Roles of thioredoxin and thioredoxin reductase in the resistance to oxidative stress in *Lactobacillus casei*

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The *Lactobacillus casei* strain Shirota used in this study has in the genome four putative thioredoxin genes designated *trxA1*, *trxA2*, *trxA3* and *trxA4*, and one putative thioredoxin reductase gene designated *trxB*. To elucidate the roles of the thioredoxins and the thioredoxin reductase against oxidative stress in *L. casei*, we constructed gene disruption mutants, in which each of the genes *trxA1*, *trxA2* and *trxB*, or both *trxA1* and *trxA2* were disrupted, and we characterized their growth and response to oxidative stresses. In aerobic conditions, the *trxA1* (MS108) and the *trxA2* (MS109) mutants had moderate growth defects, and the *trxA1 trxA2* double mutant (MS110) had a severe growth defect, which was characterized by elongation of doubling time and a lower final turbidity level. Furthermore, the *trxB* mutant (MS111), which is defective in thioredoxin reductase, lost the ability to grow under aerobic conditions, although it grew partially under anaerobic conditions. The growth of these mutants, however, could be substantially restored by the addition of dithiothreitol or reduced glutathione. In addition, MS110 and MS111 were more sensitive to hydrogen peroxide and disulfide stress than the wild-type. In particular, the stress sensitivity of MS111 was significantly increased. On the other hand, transcription of all these genes was only weakly affected by these oxidative stresses. Taken together, these results suggest that the thioredoxin–thioredoxin reductase system is the major thiol/disulfide redox system and is essential to allow the facultative anaerobe *L. casei* to grow under aerobic conditions.

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INTRODUCTION

Facultative anaerobic lactic acid bacteria can grow well under aerobic conditions and are considered to have a system to eliminate or escape from the toxicity of oxygen and reactive oxygen species such as superoxide anions, hydrogen peroxide and hydroxyl radicals. Some lactic acid bacteria have oxygen-eliminating enzymes such as NADH oxidase, pyruvate oxidase and lactate oxidase (Goffin *et al.*, 2004; Higuchi *et al.*, 1993, 1999; Miyoshi *et al.*, 2003; Sedewitz *et al.*, 1984; Seki *et al.*, 2004). Several antioxidant enzymes, including manganese superoxide dismutase (SOD) (Chang & Hassan, 1997; Sanders *et al.*, 1995), manganese catalase (Kono & Fridovich, 1983) and NADH peroxidase (Miyoshi *et al.*, 2003) have been identified in some lactic acid bacteria. In addition, several reports of genome sequences of lactic acid bacteria indicate the existence of

these stress resistance genes (Altermann *et al.*, 2005; Chaillou *et al.*, 2005; Kleerebezem *et al.*, 2003; van de Guchte *et al.*, 2006). However, the variety and number of stress resistance genes are different in each species. In *Lactobacillus casei*, five genome sequences have been reported (Ai *et al.*, 2011; Chen *et al.*, 2011; Makarova *et al.*, 2006; Mazé *et al.*, 2010; Zhang *et al.*, 2010) and comparative genome analysis has been performed (Cai *et al.*, 2009). This reveals that *L. casei* strains have genes coding for enzymes involved in oxidative stress resistance such as NADH oxidase, NADH peroxidase, lactate oxidase and SOD in common.

The inside of a living cell is generally kept in a reduced state. One of the sensitivities to oxidative stress is due to thiol oxidation and/or the inability to reduce thiol groups in enzymes. Thioredoxin is a small protein that is involved in maintaining the thiol/disulfide balance in both prokaryotic and eukaryotic cells (Holmgren, 1985), and was originally identified as the electron donor for ribonucleotide reductase in *Escherichia coli* (Laurent *et al.*, 1964). All thioredoxins identified so far possess a conserved CXXC motif at their active site. Thioredoxin plays a significant role in cellular redox processes, including protein repair and defence against oxidative stress (Arnér & Holmgren,

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Abbreviations: DTT, dithiothreitol; GSH, glutathione.

The GenBank/EMBL/DDBJ accession numbers for the nucleotide sequences of *trxA1*, *trxA2*, *trxA3*, *trxA4* and *trxB* are AB617512–AB617516.

2000). This motif is interchangeable between the oxidized disulfide and the reduced dithiol forms. Oxidized thioredoxin is reduced by a flavoenzyme, thioredoxin reductase at the expense of NADPH (Carmel-Harel & Storz, 2000; Holmgren, 1985). *E. coli* has a glutathione–glutaredoxin-dependent reduction system as well as a thioredoxin-dependent reduction system. However, glutathione is absent in extracts of many Gram-positive bacteria including *L. casei* (Fahey *et al.*, 1978), indicating that those organisms do not produce and utilize glutathione in the reduction system. Therefore, the thioredoxin–thioredoxin reductase system is considered to be the only reduction system in *L. casei*.

L. casei strain Shirota is one of the most intensively studied probiotics (Asahara *et al.*, 2001; Matsumoto *et al.*, 2005; Matsuzaki *et al.*, 2004; Shida *et al.*, 2006) and its complete genome has been sequenced (unpublished data). This strain is used in fermented milk products and has high growth and good viability under aerobic conditions, which implies that the intracellular thiol/disulfide balance and redox system is fully active in this strain. However, the mechanisms for maintenance of the thiol/disulfide balance and oxidative stress resistance in *L. casei* are largely unknown and need to be clarified. While the contribution of antioxidant enzymes to the resistance to oxygen and reactive oxygen species in lactic acid bacteria has been extensively studied (Chang & Hassan, 1997; Kono & Fridovich, 1983; Miyoshi *et al.*, 2003), few studies have examined the role of the thioredoxin–thioredoxin reductase system. In this study, we focused on the role of the thioredoxin–thioredoxin reductase system in growth and survival under aerobic conditions in *L. casei* by using gene disruption mutants of *L. casei* strain Shirota. Our findings show that the thioredoxin–thioredoxin reductase system, which controls the intracellular thiol/disulfide balance, also plays a pivotal role in aerobic growth in *L. casei*.

METHODS

Strains, plasmids, media and growth conditions. The strains and plasmids used in this study are listed in Table 1. *L. casei* strain Shirota (YIT 9029) was used as the wild-type. *E. coli* JM109 (Toyobo) was used as the competent cells for DNA transformation. Plasmid pYSSE3 (Yasuda *et al.*, 2008) was used as a cloning vector for insertion and deletion mutagenesis. Lactic acid bacteria were grown at 37 °C in MRS medium (Becton Dickinson). *E. coli* was grown at 37 °C in LB medium (Sambrook & Russel, 2001). Erythromycin (Abbott) was added to a final concentration of 20 µg ml⁻¹ for lactic acid bacteria and 500 µg ml⁻¹ for *E. coli*. The optical density of the cultures was measured at 590–660 nm by using a Klett–Summerson photoelectric colorimeter (Klett MFG). To generate aerobic conditions, the medium was dispensed into test tubes with silicon caps which allowed free air exchange (for aerobic conditions) or loose aluminium caps (for static aerobic conditions). To generate anaerobic conditions, the medium was prepared by boiling for 20 min followed by replacing the oxygen in the medium with nitrogen by bubbling it into the medium for 30 min. The medium was dispensed into test tubes, and the head space was filled with nitrogen gas. Test tubes were sealed with a butyl rubber plug to avoid oxygen contamination during the course of their anaerobic incubations. The cells were cultured with shaking at 160 r.p.m. under aerobic conditions or without shaking under anaerobic and static aerobic conditions. The *trxB* mutant was precultivated under anaerobic conditions; wild-type and other mutants were grown under static aerobic conditions. Growth analysis of wild-type and mutants under aerobic or anaerobic conditions was performed in three independent experiments. The data are shown as the mean ± SD of three independent experiments. The growth data of the wild-type and mutants were analysed by using a two-tailed Student's *t*-test. *P* < 0.05 was considered as significant.

Construction of insertion and deletion mutants. Plasmid pYSSE3 is a derivative of a shuttle plasmid vector pBE31 (Kiwaki & Shimizu-Kadota, 2002), which is devoid of the origin of replication (*ori*) of pAMβ1. Recombinant plasmids for insertional mutagenesis were constructed as follows. The truncated fragment of the target gene was amplified by PCR using *L. casei* strain Shirota genomic DNA as a template, the primer pairs listed in Table 2 and KOD Plus DNA

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype or phenotype	Source or reference
Strains		
<i>L. casei</i>		
Shirota (YIT 9029)	Wild-type	Our collection
MS108	<i>trxA1</i> ::pYSSE3-Δ <i>trxA1</i> , Em ^r	This study
MS109	<i>trxA2</i> ::pYSSE3-Δ <i>trxA2</i> , Em ^r	This study
MS110	Deletion in <i>trxA1</i> gene, <i>trxA2</i> ::pYSSE3-Δ <i>trxA2</i> , Em ^r	This study
MS111	<i>trxB</i> ::pYSSE3-Δ <i>trxB</i> , Em ^r	This study
<i>E. coli</i>		
JM109	Commercial strain purchased from Toyobo	
Plasmids		
pYSSE3	<i>E. coli</i> cloning vector carrying pUC19 <i>ori</i> region, pAMβ1 erythromycin resistance gene and multicloning sites	Yasuda <i>et al.</i> (2008)
pYSSE3-Δ <i>trxA1</i>	pYSSE3 carrying truncated fragment of <i>trxA1</i>	This study
pYSSE3-Δ <i>trxA2</i>	pYSSE3 carrying truncated fragment of <i>trxA2</i>	This study
pYSSE3-Δ <i>trxB</i>	pYSSE3 carrying truncated fragment of <i>trxB</i>	This study
pYSSE3-Δ <i>trxA1</i> -del	pYSSE3 carrying upstream region with N terminus of <i>trxA1</i> and downstream region with C terminus of <i>trxA1</i>	This study

Table 2. Primers used in this study

The restriction enzyme sites are indicated (underlined).

Target gene	Primer sequence (5'–3')		Restriction enzyme site	
	Forward	Reverse	Forward	Reverse
Construction of insertion mutants				
<i>trxA1</i>	CCGGAATTCTGGCGTCACCCTAACAGAT	TAGTCTAGATGATAGCCAACAAGCTTCTC	<i>EcoRI</i>	<i>XbaI</i>
<i>trxA2</i>	CGGGATCCGCAGATTGGTGCTCGGATTG	AAACTGCAGCGTTAAAGGCAATAAAGCTC	<i>BamHI</i>	<i>PstI</i>
<i>trxB</i>	CGGGATCCGGCTAAAGCGATTGTCATTG	AAACTGCAGAACCATGCTCATCCAGAACA	<i>BamHI</i>	<i>PstI</i>
Confirmation of insertion mutants				
<i>trxA1</i>	TAAAGTTGGGTAACGCCAGG	ACTGTGTGCCTTCATAATCG		
<i>trxA2</i>	TAAAGTTGGGTAACGCCAGG	ACGCTGGTTGATTATAACTG		
<i>trxB</i>	TAAAGTTGGGTAACGCCAGG	AATCAAGCTTTAATCTCAATA		
Construction of deletion mutants				
<i>trxA1</i> -5'	CCGGAATTCCGATAAAAATCATCAAGCAAC	CCCAAGCTTGGTTCCGTCTTGTAGTTTG	<i>EcoRI</i>	<i>HindIII</i>
<i>trxA1</i> -3'	GAGAAGCTTGTGGCTATCAAAC	TAGTCTAGACAATTACGCTTTTAGAACT	<i>HindIII</i>	<i>XbaI</i>
Confirmation of deletion mutants				
<i>trxA1</i>	AAGGACAAGGCCAACTCGTT	ACTGTGTGCCTTCATAATCG		
qRT-PCR				
<i>trxA1</i>	CGGCCCTTGCCGGATGCA	TTTCTGCCAATTTGTGCGATGAC		
<i>trxA2</i>	AACCAGCTATGCCTGAAATTGAG	GCGATCAACCGCGATGA		
<i>trxA3</i>	GGGCATTATGTCACTACCCACCT	GGTAAACACCTGTCACTTTTTCGC		
<i>trxA4</i>	GGCAAAGTGGTGACGCAGTA	CCAGCCAGCACTTTGTGCGAT		
<i>trxB</i>	CCGGCCCAGGTGGTATG	CAGCATCAGCACAGATAAATTGG		
NADH oxidase	CGACCCATGCCGGTACA	CGTACACGGTGACGTCATAATCA		
NADH peroxidase	CGGCAATCAATGCTGATGTG	CTGCGTCGTGCCGTCT		
16SrRNA	TGGTTGTCGTCAGCTCGTG	TCTTACTAGAGTGCCCAACT		

polymerase (Toyobo). The fragment was digested with relevant restriction enzymes at both ends (Table 2). The fragment was cloned into pYSSE3, which had previously been digested with the same restriction enzymes, followed by treatment with calf intestinal alkaline phosphatase (Takara). Each recombinant plasmid was purified by using the Wizard Plus SV Minipreps DNA purification system (Promega) and transferred into *L. casei* strain Shirota by electroporation (Yasuda *et al.*, 2008). Briefly, cells were grown in 20 ml MRS broth to early exponential phase and harvested by centrifugation. Cells were washed once with an equal volume of 1 mM HEPES (pH 7.0), followed by washing with a 0.5 vol. 10% (w/v) glycerol and then 1 ml 10% (w/v) glycerol. Cells were suspended in 1/200 of the original culture volume in 10% (w/v) glycerol. Electroporation was done with 40 µl competent cells and 1–2 µl plasmid DNA solutions in a 2 mm path cuvette at a 25 µF capacitance, 200 Ω resistance and 1.5 kV voltage with a Gene Pulser II Electroporation System (Bio-Rad). Cells were transferred to 1 ml MRS broth and then incubated at 37 °C for 60 min and plated onto MRS agar plates containing 20 µg erythromycin ml⁻¹ and incubated at 37 °C for 2 or 3 days. When colonies did not appear under these conditions, cells were plated onto MRS plates containing 20 µg erythromycin ml⁻¹ and 5 mM dithiothreitol (DTT), and incubated under anaerobic conditions. Erythromycin-resistant clones were selected and plasmid integration was confirmed by PCR with appropriate primers (Table 2). One primer was designed upstream of the multicloning site of pYSSE3 and the other was downstream of the target gene in the chromosome.

In order to develop the *trxA1* *trxA2* double mutant, the *trxA1* deletion mutant, which is sensitive to erythromycin, was constructed. A recombinant plasmid for deletion mutagenesis was constructed as follows: two fragments containing the 5'- and 3'-terminal ends of the target gene were amplified with the primer sets listed in Table 2. These

fragments were cloned into pYSSE3 in the same order as on the chromosome to obtain in-frame deletions within the genes. *L. casei* strain Shirota was transformed with the plasmid, and an erythromycin-resistant clone was selected first. This clone was confirmed as including the recombinant plasmid integrated into either the 5'- or 3'-terminal side of the respective gene regions by homologous recombination. After several cycles of subculturing [0.1% (v/v) inoculation into fresh medium followed by full growth], erythromycin-sensitive clones were screened and checked for the reversion or deletion by PCR with appropriate primers (Table 2) that were able to amplify the target gene. To obtain the *trxA1* *trxA2* double mutant, we transferred a recombinant plasmid containing a truncated fragment of *trxA2* gene as described above.

Inhibition of growth by hydrogen peroxide. Overnight cultures were inoculated at 1% (v/v) into 4 ml MRS medium. Strains were grown at 37 °C under static aerobic conditions (WT, MS108, MS109 and MS110) or anaerobic conditions (MS111). Hydrogen peroxide at 0.5, 1 or 2 mM was added to cultures after 7 h incubation. The turbidity of the culture was measured by using a Klett–Summerson photoelectric colorimeter. Experiments were performed three times independently. The data shown are mean ± SD of three independent experiments.

Treatments with reducing agents and diamide. Overnight cultures were inoculated at 1% (v/v) into 4 ml MRS medium with reducing agents or diamide. Strains were grown at 37 °C under static aerobic conditions or anaerobic conditions (only for diamide treatment). Reducing agents and diamide added to cultures were used at the following concentrations: DTT, 1 mM; glutathione (GSH), 1 mM; cysteine (Cys), 1 mM; diamide, 1 or 5 mM. Klett units were measured after 24 h incubation. Experiments were performed at least

three times independently. The data shown are mean $\pm$ SD of three independent experiments.

RNA isolation. An oxidative stress response was induced by cell exposure to O₂ or H₂O₂. The exposure to O₂ was performed as follows. A single 100 ml culture of *L. casei* strain Shirota, which was inoculated at 1% (v/v) with an overnight culture, was grown in MRS broth for 7 h under anaerobic conditions and divided into two 50 ml aliquots. One aliquot was grown for 30 min under anaerobic conditions as a control and the other was grown for 30 min under aerobic conditions by shaking at 160 r.p.m. in a water bath shaker. The exposure to H₂O₂ was performed as follows. A single 100 ml culture of *L. casei* strain Shirota, which was inoculated at 1% (v/v) with an overnight culture, was grown in MRS for 7 h under static aerobic conditions and divided into two aliquots. One aliquot was grown for 30 min under static aerobic conditions as a control and 0.5 mM H₂O₂ was added to the other; this culture was also grown for 30 min under static aerobic conditions. Two millilitres of each culture was added to 4 ml RNeasy Protect Bacteria Reagent (Qiagen). The mixture was incubated at room temperature for 5 min. The cells were harvested by centrifugation for 10 min at 5000 g. The cells were suspended in 200 μ l TE buffer (10 mM Tris/HCl, 1 mM EDTA, pH 8.0) containing 15 mg lysozyme ml⁻¹ (Sigma-Aldrich) and 0.05 mg N-acetylmuramidase SG ml⁻¹ (Seikagaku) and incubated at room temperature for 10 min with occasional vortexing. Total RNA was purified by using the RNeasy Mini kit (Qiagen) according to the protocol provided by the supplier. Contaminating DNA was digested by using the RNase-Free DNase set kit (Qiagen) in the purification step. RNA integrity for all samples was confirmed with an Agilent 2100 Bioanalyser (Agilent Technologies). RNA was isolated from three independent cultures.

Quantitative real-time PCR assays. cDNA was synthesized by using a PrimeScript 1st strand cDNA Synthesis kit (Takara) according to the protocol provided by the supplier. Total RNA (1 μ g) was used as a template. Quantitative real-time PCR was performed using the ABI 7500 Real-time PCR System (Applied Biosystems) with SYBR Premix ExTaq (Takara). The primers were designed to amplify products of 47–82 bp (Table 2). The reaction mixture contained 12.5 μ l 2 $\times$ SYBR Premix ExTaq, 0.5 μ l 10 μ M forward primer, 0.5 μ l 10 μ M reverse primer, 0.5 μ l ROX reference Dye II, 9 μ l dH₂O and 2 μ l diluted cDNA

template. All reactions were run in duplicate for each of three independent RNA samples. The gene expression values were normalized using 16S rRNA as internal standard. A melting curve analysis was performed after each run to confirm the amplification specificity. Data were analysed using the Sequence Detection Software (Applied Biosystems) by applying the standard-curve quantification method. Standard curves for both internal standard and target genes were generated by amplifying tenfold serial dilutions of cDNA. The gene expression data from quantitative real-time PCR were analysed by two-tailed Student's *t*-test. *P* < 0.05 was considered as significant.

Database search, homology search and multiple alignment.

Primary sequences of *L. casei* strain Shirota *trxA* and *trxB* were identified with BLASTN and BLASTP algorithms provided from the NCBI database (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). Homology searches were performed by using GENETYX software. Multiple alignment was performed by using the CLUSTALW2 programme (<http://www.ebi.ac.uk/Tools/msa/clustalw2/>).

RESULTS

Thioredoxin–thioredoxin reductase system in *L. casei*

Bioinformatic analysis found four thioredoxin genes and two thioredoxin reductase-like genes in the *L. casei* strain Shirota genome sequence. Four thioredoxins, which are designated *trxA1*, *trxA2*, *trxA3* and *trxA4*, have a common CXXC motif, which is the active site of thioredoxin and thioredoxin reductase. However, the deduced amino acid sequences of these genes showed low homology to each other except for the locus of the active site (Fig. 1). The two thioredoxin reductase-like genes identified from sequence similarity to known sequences are 29% identical in amino acid sequence to each other, but one of them does not have a CXXC motif, suggesting that this protein does not catalyse

<i>trxA1</i>	1	-----MVQAVTDSNYKTETDTGVTLTDF	23
<i>trxA2</i>	1	-----MLAMKELGSNAAILNEVKQPGKKMLFF	27
<i>trxA3</i>	1	-----MAIEATKDNLKALTAEGTVVVD	23
<i>trxA4</i>	1	-----MKHPFRHFLINTVIGVVIATASLSALYVYAPKAMINVAIPITINAQKKDQYILY	54
<i>Ec trxA</i>	1	-----MSDKIIHLTDDSFDTDLKADGAILVDF	28
<i>Ec trxC</i>	1	MNTVCTHCQAINRIPDDRIEDAAKCGRCGHDLFDGEVINATGETLD-KLLKDDLPVVDF	59
<i>trxA1</i>	24	WATWCGPCRMQSPVIDKLAESRDD-VKFKMDVDANPETPKSFGIMAFITLVIKKDGEVV	82
<i>trxA2</i>	28	SADWCSDCRFIKPAMPEIEKDFSD-YEFIAVDRDKNLEVAQDMGVFGIPSFIAFNDEKET	86
<i>trxA3</i>	24	WAPWCGPCVKVLDPLMTALEQELPA-LKVVRYNVEKDHTLPSTMGIMSVPTLVIIYQCGEAR	82
<i>trxA4</i>	55	YSPTCPVCQQVYRTY--FWRSVTGQLKFASVTNEQNRFAAALGITKVPTLVHYKKCKVV	112
<i>Ec trxA</i>	29	WAEWCGPCMKIAFILDEIADEYQGLTVAKLNIDQNPGTAPKYGIRGITLTLFLFKNGEVA	88
<i>Ec trxC</i>	60	WAPWCGPCRNFAPIFEDVAQERSGKVRFKVNTAEARELSSRFGRSIPITIMIFKNGQVV	119
<i>trxA1</i>	83	EKLVG YQT KDQLESTLNKYTA--	103
<i>trxA2</i>	87	GRFVNKDRKTKQVEDFINGLH--	108
<i>trxA3</i>	83	EKVTGVYPKDKLKRYFEQKLEA--	104
<i>trxA4</i>	113	TQYTGQREQQIDKVLGGKAGEH--	135
<i>Ec trxA</i>	89	ATKVGALS KQLKEFLDANLA--	109
<i>Ec trxC</i>	120	DMLNGAVPKAPFDSWLNESL---	139

Fig. 1. Multiple alignment of the amino acid sequences of *L. casei* strain Shirota thioredoxin and *E. coli* thioredoxin. The residues with a black background represent residues conserved in all sequences; those with a grey background represent residues conserved in at least 50% of all sequences. The CXXC active site is indicated by thick line.

reduction of oxidized thioredoxin and has some other functions. Similar sequences are found in some Gram-positive bacteria such as lactobacilli, *L. lactis* and *Bacillus subtilis*. Among these, the *B. subtilis* *yumC* product is known to function as a ferredoxin NADP⁺ reductase (Seo *et al.*, 2004) and has high amino acid identity with the corresponding protein in *L. casei* strain Shirota, implying that it may have the same function. Therefore, we designated the gene that has a CXXC motif as *trxB*, and eliminated the *yumC*-like gene from subsequent analysis in this study.

All sequenced *L. casei* strains except *L. casei* Zhang also have four thioredoxin genes and a thioredoxin reductase gene. *L. casei* Zhang does not have a *trxA4* gene. These amino acid sequences in *L. casei* strain Shirota are identical to those of BL23, BD-II and LC2W. Other strains also show high similarity with *L. casei* strain Shirota (ATCC 334: 100 % TrxA1, A2, A3, 92 % TrxA4, 99 % TrxB; Zhang: 100 % TrxA1, A2, A3, 99 % TrxB).

Construction and growth of gene disruption mutants

To elucidate the roles of thioredoxins and thioredoxin reductase in the tolerance to oxidative stresses, we constructed gene disruption mutants. We focused on the *trxA1*, *trxA2* and *trxB* genes and disrupted these as described in Methods. The *trxA1* and *trxA2* genes were chosen from the four thioredoxin genes in this study because of the high expression rates of these genes in both oxidatively stressed and unstressed conditions (M. Serata, unpublished results). The *trxA1*, *trxA2* and *trxB* mutants (MS108, MS109 and MS111, respectively) were constructed by insertional mutagenesis. The *trxA1 trxA2* double mutant (MS110) was obtained in two steps: firstly *trxA1* was disrupted by deletion mutagenesis and secondly *trxA2* was disrupted by insertional mutagenesis. Although we obtained the *trxA1* mutant and the *trxA2* mutant easily under aerobic conditions, efforts to isolate the *trxA1 trxA2* double mutant and the *trxB* mutant were unsuccessful until the selection medium was anaerobically

incubated in the presence of 1 mM DTT. The *trxB* mutant formed very small colonies even under these conditions.

Under anaerobic conditions, MS108 and MS109 grew as well as the wild-type strain, but MS110 and MS111 showed significant growth defects. While MS110 showed a slight but significant delay in growth during exponential phase, MS111 showed significant growth delay during the exponential phase and the final level of turbidity was significantly lower (Fig. 2b). Under aerobic conditions, MS108 and MS109 had a moderate growth defect, although growth of MS109 was only significantly different at one time point. MS110 had a significant growth defect in both doubling time and final turbidity level (Fig. 2a). In contrast, MS111 did not grow under aerobic conditions (Fig. 2a). These results show that TrxB has an essential role in aerobic growth, while different TrxAs contribute to aerobic growth partially and in a complementary manner.

The sensitivity of these mutants to oxidative stress was further analysed. We investigated the effect of hydrogen peroxide on the growth of the mutants. Strains were grown in MRS broth under static aerobic conditions (wild-type, MS108, MS109 and MS110) or under anaerobic conditions (MS111). Hydrogen peroxide was added after 7 h incubation. The growth of the wild-type strain and mutants was inhibited in proportion to the hydrogen peroxide concentration. MS109 grew even in the presence of 1 mM hydrogen peroxide, and the growth of MS108 was inhibited at 1 mM. The growth of MS110 was significantly delayed with 0.5 mM H₂O₂. The growth of MS111 was severely affected in the presence of 0.5 mM hydrogen peroxide even in the anaerobic conditions (Fig. 3). These results indicate that the active thioredoxin–thioredoxin reductase system is important for resisting the toxic effects of hydrogen peroxide as well.

Effects of reducing agents on the growth of mutants

In order to investigate whether the growth defects of the mutants were caused by thiol/disulfide imbalance, we

Fig. 2. Growth of *L. casei* strain Shirota and gene disruption mutants under aerobic (a) or anaerobic (b) conditions. Overnight MRS cultures were inoculated into fresh MRS cultures. ●, wild-type strain; ▲, MS108 (*trxA1* mutant); ■, MS109 (*trxA2* mutant); ◆, MS110 (*trxA1 trxA2* double mutant); ×, MS111 (*trxB* mutant). The data are means $\pm$ SD of three independent experiments. Time points when the turbidity of mutants was significantly lower than that of the wild-type were as follows: MS109 32 h (aerobic conditions), MS110 4–32 h (aerobic conditions) and 6–32 h (anaerobic conditions), MS111 4–32 h (aerobic conditions) and 6–32 h (anaerobic conditions) (Student's *t*-test $P < 0.05$).

Fig. 3. Effect of adding hydrogen peroxide on the growth of *L. casei* strain Shirota and gene disruption mutants. (a) Wild-type, (b) MS108 (*trxA1* mutant), (c) MS109 (*trxA2* mutant), (d) MS110 (*trxA1 trxA2* double mutant) and (e) MS111 (*trxB* mutant). Strains were grown in MRS broth under static aerobic (wild-type, MS108, MS109 and MS110) or anaerobic (MS111) conditions. Hydrogen peroxide was added after 7 h of incubation. ●, no hydrogen peroxide; ▲, 0.5 mM; ■, 1.0 mM; ◆, 2.0 mM. The data are mean $\pm$ SD of three independent experiments.

added thiol compounds to the medium. Fig. 4 shows the effects of DTT, GSH or Cys on the growth of the wild-type and mutants. Strains were grown in MRS broth under static aerobic conditions with 1 mM thiol compounds. Klett units were measured after 24 h incubation. The growth of the wild-type was not affected by the addition of any of the compounds. The growth defects of MS110 and MS111 were eliminated by adding DTT or GSH under static aerobic conditions. In particular, the growth phenotype of MS111 was dramatically restored under static aerobic conditions by the addition of these agents. In contrast, the addition of cysteine to the medium resulted in inhibition of the growth of MS109, MS110 and MS111, while it did not affect the growth of wild-type and MS108 (Fig. 4). This implies that cysteine has toxic effects on the growth of mutants defective in the thioredoxin–thioredoxin reductase system.

Sensitivity to diamide

Diamide is a thiol-oxidizing agent (Kosower & Kosower, 1995) that mimics damage due to oxygen exposure under anaerobic conditions. We analysed the influence of diamide

on the growth and survival of these mutants under static aerobic conditions or anaerobic conditions. We found that the growth of the wild-type strain was slightly affected by the addition of 1 or 5 mM diamide; however, MS110 and MS111 were sensitive to diamide even under anaerobic conditions (Fig. 5). MS108 and MS109 were partially affected by the addition of diamide. These results suggest that the thioredoxin–thioredoxin reductase system plays a role in maintaining the intracellular thiol/disulfide balance towards the reduced state in both static aerobic and anaerobic conditions.

Transcription analysis of *trx* genes

In order to investigate whether thioredoxin and thioredoxin reductase genes are induced by oxidative stresses, the expression of these genes after exposure to oxygen and hydrogen peroxide was examined by quantitative real-time PCR assays. In addition, in order to compare this to other antioxidant genes, the expression level of NADH oxidase (*nox2*) and NADH peroxidase (*npx*) genes, functioning as oxygen and hydrogen peroxide eliminators, respectively, were determined. These data are expressed as the mean fold

Fig. 4. Effect of adding reducing agents on the growth of *L. casei* strain Shirota and gene disruption mutants. Strains were grown in MRS cultures under static aerobic conditions with 1 mM DTT, GSH or Cys. Klett units were measured after 24 h incubation. The control had no agent added. The data are means $\pm$ SD of at least three independent experiments. Strains: WT (wild-type), MS108 (*trxA1* mutant), MS109 (*trxA2* mutant), MS110 (*trxA1 trxA2* double mutant) and MS111 (*trxB* mutant).

change compared with each unstressed control (Fig. 6). The expression of *trxA1*, *trxA2* and *trxB* after adding hydrogen peroxide was slightly but significantly increased. Furthermore, the expression of other genes did not change significantly following these treatments. On the other hand, NADH oxidase was significantly induced by oxygen stress and NADH peroxidase was significantly induced by both oxygen stress and hydrogen peroxide stress.

DISCUSSION

The thioredoxin–thioredoxin reductase system and the glutathione–glutaredoxin system control the thiol/disulfide balance in many prokaryotic and eukaryotic cells and play an important role in sustaining life (Carmel-Harel & Storz,

2000). In bacteria that do not synthesize glutathione, the thioredoxin–thioredoxin reductase system may be the only thiol/disulfide redox control system. Thioredoxin in *B. subtilis* and thioredoxin reductase in *S. aureus* were reported to be essential for their survival (Scharf *et al.*, 1998; Uziel *et al.*, 2004). Furthermore, a deletion mutant in the thioredoxin reductase gene in *B. fragilis* was unable to grow without a reductant (Rocha *et al.*, 2007). In lactic acid bacteria, it has been reported that the overproduction of thioredoxin reductase in *Lactobacillus plantarum* WCFS1 improved tolerance towards oxidative stress (Serrano *et al.*, 2007).

In this study, we showed that a thioredoxin reductase-deficient mutant of *L. casei* strain Shirota was not able to grow under aerobic conditions (Fig. 2), while the growth of *trxA1*, *trxA2* or the *trxA1 trxA2* double mutant was affected to some extent. The partial growth defect of *trxA1*, *trxA2* and the *trxA1 trxA2* double mutant indicates that *trxA3* and/or *trxA4* are also functioning.

In contrast with the inability to grow in aerobic conditions, MS111 can grow partially under anaerobic conditions. Rocha *et al.* (2007) reported that a deletion mutant in the thioredoxin reductase gene in *B. fragilis* was unable to grow in semi-defined medium but was able to grow in rich medium. This implies that the ability to grow under anaerobic conditions could be attributed to the presence of thiol compounds in the medium. Therefore, it is probable that the growth ability of MS111 of *L. casei* strain Shirota in anaerobic MRS broth can also be attributed to the presence of thiol compounds such as glutathione in the medium. Confirmation of this hypothesis, however, will require future studies in a chemically defined medium.

Both DTT and GSH are thiol compounds and can reduce disulfide bonds. In our study, DTT restored the growth of MS110 and MS111 more efficiently than GSH. DTT is known to be a strong reducing agent and can chemically reduce the other oxidized compounds containing disulfide bonds. On the other hand, the reducing power of GSH is somewhat weaker and this may involve other factors such

Fig. 5. Effect of adding diamide on the growth of *L. casei* strain Shirota and gene disruption mutants. Strains were grown in MRS under static aerobic condition (black bars) and under anaerobic condition (grey bars). Klett units were measured after 24 h incubation. The data are means $\pm$ SD of at least three independent experiments. Strains: WT (wild-type), MS108 (*trxA1* mutant), MS109 (*trxA2* mutant), MS110 (*trxA1 trxA2* double mutant) and MS111 (*trxB* mutant).

Fig. 6. Transcriptional analysis of thioredoxin and thioredoxin reductase genes. Bars represent the relative expression following exposure to oxygen compared with cells under anaerobic conditions (black bars) and the relative expression following exposure to hydrogen peroxide compared with cells under static aerobic conditions (grey bars). *L. casei* strain Shirota grown for 7 h under anaerobic condition was exposed for 30 min to oxygen provided by vigorous shaking at 160 r.p.m. *L. casei* strain Shirota grown for 7 h under static aerobic conditions was exposed for 30 min to 0.5 mM hydrogen peroxide. RNA was isolated from three independent experiments. Quantitative PCR was performed in duplicate for each of three independent samples. The gene expression values were normalized using 16S rRNA as internal standard. The results are shown as fold change compared with each control condition and are the means $\pm$ SD of three independent experiments. Significant differences compared with unstressed control are indicated by an asterisk ($P < 0.05$).

as glutathione reductase and glutaredoxins, orthologous genes of which we have found in the genome of *L. casei* strains. However, we do not know yet how GSH can act on the recovery of the thioredoxin-deficient mutants.

GSH is not produced in many Gram-positive bacteria, including *L. casei* (Fahey *et al.*, 1978). Although we have found a homologue of the GSH synthesis fusion protein (GshF) gene consisting of a γ -glutamylcysteine ligase (GshA) domain and an ATP-grasp domain as in *Listeria monocytogenes* and *Streptococcus agalactiae* (Gopal *et al.*, 2005; Janowiak & Griffith, 2005), it is still an open question whether or not *L. casei* strain Shirota can synthesize GSH. However, the fact that the growth defect of the *trxB* mutant was restored by adding GSH under static aerobic conditions (Fig. 4) indicates that *L. casei* strain Shirota may not synthesize sufficient GSH and that a glutathione-glutaredoxin system does not function without adding GSH. In addition, we demonstrated that the *trxB* mutant MS111 was sensitive to the thiol-oxidizing agent diamide even under anaerobic conditions (Fig. 5). As the thioredoxin reductase is essential to regenerate reduced thioredoxins, none of thioredoxins can function in a thioredoxin reductase-deficient mutant. Taken together, these results indicate that the thioredoxin-thioredoxin reductase system plays an essential role in maintaining the thiol/disulfide balance in *L. casei*.

Many organisms have been reported to have multiple thioredoxin genes (Akif *et al.*, 2008; Comtois *et al.*, 2003; Reott *et al.*, 2009; Serrano *et al.*, 2007). *L. casei* strain Shirota and all other sequenced *L. casei* strains except *L. casei* Zhang have four thioredoxin genes. The *trxA1* mutant MS108 and the *trxA2* mutant MS109 were slightly sensitive to oxidative stress, although the degree of sensitivity differed. The *trxA1 trxA2* double mutant MS110 was more sensitive to oxidative stress than MS108 or MS109. MS111 was more sensitive to oxidative stress than MS110. From these results, we suggest that the effects of inactivation of *trxA1* and *trxA2* were additive, and the *trxA3* and/or *trxA4* genes probably contribute in part to balance intracellular thiol/disulfide levels. In plant cells, it has been reported that they have different types of thioredoxins (Hisabori *et al.*, 2007) and that thioredoxins have different substrate specificities *in vitro* (Collin *et al.*, 2003). There is some possibility that each thioredoxin in *L. casei* strain Shirota has a different role in the cellular process, since the deduced amino acid sequences of the four thioredoxins have low amino acid identities with each other (Fig. 1), and the behaviour of MS108 and MS109 is different (Figs 3, 4 and 5). Further molecular and biochemical studies are needed to confirm this possibility in *L. casei*.

Unexpectedly, the growth of MS109, MS110 and MS111 was inhibited by adding 1 mM cysteine (Fig. 4). This is not consistent with results reported for *L. lactis*, *B. fragilis* and *B. subtilis* (Möller & Hederstedt, 2008; Rocha *et al.*, 2007; Vido *et al.*, 2005). Vido *et al.* (2005) reported that cystine did not affect the growth of *trxB* mutant in *L. lactis*. When we added cystine to the cultures of MS110 and MS111 under anaerobic conditions, the growth of the *trxA1 trxA2* double mutant and the *trxB* mutant was inhibited (data not shown). Thus, both cysteine and cystine have toxic effects on the growth of mutants defective in the thioredoxin-thioredoxin reductase system in *L. casei*. To date, we do not know the reasons for this. In order to elucidate the cysteine toxicity in *L. casei* strain Shirota, we need to carry out further studies.

In this study, we observed that the *trxA1*, *trxA2* and *trxB* genes were only weakly induced by hydrogen peroxide stress in *L. casei*, and all thioredoxin and thioredoxin reductase genes were not affected by oxygen stress (Fig. 6). However, thioredoxin and thioredoxin reductase genes were reported to be induced by oxidative stress in many bacteria. *B. fragilis* thioredoxin genes, *trxC*, *trxE*, *trxF* and *trxG*, were induced under aerobic conditions and thioredoxin reductase genes were induced by hydrogen peroxide or oxygen (Reott *et al.*, 2009; Rocha *et al.*, 2007). In order to protect against acute oxidative stress, *L. casei* has a response system that eliminates oxygen and hydrogen peroxide by immediately inducing NADH oxidase and NADH peroxidase. This may show that the facultative anaerobe *L. casei* could require enzymes such as NADH oxidase and NADH peroxidase under strong oxidative stress conditions. On the other hand, thioredoxins are always needed to maintain a certain level of reducing conditions among intracellular proteins involved in oxido-reduction reactions, irrespective of aerobic or anaerobic conditions. Taken together, the thioredoxin-thioredoxin reductase system in *L. casei* has a pivotal

role in sustaining microbial life by maintaining certain levels of the intracellular thiol/disulfide balance under both strong and weak oxidative stress conditions.

In conclusion, our study indicates that the thioredoxin–thioredoxin reductase system is essential for aerobic growth of *L. casei* strain Shirota and is the major system for maintaining the intracellular thiol/disulfide balance.

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