder · Silke Leimkühler · Franz Narberhaus · Bernd Masepohl

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Abstract To identify copper homeostasis genes in *Rhodobacter capsulatus*, we performed random transposon Tn5 mutagenesis. Screening of more than 10,000 Tn5 mutants identified tellurite resistance gene *trgB* as a so far unrecognized major copper tolerance determinant. The *trgB* gene is flanked by tellurite resistance gene *trgA* and cysteine synthase gene *cysK2*. While growth of *trgA* mutants was only moderately restricted by tellurite, *trgB* and *cysK2* mutants were severely affected by tellurite, which implies that viability under tellurite stress requires increased cysteine levels. Mutational analyses revealed that *trgB* was the only gene in this chromosomal region conferring cross-tolerance towards copper. Expression of the monocistronic *trgB* gene required promoter elements overlapping the *trgA* coding region as shown by nested deletions. Neither copper nor tellurite affected *trgB* transcription as demonstrated by reverse transcriptase PCR and *trgB–lacZ* fusions. Addition of tellurite or copper gave rise to increased cellular tellurium and copper concentrations, respectively, as determined by inductively coupled plasma-optical emission spectroscopy. By contrast, cellular iron concentrations remained fairly constant irrespective of tellurite or copper addition. This is the first study demonstrating a direct link between copper and tellurite response in bacteria.

Keywords Copper · Tellurite · Nudix hydrolase · Metal homeostasis · *Rhodobacter*

C. Rademacher · M.-C. Hoffmann · J.-W. Lackmann · R. Moser · Y. Pfänder · F. Narberhaus · B. Masepohl (✉)
Biologie der Mikroorganismen, Fakultät für Biologie und Biotechnologie, Ruhr-Universität Bochum, 44780 Bochum, Germany
e-mail: bernd.masepohl@rub.de

S. Leimkühler
Molekulare Enzymologie, Institut für Biochemie und Biologie, Universität Potsdam, 14469 Potsdam, Germany

Introduction

Copper is an important trace element for both prokaryotes and eukaryotes (Andreini et al. 2008; Banci et al. 2010). It is associated with the catalytic function of various cuproenzymes like cytochrome c oxidase, plastocyanin, superoxide dismutase, and multicopper oxidase (Solioz et al. 2010). The use of copper as a cofactor in diverse biological processes is based on its distinctive ability to alternate between oxidation states Cu(I) and Cu(II) thus rendering copper extremely reactive (Alcaraz et al. 2007; Reyes-Jara et al. 2010). However, this feature makes copper, when in excess, highly toxic to cells (Silver and Le Phung 2005; Singleton et al. 2008). Copper destabilizes iron–sulfur clusters of dehydratases and it competes with other metals for protein binding sites leading to perturbation of protein function (Chillappagari et al. 2010; Kershaw et al. 2005; Macomber and Imlay 2009; Waldron and Robinson 2009). In addition, copper has

frequently been discussed to generate highly toxic radicals causing damage of lipids, proteins, DNA, and other macromolecules (Imlay 2003; Solioz et al. 2010; Urbański and Beresewicz 2000).

To cope with copper toxicity, bacteria have evolved different defence systems mostly based on removal or detoxification of copper (Magnani and Solioz 2007; Osman and Cavet 2008; Rensing and Grass 2003). Many bacteria actively export copper by P-type ATPases or multicomponent efflux systems similar to *Escherichia coli* CopA and CusCFBA, respectively (Fan and Rosen 2002; Franke et al. 2003; Robinson 2011; Singleton and Le Brun 2009; Solioz and Stoyanov 2003; Su et al. 2011). In addition, many bacteria synthesize periplasmic multicopper oxidases similar to *E. coli* CueO (Achard et al. 2010; Singh et al. 2004; Wiethaus et al. 2006). CueO oxidizes Cu(I) to Cu(II), the latter ion having only limited potential to enter the cytoplasm (Grass et al. 2004; Outten et al. 2001; Singh et al. 2004).

Like copper ions, the tellurium oxyanion tellurite (TeO_3^{2-}) is highly toxic to bacteria, and therefore, was used as a bacterial disinfectant before the advent of the antibiotic era (Chasteen et al. 2009; Taylor 1999). In contrast to copper, however, neither tellurium nor tellurite seem to have any natural biological relevance (Ba et al. 2010; Cunha et al. 2009).

Tellurite causes oxidation of cellular thiols like cysteines and generates oxidative stress (Calderón et al. 2009; Pérez et al. 2007; Rigobello et al. 2011; Tremaroli et al. 2007; Turner et al. 1999, 2001). At sub-lethal concentrations, bacteria reduce tellurite to less toxic elemental tellurium that is accumulated as black deposits inside the cells (Borsetti et al. 2003; Chien et al. 2011; Lloyd-Jones et al. 1994; Ottosson et al. 2010). Conversion of tellurite to tellurium involves unspecific reductases, such as nitrate reductase or catalase, but also leads to production of superoxide radicals (Avazéri et al. 1997; Calderón et al. 2006; Castro et al. 2008; Chasteen et al. 2009). To date, several bacterial tellurite resistance genes have been described, but none of them was directly involved in tellurite reduction (Chasteen et al. 2009). One widely distributed tellurite resistance gene is *trgB*, which was first identified in *Rhodobacter sphaeroides* (O'Gara et al. 1997). TrgB consists of two domains exhibiting similarity to gamma-glutamyl cyclotransferases and to the ADP-ribose pyrophosphatase subfamily of nudix (nucleoside diphosphate-*x*) hydrolases (Dunn et al. 1999; Marchler-Bauer et al. 2011). To date, the specific role of TrgB in tellurite resistance remains unclear.

The α-proteobacterium *Rhodobacter capsulatus* is known for its great metabolic versatility and serves since long as a model organism to study photosynthesis and nitrogen fixation (Masepohl and Hallenbeck 2010; Onder et al. 2010). Like *E. coli*, *R. capsulatus* synthesizes a copper-inducible multicopper oxidase, CutO, which is a major copper tolerance determinant in this bacterium (Wiethaus et al. 2006). In addition, two largely uncharacterized genes flanking *cutO* were shown to be required for full copper tolerance (Rademacher et al. 2012). Up to now no further copper tolerance determinant had been identified in *R. capsulatus*.

In the present study, we searched for new copper tolerance genes in *R. capsulatus* by random transposon Tn5 mutagenesis. We identified a so far unrecognized major copper tolerance gene, the ortholog of the tellurite resistance gene *trgB* from *R. sphaeroides*. Mutational analysis revealed a role of *R. capsulatus* TrgB in tolerance towards both copper and tellurite.

Materials and methods

Bacterial strains, plasmids, primers, and growth conditions

Strains and plasmids used in this study are listed in Table 1. Primers used for reverse transcription PCR and construction of *lacZ* fusions are shown in Table 2. Media, antibiotic concentrations, growth conditions, and copper sensitivity (filter disc) assays were previously described (Wiethaus et al. 2006). *Rhodobacter capsulatus* RCV minimal medium was essentially prepared as described earlier except that no copper was added (Weaver et al. 1975). When required, appropriate concentrations of $CuSO_4$ or K_2TeO_3 were added. Unless otherwise indicated, *R. capsulatus* strains were grown under anaerobic phototrophic conditions.

Random transposon Tn5 mutagenesis

Narrow-host-range plasmid pSUP2021 carrying Tn5 was conjugationally transferred from *E. coli* S17-1 into *R. capsulatus* B10S as previously described (Klipp et al. 1988). *R. capsulatus* Tn5 mutants were selected on plates containing kanamycin (Km) and streptomycin. For details on screening for copper-sensitive Tn5

Table 1 Bacterial strains and plasmids

Strain or plasmid	Relevant characteristic(s)	Source or reference
E. coli		
JM83	Host for plasmid amplification	Vieira and Messing (1982)
S17-1	Donor for conjugational plasmid transfer	Simon et al. (1983)
R. capsulatus		
B10S	Spontaneous Sm-resistant mutant of B10	Klipp et al. (1988)
CR92-I	*trgA*::[Km>] mutant of B10S	This study
CR92-II	*trgA*::[<Km] mutant of B10S	This study
CR93-I	*trgB*::[Km>] mutant of B10S	This study
CR93-II	*trgB*::[<Km] mutant of B10S	This study
CR94-I	*cysK2*::[Km>] mutant of B10S	This study
CR94-II	*cysK2*::[<Km] mutant of B10S	This study
CR95-I	*mscS*::[Km>] mutant of B10S	This study
CR95-II	*mscS*::[<Km] mutant of B10S	This study
JW12-I	*cutO*::[Km>] mutant of B10S	Wiethaus et al. (2006)
Plasmids		
pBBR1-MCS5	Mobilizable broad-host-range vector; Gm	Kovach et al. (1995)
pBSL15	pUC18 derivative carrying [Km]	Alexeyev (1995)
pCR92-I/II	pSUP202 derivatives carrying *trgA*::[Km]	This study
pCR93-I/II	pSUP202 derivatives carrying *trgB*::[Km]	This study
pCR94-I/II	pSUP202 derivatives carrying *cysK2*::[Km]	This study
pCR95-I/II	pSUP202 derivatives carrying *mscS*::[Km]	This study
pCR205-207, pCR223	pBBR1-MCS5 derivatives carrying transcriptional *trgB–lacZ* fusions	This study
pSUP202	Mobilizable narrow-host-range vector; Tc	Simon et al. (1983)
pSUP2021	pSUP202 derivative carrying Tn5	Simon et al. (1983)
pYP35	pBSL15 derivative carrying [*lac*TeT]	Gisin et al. (2010)

Gm gentamicin resistance, *Km* kanamycin resistance, *Sm* streptomycin resistance, *Tc* tetracycline resistance

mutants, see below. To localize Tn5 insertion sites in copper-sensitive mutants, genomic DNAs were digested with BamHI and ligated into pUC18. Ligation mixtures were transformed into *E. coli* JM83 prior to selection for Km-resistant clones carrying part of Tn5 (with the Km resistance gene) and flanking sequences. Resulting hybrid plasmids were sequenced to precisely map Tn5 insertion sites within the complete genome sequence of *R. capsulatus* (Strnad et al. 2010).

Construction of *R. capsulatus* mutants by gene disruption

To construct *R. capsulatus* mutants defective for *trgA* (RCAP_rcc02957), *trgB* (RCAP_rcc02956), *cysK2* (RCAP_rcc02955), and *mscS* (RCAP_rcc02954), kanamycin resistance (Km) cassettes from plasmid pBSL15 were inserted at appropriate restriction sites within coding regions of the respective genes. For this purpose, DNA fragments of approximately 1 kbp centered around these restriction sites were PCR-amplified and blunt-end cloned into the BalI site of the mobilizable narrow-host-range plasmid pSUP202. Subsequently, Km cassettes were inserted in both orientations. Resulting mutagenic plasmids pCR92–pCR95 (Table 1) were conjugationally transferred into *R. capsulatus* B10S. Loss of pSUP202-encoded tetracycline resistance indicated marker rescue by double recombination events. Identity of mutants was in each case verified by PCR (data not shown).

Construction of *R. capsulatus* reporter strains and β-galactosidase assays

To narrow down the *trgB* promoter, different DNA fragments of the *phrB–trgA–trgB* region were

Table 2 Primer pairs

Primer pairs	Sequence (5′–3′)	Application
UP-trgAB-21	CGCCACGTCCGAACTGTCGG	RT-PCR; 314 bp *phrB* internal fragment
LP-trgAB-2	GCATCCGCCCGGTCACATACATTT	
UP-trgAB-1	CGCCCTATTTCCGCATCTTCAACC	RT-PCR; 582 bp *phrB–trgA* border fragment
LP-trgAB-7	CGTTCGATCACCTTGACCCGG	
UP-trgAB-12	CGAAGCTGTTCGGGGCGCTTG	RT-PCR; 303 bp *trgA* internal fragment
LP-trgAB-18	CGATCAGCGCCTCGCTCGGGC	
UP-trgAB-12	CGAAGCTGTTCGGGGCGCTTG	RT-PCR; 679 bp *trgA–trgB* border fragment
LP-trgAB-11	CTCTTCCTCGGCCGGGGTGACATC	
UP-trgAB-13	CGGCACTCTGGGTCATCCGCC	RT-PCR; 196 bp *trgB* internal fragment
LP-trgAB-11	CTCTTCCTCGGCCGGGGTGACATC	
Rrc-88-1-UP	AAGAGGTGCTCGACTGGTAT	RT-PCR; 1,309 bp *trgB–cysK2* border fragment
LP-trgAB-10	CCAGCCGCAGCATGTCCTTTTTCT	
UP-trgAB-5	GACCGGGCGGCGCTTTACATCATC	RT-PCR; 193 bp *cysK2* internal fragment
LP-trgAB-10	CCAGCCGCAGCATGTCCTTTTTCT	
UP-trgAB-6	CCCGGGGCACACCATCGTCAC	RT-PCR; 396 bp *cysK2–mscS* border fragment
LP-trgAB-9	CAGCGTGTCGATCTGGCTGGC	
UP-trgAB-14	GGTCCTTGCGCTGCCCGTCGC	RT-PCR; 205 bp *mscS* internal fragment
LP-trgAB-9	CAGCGTGTCGATCTGGCTGGC	
UP-trgAB-1	CGCCCTATTTCCGCATCTTCAACC	Cloning of pCR205 (*trgB–lacZ*)
LP-trgAB-4	CGATCGCCGCTTCCGCAGAGACCT	
UP-trgAB-4	TCCCGCGGCCGCTATGACG	Cloning of pCR206 (*trgB–lacZ*)
LP-trgAB-4	CGATCGCCGCTTCCGCAGAGACCT	
UP-trgAB-2	GGCGGCCGAAACAAGCAGAAG	Cloning of pCR207 (*trgB–lacZ*)
LP-trgAB-4	CGATCGCCGCTTCCGCAGAGACCT	
UP-trgAB-15	TAGGCCGCGCCCCAACCGCGG	Cloning of pCR223 (*trgB–lacZ*)
LP-trgAB-4	CGATCGCCGCTTCCGCAGAGACCT	
UP-trgAB-16	ATGTCATTGAGCAAACTCTAT	Cloning of pCR224 (*trgB–lacZ*)
LP-trgAB-4	CGATCGCCGCTTCCGCAGAGACCT	

PCR-amplified using appropriate primer pairs (Table 2) and blunt-end cloned into the SmaI site of the mobilizable broad-host-range vector pBBR1-MCS5. Subsequently, a BamHI fragment carrying a promoterless *lacZ* gene from pYP35 was added, resulting in *trgB–lacZ* reporter plasmids pCR205, pCR206, pCR207, and pCR223 (Table 1). These reporter plasmids were conjugationally transferred into *R. capsulatus* B10S, and selection for plasmid-encoded tetracycline resistance led to identification of corresponding reporter strains. After growth to late-exponential phase, β-galactosidase activities were determined as previously described (Miller 1972).

Reverse transcription PCR

To examine transcriptional organization of the *phrB–trgA–trgB–cysK2–mscS* region, reverse transcription (RT)-PCR studies were carried out. For this purpose, *R. capsulatus* cultures were grown in RCV minimal medium until late logarithmic phase, prior to RNA isolation using the PureLink™ Micro-to-Midi Total RNA Purification System (Invitrogen, Karlsruhe, Germany). Transcript analyses were carried out using DNase RQ1 (Promega, Mannheim, Germany) and Superscript II reverse transcriptase (Invitrogen, Karlsruhe, Germany) as described by the manufacturers.

Primers used for cDNA synthesis and subsequent PCR amplification reactions are shown in Table 2.

Determination of cellular metal contents

Tellurium, copper, and iron contents of *R. capsulatus* cells were quantified by inductively coupled plasma-optical emission spectroscopy (ICP-OES) with a Perkin-Elmer Optima 2100DV. For this purpose, 5-ml cultures were grown in RCV minimal medium with 10 μM K_2TeO_3 or 2 μM $CuSO_4$, or, as a control, without addition of these metals. At early stationary phase cells were repeatedly washed with 0.9 % NaCl to remove unbound tellurite or copper from the cultures. Finally, washed cells of each culture were resuspended in 1 ml 0.9 % NaCl. Aliquots were serially diluted and plated to determine numbers of colony forming units (CFU). 500-μl cell samples (in 0.9 % NaCl) were mixed with 500 μl of 65 % nitric acid, prior to wet ashing by overnight incubation at 100 °C. After addition of 4 ml of water to each sample, metal contents were determined by ICP-OES using multielement standard XVI (Merck, Darmstadt, Germany) as a reference.

Results and discussion

Screening for copper tolerance genes

Multicopper oxidase gene *cutO* (RCAP_rcc02110) and two flanking genes confer copper tolerance to *R. capsulatus* as shown in previous studies (Rademacher et al. 2012; Wiethaus et al. 2006). To identify further genes involved in copper homeostasis, *R. capsulatus* mutants were generated by transposon Tn5 mutagenesis and screened for growth defects at copper concentrations otherwise tolerated by the wild type. More than 10,000 Tn5 mutants were streaked on agar plates, prior to placement of copper-soaked filter stripes. An example is shown in Fig. 1a. Copper-sensitive phenotypes of mutant strains identified in this initial screening were verified by filter disc assays (Fig. 1b, c). Altogether 10 copper-sensitive mutants were identified (Fig. 1d). DNA sequence analyses revealed that Tn5 insertions mapped to 10 different genes thus confirming randomness of Tn5 mutagenesis (Klipp et al. 1988; Leimkühler et al. 1998). To rule out that secondary mutations rather than Tn5-induced mutations were responsible for copper sensitivity, the phenotypes of seven Tn5 mutants were verified using knock-out strains generated by plasmid integration (underlined in Fig. 1d; data not shown).

Nine Tn5 mutants exhibited moderate copper sensitivity as shown by filter disc assays (Fig. 1d) and growth curves with different copper concentrations (data not shown) suggesting that the corresponding proteins synergistically contribute to full copper tolerance. One such mutant lacks CcoH, which is essential for assembly and stabilization of a copper-containing enzyme, Cbb_3-type cytochrome oxidase (Pawlik et al. 2010). Two other mutants lack proteins belonging to different ABC transport systems, one of which may be involved in siderophore/cobalamin transport. A direct link between siderophore transport, iron homeostasis, and copper homeostasis was recently shown for *Anabaena* sp. PCC 7120 (Nicolaisen et al. 2010). Two mutants lack proteins involved in restriction-modification and thymidylate synthesis suggesting that DNA modification and DNA repair might be specifically important during copper stress. The remaining mutants lack a hemolysin-type protein or hypothetical proteins. However, the role of these proteins in copper homeostasis remain unclear to date.

In contrast to the mild phenotypes of mutants lacking either of these nine proteins, a *trgB* mutant was severely affected by copper (Fig. 1d). Annotation of *R. capsulatus trgB* reflects its similarity to tellurite resistance gene *trgB* from *Rhodobacter sphaeroides* (O'Gara et al. 1997). The *trgB* products of these two strains exhibit 46 % identity and 57 % similarity over the entire length. *R. capsulatus trgB* mutants were as copper-sensitive as *cutO* mutants lacking multicopper oxidase (Fig. 1d) (Wiethaus et al. 2006). We did not identify *cutO* mutants in this study suggesting that Tn5 mutagenesis was not saturating. In any case, mutants exhibiting severe defects during copper stress apparently are rare in *R. capsulatus*. To date, a role of TrgB in copper homeostasis has not been reported in any other bacterium.

TrgB confers tolerance to both copper and tellurite

Rhodobacter capsulatus trgB is flanked by genes predicted to code for a tellurite resistance protein (*trgA*), cysteine synthase (*cysK2*), and a mechanosensitive channel protein (*mscS*) (Fig. 2). While *trgB*-like genes are widespread in bacteria and archaea, *trgA*-like

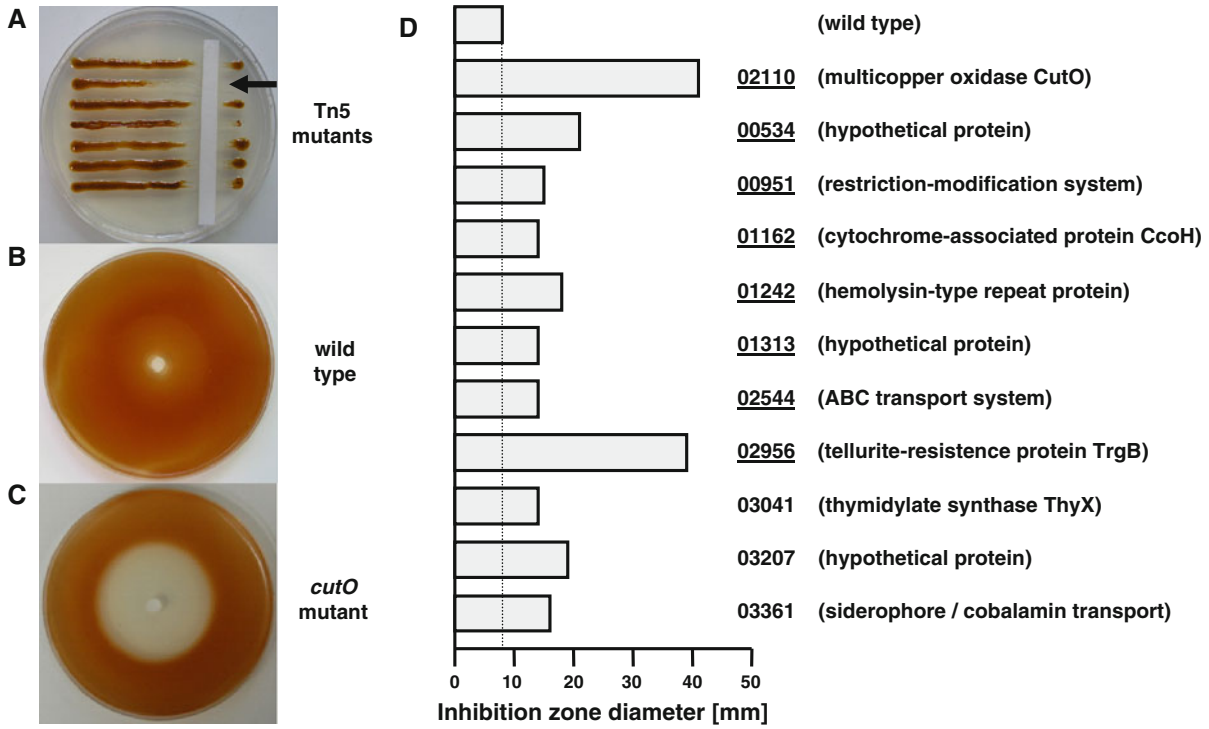

Fig. 1 Identification of copper-sensitive *R. capsulatus* mutants. To identify copper homeostasis genes in *R. capsulatus*, transposon Tn5 mutants were streaked on agar plates, before filter stripes soaked with 2.5 mM $CuSO_4$ were added. An example is shown in **a**. In total, more than 10,000 Tn5 mutants were screened by this assay. Copper-sensitive clones of this initial screening were further analyzed by filter disk assays as exemplary shown for two control strains, the wild type (**b**) and *cutO* mutant JW12-I (**c**). Inhibition zones were documented after two days of incubation. Inhibition zone diameters determined for the wild type, *cutO* mutant JW12-I, and ten newly identified copper-sensitive Tn5 mutants are shown (**d**). Five-digit accession numbers (RCAP_rcc0xxxx) of Tn5-disrupted genes are shown. To verify Tn5 mutant phenotypes, selected genes marked by *underlined numbers* were disrupted by plasmid integration

genes are unique to alphaproteobacteria (Marchler-Bauer et al. 2011). Putative *trgA* genes are mostly found directly upstream of *trgB* genes, but cotranscription of *trgA* and *trgB* genes has not been proven experimentally in any species. To examine the roles of *R. capsulatus trgA*, *cysK2*, and *mscS* in copper and tellurite tolerance, knock-out mutants were generated by insertion of kanamycin resistance (Km) cassettes. Transcription starting at the Km promoter is not terminated within the cassette, thus driving expression of downstream genes of the same operon (Drepper et al. 2006). All mutants grew as well as the wild type in standard RCV minimal medium (no copper or tellurite added) demonstrating that none of the genes was essential for viability (data not shown).

To examine effects of copper on these mutants, filter disc assays were performed (Fig. 3). The *trgB*::[Km>] and *trgB*::[<Km] mutants were copper-sensitive, thus verifying the phenotype observed for the original Tn5 mutant. In contrast to *trgB* mutants, the *trgA*::[Km>] and *trgA*::[<Km] mutants were copper-tolerant indicating that TrgA does not play a primary role in copper homeostasis in *R. capsulatus*.

Disruption of *cysK2* (*cysK2*::[Km>] and *cysK2*::[<Km]) did not cause cysteine auxotrophy indicating that loss of *cysK2* was compensated by the *cysK1* gene (RCAP_rcc02537) located upstream of the sulfate transport operon, *cysTWA*. Cysteine is of special importance during copper stress, as it acts as sulfur donor in biosynthesis of iron–sulfur clusters, which are primary targets of copper damage (Chillappagari et al. 2010). Both *cysK2* mutants were copper-tolerant suggesting that cysteine was not limiting under copper stress conditions in these strains (Fig. 3). Similar to *cysK2*, the *mscS* gene, which is cotranscribed with *cysK2* (see below), was dispensable for copper homeostasis.

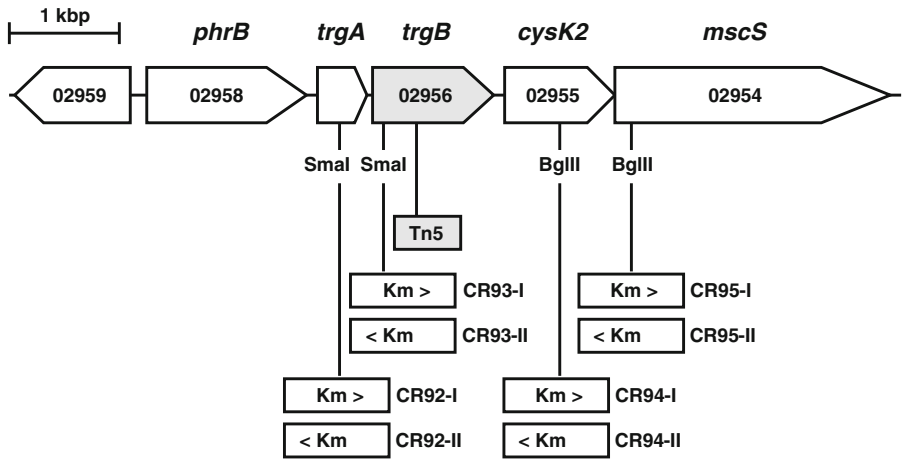

Fig. 2 Genetic map of the *R. capsulatus trgB* region. The locations of genes are given by arrows carrying their five-digit accession numbers (RCAP_rcc0xxxx) and proposed gene designations. The location of one Tn5 insertion resulting in a copper-sensitive phenotype is shown below the *trgB* gene. Restriction sites used for gene disruption by kanamycin resistance (Km) cassettes are given. Mutant designations are shown next to Km cassettes, which are not drawn to scale. The directions of Km gene transcription are symbolized by *arrowheads*, indicating polar (*lesser than arrow*) and nonpolar (*greater than arrow*) insertions

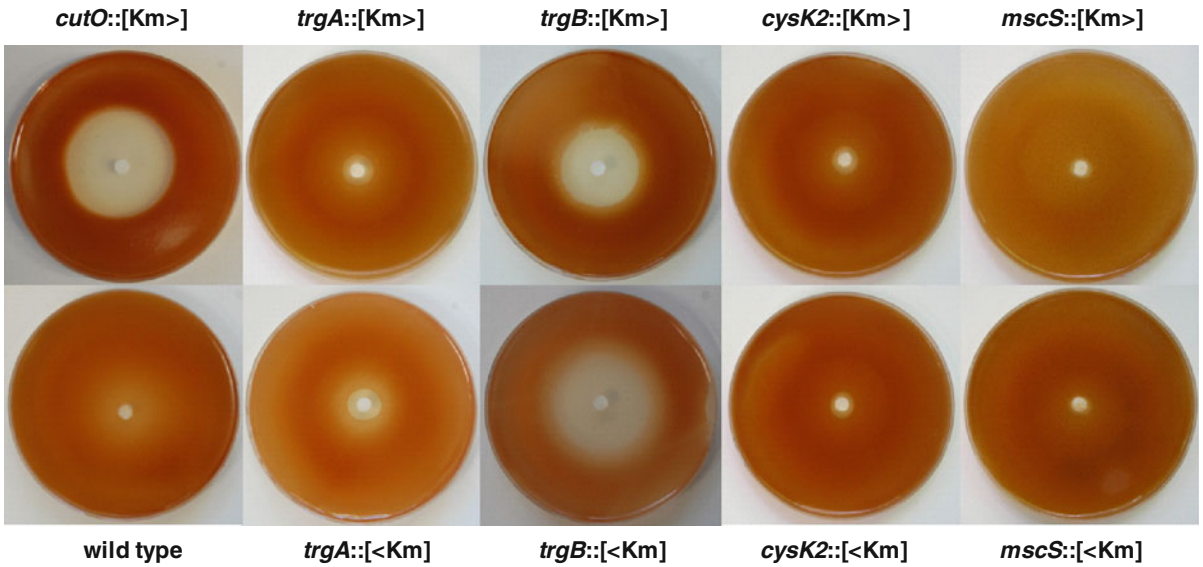

Fig. 3 Copper tolerance of *R. capsulatus* wild-type and mutant strains. Liquid cultures were plated onto RCV minimal medium plates, prior to central placement of filter discs soaked with 2.5 mM $CuSO_4$. Inhibition zones were documented after two days of incubation under anaerobic phototrophic conditions. *Data* are representative of at least three different experiments

To examine effects of tellurite on mutants defective for *trgB* and flanking genes, serial dilutions of these strains were spotted on plates containing tellurite (Fig. 4). While *R. capsulatus* typically forms red–brown colonies, colour changes to black on tellurite plates due to formation of elemental tellurium by a yet unidentified flavin-dependent reductase (Borghese and Zannoni 2010; Moore and Kaplan 1992).

Both *trgB* mutants were severely affected by tellurite (Fig. 4) implying that TrgB is a major tellurite resistance factor in *R. capsulatus*. In contrast, both *trgA* mutants exhibited only moderate tellurite sensitivity

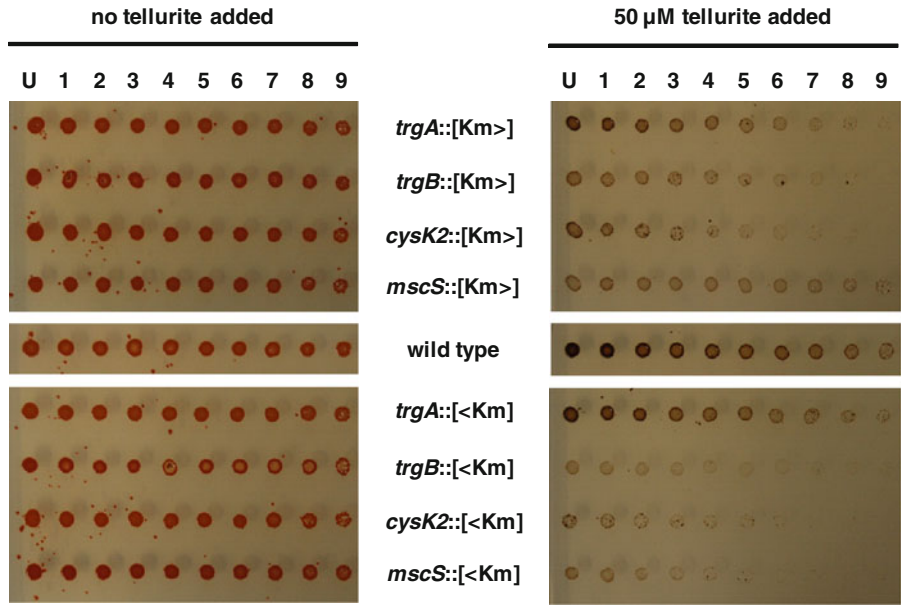

Fig. 4 Tellurite tolerance of *R. capsulatus* wild-type and mutant strains. Two microliters of undiluted (U) late-exponential phase cultures and nine serial (1:3) dilutions of wild-type and mutant strains were spotted on RCV minimal medium plates with or without 50 µM K_2TeO_3. Growth was documented after two days of incubation under aerobic conditions in the dark. Similar (but less pronounced) phenotypes were observed after growth under anaerobic phototrophic conditions (data not shown). *Data* are representative of two different experiments

indicating that TrgA is only of minor importance for tellurite tolerance. In line with the findings for *R. capsulatus trgB*, introduction of a *trgB*-like gene from *Methanococcus jannaschii* into *E. coli* increased tellurite resistance of the recombinant strain (Dunn et al. 1999). Similarly, *R. sphaeroides trgAB* genes increased tellurite resistance in *Paracoccus denitrificans* (O'Gara et al. 1997). It remains unknown, however, whether both genes contributed to tellurite resistance, since *trgA* and *trgB* were not introduced individually into *P. denitrificans*. Surprisingly, an *R. sphaeroides trgAB* double mutant was tellurite-tolerant (O'Gara et al. 1997).

Like *R. capsulatus trgB* mutants, *cysK2* mutants were strongly affected by tellurite (Fig. 4) demonstrating that *cysK1* was insufficient to maintain viability of *cysK2* mutants during tellurite stress. Although *trgB* and *cysK2* mutants exhibited comparable tellurite phenotypes, they clearly differed regarding copper sensitivity (Fig. 3). Similar to *R. capsulatus* CysK2, cysteine synthases from *R. sphaeroides*, *Azospirillum brasilense*, and *Bacillus stearothermophilus* confer tellurite tolerance (O'Gara et al. 1997; Ramírez et al. 2006; Vásquez et al. 2001).

The *mscS*::[Km>] mutant was hardly affected by tellurite (Fig. 4) indicating that MscS is per se dispensable for tellurite tolerance. By contrast, the *mscS*::[<Km] mutant exhibited severe defects in response to tellurite presumably due to uncharacterized polar effects of the Km cassette. One might speculate, however, that the *mscS*::[<Km] mutation interferes with expression of *cysK2*.

Taken together, *trgB* is the only gene in this region conferring copper tolerance, while in addition to *trgB*, *cysK2* is particularly important for tellurite tolerance. It is worth noting that a *cutO* mutant, which is strongly affected by copper (Wiethaus et al. 2006), was as tolerant towards tellurite as the wild type (data not shown). Thus, copper and tellurite stress apparently produce partly overlapping effects in *R. capsulatus*.

The monocistronic *trgB* gene is constitutively expressed

In *R. sphaeroides*, the *trgA* stop codon overlaps the *trgB* start codon (O'Gara et al. 1997) indicating transcriptional and translational coupling of the two genes. By contrast, the coding regions of *trgA* and *trgB*

are separated by 26 base pairs in *R. capsulatus*. Furthermore, *trgA*::[Km>] and *trgA*::[<Km] mutants were more tolerant towards copper and tellurite than *trgB* mutants (Figs. 3, 4) indicating that cassette-induced *trgA* mutations did not exhibit polar effects on *trgB* expression as one would have expected for two genes belonging to the same operon.

To determine the transcriptional organization of *trgB* and flanking genes, reverse transcriptase (RT)-PCR studies were carried out (Fig. 5). For this purpose, *R. capsulatus* wild-type cultures were grown in the presence of copper or tellurite or, as a control, without addition of these metals. Total RNAs from these cultures served as templates for cDNA synthesis using primers specific for *phrB*, *trgA*, *trgB*, *cysK2*, and *mscS* (Table 2). After reverse transcription, PCR amplification of DNA fragments overlapping gene borders or, as controls, internal gene fragments were undertaken.

The results of RT-PCR studies (Fig. 5) may be interpreted as follows. (i) Essentially, copper and tellurite had no effect on transcription of *phrB*, *trgB*, *cysK2*, and *mscS* as shown by analyses of internal gene fragments. (ii) We could not detect *trgA*-specific PCR products suggesting rapid degradation or low abundance of *trgA* transcripts. Control reactions with the same primer pair using chromosomal DNA as template produced PCR products of the expected size thus proving primer suitability. (iii) Failure to detect amplification products of *phrB–trgA* and *trgA–trgB* borders may either have resulted from rapid

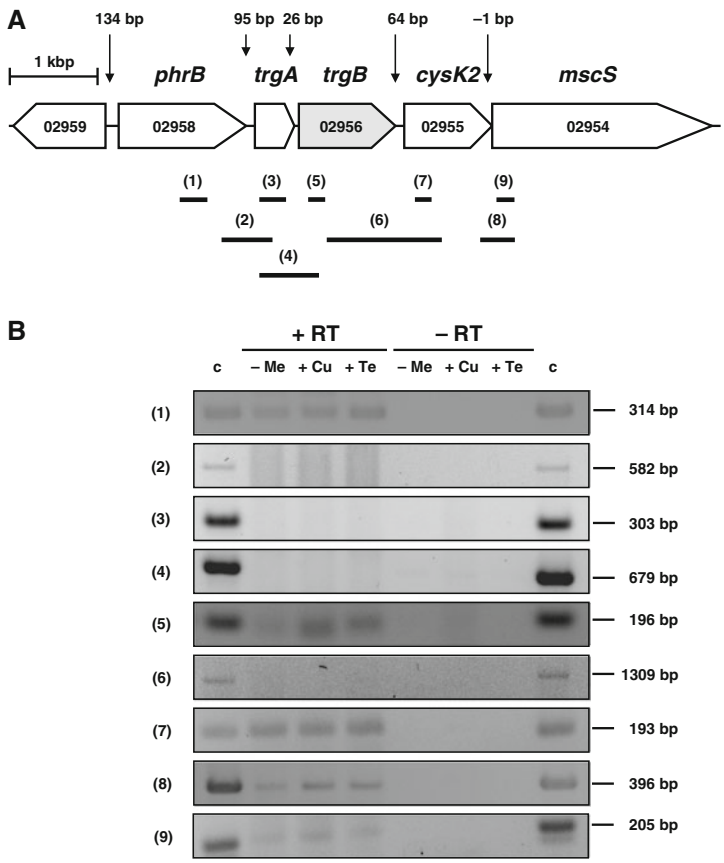

Fig. 5 Transcriptional analysis of the *R. capsulatus trgB* region. **a** The locations of genes are given by *arrows* carrying their five-digit accession numbers (RCAP_rcc0xxxx) and proposed gene designations. Distances between coding regions (in base pairs) are given above the genetic map. *Black bars* below the genetic map indicate DNA fragments emerging from reverse transcriptase (RT)-PCR. **b** Total RNA was isolated from *R. capsulatus* wild-type cells grown in RCV minimal medium in the presence of 2 μM $CuSO_4$ (+Cu) or 10 μM K_2TeO_3 (+Te) or without addition of these metals (−Me). RNA samples were either treated with reverse transcriptase to synthesize cDNA (+RT) or, as a negative control, reverse transcriptase was omitted (−RT). As a positive control (c), chromosomal DNA served as template

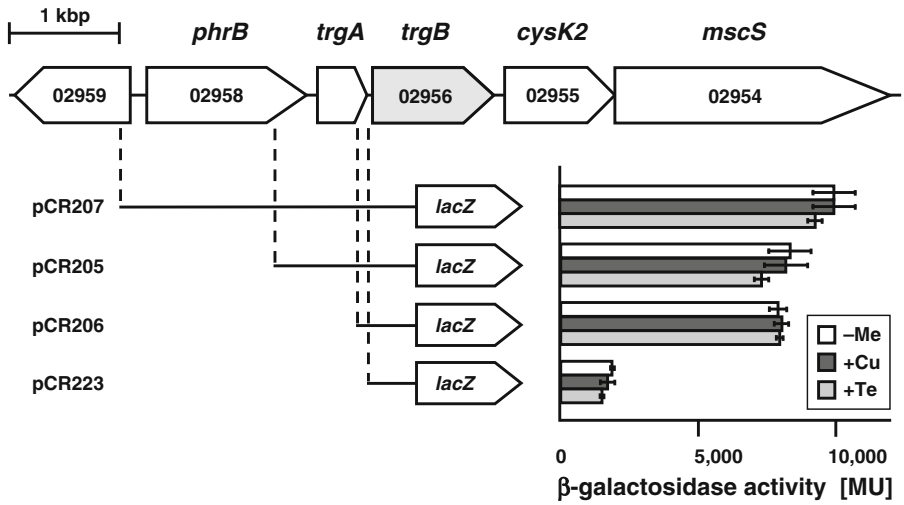

Fig. 6 Expression of *trgB–lacZ* fusions in *R. capsulatus*. The locations of genes are given by *arrows* carrying their five-digit accession numbers (RCAP_rcc0xxxx) and proposed gene designations. Reporter plasmids pCR205, pCR206, pCR207, and pCR223 carrying transcriptional *trgB–lacZ* fusions are based on the broad-host-range plasmid pBBR1-MCS5. These plasmids contain DNA fragments starting within 02959 (pCR207), *phrB* (pCR205), *trgA* (pCR206), or at the *trgA* stop codon (pCR223), respectively, as indicated. Plasmid pCR206 encompasses the last 162 bp of the *trgA* coding region. In all constructs the promoterless *lacZ* gene was fused to the same point within the *trgB* gene. *Rhodobacter capsulatus* reporter strains were grown until late-exponential phase in RCV minimal medium in the presence of 2 μM $CuSO_4$ (+Cu) or 10 μM K_2TeO_3 (+Te) or without addition of these metals (−Me) prior to determination of *β*-galactosidase activities (Miller 1972). Results (in Miller units) represent the means and standard deviations of five independent measurements

degradation of *trgA* mRNA or indicate monocistronic organization of *phrB*, *trgA*, and *trgB*. To demonstrate monocistronic organization of *trgB*, appropriate *trgB–lacZ* reporter fusions were analyzed (see below). (iv) While *trgB* and *cysK2* internal fragments were clearly detectable, no *trgB–cysK2* border-specific products were observed suggesting that the two genes belong to different transcription units. (v) The *cysK2* and *mscS* coding regions overlap by one base pair suggesting transcriptional and translational coupling. Indeed, synthesis of *cysK2–mscS* border-specific products supported cotranscription of these genes.

To narrow down the promoter region required for *trgB* expression, a series of transcriptional *trgB–lacZ* reporter fusions based on the broad-host-range plasmid pBBR1-MCS5 was constructed (Fig. 6). *Rhodobacter capsulatus* strains carrying these reporter plasmids were grown in the presence of copper or tellurite or, as a control, without addition of these metals. At late-logarithmic phase, *trgB–lacZ* expression was determined by *β*-galactosidase assays. Consistent with the RT-PCR data (see above), *trgB* transcription mediated by plasmids pCR205–pCR207 was not regulated by copper or tellurite. Like *trgB*, transcription of *cutO* did not respond to tellurite (data not shown). In contrast to *trgB*, however, *cutO* expression is regulated by copper (Wiethaus et al. 2006).

Expression of *trgB* did not depend on the *phrB* or the *trgA* promoter, but instead, *trgB* transcription was driven by sequences within the *trgA* coding region (Fig. 6). In detail, plasmid pCR206 carrying the smallest DNA fragment, which still mediated maximal *trgB–lacZ* expression, encompasses the last 162 bp of the *trgA* coding region and the *trgA–trgB* intergenic region. Plasmid pCR223, which mediated only basal *trgB–lacZ* expression, encompasses the *trgA* stop codon and the *trgA–trgB* intergenic region. These findings strongly suggest that the *trgB* promoter (or at least part of it) is located within the 3′ region of *trgA*. This region, however, did not contain obvious −35 or −10 regions, which are poorly defined in *R. capsulatus*. In any case, our findings strongly suggest that *trgB* forms a monocistronic operon, whose transcription essentially depends on DNA sequences within *trgA*.

TrgB and CutO do not influence cellular tellurium and copper contents

As described above, *R. capsulatus* produced black colonies on tellurite plates (Fig. 4) most-likely due to

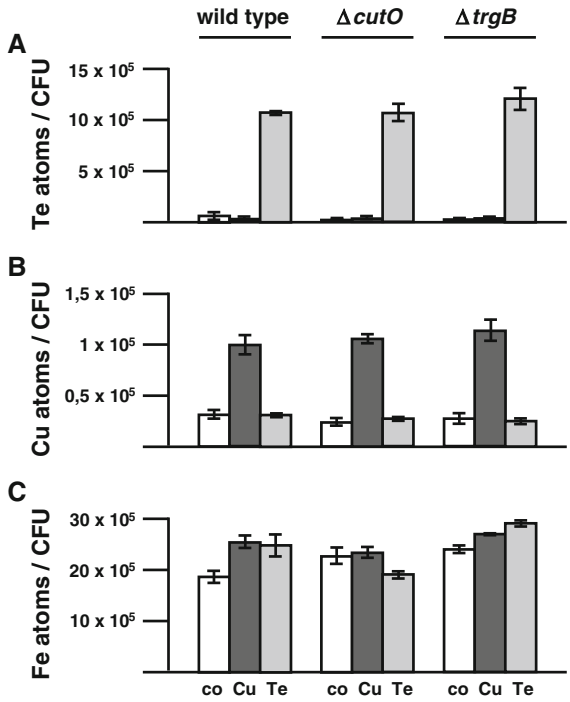

Fig. 7 Tellurium (**a**), copper (**b**), and iron (**c**) contents of *R. capsulatus* cells. Wild type, *cutO* mutant JW12-I, and *trgB* mutant CR93-I were grown to early stationary phase in RCV minimal medium in the presence of 2 μM $CuSO_4$ (Cu) or 10 μM K_2TeO_3 (Te) or, as control (co), without addition of these metals. Cells were repeatedly washed with 0.9 % NaCl, before metal contents were determined by ICP-OES. Metal contents are shown as number of atoms per colony forming unit (CFU). *Results* represent the means and standard deviations of at least three measurements from independent biological replicates

intracellular accumulation of elemental tellurium. To quantify this observation, cellular tellurium contents of the wild type, *trgB* mutant CR93-I, and *cutO* mutant JW12-I were determined by inductively coupled plasma-optical emission spectroscopy (ICP-OES) (Fig. 7a). Huge amounts of tellurium accumulated in wild-type and mutant cells during growth in the presence of 10 μM tellurite, while only background levels were detected in cells grown in standard RCV medium (no tellurite added) demonstrating that neither TrgB nor CutO were required for tellurite uptake. Tellurite uptake by *R. capsulatus* was previously shown to involve acetate permease (Borghese and Zannoni 2010). In a parallel assay, we observed increased copper accumulation by wild-type and both mutant strains during growth at elevated (2 μM) copper concentrations in the medium (Fig. 7b).

Although transcriptome studies of *Bacillus subtilis*, *E. coli*, and *Pseudomonas aeruginosa* revealed copper-induction of iron homeostasis genes including siderophore biosynthesis and uptake genes (Chillappagari et al. 2010; Kershaw et al. 2005; Teitzel et al. 2006), iron concentrations remained fairly constant in copper-stressed *B. subtilis* cells (Chillappagari et al. 2010). Likewise, iron concentrations did not change upon copper addition in *R. capsulatus* (Fig. 7c).

Conclusions

High copper and tellurite concentrations are toxic to living cells. While biological functions of copper and detoxification mechanisms against copper ions are well studied, little is known about the cellular response to tellurite. Here, we report a link between the copper and tellurite response in *R. capsulatus*. We discovered that a widely conserved tellurite resistance gene, *trgB*, confers tolerance against copper. In contrast to the periplasmic multicopper oxidase CutO (Wiethaus et al. 2006), TrgB is predicted to be localized in the cytoplasm (Yu et al. 2010) suggesting that the two enzymes protect different cellular compartments from copper toxicity.

TrgB exhibits similarity to gamma-glutamyl cyclotransferases (GGCTs) and ADP-ribose pyrophosphatases (ADPRases) (Dunn et al. 1999; Marchler-Bauer et al. 2011). GGCTs catalyze the formation of pyroglutamic acid from γ-glutamyl cysteine, which also serves as a building block for glutathione synthase to form γ-glutamyl-cysteineglycine (glutathione). Thus, GGCTs potentially influence homeostasis of glutathione and cysteine, which play central roles to maintain viability during oxidative stress (Chakravarthi et al. 2006; Helbig et al. 2008). ADPRases catalyze the hydrolysis of ADP-ribose, which accumulates during oxidative stress (Ogawa et al. 2009). Enhanced levels of free ADP-ribose lead to protein inactivation by non-specific modification (Jacobson et al. 1994; MacDonald and Moss 1994). At present, however, one might only speculate whether TrgB exhibits both GGCT and ADPRase activities.

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