INTRODUCTION

The ubiquitous thioredoxin (Trx) reduces target protein disulphides and is recycled by NADPH-dependent thioredoxin reductase (TrxR) through thiol–disulphide exchange reactions (Lu & Holmgren, 2014). The ‘classical’ Trx with a redox active CGPC motif was discovered 50 years ago as an electron donor to ribonucleotide reductase (RNR) in Escherichia coli and contains the common WCGPC active site motif, while TrxD is atypical in its oxidation state and contains an aspartate residue in the active site (WCGDC). To elucidate the physiological roles of the two Trx paralogues, deletion mutants ΔtrxA, ΔtrxD and ΔtrxAΔtrxD were constructed. In general, the ΔtrxAΔtrxD strain was significantly more sensitive than either of the ΔtrxA and ΔtrxD mutants. Upon exposure to oxidative stress, growth of the ΔtrxA strain was diminished while that of the ΔtrxD mutant was similar to the wild-type. The lack of TrxA also appears to impair methionine sulphoxide reduction. Both ΔtrxA and ΔtrxD strains displayed growth inhibition after treatment with sodium arsenate and tellurite as compared with the wild-type, suggesting partially overlapping functions of TrxA and TrxD. Overall the phenotype of the ΔtrxA mutant matches established functions of WCGPC-type Trx while TrxD appears to play a more restricted role in stress resistance of Lactococcus lactis.

Two Lactococcus lactis thioredoxin paralogues play different roles in responses to arsenate and oxidative stress

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Thioredoxin (Trx) maintains intracellular thiol groups in a reduced state and is involved in a wide range of cellular processes, including ribonucleotide reduction, sulphur assimilation, oxidative stress responses and arsenate detoxification. The industrially important lactic acid bacterium Lactococcus lactis contains two Trxs. TrxA is similar to the well-characterized Trx homologue from Escherichia coli and contains the common WCGPC active site motif, while TrxD is atypical and contains an aspartate residue in the active site (WCGDC). To elucidate the physiological roles of the two Trx paralogues, deletion mutants ΔtrxA, ΔtrxD and ΔtrxAΔtrxD were constructed. In general, the ΔtrxAΔtrxD strain was significantly more sensitive than either of the ΔtrxA and ΔtrxD mutants. Upon exposure to oxidative stress, growth of the ΔtrxA strain was diminished while that of the ΔtrxD mutant was similar to the wild-type. The lack of TrxA also appears to impair methionine sulphoxide reduction. Both ΔtrxA and ΔtrxD strains displayed growth inhibition after treatment with sodium arsenate and tellurite as compared with the wild-type, suggesting partially overlapping functions of TrxA and TrxD. Overall the phenotype of the ΔtrxA mutant matches established functions of WCGPC-type Trx while TrxD appears to play a more restricted role in stress resistance of L. lactis.

Abbreviations: DIGE, difference gel electrophoresis; EP, exponential phase; INT, iodonitrotetrazolium chloride; MetSO, methionine sulphoxide; Msr, MetSO reductase; RNR, ribonucleotide reductase; SP, stationary phase; Trx, thioredoxin; TrxR, NADPH-dependent thioredoxin reductase; TV, tetrazolium violet.

Two supplementary figures and three supplementary tables are available with the online Supplementary Material.
Escherichia coli and several other Gram-negative bacteria utilize the disulphide reductase glutaredoxin coupled to the tripeptide glutathione (GSH) and glutathione reductase as an alternative pathway to provide electrons for RNR and other target proteins (Lillig et al., 2008). In contrast, most Gram-positive bacteria lack GSH and deletion of genes encoding Trx and/or TrxR results in severe growth defects or lethal phenotypes in B. subtilis and S. aureus (Kobayashi et al., 2003; Möller & Hederstedt, 2008; Scharf et al., 1998; Uziel et al., 2004). Some species lacking GSH produce alternative low-molecular-mass thiol such as mycothiol or bacillithiol but the biological significance of these compounds has not yet been firmly established (Fahey et al., 1978; Newton et al., 1996, 2009).

Similar to most Gram-positive bacteria, the industrially important lactic acid bacterium Lactococcus lactis lacks the biosynthetic pathway for GSH but some strains can utilize exogenously supplied GSH (Fernández & Steele, 1993; Li et al., 2003; Newton et al., 1996). Furthermore, L. lactis lacks catalase and is thus likely to rely on thiol-dependent peroxiredoxins and NADH peroxidase for reduction of hydrogen peroxide (H$_2$O$_2$). L. lactis contains two Trx paralogues (TrxA, TrxD) and a glutaredoxin-like protein (NrdH), which acts as electron donor for a particular type of RNR (class Ib) that is mainly found among prokaryotes (Bjo¨rnberg et al., 2008). In addition, YtpP is proposed to be involved in amino acid metabolism (Berka et al., 2003).

Electrons from TrxA via the membrane protein CcdA (Möller & Hederstedt, 2008). In addition, YtpP is proposed to be involved in amino acid metabolism (Berka et al., 2003).

 METHODS

Strains and growth conditions. The strains used in this study are listed in Table 1. Unless stated otherwise, Lac. lactis strains were maintained under aerobic conditions on agar plates containing M17 medium (Difco) with 1% (w/v) glucose (GM17), and grown in chemically defined SA medium (Jensen & Hammer, 1993) containing 1% (w/v) glucose and 4 μg lipoic acid ml$^{-1}$ (GSAL medium). To obtain synchronized balanced cultures, colonies from fresh GM17 plates were inoculated into liquid GSAL medium, serially diluted (10$^2$, 10$^3$, 10$^4$, 10$^5$) and grown under static conditions at 30 °C overnight. The dilution with exponentially growing cells (OD$_{450}$ of 0.3–0.6) was used for further experiments. When performing phenotype screening on solid GSAL media, synchronized exponentially growing overnight cultures were used for making serial dilutions (10$^2$, 10$^3$, 10$^4$, 10$^5$) in pre-warmed GSAL medium in a 96-well plate. From each well, 10 μl was spotted on pre-warmed GSAL agar plates containing the particular stress compound (0.5 mM Na$_2$HAsO$_4$ or 0.3 mM K$_2$TeO$_4$) and incubated at 30 °C for 24 h. Alternatively, single colonies freshly grown on GM17 plates were streaked directly on GSAL agar plates followed by incubation at 30 °C for 72 h. E. coli MC1061 was grown in Luria-Broth medium (LB) at 28, 30 or 37 °C. When relevant, LB was supplemented with erythromycin (150 μg ml$^{-1}$) and GM17 by erythromycin (5 μg ml$^{-1}$) plus 1% NaCl.

Bioscreen assays. A Bioscreen C instrument (Oy Growth Curves) was used to monitor growth of Lac. lactis wt and trxA mutants exposed to a range of different stress conditions. Synchronized exponentially growing cultures were diluted in preheated GSAL medium to an OD$_{450}$ of 0.01, and then 360 μl was mixed with 40 μl of a stress compound solution (listed in Table S1, available in the online Supplementary Material) or H$_2$O in a well of a pre-warmed honeycomb plate. To monitor methionine sulphoxide (MetSO) assimilation, a freshly grown single colony from a GM17 plate was resuspended in 5 ml GSAL medium without methionine and diluted ten times in the same medium. From this culture 360 μl aliquots were pipetted into wells of a pre-warmed honeycomb plate containing 40 μl of either methionine or MetSO at 1 mg ml$^{-1}$. The plates were incubated at 30 °C without shaking. OD$_{450}$ was monitored at 40 min intervals with 10 s of intense shaking prior to measurements.

Construction of Lac. lactis ΔtrxA, ΔtrxD, ΔtrxAΔtrxD mutants and complemented strains. DNA isolation, amplification and cloning were performed according to standard procedures (Sambrook & Russell, 2001) or the manufacturers’ instructions. Upstream and downstream regions flanking the trxA and trxD genes were amplified from genomic DNA of Lac. lactis subsp. cremoris MG1363 by PCR (deletion by overlap extension) using the primers listed in Table S2 and a HotStar HiFidelity PCR kit (Qiagen). The PCR products of the upstream and downstream regions were fused and used as templates for PCR using the forward primers for the upstream regions together with the reverse primers for the downstream regions (Table S2). The PCR products were digested with BamHI and Xhol and ligated into pGHOST4 (Appligene). The resulting plasmids were used to transform E. coli MC1061, and the correct sequences were confirmed by DNA sequencing (Eurofins). Plasmids were electroporated into Lac. lactis and the transformants were selected on GM17 plates containing erythromycin at 28 °C. After homologous recombination into the chromosome, and clearing of the plasmid as described by Biwas et al. (1993), the deletions were confirmed by colony PCR amplification using the flanking primers binding to the chromosome outside the targeted region (Table S2). The ΔtrxAΔtrxD double mutant was prepared using the ΔtrxA strain as the template for homologous recombination of ΔtrxD as described above. Complemented strains were constructed by site-specific integration of trxA or trxD containing derivatives of plasmid pLB86 (Breunier et al., 2001) into the phage attachment site (attB) of the ΔtrxD mutant MK536 or the

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Different roles of two Lactococcus lactis thioredoxins
\(\Delta trxA\Delta trxD\) double mutant MK562. The mutants were transformed with plasmid pLB95 containing the gene encoding bacteriophage TP901-1 integrase, as described by Breuner et al. (2001). The complementing plasmids were constructed by inserting XhoI- and PstI-digested PCR fragments into pLB86 digested with the same enzymes. The genotypes of the complemented strains are shown in Table 1, and the primers used for amplification of the \(trxA\) and \(trxD\) genes with their own promoters, as well as the \(trxD\) gene lacking a functional promoter, are shown in Table S2.

**Preparation of polyclonal antibodies against TrxA and TrxD.** Purified recombinant *Lactococcus lactis* TrxA or TrxD produced in *E. coli* (Björnberg et al., 2014) were used for raising anti-TrxA or anti-TrxD antibodies. Prior to immunization the N-terminal His\(_6\) tags of recombinant TrxA and TrxD were removed by proteolytic digestion overnight with immobilized thrombin (Calbiochem). Cleaved His\(_6\)-tags and uncleaved His\(_6\)-Trx were subsequently removed on a HisTrap column (GE Healthcare) and 1.5 ml of non-His-tagged TrxA (120 \(\mu\)M) or TrxD (65 \(\mu\)M) equilibrated in PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na\(_2\)HPO\(_4\), 2 mM KH\(_2\)PO\(_4\), pH 7.4) was used for immunization of New Zealand white rabbits (3–3.5 kg). In the first immunization 500 \(\mu\)g of the antigen was mixed with 500 \(\mu\)g of complete Freund’s adjuvant and the solution was injected subcutaneously on five different spots on the back (0.2 ml per spot). The second and third boosters (given with 2 weeks intervals) were performed similarly but using incomplete Freund’s adjuvant instead. Blood sera containing anti-TrxA or anti-TrxD antibodies were collected 1 week after the third booster and stored at \(-80\) °C.

**Western blot analysis.** Synchronized cultures of *Lactococcus lactis* wt, \(\Delta trxA\) and \(\Delta trxD\) strains were grown under static conditions in liquid GSAL medium at 30 °C. From a total culture volume of 100 ml, 40 ml was harvested in the mid-exponential phase (EP; OD\(_{450}\) of 0.4) and in the stationary phase (SP; OD\(_{450}\) of \(\approx 2\)), respectively. Cell metabolism was quenched by pouring culture samples into pre-chilled flasks on ice and incubating for 15 min. Cultures were then centrifuged and the extracts were centrifuged (15 min at 14 000 r.p.m. at 4 °C, and supernatants were removed. Pellets were washed in 1 ml of an ice-cold sterile 0.9 % (w/v) NaCl solution, transferred into Eppendorf tubes and centrifuged again. Supernatants were discarded and pellets were stored at \(-20\) °C until extraction. Frozen pellets were dried in a SpeedVac SPD1010 concentrator (Thermo Scientific) for 1–2 h. Then, 100 and 300 \(\mu\)l of glass beads (diameter \(\leq 106 \mu\)m; Sigma) was added to the dry pellets from EP and SP cultures, respectively, followed by homogenization by aid of a micropestle (Eppendorf). Extraction buffer (0.2 M Tris/HCl, 0.2 M NaCl, 5 % glycerol, 1 mM EDTA, pH 7.6) was added to obtain a final volume of 200 and 900 \(\mu\)l for the EP and SP samples, respectively. Following centrifugation (15 min at 14 000 r.p.m., 4 °C) supernatants were collected and protein concentration was determined (Coomassie plus protein assay reagent kit; Pierce Biotechnology) with BSA as standard. SDS-PAGE was performed with 25 \(\mu\)g of total protein from each cell extract and positive controls with 200 and 100 ng of His\(_6\)-tagged TrxA and TrxD, respectively. Western blotting was performed using a X-Cell II Blot Module (Invitrogen) and Amersham Hybond ECL nitrocellulose membrane (GE Healthcare). Membranes were incubated with non-purified rabbit sera containing anti-TrxA and anti-TrxD antibodies (see above) diluted 1:2000 in TBS buffer (100 mM Tris/HCl, pH 7.5, 150 mM NaCl) containing 0.1 % (v/v) Tween-20 (TBST) for 1 h at room temperature. After several washes in TBST, alkaline phosphatase-conjugated polyclonal goat anti-rabbit IgG (0.64 mg ml\(^{-1}\); Dako) diluted 1:2000 in TBST was added and incubated for 30 min. The membrane was again washed in the same buffer followed by incubation for 10 min in 0.015 % (w/v) 5-bromo-4-chloro-3-indolyl phosphate and 0.030 % (w/v) nitro blue tetrazolium chloride in 100 mM NaCl, 5 mM MgCl\(_2\) and 100 mM Tris/HCl, pH 9.5, at room temperature. Reactions were stopped by transferring the membrane into 20 mM EDTA.

**Tetrazolium salt reduction assay.** Samples of synchronized cultures (0.9 ml) of *Lactococcus lactis* wt, \(\Delta trxA\) and \(\Delta trxD\) grown under static conditions at 30 °C were collected in the mid-EP (OD\(_{450}\) of 0.4) and in SP (OD\(_{450}\) of \(\approx 2\)), mixed with 100 \(\mu\)l of 5 mM tetrazolium violet (TV) or iodonitrotetrazolium chloride (INT) and incubated for 15 min at room temperature in the dark. Samples were centrifuged (20 000 g, 15 min, room temperature) and supernatants discarded. Pellets were resuspended in 1 ml DMSO and centrifuged again (20 000 g, 15 min, room temperature). The absorbances at 510 nm (reduced TV) and 468 nm (reduced INT) in the supernatants were determined and divided by cell density (OD\(_{450}\) values of the cultures at harvest).

**Difference gel electrophoresis (DIGE).** Synchronized cultures of *Lactococcus lactis* wt, \(\Delta trxA\) and \(\Delta trxD\) strains were grown in GSAL medium under static conditions at 30 °C and samples were harvested in the mid-EP (OD\(_{450}\) of 0.4). Cell pellets from 80 ml cultures washed in 0.9 % NaCl were freeze-dried (Scanvac CoolSafe; LaboGene) for 2 h. Thereafter, 500 \(\mu\)l extraction buffer (0.2 M Tris/HCl, 0.2 M NaCl, 5 % glycerol, 1 mM EDTA, pH 7.6) and 500 \(\mu\)l of glass beads (diameter \(\leq 106 \mu\)m) were added and cells were disrupted by three cycles in a FastPrep FP120 homogenizer (Qiagen) set up at speed 4 and time 45 s (samples were kept on ice for 2 min between the cycles). The extracts were centrifuged (15 min at 14 000 r.p.m. at 4 °C), supernatants were collected, treated using Benzonase (0.25 U extract \(\mu\)l\(^{-1}\)) and proteins concentrations were determined (Coomassie plus protein assay reagent kit; Pierce Biotechnology) with BSA as standard. Four biological replicates each of *Lactococcus lactis* wt, \(\Delta trxA\) and \(\Delta trxD\) were compared. For each replicate, 30 \(\mu\)g protein was precipitated by chloroform/methanol extraction (Wessel & Flugge, 1984). Pellets were dissolved in 105 \(\mu\)l rehydration buffer (7 M urea, 2 M thiourea, 10 mM Tris/HCl, pH 8.5, 4 % CHAPS) and 70 \(\mu\)l of each sample was labelled with 100 pmol (1 \(\mu\)l of 100 \(\mu\)M) of either of the fluorescent dyes Cy3 or Cy5 (CyDye DIGE Fluor minimal; GE Healthcare) in

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant genotype</th>
<th>Reference</th>
</tr>
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<td>Gasson (1983)</td>
</tr>
<tr>
<td>LB504</td>
<td>MG1363/pLB95</td>
<td>Breuner et al. (2001)</td>
</tr>
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<td>MG1363 (\Delta trxA)</td>
<td>This work</td>
</tr>
<tr>
<td>MK556</td>
<td>MG1363 (\Delta trxD)</td>
<td>This work</td>
</tr>
<tr>
<td>MK562</td>
<td>MG1363 (\Delta trxA \Delta trxD)</td>
<td>This work</td>
</tr>
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<td>MK745</td>
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anhydrous N,N-dimethylformamide. In addition, an internal standard containing 35 μl from each sample was labelled with 600 pmol (6 μl of 100 μM) Cy2 dye (CyDye DIGE Fluor minimal) in anhydrous N,N-dimethylformamide. Fluorophore labelling was carried out on ice in the dark for 30 min followed by addition of 2 μl lysine (100 mg ml⁻¹) and incubation for 10 min on ice in the dark. Samples were mixed according to the set-up detailed in Table S3, 6 μl of 100 mg DTT ml⁻¹ and 1 μl iPG buffer, pH 4–7 (GE Healthcare), was added and isolectric focusing with Immobiline DryStrip, pH 4–7, 11 cm strips (GE Healthcare) was performed according to the following programme: 6 h at 30 V, 6 h at 60 V, 1 h at 200 V, 1 h at 500 V, 1 h at 1000 V, 1 h gradient from 1000 to 8000 V followed by a constant 8000 V until reaching 20000 Vb. Prior to the second dimension, strips were incubated for 15 min in equilibration buffer [6 M urea, 30 % (v/v) glycerol, 0.01 % bromophenol blue, 2 % (w/v) SDS, 100 mg DTT ml⁻¹, 50 mM Tris/HCl, pH 8.8] followed by 15 min in the same buffer containing iodoacetamide (250 mg ml⁻¹) instead of DTT. The second dimension was performed using Criterion Precast 12.5 % polyacrylamide gels (Bio-Rad) with MES running buffer (50 mM MES, 50 mM Tris base, 0.1 % SDS, 1 mM EDTA, pH 7.3). Gels were fixed for 30 min in 30 % (v/v) ethanol containing 2 % (v/v) phosphoric acid, scanned using a Typhoon Trio (GE Healthcare) at 100 μm resolution at excitation/emission wavelengths of 488/520 nm (Cy2), 532/580 nm (Cy3) and 633/670 nm (Cy5), and subsequently stained by Coomassie brilliant blue G-250 (Merck) as described previously (Candiano et al., 2004). Fluorescence images were analysed by Progenesis SameSpots software (Non-linear Dynamics). Only spots displaying volume fold change >1.5 and an ANOVA P value <0.05 were selected for identification by MS.

In-gel trypsin digestion and MS. Spot gel-plugs were manually picked from Coomassie-stained gels and subjected to in-gel trypsin digestion as described previously (Majumder et al., 2011). Briefly, the gel-plugs were washed using 40 % ethanol, dried with 100 % acetonitrile, added to 250 ng porcine trypsin (Promega) in 10 μl 10 mM NH₄HCO₃ and incubated overnight at 37 °C. Samples of 1 or 2 μl were loaded on an AnchorChip target plate (Bruker Daltonics) followed by 1 μl of 0.5 μg matrix solution ml⁻¹ (a-cyano-4-hydroxycinnamic acid in 70 % acetonitrile, 0.1 % trifluoroacetic acid). In some cases, samples were desalted and concentrated by using a POROS R2 (Applied Biosystems) microcolumn prior to analysis (Gobom et al., 1999). Samples were analysed using an Ultraflex II MALDI-TOF/TOF mass spectrometer (Bruker Daltonics), and spectra were processed by FlexAnalysis (v3.3) and BioTools (v3.2) software provided by the instrument manufacturer. Combinations of MS and MS/MS data were used as input for the Mascot search engine (www.matrixscience.com) with the following parameters: NCBI_nr database; trypsin digestion (one partial cleavage), carbamidomethylation of Cys (global modification), oxidation of Met (variable modification), and MS and MS/MS mass tolerance of 0.8 p.p.m. and 0.6 Da, respectively. Alternatively, the trypsin digests were analysed on an LC-MS system composed of an EASY nLC 1000 chromatograph coupled online to a Q-Exactive spectrometer (Thermo Scientific) and spectra were processed using Proteome Discoverer (Thermo Scientific). The set-up of the Mascot database searching for LC/MS data was as follows: Swiss-Prot database, trypsin digestion (one partial cleavage), carbamidomethylation of Cys (global modification), oxidation of Met (variable modification), and peptide and fragment mass tolerance of 10 p.p.m. and 20 mDa, respectively. The significance threshold for protein identifications was P<0.05.

RESULTS AND DISCUSSION

TrxA can compensate for the loss of TrxD under non-stressed growth conditions

Lactococcus lactis MG1363 contains two thioredoxins encoded by trxA (llmg_0779) and trxD (llmg_0406; annotated as trxH), organized in two separate operons (Fig. S1). Expression of the genes was confirmed by Western blot analysis, which further demonstrated that TrxA and TrxD were present in the mid-EP and in the SP (Fig. 1). Deletions of trxA and trxD were constructed by overlap extension PCR, followed by homologous recombination into the chromosome, as verified by colony PCR (data not shown). The success of the deletions and the correct identification of TrxA and TrxD in the Western blots were confirmed by the absence of signal in protein extracts from ΔtrxD and ΔtrxA mutants, respectively using the appropriate antibodies (Fig. 1).

Changes in proteome profiles of ΔtrxA and ΔtrxD mutants

Protein profiles of the wt strain and ΔtrxA and ΔtrxD mutants in mid-EP under standard (non-stressed) conditions were compared using DIGE. In general most differences were observed between the ΔtrxA mutant and wt (Fig. 3). Several proteins involved in the oxidative stress response were upregulated in the ΔtrxA proteome compared with the wt, including TrxR (TrxB) and glutathione peroxidase (Table 3). Despite its name, glutathione peroxidase is likely to be

![Fig. 1. Western blot of protein extracts from wt, ΔtrxA and ΔtrxD strains harvested in mid-EP or SP. Recombinant TrxA and TrxD were used as positive controls. 1, wt EP; 2, wt SP; 3, ΔtrxA EP; 4, ΔtrxA SP; 5, ΔtrxD EP; 6, ΔtrxD SP; 7, 200 ng TrxA; 8, 100 ng TrxD. No apparent cross-reactivity between anti-TrxA and anti-TrxD was observed. The observed mass shift is attributed to the N-terminal His-tags on the recombinant proteins.](https://www.microbiologyresearch.org)
thioredoxin-dependent, as has been demonstrated for the homologous proteins from plants, fungi and bacteria (Lee et al., 2008). The protein encoded by llmg_1475 was the most highly upregulated (3.7-fold) in ΔtrxA versus the wt (Table 3). YnzC, a homologous protein from Bcl. subtilis (41% identity, 58% similarity), was previously suggested to be involved in the SOS DNA damage response and was upregulated upon H2O2 treatment in Bacillus licheniformis (Kawai et al., 2003; Schroeter et al., 2011). The upregulated protein encoded by llmg_2273 contains a histidine triad active site motif and is tentatively annotated as a diadenosine tetraphosphate hydrolase. No bacterial homologue of Llmg_2273 has been characterized, but eukaryotic proteins containing histidine triad motifs influence the cell cycle through interactions with regulatory proteins (Huebner et al., 2011; Nishizaki et al., 2004), and are associated with oxidative stress defence and DNA repair.

Downregulated proteins in ΔtrxA versus the wt include pyruvate kinase (Pyk), formate-tetrahydrofolate ligase (Fhs), tyrosyl-tRNA synthetase (TyrS), as well as a putative tellurium resistance protein (TelB) and Llmg_0304, annotated as a potential RNA-binding protein. Both Pyk and Fhs appear in two spots with different pI, suggesting that these proteins are subjected to some type of post-translational modification (Fig. 3). It is noteworthy that Fhs is overexpressed in Lac. lactis under respiratory conditions, and was highly overexpressed in Porphyromonas gingivalis subjected to oxidative stress (Lewis et al., 2009; Vido et al., 2004). The most downregulated protein in the trxA mutant (twofold) was TelB. Lac. lactis TelB is a homologue of TerD, a metal-binding protein proposed to be involved in calcium signalling (Pan et al., 2011). Overall, DIGE analysis demonstrated that deletion of TrxA influences the expression profiles of proteins implicated in oxidative stress resistance.

**Reduction of TV and INT is dependent on TrxA**

Tetrazolium salts are weakly coloured compounds that turn into strongly coloured formazans upon reduction and are widely used for spectrophotometric determinations of cellular redox activities (Berridge et al., 2005). Here, Lac.

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**Fig. 2.** Trx mutant phenotypes. Growth curves display wt (●), ΔtrxA (▲), ΔtrxD (○) and ΔtrxAΔtrxD (×) strains in an unstressed control (a), or exposed to 0.3 mM H2O2 (b) or 1.25 mM sodium arsenate (c). (d) Serial dilutions of three biological replicates of exponentially growing wt, ΔtrxA and ΔtrxD strains spotted on regular GSAL agar plates (control) or corresponding plates supplemented with 0.5 mM sodium arsenate and 0.3 mM potassium tellurite (see Methods for details). Dilution factors are indicated at the bottom of the figures. In the presence of tellurite, black colonies appear due to formation of metallic Te⁰.

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lactis DtrxA and DtrxD mutants were exposed to TV and INT, which are correlated with dehydrogenase activity in bacterial cells (Smith & McFeters, 1996). Overall, the extent of INT and TV reduction was similar in the wt and the DtrxD mutant (Fig. 4). By contrast, the DtrxA mutant exhibited significantly increased reduction of INT in mid-EP compared with the wt. Both TV and INT were reduced significantly more efficiently by the DtrxA mutant in SP, but TV reduction during exponential growth was similar for the wt and the DtrxA mutant (Fig. 4). It is also notable that the extent of tetrazolium salt reduction was five- to 10-fold higher in mid-EP than in SP for the three strains. Probably reflecting an overall higher rate of metabolic turnover in the EP. Reduction of TV in Lac laci has been linked to menaquinones and the membrane-bound NADH dehydrogenases NoxA and NoxB in an NADH-dependent manner (Tachon et al., 2009). It may thus be suggested that depletion of TrxA causes an increased availability of NADH, menaquinones or NoxA/NoxB through: (i) shifts in metabolic fluxes, (ii) transcriptional regulation and/or (iii) thiol/disulphide exchange control mechanisms.

**Table 2.** Growth rates and lag phases of wt, ΔtrxA, ΔtrxD and ΔtrxAΔtrxD strains exposed to selected compounds in the Bioscreen assay format.

Standard deviations are based on three biological replicates. Table S1 displays corresponding data for all stress compounds tested. NA, Not applicable.

<table>
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<th>Compound (mM)</th>
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<th>ΔtrxA</th>
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<tr>
<td></td>
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<td>Lag phase (h)</td>
<td>Relative μ* (%) ctrl</td>
<td>Lag phase (h)</td>
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<tr>
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*Relative growth rates (μ) were calculated by dividing μ of a strain at a stress condition by μ of non-stressed control (ctrl) or μ of wt at the same stress condition. At non-stressed conditions, 100% (wt)=0.506 h⁻¹, 100% (ΔtrxA)=0.365 h⁻¹, 100% (ΔtrxD)=0.479 h⁻¹ and 100% (ΔtrxAΔtrxD)=0.167 h⁻¹.

†Methionine replaced by MetSO in growth medium.
Table 3. Up- and downregulated proteins identified by DIGE of non-stressed ΔtrxA mutant versus wt in mid-EP

| Spot no. | Protein name                        | Fold change | pI/Mr [kDa] | Gene  | Accession no. | P*  | Score§ | SC (%)|| | M¶ | Peptide sequences identified by MS/MS | G#  |
|---------|-------------------------------------|-------------|-------------|-------|---------------|-----|-------|------|---|----|--------------------------------------|-----|
| 1       | Hypothetical protein                | +3.7        | 5.6/9.2     | ilmg_1475 | gii125624282  | 0.0002 | 87    | 21   | 2 |    | KAEGLSEAELEEQALLRR                    | 4   |
| 2       | Glutathione peroxidase              | +2.1        | 5.2/18.1    | gpo    | gii125623919  | 0.0002 | 84    | 28   | 6 |    | KFLIDRDGOVIERF                        | 1   |
| 3       | 30S ribosomal protein S5            | +1.7        | 10.2/17.6   | rpsE   | gii125625124  | 0.0030 | 204   | 56   | 11|    | RAFALVVVGDRN                           | 3   |
| 4       | Pyruvate dehydrogenase E1           | +1.6        | 4.8/35.1    | pdhB   | gii125622952  | 0.0003 | 292   | 63   | 19|    | KAQEVEPAIRKA KSLGNSTPINVYRA            | 2   |
| 5       | Thioredoxin reductase               | +1.5        | 4.8/34      | trxB1  | gii125624390  | 0.0008 | 156   | 18   | 4 |    | RNQEILVIGGGDSAVEEALYLFTR               | 1   |
| 6       | Hypothetical protein                | +1.5        | 5.4/14.9    | ilmg_2273 | gii125625038  | 0.0030 | 114   | 49   | 5 |    | KFTAHYDLAEIAKQ 4                       | 4   |
| 7       | Formate-tetrahydrofolate ligase     | −1.6        | 5.7/59.7    | fhs    | gii125623054  | 0.0050 | 615   | 53   | 34|    | KSTVTVGLADAFARQ RIVIAQNYDRK; KTVSFSQANLAAPEGEVTVRE | 4   |
| 8       | Pyruvate kinase                     | −1.6        | 5.2/54.3    | pyk    | gii125623950  | 0.0050 | 465   | 56   | 23|    | KIVSTLGPAAEIRG RTELFTDGADISVVTGDFKRV | 2   |
| 9       | Tyrosyl-tRNA synthetase             | −1.6        | 5.38/47.3   | tyrS   | gii125624396  | 0.0090 | 323   | 37   | 16|    | KTVSFSQANLAAPEGEVTVRE KLIVALTESGNTARL | 3   |
| 10      | Pyruvate kinase                     | −1.6        | 5.2/54.3    | pyk    | gii125623950  | 0.0060 | 335   | 57   | 31|    | KTVSFSQANLAAPEGEVTRE KLIVALTESGNTARL  | 2   |
| 11      | Hypothetical protein                | −1.7        | 4.5/8.8     | ilmg_0304 | gii125623269  | 0.0110 | 236   | 88   | 8  |    | RVTTLGVLDAFARK RIVIAQNYDRK              | 4   |
| 12      | Formate-tetrahydrofolate ligase     | −1.7        | 5.7/59.7    | fhs    | gii125623950  | 0.0010 | 169   | 28   | 15|    | RVTTLGVLDAFARK RIVIAQNYDRK             | 4   |
| 13      | Putative tellurium resistance protein| −1.9      | 4.38/21.1   | telB   | gii125624170  | 0.0310 | 241   | 48   | 9  |    | KVRNDDDFIYNIKHI RNDDDDFIYNIKHI         | 1   |

*Spot volume ratio of ΔtrxA to wt; plus and minus indicate up- and downregulation, respectively.
†Calculated values.
‡ANOVA P value from the image analysis; applied threshold was P<0.05.
§Mascot score.
||Sequence coverage in peptide mass fingerprinting (PMF).
¶Number of matched peptides in PMF.
#Groups: 1, stress; 2, carbon metabolism; 3, translation; 4, other.
**MetSO reduction is dependent on TrxA**

Methionine residues are highly susceptible to oxidative damage resulting in a racemic mixture of (S)- and (R)-enantiomers of MetSO. This reaction is reversed by Trx-coupled MetSO reductase (Msr) activity. (S)- and (R)-MetSO are reduced by two distinct types of MetSO reductases, MsrA and MsrB, respectively (Boschi-Muller et al., 2008). In *E. coli*, MsrA efficiently reduces both bound and free (S)-MetSO while MsrB reduces only peptide- or protein-bound (R)-MetSO (Grimaud et al., 2001). In addition, MsrA-independent reduction of free (S)-MetSO is catalysed in a Trx-independent manner by BisC in *E. coli* (Ezraty et al., 2005). The genome of *Lactococcus lactis* contains genes encoding putative MsrA, MsrB and free methionine-(R)-sulphoxide reductase (*llmg_2480*), but no BisC homologue. *Lactococcus lactis* MG1363 is auxotrophic for methionine (Jensen & Hammer, 1993; Seefeldt & Weimer, 2000) and the capacity of the *trx* mutants to generate Msr activity was thus tested in a Bioscreen assay using *(R/S)*-MetSO as sole methionine source. No significant difference was observed between growth of the wt and the *ΔtrxD* mutant (Table 2). However, both the *ΔtrxA* and the *ΔtrxAΔtrxD* mutants exhibited significantly prolonged lag phases, and growth rates were reduced by 78 and 82%, respectively, compared with the wt. Thus, it appears likely that TrxA functions as the major electron donor for Msr in *L. lactis*. However, because all the *trx* mutants were viable, *Lactococcus lactis* might also utilize a less efficient alternative Trx-independent MetSO reduction pathway.

**TrxA is important while TrxD is dispensable for recovery from oxidative stress.**

It is well established that thioredoxins provide reducing equivalents to thiol-based peroxiredoxins that in turn catalyse reduction of H₂O₂ (Hofmann et al., 2002). This function is likely to be of particular importance for stress resistance in organisms such as *Lactococcus lactis* lacking catalase, a highly efficient haem-based peroxidase. To analyse the involvement of TrxA and TrxD in the response towards oxidative stress, the *Lactococcus lactis* wt strain and the *trx* mutants were exposed to oxidizing reagents. In the presence of 313 μM H₂O₂, the lag phases before reaching maximal growth rate were prolonged by 5 h for the wild-type and *ΔtrxD* mutant, and >24 h for the *ΔtrxA* and *ΔtrxAΔtrxD* mutants (Fig. 2b). Following the lag phase the *ΔtrxA* and *ΔtrxD* mutants showed similar growth rates as prior to exposure while the *ΔtrxAΔtrxD* mutant grew very slowly. With 1.25 mM diamide a similar pattern was observed except that the *ΔtrxA* mutant displayed a decreased growth rate after the lag phase while the *ΔtrxD* mutant and wt did not (Table 2). At high concentrations of paraquat (5–20 mM), the *ΔtrxA* mutant was impaired to a greater extent than the wt and *ΔtrxD* cultures, and the *ΔtrxAΔtrxD* mutant was the most severely affected (Table 2). Surprisingly, concentrations of the superoxide-inducing reagent paraquat <1 mM had no effect on growth of the wt strain and the *ΔtrxA* and *ΔtrxD* mutants, but a positive effect on growth of the *ΔtrxAΔtrxD* mutant was observed. Formaldehyde is a reactive electrophilic species and has been shown to interact with thiol-based redox sensors and induces a disulphide stress response, including upregulation of Trx and TrxR (Antelmann & Helmann, 2011; Nguyen et al., 2009). No significant difference between the effects on growth of *ΔtrxA*, *ΔtrxD* and wt strains was, however, observed upon exposure to formaldehyde (80–5000 μM), but the *ΔtrxAΔtrxD* mutant was severely impaired (Table S1). In conclusion, TrxA appears to be more important for oxidative stress resistance than TrxD. These results suggest
that TrxA rather than TrxD acts as preferential electron donor to thiol-based enzymes such as peroxiredoxins involved in decomposition of reactive oxygen species.

The Trx system influences resistance to metal ion toxicity

The toxic effects of various divalent metal ions are related to their propensities to react with thiol groups in general (Lemire et al., 2013) and Trx in particular (Hansen et al., 2006). In addition, Fe(II) and Cu(II) react with H₂O₂ through the Fenton reaction and thus contribute to production of reactive oxygen species (Imlay, 2003). Selected metal ions were investigated to determine their influence on growth of the trx mutants. Cd(II) is highly toxic and was found to be lethal for Lac. lactis at 20 μM (Table S1), in good agreement with previous observations in E. coli (Ferianc & Farewell, 1998). At 5 μM Cd(II), the ΔtrxA mutant displayed a pronounced growth defect while no apparent growth of the ΔtrxAAΔtrxD mutant was detected. These results suggest that TrxA is important for cadmium resistance, in agreement with previous observations in Saccharomyces cerevisiae (Vido et al., 2001). Compared with Cd(II), Lac. lactis was more tolerant to Zn(II) and no pronounced effects of the trx genes were observed at 78 μM (Table S1). At 313 μM Zn(II), all strains were inhibited to a similar extent and no growth was observed at 1.25 mM. Interestingly, lower concentrations (5–20 μM) of Zn(II) had a slightly positive effect on the growth rate of the ΔtrxAAΔtrxD mutant. In this context it is relevant to mention that Zn(II) has been suggested to have a thiol-protective function and Lac. lactis strains with impaired Zn uptake are hypersensitive to oxidative stress (Scott et al., 2000).

Addition of Fe(II) in the range of concentrations tested (5–1250 μM) had only minor effects on growth, except that it led to a prolonged lag phase for the ΔtrxAAΔtrxD mutant (Table S1). The high tolerance may reflect the presence of proteins such as Dps sequestering cytosolic Fe ions and thus preventing formation of hydroxyl radicals through the Fenton reaction (Imlay, 2003; Stillman et al., 2005). Cu(II) was more toxic than iron, causing complete growth inhibition at 313 μM for the ΔtrxA, ΔtrxD strains and wt and at 78 μM for the ΔtrxAAΔtrxD mutant (Table S1). In general a copper-dependent growth defect was observed for ΔtrxA compared with the ΔtrxD mutant and wt.

Resistance towards arsenate and tellurite is dependent on TrxD

Arsenate is a highly toxic analogue of phosphate that reacts with thiol groups and induces oxidative stress (Hughes, 2002). Arsenate(V) is reduced to arsonite(III) by arsenite reductase (ArsC) and exported by ArsAB, an ATPase-driven transporter (Turner et al., 1992). In Gram-positive bacteria such as Bcl. subtilis, ArsC receives reducing equivalents from Trx, but a range of alternative electron donors have been described; for example, ArsC from E. coli is reduced by Gsr (Messens & Silver, 2006). Here, we investigated the influence of the two Lac. lactis Trxs on arsenate stress. The wt, ΔtrxA and ΔtrxD cultures subjected to 1.25 mM arsenate had similar growth rates in the Bioscreen assay but the lag phases were prolonged by 3, 6 and 9 h, respectively, compared with an unstressed control (Fig. 2a, c). The ΔtrxAAΔtrxD mutant did not recover from exposure to 1.25 mM arsenate, suggesting that at least one Trx is required to counteract arsenate stress. Sensitivity to arsenate stress was also studied in serially diluted cultures plated on GSAL agar plates containing arsenate. Growth was detected in a spot of 10²-fold diluted cultures of both the wt and the ΔtrxA mutant, but no growth was detected for the ΔtrxD mutant in spots of 10²-fold diluted cultures (Fig. 2d). The increased arsenate sensitivity of the trx deletion mutants suggests that Lac. lactis TrxD and TrxA play important roles in detoxification, for example as electron donors to ArsC.

Tellurite causes intracellular production of superoxide (Pérez et al., 2007) and is correlated with arsenate detoxification in E. coli (Turner et al., 1992). Tellurite-resistant Lac. lactis strains were found to contain mutations in, for example, high-affinity phosphate (particularly pstA and pstD) and iron transporters (mntH), and in trmA, a homologue of the disulphide stress sensor spx (Turner et al., 2007). Here, tests with agar plates containing tellurite indicated similar sensitivity of the ΔtrxD and ΔtrxA mutants (Fig. 2d). In the Bioscreen assay the ΔtrxA mutant displayed a prolonged lag phase following addition of 1.25 mM potassium tellurite compared with the wt. The ΔtrxD mutant, by contrast, showed a similar lag phase as the wt but the growth rate was reduced to 64% that of the wt following tellurite stress (Table 2). These observations indicate that TrxA is important in the phase immediately after tellurite exposure, suggesting involvement in adaptive responses such as transcriptional regulation. The more long-term effects of the ΔtrxD mutant strain, by contrast, indicate direct involvement in detoxification reactions.

To confirm that the phenotypes were caused by the loss of the trxA or trxD genes, the ΔtrxAAΔtrxD mutant was complemented with either trxA or trxD. Either of the two genes restored growth of single colonies on GSAL plates containing 1.5 mM potassium tellurite (Fig. S2). In contrast, a ΔtrxAAΔtrxD strain complemented with trxD lacking a functional promoter was not able to form single colonies on the same type of plates.

CONCLUSIONS

The observed phenotypes of the trx mutants suggest that the two thioredoxins have different functions in stress resistance in Lac. lactis. TrxA seems to be involved in responses to oxidative stress while TrxD appears to be important for resistance towards arsenate and tellurite. The role of TrxD in these processes is unknown but it is speculated that TrxD may act as an alternative electron donor for arsenate reductase, an established Trx target in Bcl. subtilis.
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REFERENCES


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