Thioredoxin can influence gene expression by affecting gyrase activity

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ABSTRACT

The expression of many genes of facultatively photosynthetic bacteria of the genus Rhodobacter is controlled by the oxygen tension. Among these are the genes of the puf and puc operons, which encode proteins of the photosynthetic apparatus. Previous results revealed that thioredoxins are involved in the regulated expression of these operons, but it remained unsolved as to the mechanisms by which thioredoxins affect puf and puc expression. Here we show that reduced TrxA of Rhodobacter capsulatus and Rhodobacter sphaeroides and oxidized TrxC of R. capsulatus interact with DNA gyrase and alter its DNA supercoiling activity. While TrxA enhances supercoiling, TrxC exerts a negative effect on this activity. Furthermore, inhibition of gyrase activity strongly reduces puf and puc expression. Our results reveal a new signaling pathway by which oxygen can affect the expression of bacterial genes.

INTRODUCTION

Thioredoxins, ubiquitous small proteins (~12 kDa) containing an extremely reactive dithiol-disulfide in their active center, are implicated in many cellular processes. Originally discovered as electron donors for ribonucleotide reductase in Escherichia coli (1), thioredoxins were later identified as key players in keeping intracellular protein disulfides generally reduced (2). Their role in defense against oxidative stress is well established for E.coli and Saccharomyces cerevisiae (3). Thioredoxins are known as a subunit of T7 DNA polymerase; they are involved in phage assembly, in the light-dependent regulation of chloroplast photosynthetic enzymes and in the regulation of apoptosis (4). In mammals, thioredoxins are also implicated in a redox regulation of transcription factors such as NF-κB or AP-1 (5). The thiol-reducing activities of thioredoxins and glutaredoxins have been best characterized in E.coli, which has two genes encoding soluble thioredoxins (trxA and trxC) and three genes encoding glutaredoxins (grxA, grxB and grxC). A trxAtrxC mutant of E.coli is viable, suggesting that the glutaredoxin system can functionally replace the thioredoxin system (6). Thioredoxin is, however, essential in other organisms such as Anacystis nidulans (7), Synechocystis sp. PC6803 (8) and Rhodobacter sphaeroides (9).

More recently, thioredoxin was also shown to be involved in the oxygen-dependent regulation of photosynthesis genes in R.sphaeroides and Rhodobacter capsulatus (10,11). Members of the genus Rhodobacter are facultatively photosynthetic bacteria that synthesize photosynthetic complexes only if the oxygen tension in the environment is low. Oxygen affects expression of photosynthesis genes at transcriptional and post-transcriptional levels. The genes for pigment binding proteins are organized in two polycistronic operons. The puf operon encodes the pigment binding proteins of the light harvesting (LH) I complex and of the reaction center. The puc operon encodes proteins of the LHII complex. Many proteins are involved in oxygen-dependent transcription of these operons (12,13). The two-component system PrrB/PrrA has a central role in this regulation in R.sphaeroides, and its counterpart RegB/RegA in the close relative R.capsulatus was also shown to be involved in oxygen-dependent regulation of genes for nitrogen fixation, CO2 fixation, hydrogenase synthesis (14), enzymes of the respiratory chain (15) and dimethylsulfide reductase (16). The sensor kinase RegB autophosphorylates in a redox-dependent manner (17,18). A highly conserved cysteine, which forms an intermolecular disulfide bond in oxidizing condition, converts the active RegB kinase to an inactive tetramer (19). RegA is phosphorylated by RegB and activates transcription under low oxygen tension after binding to the puf and puc promoter regions (20). Under high oxygen tension PpsR (CrtJ in R.capsulatus) binds to the puf and puc promoter regions and represses transcription. The activity of PpsR is regulated by the AppA protein, which not only transmits a redox signal but also functions as a blue light receptor (21–23).
**Materials and Methods**

Strains, plasmids and growth conditions

The strains and plasmids used in this study are listed in Table 1. *E. coli* cultures were grown in Luria–Bertani (LB) broth at 37°C, while *R. sphaeroides* and *R. capsulatus* were grown at 32°C in a malate minimal salt medium (26). The cells were grown aerobically by incubating 100 ml of culture in 500 ml baffled flasks under vigorous shaking. The oxygen partial pressure was determined to be 20% by using a Pt/Ag oxygen electrode (Bachofer). For semiaerobic growth conditions 40 ml pressure was determined to be 20% by using a Pt/Ag oxygen electrode (Bachofer). For semiaerobic growth conditions 40 ml of culture was incubated in 50 ml flasks (oxygen partial pressure of 1–2%). For oxygen shift experiments, cells were grown aerobically to an OD660 of 0.4–0.5 and then transferred to the 50 ml flasks or vice versa. Cells were grown in screw-cap flasks filled to the top for anaerobic phototrophic growth in the light (45 W m⁻²). Incubation was in the dark for aerobic and semiaerobic growth.

Yeast two-hybrid screening

Thioredoxin A and/or C of *R. capsulatus* and *R. sphaeroides* were cloned into pAS2-1 or pGBK7, and the constructs were introduced into yeast strain *Saccharomyces cerevisiae* AH109 as described in MATCHMAKER Two-Hybrid System 2 or 3. Retransformation was carried out with *R. capsulatus* and *R. sphaeroides* genomic libraries into strain AH109, which already contained the pAS-RstrA or pGBK7-RTrxA/C plasmid. The libraries were constructed from chromosomal DNA partially digested by Sau3A I and ligated into BamHI-digested pACT2 or pGADT7. The transformants were plated onto synthetic complete medium without adenine, tryptophan, leucine and histidine, but containing 6 mM aminotriazole. After incubation for 5 days, colony lift-filter assays to determine β-galactosidase activity were performed on each plate, as described in the yeast protocols handbook (Clontech). For each positive colony, the library plasmid was isolated. Confirmation of positive interactions (secondary screening) was performed by cotransforming the library plasmid and pAS-RstrA or pGBK7-RTrxA/C into strain AH109. Cotransformants which can grow on selective medium were chosen and β-galactosidase activity was measured. Library clones that tested positive were sequenced.

**Mutant construction**

In order to generate a *trxA* mutant of *R. capsulatus*, a DNA fragment containing the full-length *trxA* gene was amplified by PCR with primers: 5′-GAGAATCCCATGGCTACCGTGCGG-3′ and 5′-GGTACGCTTTGCCCCCAGTTGC-3′ from genomic DNA. The PCR product (447 bp) was cloned into plasmid pUT5C (27), replacing 421 bp of the *trxA* gene of *R. sphaeroides*. Then a 1.7 kb DNA fragment containing the first 144 bp of the *trxA* gene of *R. capsulatus*, the hybrid pTR promoter (28) and the lacI Q element was removed from the resulting plasmid by PstI and SacI and cloned into pPHU281 (29) to generate plasmid pPHURcTrxA (Table 1), followed by transformation into the *E. coli* strain SM10 (30). This plasmid was conjugated diparentally into SB1003. Candidates for single crossover recombination events were first screened for tetracycline resistance which is carried by plasmid pPHURcTrxA. The resulting mutant has one intact *trxA* gene but under the control of the *trc* promoter. The pTR promoter is constitutively expressed in *Rhodobacter*, independently of oxygen tension (10). The native *trxA* gene was partially deleted and inactivated in the mutant. The correct insertion was confirmed by Southern blot analysis and PCR.

**Southern and northern hybridization analysis**

Total RNA and genomic DNA were isolated from wild-type *R. capsulatus* SB1003 and *trxA* and *trc* mutants. Southern and northern blot analyses were performed as described previously (11). To ensure that equal quantities of RNA were present in each lane of the northern blot, the membrane was stripped and reprobed with an oligo 5′-CTTAGATGTATT-CAGTTCCC-3′, corresponding to the 23S rDNA positions 187-205 (*E. coli* numbering) of *Rhodobacter* as a control. Each northern analysis was performed at least 3 to 4 times.

**RT-PCR**

For RT–PCR analysis the RT reaction was carried out in a final volume of 25 μl containing 30 pmol of reverse primer, 2 μg of total RNA, 1 mM each of the four deoxyribonucleoside triphosphates and 1.5 U of 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 of reverse transcription reaction product, 1 U of *Taq* polymerase (Qiagen) and 2 pmol each of the oligonucleotide primers 5′-GGTACGCTTTGCCCCCAGTTGC-3′ and 5′-GATGAATTCGCCGCCCGTCTTAC-3′ and 5′-GGTACGCTTTGCCCCCAGTTGC-3′ for gyrB. Amplification was carried out by an initial denaturation step at 96°C for 3 min followed by 28 cycles of 96°C for 1 min, 62°C for 40 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 10% PAGE.

**Bacteriochlorophyll measurements**

An aliquot of 0.5 ml of culture was sedimented at 10 000 g 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 at ≥ 10 000 g 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.

**Enzyme assays**

Supercoiling assays were carried out as described previously (31,32). Plasmid pBluescript SK (+) (Stratagene) was used as the substrate. Novobiocin or ciprofloxacin (Sigma) was added at a final concentration of 75 nM (50 ng/ml) or 0.3 mM (100 μg/ml), respectively. The reaction was trapped by adding 0.2% SDS and the reaction mixtures were resolved on 0.8% agarose gels in 40 mM Tris-acetate buffer containing 1 mM EDTA. For in vitro assays, purified gyrase (John Innes Enterprise Ltd) and purified E.coli thioredoxin 1 (Promega) were used. The gel was then run overnight.

**In vitro GST pulldown assay**

A DNA fragment carrying the complete gyrB gene of R.capsulatus was amplified by PCR with the primers 5’-CG GATCCATGACCGAATCCTGACGAGGCTTCCAGGC-3’ and 5’-CCCTCGAGGTCGACAAATCAGGTTCGTTTTCCAGGC-3’, and cloned into the BamHI site of pGEX-4T-1, to generate pGEX-RcGyrB, followed by transformation into E.coli strain BL21 (Amersham). Glutathione S-transferase (GST) or GST fusion proteins were purified with glutathione-conjugated agarose beads (Pharmacia) from BL21 following the manufacturer’s instruction (Amersham). (His)_6-tagged thioredoxin 2 of R.capsulatus was purified from E.coli M15(pREP4) as

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described in (11). In order to detect the interaction of GyrB with TrxA, anaerobic and aerobic cell extracts of wild-type SB1003 were prepared. An aliquot of 30 ml of fresh overnight culture were harvested and resuspended in sonication buffer I (50 mM Tris–HCl, pH 7.5, 2 mM DTT, 20 mM KC1, 1.5 mM ATP, 5 mM spermidine, 10 mM MgCl2, 50 µg/ml BSA). After sonication with a Bandelin SONOPULS GM 70 at a 50% duty cycle on ice for 40 s 4 times with 30 s interval break, crude cell extracts were obtained by centrifugation at 12 000 g for 15 min to remove cell debris. An aliquot of 20 µg of total proteins determined with Bradford was additionally treated with 20 mM β-mercaptoethanol or 1 mM H2O2 to reduce or oxidize TrxA, which was then added to the GST–GyrB protein. GST pulldown assays were performed as described (Amersham) for TrxC–GyrB interaction using His-tagged TrxC, for TrxA–GyrB interaction, using cell extracts. Samples were separated on reducing SDS–15% polyacrylamide gels (SDS–PAGE), followed by immunological analysis.

Immunological analysis

Samples were electrophoresed in SDS–15% polyacrylamide gels and transferred to Immobilon™-P membrane (Millipore). Western blotting was performed according to the Western Exposure Chemiluminescent Detection system (Clontech). Thioredoxin antibodies (11) were used to detect GST fusion protein-associated TrxC. A monoclonal N-terminal antibody of GyrB (John Innes Enterprises Ltd) was used to detect the GyrB level in the cells.

Thiol-redox state analysis

Oxidized and/or reduced cell extracts (20 µg total protein) or TrxC protein (50 µM) were incubated at 34°C for 2 h in 50 mM Tris–HCl (pH 8.0) containing 0.1% SDS and 15 mM 4- acetamido-4’-maleimidylstibene-2,2’-disulfonic acid (AMS) (Molecular Probes). The mixtures were then separated by 15% non-reducing SDS–PAGE, and TrxA or TrxC were detected by immunological analysis.

Redox states of TrxA in vivo were detected as described previously (23). Briefly, whole-cell proteins of the strain SB1003 were precipitated by direct treatment with trichloroacetic acid to give a final concentration of 10%. Protein precipitates were collected by centrifugation, washed with 500 mM Tris–HCl (pH 8.0), and dissolved in 50 mM Tris–HCl (pH 8.0) containing 1% SDS and 15 mM AMS. After incubation at 34°C for >3 h, the mixture was subject to 15% SDS–PAGE and western analysis.

RESULTS

Use of the yeast two-hybrid system to identify putative interaction partners of thioredoxin

Since mutants of Rhodobacter that lack the central oxygen-responsive two-component system PrrB/PrrA (RegB/RegA) still show some residual oxygen-dependent expression of photosynthesis genes (33), we were looking for other oxygen sensors in this genus. As reported previously, trx mutant strains of R.sphaeroides or R.capsulatus show altered oxygen-dependent transcription of the puf and puc operons (10,11). Since thioredoxin is not known as a DNA binding protein, we assumed that it would act on gene transcription indirectly by affecting the DNA binding affinity or the activity of a transcription factor. Therefore, we tested the interaction of TrxA from R.sphaeroides, and TrxA or TrxC from R.capsulatus, respectively, with such candidate proteins by applying the yeast two-hybrid system. We did not detect interaction of any of the Rhodobacter thioredoxins to the R.capsulatus CrtJ protein or the R.sphaeroides PpsR (CrtJ homolog) or AppA protein. All these proteins are known to be involved in the redox regulation of the photosynthesis genes in Rhodobacter (34).

We also screened libraries of the R.sphaeroides and R.capsulatus chromosome for putative interaction partners of thioredoxin by using the yeast two-hybrid system in order to unravel the pathway of signaling from thioredoxin to puf and puc transcription. Considering the numerous functions of thioredoxin, we were not surprised to isolate a number of different positive clones from this screening. One positive clone from the screen with the R.sphaeroides library was identified to carry a DNA fragment encoding part of the biotin carboxylase. Biotin carboxylase is a subunit of acetyl CoA carboxylase which is known to be affected by thioredoxin (35). Additional proteins or protein domains identified as thioredoxin targets by our two-hybrid screen and also by proteomic-based screens (36,37) are arginosuccinate synthase, malonyl CoA acyl carrier protein transacylase, translational elongation factor Tu and sugar epimerase. These results confirmed that the yeast two-hybrid screen is a suitable system to find interaction partners for a bacterial thioredoxin.

Our further interest focused on a protein that was identified as a putative interaction partner for TrxA of R.sphaeroides and the R.sphaeroides genomic library as well as for TrxC of R.capsulatus and the R.capsulatus genomic library, the DNA gyrase B subunit (GyrB). An influence of thioredoxin on gyrase activity is an attractive model to explain the effect of thioredoxin on the expression of photosynthesis genes. The plasmid isolated from the R.sphaeroides genomic library harbored a 136 nt DNA fragment (clone 280) encoding the amino acid residues 342–387 of GyrB. The plasmid isolated from the R.capsulatus genomic library contained a 388 nt DNA fragment (clone 29) encoding the amino acid residues 69–198 of GyrB (Figure 1A). This part of GyrB is also known to bind the gyrase inhibitor novobiocin (38). Yeast strains expressing only TrxA or TrxC from Rhodobacter or harboring only one of the gyrB plasmids were unable to grow on selective agar.

When the β-galactosidase assay was used to quantify the interaction of the different Trx proteins to gyrase B, the strongest interaction was found for TrxC from R.capsulatus (Figure 1B). The interaction of TrxA from R.capsulatus with the peptide of its cognate gyrase was considerably weaker, but still stronger than the interaction between TrxA from R.sphaeroides and the peptide of its cognate GyrB (Figure 1B). Plasmid pGADT7gyr392 carries a 392 nt DNA fragment of the R.capsulatus gyrB gene. This fragment contains the DNA sequence homologous to the fragment present in clone 280, which was isolated from the R.sphaeroides genomic library. When pGADT7gyr392 was cotransformed into yeast strain AH109 with plasmid pGBK7-TrxA or pGBK7-TrxC, a strong interaction was observed (Figure 1B), indicating that the N-terminal domain of GyrB interacts with both thioredoxins, TrxA and TrxC, in R.capsulatus.
pulldown assays were performed using recombinant GST-tagged gyrase B from \textit{R.\textit{capsulatus}}. Since the function of thioredoxins can depend on the redox state, we monitored disulfide bond formation by covalent modification of Cys sulphydryl groups with AMS. This modification results in retardation of the electrophoretic mobility of the proteins. The formation of a disulfide bond between the two active site cysteines of thioredoxin renders the protein resistant to AMS modification. As shown in Figure 2A (lane 2) and Figure 2C (lane 2 and 3, upper panel), treatment of reduced TrxA or TrxC with AMS resulted in slower electrophoretic migration than migration of AMS-treated oxidized TrxC (Figure 2C, lane 2, upper panel) or TrxC (Figure 2A, lane 3). There is no difference in the electrophoretic migration pattern between oxidized TrxC that was treated with AMS from that of untreated oxidized TrxC (Figure 2A, lanes 1 and 3, respectively) confirming that oxidized TrxA and TrxC have no available Cys for AMS modification.

When protein affinity pulldown assays were performed using recombinant GST-tagged GyrB and (His)\textsubscript{6}-tagged \textit{R.\textit{capsulatus}} TrxC, only oxidized TrxC showed binding (Figure 2B, lane 3, upper panel). We could not see any binding using recombinant (His)\textsubscript{6}-tagged TrxA of both \textit{Rhodobacter} species to gyrase. It is conceivable that the (His)\textsubscript{6}-tag at the N-terminus of TrxA changes its local conformation. However, when we used cell extracts for the pulldown assay, native reduced TrxA of \textit{R.\textit{capsulatus}} showed interaction to GyrB (Figure 2D, lane 4, upper panel), as well as TrxA of \textit{R.\textit{sphaeroides}} (data not shown). TrxC or TrxA did not bind to the GST–sepharose (Figure 2B and D, both lanes 1 and 2, upper panel).

#### Generation and characterization of a \textit{trxA} mutant strain from \textit{R.\textit{capsulatus}}

The results from the yeast two-hybrid screen and the GST pulldown demonstrate an interaction of \textit{Rhodobacter} TrxA with GyrB. If the effect of thioredoxin on the transcription of photosynthesis genes is indeed mediated by gyrase, \textit{trx} mutant strains should show altered gyrase activity. To directly compare the effect of TrxA and TrxC on gyrase activity and expression of photosynthesis genes, it was necessary to generate mutants from the identical parental strain. The \textit{trxA} gene of \textit{R.\textit{capsulatus}} could not be inactivated completely (data not shown), suggesting that it is essential as observed for \textit{R.\textit{sphaeroides}} (9). The \textit{trxA} mutant of \textit{R.\textit{capsulatus}} was generated by single crossover through recombination of part of the \textit{trxA} gene with one intact copy of \textit{trxA}. In the final strain, the \textit{trxA} gene is under the control of the \textit{trx} promoter on the chromosome. Using antibodies directed against \textit{R.\textit{capsulatus}} TrxA, we detected very low levels of the protein in the mutant strain, independent of growth conditions (data not shown). No significant difference of the doubling times was observed under high oxygen tension (2 h ± 10 min doubling time for wild type and \textit{trxA} mutant) and low oxygen tension (3 h ± 10 min for wild type, 3 h 27 min ± 10 min for \textit{trxA} mutant), while a severe growth deficiency of the \textit{trxA} mutant is observed under anaerobic phototrophic condition with much longer doubling time (6.0 h ± 20 min) than that of the wild-type strain (2.5 h ± 10 min).

We have previously characterized the \textit{trxA} mutant TK1 from \textit{R.\textit{sphaeroides}}, which accumulates less bacteriochlorophyll
and less puf and puc mRNAs after a transition from growth under high oxygen tension to growth under low oxygen tension when compared with its parental wild-type strain (10). Similar results were observed for the trxA mutant of R. capsulatus. However, compared with strain TK1, the R. capsulatus trxA mutant accumulated much less bacteriochlorophyll (34–3% of the level of wild type) due to the much lower TrxA protein level (<5% of the level of wild type) as detected by western analysis (data not shown). Intriguingly, we did not observe puc mRNA accumulation in the R. capsulatus trxA mutant after the transition from high to low oxygen tension, even though 20 μg of total RNA was loaded onto the gel for northern hybridization. Much less puf mRNA was detected in the R. capsulatus trxA mutant than in the wild-type strain (Figure 3A and B) after the transition of the culture. A very faint band for puc mRNA appeared only when the trxA mutant was constantly cultivated under low oxygen tension (data not shown). Accordingly, the reduced bacteriochlorophyll level in the trxA mutant of R. capsulatus was reflected by lower absorption peaks of photosynthetic complexes in whole-cell spectra (Figure 3C).

Gyrase supercoiling activity in trxA mutant strains

Gyrase catalyzes the introduction of negative supercoils into DNA (24). The protein binds to DNA as a tetramer of two A and two B subunits and wraps DNA into a positive supercoil. One region of duplex DNA is passed through another by means of DNA breakage and religation. The binding of ATP drives the supercoiling reaction, with ATP hydrolysis serving to reset the enzyme for a second round of catalysis. In the absence of ATP, gyrase also catalyzes the relaxation of negatively supercoiled DNA in an ATP-independent reaction. Since the ratio of ATP to ADP determines the final level of supercoiling (39,40), this makes gyrase activity sensitive to changes in intracellular energetics and, as a consequence, to the extracellular environment. Gyrase also facilitates the movement of the transcription complexes through DNA by introducing negative supercoils ahead of it and can influence transcription by bending and folding of DNA (41).

In order to study the effect of thioredoxin on gyrase activity, we investigated the gyrase supercoiling activities in vitro with cell extracts. We isolated extracts from R. capsulatus in order to study the effect of both TrxA and TrxC on supercoiling. We used R. sphaeroides extracts since this bacterium does not express TrxC and therefore allows direct conclusions on TrxA function. Aerobic extracts from R. sphaeroides strain TK1, which produces reduced amounts of TrxA, showed no significant supercoiling activity (Figure 4A). When higher amounts of cell extracts from mutant TK1 were used supercoiling was observed (data not shown), but at much lower levels than with extracts from wild type. This result was
similar to that for the trxA mutant of *R. capsulatus* (Figure 5A and D). This suggests that strain TK1 indeed shows reduced supercoiling activity rather than increased topoisomerase I or nuclease activity. The supercoiling activity varied under different growth conditions and in different growth phases; however, the protein levels of TrxA mostly correlated well to the supercoiling activity (Figure 4B). Extracts from semiaerobically grown wild-type cells exhibited similar supercoiling activity as extracts from aerobically grown cells, despite the fact that the total amount of TrxA in semiaerobic extracts was only 50% of that in aerobic extracts. These results imply that not only the level of thioredoxin but also its redox state influences the supercoiling activity.

When we tested cell-free extracts from *R. capsulatus* strains, the extracts from the trxC mutant strain SBrtrxC showed higher supercoiling activity than extracts from the parental strain SB1003 in exponential phase under semiaerobic conditions (Figure 5A). We did not observe marked differences in supercoiling activity with extracts from the wild type and the trxC mutant strain which were cultivated under high oxygen tension (data not shown). Supercoiling activity of extracts from the trxA mutant was always lower than activities from extracts of the wild type and the trxC mutant, at high or low oxygen tension and in exponential or stationary phases (data not shown). Our results reveal that TrxC and TrxA have opposite effects on supercoiling activity. Under semiaerobic conditions TrxA enhances the supercoiling activity, while TrxC reduces gyrase activity.

The coumarin novobiocin inhibits the supercoiling activity of gyrase without affecting its ATP-independent relaxation activity by binding to the N-terminal fragment of gyrase. The coumarin novobiocin inhibits the supercoiling activity of gyrase without affecting its ATP-independent relaxation activity by binding to the N-terminal fragment of gyrase.

The coumarin novobiocin inhibits the supercoiling activity of gyrase without affecting its ATP-independent relaxation activity by binding to the N-terminal fragment of gyrase.

**Figure 3.** Characterization of the trxA mutant of *R. capsulatus*. (A) Effect of TrxA on the expression of photosynthesis genes, i.e., the *puf* and *puc* operons. Cultures were grown aerobically until the optical density (OD600) reached 0.4 to 0.5, and then shifted to low oxygen tension. Total RNA was prepared and northern blot analysis was performed as described in Materials and Methods. (B) Quantification of the effect of TrxA on *puf* expression from the northern result (A). Closed square: trxA mutant; open square: wild type. (C) Absorption spectra of whole cells of wild type (solid line) and trxA mutant (dotted line). Cultures were grown semiaerobically in malate medium supplemented with appropriate antibiotics overnight. Sample preparation and measurement were carried out as described in Materials and Methods.

**Figure 4.** TrxA affects gyrase supercoiling activity in *R. sphaeroides*. Exponential (OD600 = 0.6) or stationary (OD600 = 1.4) phase cells were harvested. Half of the cells were resuspended in sonication buffer I [25 mM Tris–HCl, pH 7.5, 150 mM KCl, 2 mM phenylmethylsulfonyl-fluoride (PMSF), 10% glycerol] for the supercoiling assay, and the other half in sonication buffer II (50 mM Tris–HCl, pH 8.0, 250 mM NaCl) for detection of TrxA protein levels. Cell extracts were prepared as described in Materials and Methods. (A) Supercoiling activity of gyrase in cell extracts of wild-type WS8 and trxA mutant TK1. Two micrograms of total protein (cell extract) as determined by Bradford assay were incubated at room temperature in buffer (50 mM Tris–HCl, pH 7.5, 2 mM DTT, 20 mM KCl, 1.5 mM ATP, 5 mM spermidine, 10 mM MgCl2, 50 μg/ml BSA) with 0.2 μg of relaxed plasmid pBluescript DNA, for 2 h or overnight at 37°C. SDS (0.2%) was added to stop the reaction, and the samples were analyzed by 0.8% agarose gel electrophoresis. Odd numbers: 2 h incubation; even numbers: overnight incubation. A, aerobic condition; SA, semiaerobic condition; Exp, exponential phase; Sta, stationary phase. M, DNA marker; C, control without cell extract. (B) TrxA levels of WS8 and TK1 in different growth condition analyzed with western blot.
confirmed that the supercoiling observed after addition of cell-free extracts is catalyzed by gyrase.

To test whether the higher or lower supercoiling activity of gyrase in the cell extracts of trxA or trxC mutants arises from the higher or lower levels of gyrase protein, western blot analysis was performed with monoclonal antibodies directed against the N- or C-terminus of the gyrase A or B subunit of E.coli. The R.capsulatus trxC mutant contained a similar level of gyrase A and B subunits as the wild type, while the trxA mutant contained much higher levels of gyrase A and B subunits than the wild type or the trxC mutant (Figure 5B, gyrase A subunit not shown). The fact that the cell extracts of the trxA mutant exhibited lower supercoiling activity than that of the wild type despite higher levels of gyrase indicates that TrxA is very important for gyrase to change the DNA supercoiling status in the cells.

We also monitored the TrxA and TrxC protein levels in wild-type and mutant strains by the use of specific antibodies. In wild-type SB1003, TrxC was not detectable under any growth condition and in the different growth phases. However, TrxC was abundantly induced in the trxA mutant of R.capsulatus (Figure 5B). The higher expression of TrxC at low TrxA concentration suggests that TrxC can substitute some of the TrxA functions in R.capsulatus as previously reported for E.coli; however, no induction of TrxC is observed in a TrxA mutant of E.coli (44). This result indicates that the much lower supercoiling activity of cell extracts of trxA mutant strains may be at least partially due to the high abundance of TrxC in R.capsulatus.

In order to see whether the effect of thioredoxins on gyrase activity is a special feature in Rhodobacter or rather a general signaling pathway in bacteria, we also analyzed E.coli strains lacking either trxA or trxC. Extracts from an E.coli strain lacking trxA exhibited lower supercoiling activity than the parental wild type, while extracts from a strain lacking trxC exhibited stronger supercoiling activity (Figure 5C). These results suggest that TrxA and TrxC have the same opposite effect on gyrase activity in E.coli as observed in R.capsulatus.

In order to demonstrate that the different supercoiling activities were indeed caused by a direct effect of thioredoxins on gyrase, we performed additional assays with purified gyrase from E.coli and purified thioredoxin 1 from E.coli and R.sphaeroides. As shown in Figure 5D, an enhancement of the supercoiling activity was observed by the presence of increasing amounts of thioredoxin 1. This is in agreement with the lower supercoiling activity that we observed in the trxA mutants of Rhodobacter and E.coli, and suggests that no additional proteins are involved in the thioredoxin gyrase interaction.

The effect of gyrase inhibitors on bacteriochlorophyll accumulation and the expression of photosynthesis genes

It was previously reported that novobiocin specifically inhibits the expression of certain photosynthesis genes in R.capsulatus (25). We tested the effect of novobiocin on bacteriochlorophyll accumulation and puf and puc mRNA levels in the different wild-type and trxA mutant strains. The concentration (30 or 75 μM) of novobiocin applied did not affect the growth rate of the R.sphaeroides wild-type strain WS8 and trxA mutant TK1 after the transition of the cultures from high to low oxygen tension. However, the bacteriochlorophyll accumulation was inhibited, more severely in mutant TK1 than in WS8, even if the drug was added at the same time as the oxygen tension was reduced (Figure 6A). Quantification of mRNA levels by northern blot analysis revealed that there was still accumulation of the 0.5 kb pufBA mRNA in R.sphaeroides wild-type WS8, but not in strain TK1 in the presence of 75 μM novobiocin after lowering of oxygen tension (Figure 6B). The addition of novobiocin completely abolished the expression of puc mRNA in the wild-type WS8 and the trxA mutant TK1 (data not shown). When the oxygen tension was reduced without the addition of novobiocin, pufBA and pucBA
mRNA levels increased by factors of about 35 and 75 in the wild
type, and by factors of about 6–8 and 25, respectively, in the
TK1 mutant (Figure 6B for \( puf \), and figure for \( puc \) not shown).

The effect of novobiocin on bacteriochlorophyll accumulation and \( puf \) and \( puc \) expression in \( R \).capsulatus was similar as observed for \( R \).sphaeroides. The addition of the identical
centrations of novobiocin did not affect the growth of the
\( R \).capsulatus wild-type SB1003 or the \( trxC \) mutant to the same extent, but more
severely in the \( trxA \) mutant (Figure 7A), in which 75 \( \mu \)M of novobiocin abolished the bacteriochlorophyll accumulation. When mRNA levels of \( puf \) and \( puc \) were analyzed by
northern blot after lowering the oxygen tension in the presence
of novobiocin (75 \( \mu \)M), no expression of \( puf \) and \( puc \) was
detectable in the \( trxA \) mutant (Figure 7B), \( puf \) and \( puc \) expression was still induced in the \( trxC \) mutant (3-fold induction of \( puf \), 18.4-fold induction of \( puc \)) and in the wild type (2.9-fold induction of \( puf \), 10-fold induction of \( puc \)) (Figure 7B), but to a
much lower extent than in the control cultures without novobiocin treatment [\( trxC \) mutant: 9-fold induction of \( puf \), 33-fold induction of \( puc \); wild type: 6-fold induction of \( puf \), 25-fold induction of \( puc \) (11)].

The same effects on gene expression of \( puf \) and \( puc \) operons and the formation of photosynthetic complexes as for
novobiocin were observed after addition of ciprofloxacin to a \( Rhodobacter \) culture (data not shown). The finding that
growth of the strains used in this study was not affected by the
presence of novobiocin (30 or 75 \( \mu \)M), but \( puf \) and \( puc \) expression was strongly reduced, suggests that the transcription of the \( puf \) and \( puc \) genes is very sensitive to the DNA conformation. In order to exclude the possibility that the inhibiting
effect of novobiocin on gene expression is general, we mon-
tored the mRNA level for gyrase B (\( gyrB \)) after novobiocin
treatment by RT–PCR. Expression of \( gyrB \) increased 4-fold
within 20 min after the addition of novobiocin (30 \( \mu \)M) (Figure 7C). Our inhibition studies verified that novobiocin
inhibits the expression of photosynthesis genes by the action of
gyrase and that \( TrxA \) and \( TrxC \) influence this inhibiting effect.

**DISCUSSION**

Thioredoxin influences many cellular processes by affecting
the activity of other proteins by means of its thiol-redox control, as a hydrogen donor or by formation of protein complexes
(45). Among its various cellular functions thioredoxin can also
affect gene transcription. This effect can be mediated by a
redox regulation of transcription factors as shown for NFxB
or AP-1 in mammals (5). We have previously shown that
thioredoxin also affects the transcription of the \( puf \) and \( puc \)
genes in *R. sphaeroides* and in *R. capsulatus* which encode proteins of the photosynthetic apparatus (9,11). The signal chain from thioredoxin to *puf* and *puc* transcription, however, remained to be elucidated.

In order to unravel this signal chain we attempted to identify proteins interacting with thioredoxin. A model for an effect of thioredoxin on *puf* and *puc* transcription emerged, when we identified GyrB as a putative interaction partner of thioredoxin. Gyrase can affect transcription rates by its supercoiling or relaxation activities, since the local supercoiling of DNA can influence promoter activities (46–48). An effect of environmental factors such as temperature, oxidative stress, osmotic stress and pH changes on DNA supercoiling is well established (49). The influence of DNA supercoiling on gene expression can differ significantly for individual genes. Higher supercoiling can stimulate gene expression (e.g. *topA* in *E. coli; ospAB* promoter in *Borrelia burgdorferi*, osmotic stress responsive genes in *E. coli*, the *fis* gene of *E. coli*, *ompR* gene in *Salmonella enterica*) and can also decrease gene expression (e.g. *ospC* of *B. burgdorferi*, the *sol* and *ade* operons of *Clostridium acetobutylicum*, *gyr* expression in *E. coli*) (50–58). It is worth mentioning that it is difficult to accurately measure changes in the effective supercoiling density of the bacterial chromosome *in vivo*. This can be done by monitoring the products of the genes whose expression is correlated with the level of supercoiling. One such gene is *gyrB*, and the increased *gyrB* expression observed in *R. capsulatus* after addition of novobiocin (Figure 7C) and the increased levels of the GyrB protein in the *trxA* mutant (Figure 5B) strongly suggest that the supercoiling level in this mutant is significantly decreased. It has been reported previously that *puf* and *puc* expression depend on DNA supercoiling (25). Based on a new assay to measure supercoiling, a later study from the same group suggested that induction of anaerobic gene expression in *R. capsulatus* is not accompanied by local change in chromosomal supercoiling (59). However, our data using inhibitors of gyrase activity in wild-type and mutant strains of *Rhodobacter* strongly suggest an effect of gyrase on *puf* and *puc* expression. Whether gyrase affects *puf* and *puc* expression rather by change in superhelicity or by modulating DNA bending (60), and whether RNA polymerase activity is influenced directly or indirectly by binding of other proteins to the promoter region remains to be elucidated.

By analyzing wild-type and *trx* mutant strains of *Rhodobacter* with regard to gyrase activity and in response to gyrase inhibitors, we can demonstrate that gyrase activity is indeed influenced by thioredoxin. Considering the stimulatory effect of TrxA on gyrase activity, it is understandable that the *trxA* mutants, which exhibit lower gyrase activity, are more sensitive to novobiocin as observed by zone inhibition assays (data not shown), since novobiocin further reduces gyrase activity. The fact that the *trxC* mutant, which exhibits higher gyrase activity, shows the same sensitivity to novobiocin as the parental wild type indicates that the lack of the inhibiting TrxC cannot compensate the inhibitory effect of novobiocin. Our data demonstrate a role of thioredoxin in controlling DNA supercoiling. An effect of thioredoxin from *Streptomyces aureofaciens* on coiling of plasmid DNA was reported previously (61). From a number of *in vitro* experiments, these authors concluded that thioredoxin directly binds to the DNA randomly and introduces single-strand breaks allowing relaxation of the DNA. The correlation of TrxA levels and the supercoiling activity and the opposite effects of TrxA and TrxC on supercoiling activity as reported
here argue against the application of this model for *Rhodobacter*.

Interestingly, mutations in *trxA* or *trxC* showed opposite effects on gyrase activity. While extracts from the two *trxA* mutants showed lower supercoiling activity than extracts from wild-type cells, extracts from the *R. capsulatus trxC* mutant showed higher supercoiling activity. Even the higher levels of gyrase that we determined in the *trxA* mutant could not compensate the deficiency of the supercoiling activity of gyrase. Our data reveal that TrxC has an inhibiting effect on gyrase activity while TrxA has a stimulating effect. The gyrase domain encoded by the DNA fragment isolated from the *Rhodobacter* libraries overlaps the novobiocin and also the ATP binding site of GyrB (62). Nevertheless, the effect of thioredoxin on gyrase activity cannot be attributed to competition with ATP binding since TrxA has a stimulating effect. As also revealed by our *in vitro* binding studies, the thioredoxin effect on gyrase activity depends on its redox status. We present a model (Figure 8) in which a change in oxygen tension influences the redox state of thioredoxins, which consequently alters gyrase activity. Since oxidized TrxC inhibits gyrase activity while reduced TrxA stimulates gyrase activity, both thioredoxins mediate a signal leading to higher supercoiling activity at reduced redox potential. The opposite effects of TrxA and TrxC on supercoiling activity of gyrase correlate well with the opposite effect of *trxA* and *trxC* mutations on the expression of the photosynthesis genes and bacteriochlorophyll accumulation after reduction of oxygen tension in *Rhodobacter* (9, 11). It has been reported that the redox potential in aerobically or anaerobically grown *E. coli* cells remains between −260 and −280 mV (63, 64). Masuda and Bauer (23) demonstrated that the redox potential in aerobically or anaerobically grown cells of *R. capsulatus* is identical (−224 and −222 mV, respectively). Those indirect measurements of the internal ambient potential may, however, not reflect the *in vivo* situation since thioredoxin 1 changes its redox state in *R. capsulatus* grown at different oxygen tensions (Figure 2C). These data strongly suggest that cellular redox potentials are different in these two different growth conditions and that the signal chain from thioredoxin to gyrase involves a redox switch of thioredoxin.

Nevertheless, we have to consider that the redox-dependent influence of thioredoxins on gyrase activity is only part of the redox signal chain regulating the expression of photosynthesis genes in *Rhodobacter*. As demonstrated before, expression of TrxA and TrxC in *Rhodobacter* are oppositely regulated by oxygen. In both *Rhodobacter* wild types, *trxA* expression is upregulated by oxygen, while *trxC* expression in *R. capsulatus* is downregulated (9, 11). Furthermore, we observed an effect of TrxA on the gyrase level. Since we observed increased gyrase protein levels when supercoiling is decreased, this effect of TrxA on gyrase expression may also involve the thioredoxin–gyrase pathway, but other regulatory mechanisms can presently not be excluded. In *Rhodobacter* the thioredoxin–gyrase signaling pathway overlaps with RegB/RegA and CrtJ redox-dependent regulation.

Our data support a model in which thioredoxins of *Rhodobacter* act on photosynthesis gene expression by the action of gyrase. Although the expression of individual genes is differentially affected by the DNA topology, the differences in gyrase activity will certainly affect not only *puf* and *puc* expression, but also the expression of a number of *Rhodobacter* genes. Even the closely related species *R. sphaeroides* and *R. capsulatus* significantly differ in the composition of their thioredoxin systems, suggesting that the thioredoxin–gyrase signaling pathway may vary among individual species. We found the same effect of thioredoxins on gyrase activity in *R. capsulatus* and in *E. coli*. We, therefore, propose that the action of thioredoxins on gyrase activity is a general signaling pathway in bacterial systems.

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