Thioredoxin 2 is involved in oxidative stress defence and redox-dependent expression of photosynthesis genes in *Rhodobacter capsulatus*

Kuanyu Li, Elisabeth Härtigt and Gabriele Klug

Thioredoxins are small ubiquitous proteins that display different functions mainly via redox-mediated processes. The facultatively photosynthetic bacterium *Rhodobacter capsulatus* harbours at least two genes for thioredoxin 1 and 2, *trxA* and *trxC*. It is demonstrated that thioredoxin 2 of *R. capsulatus* can partially replace the thioredoxin 1 function as a hydrogen donor for methionine sulfoxide reductase but cannot replace thioredoxin 1 as a subunit of phage T7 DNA polymerase. By inactivating the *trxC* gene in *R. capsulatus*, it is shown that thioredoxin 2 is involved in resistance against oxidative stress. As thioredoxin 1 of *Rhodobacter sphaeroides*, *R. capsulatus* thioredoxin 2 affects the oxygen-dependent expression of photosynthesis genes, albeit in an opposite way. The *trxC* mutant of *R. capsulatus* shows a stronger increase in photosynthesis gene expression after a decrease in oxygen tension than the isogenic wild-type strain. The expression of the *trxC* gene is downregulated by oxygen.

**INTRODUCTION**

Thioredoxins are found in all living organisms. Together with glutaredoxins, they are the major enzymes responsible for maintaining disulfide bonds in cytoplasmic proteins in a reduced state (Ritz & Beckwith, 2001). These small proteins are capable of catalysing thiol-disulfide redox reactions by a common active site sequence: Cys-X1-X2-Cys. The oxidized thioredoxins are reduced by thioredoxin reductase under consumption of NADPH.

The electron transfer through disulfide bond exchange reactions in the cytoplasm recycles essential enzymes such as ribonucleotide reductase (Orr & Vitols, 1966), which provides deoxyribonucleotides for DNA synthesis. Other metabolic enzymes that are recycled by thiol-disulfide reactions are phosphoadenosine-phosphosulfate reductase (Lillig *et al.*, 1999), methionine sulfoxide reductase (Bosch-Muller *et al.*, 2000, 2001) and arsenate reductase (Shi *et al.*, 1999).

Furthermore, thioredoxin is an essential subunit of the DNA polymerase of bacteriophage T7 (Huber *et al.*, 1987; Mark & Richardson, 1976) and is essential for the assembly of several filamentous phages (Russel & Model, 1985). In eukaryotic organisms, many additional roles of thioredoxin have been reported, among which are the regulation of transcription factors such as NF-κB (Schulze-Osthoff *et al.*, 1995) and the regulation of apoptosis (Saitoh *et al.*, 1998). Thioredoxin is also involved in the regulation by light of photosynthetic enzymes in plant chloroplasts (Buchanan, 1984; Buchanan *et al.*, 1994).

Because of its extremely low redox potential and free thiol in its reduced form, which can readily form a disulfide bridge, thioredoxin is considered to be involved in defence against oxidative stress not only by regeneration of oxidatively damaged proteins (Fernando *et al.*, 1992; Natsuyama *et al.*, 1992), but also by its ability to reduce hydrogen peroxide (Mitsui *et al.*, 1992; Nakamura *et al.*, 1994; Spector *et al.*, 1988; Tomimoto *et al.*, 1993) or by acting as a hydrogen donor for peroxidase (Chae *et al.*, 1994). Thioredoxin also functions as a singlet oxygen quencher and hydroxyl radical scavenger independently of its redox state (Das & Das, 2000).

Two thioredoxin genes, *trxA* and *trxC*, encoding thioredoxin 1 and 2, respectively, were identified in *Escherichia coli* (Laurent *et al.*, 1964; Miranda-Vizuete *et al.*, 1997). Thioredoxin 1 and thioredoxin 2 have 29% sequence identity, with the greatest difference being a 32 aa extension of thioredoxin 2 at its N terminus. Thioredoxin 2 possesses additional cysteine thiols apart from those of the active site, which, when oxidized, downregulate its activity in the reduction of insulin disulfides. Thioredoxin 2 is also less heat-stable than thioredoxin 1 and does not reduce ...
ribonucleotide reductase as efficiently as thioredoxin 1. Thioredoxin 2 participates in the OxyR-orchestrated antioxidant response (Ritz et al., 2000). Thioredoxin 1 expression is not controlled by OxyR but in the stationary phase is controlled by ppGpp (Lim et al., 2000).

The facultatively phototrophic purple bacteria of the genus *Rhodobacter* can adapt rapidly to changes in their environment. As long as oxygen is available, they perform oxidative respiration. If the oxygen tension drops below a threshold value, the formation of pigment protein complexes is induced. When oxygen is no longer available, *Rhodobacter* gains energy by anoxygenic photosynthesis in the presence of light. Many different proteins are involved in the oxygen-dependent regulation of photosynthesis genes in *Rhodobacter* (e.g. Gregor & Klug, 2002). A central component of the redox control system of *Rhodobacter capsulatus* is the two-component system RegB/RegA (PrrB/PrrA in *Rhodobacter sphaeroides*) (Senga & Bauer, 1992). The phosphorylated response regulator RegA activates the expression of photosynthesis genes at a low oxygen tension (Masuda et al., 1999; Senga & Bauer, 1992). Some photosynthesis genes are repressed at a high oxygen tension by the CrtJ protein (PpsR in *R. sphaeroides*). CrtJ binds to its target DNA sequences in a redox-dependent manner (Ponnampalam & Bauer, 1997). It was shown recently that the CrtJ protein forms an intramolecular disulfide bond when exposed to oxygen, which is critical for binding to its target promoters (Masuda et al., 2002). In addition to these DNA-binding proteins, thioredoxin 1 was shown to be involved in the redox-dependent expression of photosynthesis genes in *R. sphaeroides* by a yet unidentified mechanism. A mutant strain, which harbours reduced levels of thioredoxin 1, shows a lower degree of induction of the *puf* and *puc* genes encoding pigment-binding proteins after a decrease in oxygen tension than the wild-type strain (Pasternak et al., 1999). In *R. sphaeroides*, the single thioredoxin 1 is essential for growth (Pasternak et al., 1997). The expression of *trxA* increases during an increase in oxygen tension (Pasternak et al., 1999).

The genome of *R. capsulatus*, a close relative of *R. sphaeroides*, harbours at least two genes encoding thioredoxins, *trxA* and *trxC*. Since thioredoxin 2 resembles the thioredoxin 1, it is possible that thioredoxin 2 of *R. capsulatus* is also involved in the redox-dependent regulation of photosynthesis genes. To learn more about the function of thioredoxin 2 in *R. capsulatus*, we constructed a *trxC* mutant of this strain and constructed plasmids with wild-type and mutated thioredoxin genes, which were expressed in *E. coli*. We demonstrate (1) that it can partially replace the thioredoxin 1 function as hydrogen donor for methionine sulfoxide reductase, but (2) that it cannot replace thioredoxin 1 as a subunit of phage T7 DNA polymerase independent on its redox potential, (3) that *R. capsulatus* thioredoxin 2 is involved in defence against oxidative stress and (4) that *R. capsulatus* thioredoxin 2 affects the oxygen-dependent expression of photosynthesis genes as thioredoxin 1 from *R. sphaeroides*, albeit in an opposite way. Thus, the *trxA* gene from *R. sphaeroides* and the *trxC* gene from *R. capsulatus* are reversely affected by oxygen, and the corresponding thioredoxins exert an opposite effect on the expression of photosynthesis genes.

**METHODS**

**Strains, plasmids and growth conditions.** The strains and plasmids used in this study are listed in Table 1. Thioredoxin sequences from *R. capsulatus* and *R. sphaeroides* genomes were used to design the primers KH8 (5’-GGCCACCATGGCGGAATC-3’) and HE26 (5’-TCAAGCTTCCAAACCC-3’) for *trxA* (RCC00979), encoding the 147 aa thioredoxin 2 protein of *R. capsulatus*, RtrxAsart (5’-GAGAATCCCATGGCTACCGTCG-3’) and RtrxAEndHind (5’-GGTAAGCTTGGCCTCCCGTTGC-3’) for *trxA* (RCC03345), encoding the 106 aa thioredoxin 1 protein of *R. capsulatus*, and HE7 (5’-CTCGGAAGCTTGTCAAGGAA-3’) and HE12 (5’-AATGGATCATGAGCATT-3’) for the 106 aa thioredoxin 1 protein of *R. sphaeroides*. The PCR products were restricted with *NcoI* and *HindIII*, ligated to appropriately digested pUTC51 and transformed into *E. coli* strain BH216 (Table 1).

*E. coli* cultures were grown in Luria–Bertani (LB) or M9 minimal medium as indicated, *R. capsulatus* was cultivated at 32 °C in a malate minimal salt medium (Drews, 1983). Cells were grown aerobically by shaking 100 ml of culture at 140 r.p.m. in 1 l baffled flasks (about 20 % oxygen saturated, as determined using an Ag/Pt oxygen electrode). For semi-aerobic cultures, 40 ml of culture was shaken in 50 ml Erlenmeyer flasks at 140 r.p.m. (1–2 % dissolved oxygen, as determined using an Ag/Pt oxygen electrode). For oxygen shift experiments, cells were grown aerobically to an OD600 of 0.5–0.6 and then transferred to the 50 ml flasks or vice versa. Incubation was in the dark.

**Determination of survival rates.** Cultures were grown under semi-aerobic conditions until the optical density reached 0.4–0.5; then, oxidative stress agents were added at indicated concentrations for 1 h and dilutions were plated. Survival of 100 % corresponds to the viable cell number determined immediately before the addition of the oxidative agents. The percentage of colonies grown from the treated cultures is given as the percentage survival in Table 2. The values are the mean of three experiments. Hydrogen peroxide, tert-butyl hydroperoxide (t-BOOH), diamide and methyl viologen (Parquat) were purchased from Sigma.

**Zone of inhibition assays.** For zone of inhibition assays, cells were grown semi-aerobically overnight at 32 °C and then diluted in the same medium to an OD600 of 0.2. Cells were then grown to an OD600 of 0.5 (mid-exponential phase samples) and 100 µl of culture was added to 2 ml prewarmed (47 °C) top agar (0.7 % agar) and layered onto malate minimal salt medium plates. After the top agar had hardened, a 5-5 mm filter paper disk, containing 5 µl of oxidative agent as indicated, was placed on the plate. The plates were then incubated overnight at 32 °C and the radio of the zone of growth inhibition was examined to determine the sensitivity of the cell against the agents. All assays were performed three or four times in duplicate.

**Phage assay.** To determine whether TrxC of *R. capsulatus* can substitute for *E. coli* TrxA as an essential subunit of T7 DNA polymerase, T7 infection tests were performed as described by Sambrook et al. (1989). Bacteria were grown overnight in LB broth supplemented with the corresponding antibiotics. Appropriate culture dilutions were mixed with corresponding-titre T7, incubated for 20 min at
Table 1. Bacterial strains and plasmids

<table>
<thead>
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<th>Strain/plasmid</th>
<th>Relevant characteristic(s)*</th>
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<td>JM109</td>
<td>recA1 supE44 endA1 hisD177 gyrA96 relA1 thi (lac-proAB)</td>
<td>Stratagene</td>
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<tr>
<td></td>
<td>F' [traD36 proAB+ lacIq lacZ M15]</td>
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<td>BH216</td>
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<tr>
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<td>Host strain for protein overexpression</td>
<td>Qiagen</td>
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<td>Keen &amp; Tamaki (1988)</td>
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<td>Assemat et al. (1995)</td>
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<td>This study</td>
</tr>
<tr>
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<td>Tc' lacZa mob(LP4)</td>
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<tr>
<td>pILA</td>
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<td>Kunert et al. (2000)</td>
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<td>PBBR1MCS with upstream fragment of trxC gene</td>
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<tr>
<td>pBBR502lux</td>
<td>PBBR502 with 2·45 kb luxAB fragment</td>
<td>This study</td>
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*Ap, ampicillin; Km, kanamycin; Tc, tetracycline.

room temperature for attachment then added to 0·7% agar LB broth and spread on to 1% LB plates, which were incubated at 37°C for 4 h to monitor T7 development.

**Cloning of R. capsulatus and R. sphaeroides trx genes.** The trxC sequence (RCC00979) was used to design the specific mutagenic primers RtrxCex> A, 5'-GGATCCCGGAGAACGGCTTGGAGA-3' (forward, introduction of BamHI site), and RtrxCex> B, 5'-AAGCTTTCTGCGGCCGAGTGTTCTC-3' (reverse, introduction of HindIII site). The PCR product produced with the above primers was cloned into pGEM T-vector (Promega) and recloned into pQE32 His-tag vector (Qiagen) digested with HindIII and BamHI. The recombinant plasmid designated pQEtrxC was transformed into E. coli strain JM109. The correct construct as confirmed by sequencing was transformed into E. coli strain M15 (pREP4) for overexpression of thioredoxin 2 induced by the addition of 1 mM IPTG at 32°C for 4 h.

For mutagenesis of the first cysteine of thioredoxin 1 and/or 2 active site -C-X-C-, the following primers were designed: RsC29A > A (forward), 5'- GGGGCCGGCCTGGCGGAGAT-3', and RsC29A > B (reverse, complementary to RsC29A > A), 5'- ATCTGCGGGCCGGCCGGCCG-3', for trxA of R. sphaeroides (RtrxA) and trxC of R. capsulatus (RtrxC); RsC29A > A, 5'- GAATTGGCGTGCCGGCCGCCCAGAT-3', and RsC29A > B (reverse, complementary to RsC29A > A), 5'- CATCTTGCGGGCCGCGGCCCATTCC-3', for trxA of R. capsulatus (RtrxA). The upstream fragments of RtrxA from primers EH12 and RsC29A > B, RtrxA from RtrxAstart and RsC29A > A, RtrxC from KH8 and RsC29A > B and the downstream fragments of RtrxA from primers EH7 and RsC29A > A, RtrxA from RtrxAend and RsC29A > A, RtrxC from EH26 and RsC29A > A were amplified by PCR. The diluted overlapped PCR products of upstream and downstream fragments, which acted as primers and templates, were amplified to produce the full-length thioredoxin fragments with the mutation of cysteine into alanine. The full-length trx fragments were cloned into plasmid pUTC5 (Assemat et al., 1995) replacing wild-type trxA of R. sphaeroides.

**Mutant construction.** R. capsulatus strain SB1003trxC− was generated by transferring the suicide plasmid pPHARtrxC::Km into wild-type SB1003 and screening for insertion of the kanamycin cassette into the chromosome by double crossover. To this end, parts of the
### Table 2. Survival rates of wild-type SB1003 and a *trxC* mutant from *R. capsulatus* after the addition of substances which generate oxidative stress

The number of viable cells before change in oxygen tension or addition of oxidative-stress-generating agents equals 100% (column 2). All other columns refer to the number of viable cells 1 h after the change in culture conditions. The values give the mean of three experiments with maximal deviation. SA, Semi-aerobic conditions; SA–A, shift from semi-aerobic to anaerobic conditions; SA–nBOOH, shift from semi-aerobic to anaerobic conditions with 10 mM H$_2$O$_2$; SA–nParaquat, shift from semi-aerobic to anaerobic conditions with 50 mM paraquat; SA–nDiamide, shift from semi-aerobic to anaerobic conditions with 11 mM diamide; SA–nDiphenyleneiodonium, shift from semi-aerobic to anaerobic conditions with 50 mM diphenyleneiodonium; SA–nBu$_2$I, shift from semi-aerobic to anaerobic conditions with 50 mM Bu$_2$I.

<table>
<thead>
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<th>Strains</th>
<th>SA (t=0)</th>
<th>SA (t=1 h)</th>
<th>SA–A</th>
<th>SA–nBOOH</th>
<th>SA–nParaquat</th>
<th>SA–nDiamide</th>
<th>SA–nBu$_2$I</th>
<th>SA–nDiphenyleneiodonium</th>
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<tr>
<td>SB1003ΔtrxC</td>
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<td>100⁄±7</td>
<td>100</td>
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</table>

*trxC* gene together with upstream and downstream sequences were amplified by PCR using the primers P1 and P2 and primers P3 and P4, respectively. The primer sequences were as follows: P1, 5'-GGGTAGACCCAGGCGGCTTACG-3'; P2, 5'-GGATCCGCCGCAAGGCGGGTATG-3'; P3, 5'-CGGGGATCTTGRGCGGCTGT-3'; P4, 5'-CAGACGTTATCGGGCCTAGTCT-3'. The PCR products were digested with appropriate restriction enzymes (Kpn I and Bam HI for the upstream fragment, Bam HI and HindIII for the downstream fragment) and cloned into pPHU281 (Hubner et al., 1993) to give plasmid pPHRctrxC and generating a Bam HI site at the junction. Then, a 1:3 kb Bam HI fragment containing the kanamycin cassette from pUC4KSAC (Barany, 1985) was inserted into the Bam HI site of pPHRctrxC to generate pPHRctrxC::Km, which was transformed into *E. coli* strain SM10. For the purpose of generating strain SB1003ΔtrxC, diparental conjugation was carried out with *R. capsulatus* SB1003 as a recipient strain. A Southern hybridization was performed to confirm the correct insertion of the kanamycin cassette into the chromosome.

#### RT-PCR.
For RT-PCR analysis, the reverse transcription reaction was carried out in a final volume of 25 µl containing 0·125 µg random primer (Promega), 5 µg total RNA, 1 mM each of four deoxyribonucleoside triphosphates and 1·5 units reverse transcriptase (Promega) at 42°C for 1 h and 99°C for 2 min to inactivate the reverse transcriptase. PCR was carried out in a final volume of 50 µl containing 5 µl reverse-transcribed cDNA solution, 1 unit Taq polymerase (Qiagen) and 2 pmol each of the oligonucleotide primers described previously (Engler-Blum et al., 1993; Pasternak et al., 1997). Amplification was carried out by an initial denaturation step at 96°C for 1 min followed by 38 cycles for *trxC* or 23 cycles for *trxA* at 96°C for 1 min, 62°C (for *trxC*) or 57°C (for *trxA*) for 30 s and 72°C for 30 s. A sample lacking reverse transcriptase was included for each reaction as a control for DNA contamination. Reaction products were subjected to electrophoresis on 4% agarose gels (Biozym).

#### Southern, Northern and colony hybridization analysis.
Southern and Northern blot hybridizations were performed as described previously (Engler-Blum et al., 1993; Pasternak et al., 1997). Colony hybridization was carried out according to the manufacturer’s recommendation (PALL).

#### Production of antibodies and Western blot analysis.
For immunological detection of thioredoxin 1 or 2, 20 µg crude cell extracts as determined by Bradford (1976) were separated by SDS-PAGE (Laemmli, 1970) on 15% polyacrylamide gels and transferred to Immobilon-P membrane (Millipore). The *R. sphaeroides* thioredoxin 1 and *R. capsulatus* thioredoxin 2 proteins were expressed as His-tag fusion proteins from pQE32-derived plasmids (Qiagen) in *E. coli* M15(pREP4) and the antibodies against the gel-purified proteins were raised in rabbits (Clontech). The antibodies were purified from the serum by protein A Sepharose. Western blotting was performed according to the Western Exposure Chemiluminescent Detection system (Clontech).

#### Bacteriochlorophyll measurements.
A sample (0·5 ml) of culture was sedimented and resuspended in 0·5 ml of acetone–methanol (7·2, v/v). The absorbance of the supernatant at 770 nm was determined after spinning in a microcentrifuge for 3 min. The relative bacteriochlorophyll content of the cells is given as the absorbance at 770 nm divided by the optical density at 660 nm.

#### Construction of the reporter genes for luciferase assays.
The luciferase reporter plasmids were constructed by cloning the *Kpn*–*Stu* PCR fragments coding the 5’-flanking region of the *trxC* gene of *R. capsulatus* into restriction sites *Kpn* I and *Smal* of vector pBBR1MCS-5 (Kovach et al., 1994) to generate pBBS02.
generate pBBR502lux, the luxAB Xbal fragment from plasmid pILA (Kunert et al., 2000) was cloned into the appropriate restriction sites of pBBR502. Plasmid pBBR502lux was transferred into R. capsulatus SB1003 by diparental conjugation to obtain SB1003 (pBBR502lux) (Table 1).

Luciferase assays were performed as described by Kunert et al. (2000) at room temperature with some modifications. In brief, luminescence of the luciferase reaction was induced by the addition of decanal (Sigma) to 1 ml culture (final concentration of decanal 0.5 mM). Light emission was monitored in a photomultiplier-based luminometer (BioOrbit; Labsystems). The mean value of four to five data near the maximum of the peak was used as the luminescence output. All the measurements were made in duplicate and experiments were performed at least twice using independent cultures.

RESULTS

Thioredoxin 2 from R. capsulatus can replace the thioredoxin 1 from E. coli as an electron donor for methionine sulfoxide reductase, but not as a functional subunit of phage T7

Before analysing the effect of a trxC mutation in R. capsulatus, we wanted to confirm that thioredoxin 2 from this organism is functionally similar to thioredoxin 2 from E. coli. The E. coli metE strain BH216 is a methionine auxotroph and lacks a functional trxA gene (Pille et al., 1990). The parental metE strain can grow on minimal medium supplemented with methionine sulfoxide, since it is able to convert methionine sulfoxide to methionine. Since thioredoxin 1 is required as a hydrogen donor for methionine sulfoxide reductase, BH216 can grow on minimal medium only if methionine is added. Different plasmids, in which overexpression of thioredoxin was regulated by an IPTG-inducible promoter, were constructed and transformed into BH216 to complement trx deficiency, as evidenced by growth on minimal medium with methionine sulfoxide (Table 1).

Affinity-purified polyclonal antibodies were used to determine the presence of thioredoxin of R. sphaeroides or R. capsulatus in E. coli strains, which harbour the plasmids with trx genes of R. sphaeroides or R. capsulatus. Fig. 1 shows that the anti-thioredoxin 1 or anti-thioredoxin 2 antibodies reacted with one band of the expected size of thioredoxin 1 (11–12 kDa) or thioredoxin 2 (15–3 kDa) in a total crude extract of the E. coli transformants. Thus, thioredoxin 1 of R. sphaeroides or R. capsulatus and thioredoxin 2 of R. capsulatus are expressed in E. coli strain BH216. Thioredoxin 1 proteins of E. coli, R. sphaeroides and R. capsulatus were not detectable with anti-thioredoxin 2 antibody of R. capsulatus; only one clear band was observed in BH216(pUTRctrxC) cell extracts (Fig. 1b). In contrast, antibodies raised against E. coli thioredoxin 2 could react with E. coli thioredoxin 1, while E. coli thioredoxin 1 antibodies did not cross-react with thioredoxin 2 (Miranda-Vizuete et al., 1997).

Since thioredoxin 1 is required as hydrogen donor for methionine sulfoxide reductase, BH216 can form colonies overnight on minimal medium only if methionine is added. Strain BH216, which contains a plasmid harbouring the E. coli trxA gene, can grow on minimal medium containing methionine sulfoxide but no methionine. Strain BH216 containing either a plasmid harbouring the trxA gene from R. sphaeroides or from R. capsulatus can also grow under these conditions, indicating that the thioredoxin 1 protein from R. sphaeroides or R. capsulatus can act as a hydrogen donor for methionine sulfoxide reductase in E. coli (Fig. 2).

Fig. 1. Test for thioredoxin expression of R. sphaeroides or R. capsulatus in E. coli using a Western blot. Samples were probed with the anti-TrxA antibody of R. sphaeroides (a), then the blot was stripped and reprobed with anti-TrxC antibody of R. capsulatus (b). (a) Identification of TrxA in cell extracts of E. coli. Lanes: 1, BH216 control, no TrxA expression; 2, BH216(pUTC51) expressing RsTrxA (11.3 kDa); 3, BH216 (pUTECTX) expressing EcTrxA (11.8 kDa); 4, BH216(pUTRctrxA) expressing RcTrxA (11.2 kDa); 5, BH216(pTURctrC) expressing RcTrxC, no signal with anti-RsTrxA. Note that the relative migration of the different thioredoxin proteins in 15% SDS–PAGE is not in accordance with the calculated molecular masses. (b) Identification of TrxC in cell extracts of E. coli BH216(pUTC51) expressing RsTrxC (15.3 kDa). No TrxC signal was observed in other BH216 derivative strains.

Fig. 2. Test for thioredoxin function as an electron donor of methionine sulfoxide reductase. Strain BH216 with plasmids harbouring trxC (b), trxA (c) of R. capsulatus, or trxA of R. sphaeroides (d) or E. coli (e) exhibited growth on minimal medium overnight with 30 μg methionine sulfoxide ml⁻¹ as the methionine source. Parental strain BH216 used as a control did not show any growth on the same medium (a).
Strain BH216 containing a plasmid that allows overexpression of the \( R. \ capsulatus \) trxC gene shows only very weak growth on minimal medium with methionine sulfoxide, indicating that thioredoxin 2 cannot fully replace this thioredoxin 1 function.

To see whether the function of thioredoxin of \( R. \ sphaeroides \) or \( R. \ capsulatus \) as hydrogen donor for methionine sulfoxide reductase is dependent on the cysteine at the active site, the first cysteine of the active site was mutated into alanine in the three thioredoxins (C29A for thioredoxin 1 of \( R. \ sphaeroides \) and \( R. \ capsulatus \), C70A for thioredoxin 2 of \( R. \ capsulatus \)). As expected, the mutant thioredoxins were no longer able to function as a hydrogen donor for methionine sulfoxide reductase. BH216 derivatives harbouring the plasmid, which enables overexpression of the C29A or C70A mutant thioredoxin, could not form colonies on M9 minimal medium with the addition of methionine sulfoxide after overnight incubation (data not shown). But with the addition of methionine in M9 medium, colonies of a normal size were formed. These results confirmed that this function of thioredoxin as a hydrogen donor is dependent on the cysteine of the active site and that the two additional pairs of cysteines at the N terminus of thioredoxin 2 from \( R. \ capsulatus \) cannot donate hydrogen to methionine sulfoxide reductase.

Reduced thioredoxin complexes with bacteriophage T7 DNA polymerase, resulting in an enzyme with a high processivity (Huber et al., 1987). T7 phage cannot be propagated in \( E. \ coli \) strain BH216, which is unable to express thioredoxin 1. T7 phage can be propagated in \( E. \ coli \) strain BH216 containing a plasmid harbouring the \( E. \ coli \) trxA gene, the \( R. \ sphaeroides \) trxA gene or the \( R. \ capsulatus \) trxA gene (data not shown). Thus, thioredoxins 1 from \( R. \ sphaeroides \) and \( R. \ capsulatus \) can function as subunits of T7 DNA polymerase in \( E. \ coli \). However, we observed no propagation of phage T7 in BH216 containing a plasmid, which allows expression of the \( R. \ capsulatus \) thioredoxin 2 (data not shown). We conclude that \( R. \ capsulatus \) thioredoxin 2 cannot function as a subunit of T7 DNA polymerase.

The ability of T7 to grow on the \( E. \ coli \) trxA mutant strains in which one or both active site cysteine residues of thioredoxin had been changed to serine or alanine definitively demonstrated that the redox capacity of thioredoxin is not required for stimulation of DNA polymerase activity (Huber et al., 1986). In contrast, neither thioredoxin-S2 nor modified thioredoxin in which a single cysteine had been methylated formed a complex with T7 DNA polymerase (Adler & Modrich, 1983), suggesting that the conformation of the active site is very important for this interaction. To test the effect of thioredoxin 1 mutation C29A, the identical constructs as described above for the \textit{in vivo} methionine sulfoxide assay were used for T7 infection. Interestingly, clear plaques were observed in the plate with strain BH216(pUTRctrxC29A), but not in the plate with strain BH216(pUTRstrxC29A). Thus, the mutated thioredoxin 1 (C29A) from \( R. \ capsulatus \) still acts as a subunit of T7 DNA polymerase to allow T7 phage to amplify, while mutated thioredoxin 1 of \( R. \ sphaeroides \) does not. Both thioredoxins 1 of the closely related species, \( R. \ sphaeroides \) and \( R. \ capsulatus \), share an amino acid sequence identity of 82%. However, the two active sites have different amino acids surrounding the cysteines. The mutation in the active site may lead to different conformational changes in the two thioredoxins (see Discussion).

**Construction of a trxC mutant of \( R. \ capsulatus \)**

To learn more about the role of thioredoxin 2 in \( R. \ capsulatus \) and to test for a function in redox-dependent regulation of photosynthesis genes, we inactivated the trxC gene of \( R. \ capsulatus \). The trxC gene is positioned between the genes for thymidine kinase and Ala-tRNA on the \( R. \ capsulatus \) chromosome (Fig. 3). All these genes are oriented in the same direction as the trxC gene. To inactivate the trxC gene in \( R. \ capsulatus \), we cloned the trxC upstream and downstream regions including the N-terminal and C-terminal segments of the coding regions into plasmid pPHU281 (Hübner et al., 1993), which is unable to replicate in \textit{Rhodobacter}. We then inserted the kanamycin cassette gene lacking transcriptional terminators between the trxC upstream and downstream segments. After conjugational transfer into \( R. \ capsulatus \), about 400 colonies were screened for insertion of the resistance gene into the chromosome via a double crossover by testing for resistance to kanamycin but a lack of the plasmid encoded tetracycline resistance. The fact that all of the kanamycin-resistant colonies were tetracycline-sensitive indicated that insertion of the tetracycline resistance gene into the chromosome by a single crossover may not lead to expression of tetracycline resistance. We therefore analysed those kanamycin-resistant clones by colony hybridization. Twenty-nine clones did not show hybridization to plasmid pPHU281. Isolated chromosomal DNA from these clones was used for Southern hybridization to confirm the presence of the resistance cassette and to test for the interruption of trxC gene. Two trxC mutant clones were identified, which had the kanamycin cassette integrated into the chromosomal trxC gene by a double crossover.

**Fig. 3.** Schematic representation of the thioredoxin 2 gene, trxC, and its neighbouring genes in strain SB1003 and the trxC replacement by a kanamycin cassette. The arrows show the orientation of the genes on the chromosome. The size of the hypothetical protein is not to scale, as indicated by slashes.
Doubling times and survival rates of the \textit{trxC} mutant under different growth conditions

To see whether the \textit{trxC} mutation affects the doubling time of \textit{R. capsulatus} cultures, we determined the growth rates of the mutant and the isogenic wild-type strain during growth under a high oxygen tension (about 20\% dissolved oxygen), low oxygen tension (1–2\% dissolved oxygen), anaerobic incubation in the light and after a shift from a high to low oxygen tension or vice versa. Under all conditions tested, the maximal doubling times of the wild-type and the \textit{trxC} mutant showed no significant differences, while some difference was observed during the sudden change in oxygen tension. When exponential-growth cultures (OD$_{600}$ = 0.4–0.5) were shifted from low to high oxygen tension, in the first hour the \textit{trxC} mutant showed a transient decrease in optical density, which did not occur in the wild-type strain (Fig. 4a). Later on, the mutant and the wild-type grew with identical rates. The number of viable cells determined in the culture of the \textit{trxC} mutant 1 h after the increase in oxygen tension was similar to that directly before the transition (Table 2). In contrast, the number of viable cells in the wild-type culture increased to 136\% during the same time (Table 2). These results indicate that thioredoxin 2 of \textit{R. capsulatus} is required for a fast adaptation to increasing oxygen tension in the environment.

To test whether thioredoxin 2 is involved in the oxidative stress response of \textit{R. capsulatus}, we determined the survival rates after treatment with compounds that can induce oxidative stress artificially. We used Paraquat as a superoxide-generating compound, H$_2$O$_2$ as a direct oxidant and compounds leading to glutathione depletion: the membrane-permeable thiol-specific oxidizing agent diamide and the organic hydroperoxide t-BOOH. t-BOOH serves also as a non-physiological model alkylhydroperoxide. The results of these experiments are summarized in Table 2.

The addition of 1 mM H$_2$O$_2$ inhibited growth of the \textit{trxC} mutant to a larger extent than growth of the wild-type strain. One hour after addition of 1 mM H$_2$O$_2$ to wild-type cells, the number of viable cells was increased to a mean of 134\% of the number before the treatment, while the number of viable cells increased to a mean of 157\% in the untreated control. In the culture of the \textit{trxC} mutant, the number of viable cells was decreased to 73\% after 1 h of H$_2$O$_2$ treatment. There were no significant differences in the survival rates of wild-type and mutant strain after treatment with lower concentrations of H$_2$O$_2$. The \textit{trxC} mutant was very sensitive against treatment with 1 mM Paraquat. The survival rate after 1 h of treatment with Paraquat was

Fig. 4. Effect of the \textit{trxC} mutation on growth of \textit{R. capsulatus} after (a) an increase in oxygen tension and (b) in the presence of agents generating oxidative stress. (a) Cultures of the wild-type SB1003 and the \textit{trxC} mutant were shifted from semi-aerobic growth (1–2\% dissolved oxygen) to aerobic growth (about 20\% dissolved oxygen) at time point zero or were further incubated without any transition. Filled diamonds, semi-aerobic conditions for SB1003; filled squares, shift from semi-aerobic to aerobic conditions for SB1003; filled triangles, semi-aerobic conditions for \textit{trxC} mutant; open circle, shift from semi-aerobic to aerobic conditions for \textit{trxC} mutant. The growth of both strains was identical under semi-aerobic conditions. (b) Zone of growth inhibition assay. SB1003 or the \textit{trxC} mutant cells were plated in top agar. A disk (5.5 mm) containing t-BOOH (5 \, \mu l of 100, 50, 20 and 10 mM stocks from left to right), H$_2$O$_2$ (5 \, \mu l of 100, 50, 20 and 10 mM stocks) or diamide (6 \, \mu l of 50, 20, 10 and 5 mM stocks) or Paraquat (5 \, \mu l of 10, 5, 2 and 1 mM stocks) was added to the plates. The zone of growth inhibition is shown in the plates after overnight growth. For Paraquat, no clear inhibition zones were formed on the plates. Similar results were obtained from three independent experiments in duplicate. Mean values (mm) of the radius of the inhibition zone are displayed in the table. t, Turbid zone, some cells grown in the zone; c, clear zone, no cells grown in the zone; --, no obvious inhibition zone.
70% in a wild-type culture, but only 20% in a culture of the trxC mutant. The trxC mutant also showed a higher sensitivity against 0.5 mM Paraquat, but survival of both the mutant and the wild-type strain was affected to the same extent by 0.1 mM Paraquat. One hour after the addition of 1.5 mM diamide, 62% of the cells of a wild-type culture were viable, but only 46% of the cells of the trxC mutant were viable. The trxC mutant also showed lower survival rates after treatment with 1 or 0.5 mM diamide. One hour after the addition of 0.6 mM t-BOOH, the wild-type and the mutant strain showed survival rates of about 87 and 53%, respectively, while the survival rates of the mutant and wild-type were similar at lower concentrations of t-BOOH. These results show that trxC mutant cells are more sensitive to the agents generating oxidative stress than the wild-type SB1003.

The observation from the determination of survival rate was verified by the zone of growth inhibition (Fig. 4b). The trxC mutant showed a decreased resistance to all oxidative agents applied. While clear zones of growth inhibition were observed when the disks were treated with H2O2 or diamide, turbid inhibition zones formed around the disks treated with t-BOOH or Paraquat.

Our results imply a function of thioredoxin 2 of R. capsulatus in the defence against oxidative stress.

Role of thioredoxin 2 in expression of genes for pigment-binding proteins and formation of photosynthetic complexes in R. capsulatus

We have previously characterized the trxA mutant TK1 of R. sphaeroides, which produces less thioredoxin than a wild-type strain. Strain TK1 accumulates less bacteriochlorophyll and less puf and puc mRNAs encoding pigment-binding proteins after a transition from growth under high oxygen tension to growth under low oxygen tension compared with its parental wild-type strain (Pasternak et al., 1999). When the trxC mutant of R. capsulatus was subjected to the same change in growth conditions, it accumulated similar levels of bacteriochlorophyll to that of the parental wild-type strain, indicating that the same amounts of photosynthetic complexes are formed in both strains. However, the bacteriochlorophyll content increased more rapidly in the trxC mutant than in the isogenic wild-type strain (Fig. 5). We also analysed the levels of puf and puc mRNA in the trxC mutant. puf and puc are both polycistronic operons, which harbour genes required for the formation of pigment protein complexes (Alberti et al., 1995; Choudhary & Kaplan, 2000; Zsebo & Hearst, 1984). Using Northern blot analysis, we determined the level of the 0.5 kb pucBA mRNA that encodes the two pigment-binding proteins of the light-harvesting II complex. This mRNA species is a stable processing product of the 2.3 kb primary puc transcript. We also determined the level of the 0.5 kb pufBA mRNA, which is a processing product of the pufQRALMX primary transcript and encodes the proteins of the light-harvesting I complex.

The expression of puf and puc genes after a decrease in oxygen tension differed in the trxC mutant and the wild-type (Fig. 6). The amount of the 0.5 kb pucBA mRNA increased by a factor of 25 in the wild-type and a factor of about 33 in the trxC mutant. The total pucBA mRNA amount was about twice as high in the mutant, before and after the transition to low oxygen tension. The amount of the 0.5 kb pufBA mRNA increased by a factor of approximately 6 in the wild-type but by a factor of approximately 9 in the trxC mutant. The puf mRNA levels during aerobic growth were about 1.5-fold higher in the trxC mutant than in the wild-type strain (Fig. 6).

Our results reveal that the effect of the deletion of trxC in R. capsulatus on the expression of puf and puc genes is opposite to the effect of the trxA mutation in R. sphaeroides (Pasternak et al., 1997).

Effects of oxygen on trxC expression

Our data show that thioredoxin 2 of R. capsulatus affects the oxygen-dependent expression of photosynthesis genes. To test how trxC expression reacts to changes in oxygen tension in the environment, we monitored trxC expression under different growth conditions. Since the trxC mRNA does not show up on Northern blots as a distinct band (unpublished), we used a semiquantitative RT-PCR approach for directly quantifying trxC transcript levels. In addition, we used a trxC–luxAB fusion harbouring 450 nt of sequence upstream of trxC for following the expression of trxC.

As shown in Fig. 7(b), the trxC mRNA level strongly increased after a shift of the cultures from high to low oxygen tension. It has been reported that the trxA mRNA level in R. sphaeroides shows a slight decrease under these conditions (Pasternak et al., 1996). Likewise, we observed a
significant decrease for trxA expression in R. capsulatus in the Northern blot analysis (Fig. 7a) and by semiquantitative RT-PCR (Fig. 7b). Thus, trxA and trxC expression are regulated by oxygen tension in an opposite way. When we expressed the trxC–luxAB fusion in the wild-type strain, we were able to confirm this effect of oxygen on trxC expression. The luciferase activity increased by a factor of about eight to nine 1–5 h after the transition and then dropped again. When the cultures were shifted from low to high oxygen tension, we observed a strong decrease in luciferase activity (Fig. 7c).

It has been reported that reduced levels of R. sphaeroides thioredoxin 1 correlate with lower puf and puc mRNA levels at low oxygen tension (Pasternak et al., 1999). The R. capsulatus trxC mutant, however, showed higher amounts of puf and puc mRNA. Thus, the opposite effects of thioredoxin 1 of R. sphaeroides and thioredoxin 2 of R. capsulatus on oxygen-dependent expression of photosynthesis genes correlate with an opposite effect of oxygen on expression of the corresponding genes.

**DISCUSSION**

Thioredoxins are present in all living organisms, and have been isolated and characterized from a variety of prokaryotic and eukaryotic cells. R. sphaeroides is a facultatively phototrophic bacterium that can rapidly adapt to changing environmental conditions. It contains a single gene for thioredoxin, trxA, which is essential (Pasternak et al., 1997) and affects the oxygen-dependent expression of photosynthesis genes (Pasternak et al., 1999). The molecular mechanisms by which thioredoxin affects the transcription of photosynthesis genes remain to be elucidated.

R. capsulatus, a close relative of R. sphaeroides, contains at least two thioredoxin genes, trxA and trxC. To determine whether thioredoxin 2 from R. capsulatus functionally resembles thioredoxin 2 of E. coli and to test its involvement in the oxygen-dependent expression of photosynthesis genes, we inactivated the trxC gene of R. capsulatus and expressed it in the E. coli strain BH216. Since strain BH216, which lacks a functional trxA gene but harbours the intact trxC gene, cannot grow on minimal medium with methionine sulfoxide and cannot propagate phage T7, E. coli TrxC is not able to replace TrxA for its functions as hydrogen donor for methionine sulfoxide reductase and as a subunit of T7 DNA polymerase when expressed at a normal cellular level. When the trxC gene is overexpressed in an E. coli metEtrxA background, it confers weak growth to this strain on minimal medium with methionine sulfoxide (Ritz et al., 2000; Stewart et al., 1998), indicating that it can complement the defect poorly. We made the identical observation after expressing the trxC gene from R. capsulatus in an E. coli metEtrxA background. This strain was unable to allow propagation of phage T7, indicating that thioredoxin 2 is unable to function as a subunit for T7 DNA polymerase, even when the trxC gene is overexpressed.

The inactivation of the trxC gene had little effect on the growth of R. capsulatus, even under a high oxygen tension. An extended lag phase of the mutant strain after increasing the oxygen tension in the culture indicates the involvement of thioredoxin 2 in adaptation to high oxygen tension. This assumption is supported by the fact that no growth of the mutant was observed 1 h after a transition from low to high oxygen tension. More pronounced differences between the wild-type strain and the trxC mutant of
Our data suggest that thioredoxin 2 of *R. capsulatus* were observed in the presence of oxidative stress-generating agents (Table 2). The mutant strain *R. capsulatus* were observed in the presence of oxidative stress defence in *E. coli* thioredoxin 1 has the same amino acids KM directly behind the second cysteine of the active site as *E. coli* thioredoxin 1. However, *R. sphaeroides* thioredoxin 1 has the amino acids RQ following the second cysteine of the active site, as *R. capsulatus* and *E. coli* thioredoxin 2, which were found not to act as a subunit of T7 DNA polymerase. These two residues KM are important in protein interactions and may stabilize thiolate in the active site (Eklund *et al.*, 1991). This suggests that the thioredoxin 1 mutation C29A of *R. capsulatus* might not change the conformation at the active site, probably partly contributed by the following residues KM. The *R. sphaeroides* thioredoxin 1 C29A mutation might change it. It is conceivable that the flexibility of the active site region to allow the reduced form of the protein to take up functionally significant conformations of a slightly higher energy than the oxidized form is more subtle in *R. sphaeroides* thioredoxin 1 than in *R. capsulatus* thioredoxin 1.

Although thioredoxin 1 and 2 have similar functions in some regards, the two genes respond to changes in oxygen tension in an opposite way. While a reduction in oxygen tension in cultures of *R. capsulatus* resulted in a significant increase in *trxC* mRNA level, the *trxA* mRNA level decreased. Interestingly, the opposite response of the two genes to oxygen tension correlates with an opposite effect on the oxygen-dependent expression of photosynthesis genes. A *trxA* mutant of *R. sphaeroides* harbouring lower levels of thioredoxin 1 than the isogenic wild-type showed a diminished increase in *puf* and *puc* mRNA levels after such a transition when compared with the parental wild-type strain (Pasternak *et al.*, 1999). Until now we were unable to construct a *trxA* mutant of *R. capsulatus* even by applying exactly the same strategy as that used for constructing the *R. sphaeroides* strain with an altered thioredoxin 1 level. The *trxC* mutant of *R. capsulatus* showed a faster increase and a higher accumulation of *puf* and *puc* mRNA levels after a reduction in oxygen tension than the isogenic wild-type strain. We conclude that the two thioredoxins have opposite effects on some cellular functions. At present, it is unknown how the effect of thioredoxin on the expression of photosynthesis genes is exerted. Thioredoxins may influence the activity of some of the proteins (RegB/RegA, CrI; see introduction), which have been identified as regulators of photosynthesis gene expression in *R. capsulatus*. We are in the process of unravelling the molecular basis for the effect of thioredoxin on the expression of photosynthesis genes to learn more.
about such differing functions of thioredoxin 1 and thioredoxin 2, which may also be of importance for other cellular functions.

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