Molecular genetic analysis of a thioredoxin gene from *Thiobacillus ferrooxidans*

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The *Thiobacillus ferrooxidans* thioredoxin gene, *trxA*, was isolated by its ability to complement an *Escherichia coli* *gshA trxcl* mutant which was otherwise unable to grow on minimal medium lacking glutathione. The *T. ferrooxidans* thioredoxin also enabled the *in vivo* reduction by *E. coli* of methionine sulfoxide to methionine, as well as the *in vitro* reduction of insulin. When present in *E. coli*, the *T. ferrooxidans* thioredoxin supported the replication of phage T7, but not the growth of phage M13. The *T. ferrooxidans* *trxA* gene was sequenced and the thioredoxin was found to be most like that of *E. coli* (71% identity) and *Chromatium vinosum* (70% identity). As in the case of *E. coli*, the gene was located immediately upstream of the gene for the *rho* transcriptional terminator. DNA:RNA blot hybridization and primer-extension analysis of the *trxA* gene in *T. ferrooxidans* and the cloned gene in *E. coli* indicated that it was transcribed as an independent unit and that the major transcriptional start sites were the same in both organisms.

**Keywords:** *Thiobacillus ferrooxidans*, thioredoxin, molecular cloning

**INTRODUCTION**

*Thiobacillus ferrooxidans* is an autotrophic, chemolithotrophic, Gram-negative bacterium that obtains its energy by oxidizing Fe$^{2+}$ to Fe$^{3+}$ or reduced sulfur compounds to sulfuric acid. It is highly acidophilic and grows optimally within the pH range 1.5-3.5. Much interest has been shown in *T. ferrooxidans* because of its use in industrial mineral processing and because of its unusual physiology.

Thioredoxins are small, heat-stable, ubiquitous proteins that serve as a source of electrons in numerous metabolic processes (Holmgren, 1989). The oxidized form of thioredoxin contains a disulfide bridge that is reversibly reduced by NADPH and thioredoxin reductase (Moore et al., 1964). The reduced form is a strong protein disulfide oxidoreductase that is involved in the reduction of ribonucleotide reductase, an essential enzyme in DNA synthesis (Laurent et al., 1964), and in the reduction of enzymes that reduce sulfate and methionine sulfoxide (Gonzalez Porquier et al., 1970). Thioredoxin is also a highly efficient disulfide reductase of wide specificity, catalysing many dithiol-disulfide redox reactions. A characteristic of thioredoxins is the presence of the well-conserved redox active site, -Trp-Cys-Gly-Pro-Cys-(Holmgren, 1968).

Thioredoxin has been identified as an essential subunit of T7 phage DNA polymerase (Mark & Richardson, 1976). It forms a stable 1:1 complex with the T7 polymerase gene 5 protein, increasing the processivity of the enzyme several hundredfold (Tabor et al., 1987). Thioredoxin is also required for the growth of the filamentous phages M13, f1 and fd, where the role of thioredoxin is different from that of phage T7. Although the exact mechanism is not fully understood, thioredoxin is thought to interact with the gene 1 protein and to be involved in filamentous phage assembly (Russel & Model, 1986).

In plant cells, thioredoxin is involved in the regulation of enzymes of carbon dioxide fixation. During photosynthesis, electrons are passed from chlorophyll to ferredoxin and then to thioredoxin via ferredoxin-thioredoxin reductase (Buchanan, 1980). The reduced thioredoxin then activates enzymes such as fructose-1,6-bisphosphatase and NADP-malate dehydrogenase. All plant tissues analysed have two or more thioredoxin species (Buchanan et al., 1979). As an obligate autotroph, an efficient fructose-1,6-bisphosphatase is essential for the growth of *T. ferrooxidans* and two genes encoding this enzyme have been cloned from the bacterium (Kusano et al., 1991). Whether thioredoxin plays a role in the regulation of these enzymes is still not known. Thioredoxin may be used in other roles...
associated with the habitat of *T. ferrooxidans*. For example, thioredoxin is a cofactor used for the detoxification of arsenic by the ArsC protein (Ji & Silver, 1992) found on several plasmids and the chromosome of *E. coli* (Carlin et al., 1995). When growing in arsenopyrite ores, *T. ferrooxidans* is able to tolerate high concentrations of arsenic, although it has not yet been shown that arsenic resistance is due to a mechanism similar to that of ArsC.

In this paper we report the isolation of the *T. ferrooxidans* trxA gene, its characterization, and the ability of the thioredoxin to complement *E. coli* trxA mutants and to reduce insulin.

**METHODS**

**Bacterial strains.** Genotypes of the strains used are: *E. coli* JM109, endA1 recA1 gyrA96 thi hsdR17 (rK- mK+) relA1 supE44 Δ(lac-proAB) (F’ traD36 proAB lacF2ΔM15); *E. coli* BH5262, K12 F’ araD1397 galU galK hsdR rpsL argH1 trxA7004 gshA slr::Tn10; *E. coli* BH2012, K12 F’ araD1397 galU galK hsdR rpsL metA46 argH1 thrA7004 ilvC A(lac-proAB) (F’ traD36 proAB lacF2ΔM15) (Heidecker et al., 1980). The above strains and phage T7 were kindly donated by J. A. Fuchs. *E. coli* K12 F- araD1391 galU galK hsdR rpsL metA46 argH1 trxA7004 gshA supE (F’ proAB lac195174) and *E. coli* MC1061 (K12 F’ araD1397 galU galK hsdR rpsL metA46 argH1 trxA7004 ilvC::Tn5) (Lim et al., 1986) is an iacC::Tn5 trxA7004 derivative of strain 71/18 [Δ(lac-proAB) thi supE (F’ proAB laclzAM15)] (Heidecker et al., 1980). Strain BH2012 trxA was kindly donated by B. C. Persson. 

**METHODS**

**Media.** Tetrathionate medium was made from mineral salts solution (g l⁻¹): (NH₄)₂SO₄, 3.0; KCl, 0.1; KH₂PO₄, 0.5; Ca(NO₃)₂, 0.01; the pH was adjusted to 2.5 with H₂SO₄ and it was autoclaved. Trace elements solution was prepared (mg l⁻¹): FeCl₂·4H₂O, 110; CaSO₄·H₂O, 0.5; HOB₈, 20; Na₂MoO₄·2H₂O, 0.8; CoCl₂·6H₂O, 0.6; ZnSO₄·7H₂O, 0.9; filter sterilized. One millilitre trace elements solution was added to 100 ml minimal medium solution and to this was added either 50 mM K₂S₂O₃ or 100 mM FeSO₄, and the final pH was adjusted to 2.5.

**Cloning and genetic manipulations.** A cosmids bank of the *T. ferrooxidans* genome was constructed by cloning sized (36-45 kb) fragments, generated by partial digestion with Sau3A1 into the BamHI restriction site of cosmid pHC79 (Ramesar, 1988). The bank was transduced into the *E. coli* mutant BH5262 according to the method of Sambrook et al. (1989). Possible thioredoxin-positive clones were identified by their ability to grow on M9 minimal medium (Sambrook et al., 1989). Strain BH5262 is unable to grow on minimal medium lacking glutathione whereas TrxA⁻ or GshA⁻ colonies can grow (Lim et al., 1986). *E. coli* strain BH2012 was used to confirm the TrxA phenotype, as this methionine auxotroph is unable to synthesize methionine from methionine sulfoxide in the absence of a methionine auxotroph. Positive colonies were re-transformed into *E. coli* BH2012 and cosmids were isolated from colonies able to grow on minimal medium plus methionine sulfoxide. Subclones of one of the TrxA⁺ cosmids were made and tested according to the method of Ausubel et al. (1993).

**DNA techniques, sequencing and analysis.** Standard methods were used for plasmid preparation, restriction enzyme digests, gel electrophoresis, ligations and Southern blot hybridizations (Sambrook et al., 1989). Labelling of probes, hybridization, detection and clone isolation were done with the digoxigenin-DUTP non-radioactive DNA labelling and detection kit (Boehringer Mannheim). DNA sequencing was by the dideoxy chain-termination method (Sanger et al., 1977), using the Sequenase version 2.0 kit (USB).

The sequence of the 1.1 kb HindIII-PstI fragment from pTRX32 (Fig. 3) was determined from both strands. The Genetics Computer Group (GCG) software package was used for sequence analysis (Devereux et al., 1984).

**In vitro synthesis of thioredoxin.** The synthesis of polypeptides from cosmid 32, plasmids pTRX32, pTRX6, pTRX14A, pTRX9 and pBluescript SK was determined using the prokaryotic DNA-directed transcription kit, *E. coli* S30 system from Promega. Reactions were performed according to the manufacturer’s specifications and the proteins were separated by SDS-PAGE using 10–20% (w/v) gradient gels.

**Phage growth.** A growth curve of phage T7 was determined in *E. coli* host strains MC1061, BH2012 and BH2012(pTRX6). Phage titres were estimated using *E. coli* MC1061 as the plating bacterium, at different time intervals during the growth curve. Growth of phage T13 was monitored in *E. coli* strains 71/18, JF501 and JF501(pTRX6). Cultures were inoculated with M13 phage to 5 x 10⁸ p.f.u/ml and grown overnight with vigorous aeration at 37 °C. The cultures were titred for M13 phage using *E. coli* 71/18 as the plating bacterium, at the time of inoculation and again 20 h later.

**Insulin assay.** The insulin assay of Holmgren (1979) was used to measure the rate of disulfide reduction, by recording the rate of precipitation at 650 nm. Crude cell extracts were prepared as follows. Mid-exponential phase bacterial cells were harvested from 100 ml minimal medium and resuspended in 1 ml assay buffer (100 mM potassium phosphate, pH 7.5, 1 mM EDTA, 1 mM DTT and 1 mM Pefabloc). Pefabloc [N-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride] was from Boehringer Mannheim. After sonication, lysed cells were precipitated and the supernatant was heated at 85 °C for 5 min. Denatured proteins were removed by centrifugation and the supernatant was used in the assay. Insulin was added to 900 µl crude extract (final concentration 1 mg ml⁻¹), the reaction was initiated by adding 3 µl 100 mM DTT and monitored by measuring the increase in optical density at 650 nm (Beckman DU64 spectrometer). Crude extract prepared from *E. coli* BH2012 trxA mutant was used as a negative control.

**Analysis of transcripts.** *T. ferrooxidans* total RNA was prepared from cultures grown on tetrathionate medium, and *E. coli* total RNA from cultures grown on M9 minimal medium, by the method of Aiba et al. (1981). For DNA:RNA hybridization blots, total RNA was separated on a 1.5% (w/v) agarose gel containing 6% (v/v) formamide. The DNA was transferred to an Amersham Hybond N⁺ membrane and hybridization and washes were carried out according to the manufacturer’s protocol. The HindIII fragment of pTRX9, labelled with ³²PdCTP, using a Random Primed DNA labelling kit from Boehringer Mannheim, was used as a probe. Transcript starts were determined by primer-extension analysis. A synthetic 27 bp DNA oligomer primer was end-labelled using the polynucleotide kinase/³²P method (Ausubel et al., 1993), and hybridized to the 5' end of the thioredoxin mRNA. Primer extension was carried out using total RNA derived from *E. coli* BH5262(pTRX6) and total RNA from *T. ferrooxidans* according to the method of Ausubel et al. (1993).

**RESULTS**

Isothermal and localization of the *T. ferrooxidans* thioredoxin gene

Transduction of the *T. ferrooxidans* genome cosmid library into *E. coli* BH5262 resulted in approximately 100 colonies that were able to grow on minimal medium lacking
glutathione. DNA was prepared from 16 of these colonies. These cosmids had several fragments in common and could be divided into two groups that appeared to contain overlapping pieces of two regions of the *Thiobacillus ferrooxidans* chromosome. Only one of the groups of cosmids, on transforming into *E. coli* BH2012, enabled cells to grow on minimal medium plus methionine sulfoxide. One cosmid from this group, containing a 37 kb genomic insert (cosmid 32), was chosen for further study.

PstI fragments from cosmid 32 were cloned into the vector pBluescript SK and tested for complementation of *E. coli* BH5262, resulting in subclone pTRX32A. A restriction map of pTRX32A was constructed and smaller fragments were subcloned into pBluescript SK (Fig. 1). Plasmids pTRX32 and pTRX6 complemented *E. coli* mutants BH5262 and BH2012, while plasmids pTRX13A, pTRX14A and pTRX9 did not, indicating that the thioredoxin gene was located between the Sful and PstI sites of pTRX32.

The source of the cloned thioredoxin-complementing DNA was confirmed by hybridization of the labelled *SacI–Kpni* fragment from pTRX32 to *T. ferrooxidans* ATCC33020 chromosomal DNA, cosmid 32, pTRX32 and pTRX6 (Fig. 2). The 685 bp HindIII fragment that is internal to the cloned *T. ferrooxidans* chromosomal DNA present on pTRX32 corresponded exactly to a HindIII fragment present on the *T. ferrooxidans* chromosome, cosmid 32 and pTRX32. The hybridization signal at 1.8 kb represents the adjacent HindIII fragment which contains part of the trxA and rho genes and is present in the chromosomal and the cosmid DNA only. Similarly, when the *T. ferrooxidans* chromosomal DNA was digested with both *SacI* and *Kpni*, a single 530 bp hybridization signal, which was also present on cosmid 32, pTRX32 and pTRX6 was detected. This indicated that the source of the cloned gene originated from *T. ferrooxidans* ATCC33020 and that there was only one copy of the thioredoxin gene per genome. Over-exposure of the autoradiogram (not shown) failed to indicate any additional chromosomal bands. The faint upper bands in lanes 2, 7, 8 and 9 (Fig. 2) are due to a low concentration of vector DNA which remained in the incompletely purified *SacI–Kpni* probe.

**Fig. 1.** Restriction map of pTRX32A and derivatives. The trxA gene and the start of the rho gene are indicated by arrows. The dotted line indicates the region of DNA sequenced from both strands. TrxA+ activity indicates that the transformed plasmid was able to complement the thioredoxin mutant *E. coli* BH2012.

**Fig. 2.** Hybridization of the labelled 517 bp *Kpni–SacI* fragment of pTRX32 to HindIII digests (lanes 1–4) and *Kpni–SacI* digests (lanes 6–9) of: lanes 1 and 6, *T. ferrooxidans* chromosomal DNA; lanes 2 and 7, cosmid 32; lanes 3 and 8, pTRX32; lanes 4 and 9, pTRX6. Lane 5, blank.
Sequence analysis

Analysis of the sequence data revealed one complete and one partial ORF (Fig. 3). The complete ORF is preceded by a strong RBS and encodes a protein of 101 amino acids, corresponding to a polypeptide of 11.2 kDa. The predicted amino acid sequence was closely related to thioredoxins from other prokaryotic and eukaryotic organisms and contains the highly conserved active site sequence, -Trp-Cys-Gly-Pro-Cys- (Holmgren, 1968). T. ferrooxidans thioredoxin was most similar to the E. coli (56% identity, accession no. M91384) and Saccharomyces cerevisiae (56% identity, accession no. M62647). An incomplete ORF was situated 126 bp downstream of the thioredoxin gene in pTRX32 and although there is no clear RBS, the predicted polypeptide has similarity to the rho termination protein of other prokaryotes. Only the first 58 amino acids of the predicted T. ferrooxidans rho protein were analysed and this sequence shows clear similarity (59% identity, accession no. J01673) to the E. coli rho protein.

Synthesis of a protein corresponding to the T. ferrooxidans thioredoxin was confirmed using an E. coli-derived in vitro transcription-translation system. A protein of approxi-
Thioredoxin of *Thiobacillus ferrooxidans*

Table 1. Growth of phages T7 and M13 in different hosts

Values are expressed as p.f.u. ml⁻¹. Data are the means of three different experiments ± sd.

<table>
<thead>
<tr>
<th>Phage</th>
<th><em>E. coli</em> host strain</th>
<th>Time (min)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>T7</td>
<td>MC1061</td>
<td>1·26 ± 0·60 x 10⁸</td>
</tr>
<tr>
<td></td>
<td>BH2012 (pTRX6)</td>
<td>1·58 ± 0·64 x 10⁹</td>
</tr>
<tr>
<td></td>
<td>BH2012</td>
<td>1·58 ± 1·00 x 10⁹</td>
</tr>
<tr>
<td>M13</td>
<td>71/18</td>
<td>1·69 ± 0·13 x 10⁹</td>
</tr>
<tr>
<td></td>
<td>JF510 (pTRX6)</td>
<td>1·55 ± 0·39 x 10⁹</td>
</tr>
<tr>
<td></td>
<td>JF510</td>
<td>1·52 ± 0·45 x 10⁹</td>
</tr>
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![Fig. 5. Hybridization of labelled HindIII fragment of pTRX9 to total RNA prepared from: lane 1, *E. coli* BH5262 (~20 μg); lane 2, *E. coli* BH5262 (pTRX6) (~40 μg); lane 3, *T. ferrooxidans* (~25 μg); lane 4, *T. ferrooxidans* (~5 μg).](image)

T7 and M13 phage complementation

The ability of the *T. ferrooxidans* thioredoxin expressed from pTRX6 in *E. coli* BH2012 to support phage T7 replication was compared with the thioredoxin expressed from the chromosome of the *E. coli* parental strain MC1061 (Table 1). Titres of phage T7 reached almost the same level when the *T. ferrooxidans* thioredoxin was provided as the titre obtained with the natural *E. coli* thioredoxin. When *E. coli* strain BH2012 (pTRX6) was used as the plating bacterium, the phage T7 plaques were more variable in size and slightly smaller than when strain MC1061 was used. The cloned *T. ferrooxidans* trxA gene was clearly able to complement the *E. coli* BH2012 trxA mutant to support growth of phage T7 although with a slightly reduced efficiency. The *T. ferrooxidans* thioredoxin, therefore, appears to be able to form a functional association with the gene 5 protein of the phage T7 DNA polymerase complex.

The ability of the *T. ferrooxidans* thioredoxin to support the growth of phage M13 is shown in Table 1. Although there was a slight increase in the titre of phage M13 in *E. coli* JF510 (pTRX6) relative to the *E. coli* JF510 control when grown in liquid medium, no plaques were detected on solid medium when JF510 (pTRX6) was used as the plating bacterium. The *T. ferrooxidans* thioredoxin was not able to satisfy the thioredoxin requirement of the filamentous phage.

Insulin reduction

Thioredoxin has been shown to catalyse the reduction of the insulin disulfide bridge by dithiothreitol (Holmgren, 1979). This reduction results in the precipitation of the insulin B chain which can be readily measured as an increase in optical density. We compared crude extracts of *E. coli* trxA mutants with and without the cloned *T. ferrooxidans* trxA gene for the ability to reduce insulin. Extracts prepared from *E. coli* BH2012 (pTRX6) cells were able to reduce insulin at a greatly enhanced rate compared to extracts from *E. coli* BH2012 cells (results not shown). This clearly indicates that the thioredoxin from the cloned *T. ferrooxidans* trxA gene was active in *E. coli*.

Transcript analysis

To determine whether the *T. ferrooxidans* trxA gene was independently transcribed, or was co-transcribed with an unidentified upstream gene or with the downstream rho gene, DNA:RNA hybridization analysis was carried out on RNA transcripts prepared from *E. coli* BH5262 trxA mutants, *E. coli* BH5262 (pTRX6) and *T. ferrooxidans* cells and probed with the HindIII fragment from pTRX9 (Fig.
Fig. 6. Primer-extension analysis of the 5’ transcription start sites of the trxA gene cloned in E. coli and in T. ferrooxidans. The letters above each lane indicate the dideoxynucleotide used to terminate the sequencing reaction. Extension products from E. coli BH5262(pTRX6) (lane 1) and T. ferrooxidans (lane 2) RNA. The first two transcription start sites of the gene in T. ferrooxidans are indicated by arrows.

1). A single transcript of about 0.5 kb was obtained for RNA from T. ferrooxidans cells (Fig. 5, lanes 3 and 4), which would correspond to the predicted size of the thioredoxin gene and indicate that the T. ferrooxidans trxA gene is transcribed on its own. Several transcripts were produced from E. coli BH5262(pTRX6) cells (Fig. 5, lane 2) and one of these corresponded exactly in size to the 0.5 kb signal from T. ferrooxidans, while the other transcripts were much larger (approximate sizes 1-35, 1.4, 28, 25, 24, 23, 22 and 18 kb). These presumably represent transcription products that originate from the lasZ promoter of the vector. A very weak signal at about 0.45 kb was produced from RNA isolated from E. coli BH5262 cells which was smaller in size than the T. ferrooxidans trxA transcript and may represent a low amount of trxA transcription from the E. coli BH5262 trxA mutant.

Primer-extension analysis was used to compare the transcriptional start sites of the T. ferrooxidans trxA gene in its natural host with those of the gene cloned in E. coli. RNA prepared from T. ferrooxidans gave three possible transcriptional start sites, at positions 274, 298 and 345 (Fig. 6, lane 1). In the case of RNA from E. coli BH5262, only two transcription start sites were detected which were identical to those of T. ferrooxidans at positions 298 and 345 (Fig. 6, lane 1).

**DISCUSSION**

As an obligately autotrophic, chemolithotrophic and acidophilic bacterium, T. ferrooxidans has a unique physiology and occupies a very different ecological niche compared with E. coli. Based on 16S rRNA sequences, T. ferrooxidans is grouped with the β-proteobacteria (Lane et al., 1992), whereas E. coli is a γ-proteobacterium. In spite of these differences, the two bacteria share a remarkable amount of similarity at the genetic level (Rawlings & Kusano, 1994). Analysis of the trxA genes and flanking regions is an illustration of this. In both bacteria, the trxA genes are independently transcribed (Wallace & Kushner, 1984), present in a single copy and have a rho gene located immediately downstream. Furthermore, two of the three trxA transcriptional start sites detected in T. ferrooxidans were also functional in E. coli. A minor difference is that in E. coli approximately 10% of the rho gene mRNA occurs as a 2.1 kb transcript (Matsumoto et al., 1986), which is a result of transcriptional read-through from the trxA gene. This does not appear to be the case in T. ferrooxidans.

The T. ferrooxidans thioredoxin was clearly functional in E. coli as it enabled the growth of the E. coli BH5262 gshA trxA mutant on minimal medium lacking glutathione and the E. coli BH2012 trxA met mutant to reduce methionine sulfoxide to methionine. The ability of the T. ferrooxidans thioredoxin in E. coli to support growth of phage T7, but not the filamentous phage M13, is different to what was found with the thioredoxin from Anabaena sp. strain PCC7119. No growth of wild-type phage T7 occurred in the presence of the Anabaena thioredoxin, indicating that it was unable to form an active DNA polymerase complex with the gene 5 protein in vitro (Lim et al., 1986). It has been suggested that the regions around amino acids 74–77 and 91–93 of the E. coli protein are critical for the interaction of thioredoxin with the gene 5 protein (Huber et al., 1986). The Anabaena thioredoxin differs by a single amino acid in one of these regions (E. coli G74 to Anabaena S74), whereas the T. ferrooxidans thioredoxin is identical to that of E. coli. This may explain why the T. ferrooxidans thioredoxin can support the growth of phage T7 whereas the Anabaena thioredoxin failed to do so.

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