**Introduction**

Aerobic organisms must withstand reactive oxygen species (ROS) released as a result of the incomplete reduction of molecular oxygen, or various environmental insults. ROS contains superoxide, hydroxyl radicals, \( \text{H}_2\text{O}_2 \), and organic hydroperoxides (OHPs). Excessive production of ROS can consume the cellular thiol pool, resulting in oxidative stress. Although excess ROS can directly affect other components of a cell including nucleic acids and lipids, a change of cellular redox homeostasis through post-translational modification of proteins is one of the primary effects of oxidative stress (Nystrom, 2005; Rietsch and Beckwith, 1998). Oxidative modification of cysteine thiol groups in proteins usually leads to protein disulfide bond formation, causing alteration of protein function and loss of enzymatic activity (Nystrom, 2005; Rietsch and Beckwith, 1998). Therefore, maintaining intracellular redox homeostasis and facilitating the proper folding of proteins were vital.

Two families of enzymes with a redox-active disulfide motif had been identified to be associated with reducing disulfide bonds of target proteins in bacteria (Daily et al., 2001; Rouhier et al., 2003). Thioredoxins (Trxs) and protein-disulfide isomerases (PDIs) have been annotated in *Corynebacterium glutamicum*. However, nothing is known about their functional diversity in the redox regulation of proteins. Thus, we here analyzed the Trx- and PDI-dependent redox shifts of ribonucleotide reductase (RNR), insulin, 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB), and several thiol-dependent peroxidases by measuring enzyme activity and thiol status *in vitro*. We found that the two Trxs and the three PDIs had activities in the cleavage of the disulfide bond, whereas the PDIs had a lower efficiency than the two Trxs. Trx2 could activate thiol-dependent peroxidases with an efficiency comparable with that of Trx1, but the PDIs were inefficient. The redox-active Cys-X-X-Cys motif harbored in both Trxs and PDIs was essential to supply efficiently the donor of reducing equivalents for protein disulfides. In addition, stress-responsive extracytoplasmic function (ECF)-sigma factor H (SigH)-dependent Trxs and PDIs expressions were observed. These results contributed importantly to our overall understanding of reducing functionality of the Trx and PDI systems, and also highlighted the complexity and plasticity of the intracellular redox network.

**Key Words:** *Corynebacterium glutamicum*; protein-disulfide isomerase (PDI); SigH; thiol status; thioredoxin (Trx)
merase (PDI) was another type of disulfide-reducing enzyme. PDI was initially considered as in vivo catalyzing the formation and isomerization of disulfide bonds during protein folding (Lyles and Gilbert, 1991; Morin and Dixon, 1985). However, many PDIs protein had been shown to share structural and functional identity with Trxs. Experiments in some bacterial species verified that PDIs containing the CXXC motif near an N-terminal region, which was homologous to the redox center of Trx (Van et al., 1993; Wilkinson et al., 2005), had the strong ability to reduce disulfides in protein, like Trx. In contrast, they had significantly lower disulfide isomerase activity (Lyles and Gilbert, 1994; Van et al., 1993). PDI was also a disulfide substrate for NADPH and TrxR (Wilkinson et al., 2005). Thus, PDIs containing the CXXC motif near an N-terminal region were also named Trx. Interestingly, some organisms expressed multiple Trx and PDI containing the CXXC motif near an N-terminal region homologous, which had an overlapping and distinct function. For example, the Trx system in plants was characterized by a large Trx family composed of Trx-α, -m, -x, -y, and -z (Serrato et al., 2013). Trx-m was identified as an effective activator of malate dehydrogenase (NADP-MDH), while Trx-f was the donor of reducing equivalents for FBPase and NADP-MDH (Collin et al., 2003; Geck et al., 1996; Hodges et al., 1994; Schürmann et al., 1981; Si et al., 2018). Trx-α, Trx-γ, and Trx-z were reported to be efficient in donating reducing equivalents to antioxidiant defense systems (Chibani et al., 2011; Collin et al., 2003). Trx-z was also shown to act as a decisive regulator for plastidial transcription (Arsava et al., 2010).

Corynebacterium glutamicum is a widespread Gram-positive bacterium of industrial importance. C. glutamicum produces significant amounts of various L-amino acids, including L-lysine and L-glutamate, and vitamins (Bröer et al., 1993). During culture, C. glutamicum inevitably encounters adverse circumstances, such as high osmotic pressure, low pH, and oxidation (Lee et al., 2013), causing a serious problem-excitatory stress (Bott and Niebisch, 2003). But surprising observations in the previous reports showed that C. glutamicum robustly survived the various adverse stresses of the culture process (Liu et al., 2013; Newton et al., 2008). Thus, the existence of redox active molecules, such as Trxs and PDIs, in C. glutamicum was suggested. Bioinformatics analysis revealed that C. glutamicum had two Trxs, two TrxRs and three PDIs containing the CXXC motif near N-terminal region homologs (NCgl2988 for trx1; NCgl2874 for trx2; NCgl2984 for trxR1; NCgl0663 for trxR2; NCgl0289 for pdi1; NCgl2339 for pdi2; NCgl2478 for pdi3). Moreover, these genes in this genome-streamlined bacterium were highly conservative. These phenomena suggested important functions of Trxs and PDIs. Previously, trxl and trxR1 were shown to play an important role in reducing mycothiol peroxidase (MPx)/thiol peroxidase (Prx)/methionine sulfoxide reductase A (MsrA)/peroxiredoxin Q (PrxQ) in C. glutamicum (Si et al., 2015a, 2015b, 2017; Su et al., 2018). However, the biological role of other Trxs, TrxR and PDIs remained unknown. Moreover, functional specificity and redundancy of different Trxs and PDIs subtypes also needed to be revealed. Here, we have focused on a description of the functional diversity of Trxs, providing an important understanding toward the intracellular redox network of C. glutamicum.

Materials and Methods

Bacterial strains and culture conditions. Bacterial strains and plasmids used in this study are listed in Table S1 (Jacoby et al., 1999; Schäfer et al., 1994; Tauch et al., 2002). C. glutamicum and Escherichia coli strains were cultured in Luria-Bertani (LB) broth aerobically on a rotary shaker (220 rpm), or on LB plates, at 30°C and 37°C, respectively. For complementation in various C. glutamicum strains, the pXJM19 derivatives were transformed into relevant C. glutamicum strains by electroporation and the expression in C. glutamicum was induced by the addition of 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). When needed, antibiotics were used at the following concentrations: Kanamycin, 50 µg ml⁻¹ for E. coli; Nalidixic acid, 40 µg ml⁻¹ for C. glutamicum; Ampicillin, 100 µg ml⁻¹ for E. coli; chloramphenicol, 20 µg ml⁻¹ for E. coli and 10 µg ml⁻¹ for C. glutamicum.

Construction of expression plasmid. Gene fragments encoding for C. glutamicum TrxR2 (NCg10063), PDI2 (NCgl2339), PDI3 (NCgl2478) were amplified by PCR using genomic DNA of C. glutamicum RES167 as template, and TrxR2-F/TrxR2-R, PDI2-F/PDI2-R, PDI3-F/PDI3-R as primer pair (Table S2). The resulting PCR products were used to amplify segments 1 and 2, respectively. The second round of PCR was carried out by using TrxR1-F/TrxR1-R as a primer pair while fragment 1 and fragment 2 were used to amplify segments 1 and 2, respectively. The second round of PCR was carried out by using TrxR1-F/TrxR1-R as a primer pair while fragment 1 and fragment 2 as templates to get the trxR1-SXXC segment, which contained a mutation in the C₁₄₂ site of TrxR1. The trxR1-SXXC DNA fragment was digested and cloned into similarly digested pET28a (for TrxR2, PDI2, and PDI3) expression vector (Novagen), obtaining pET28a-trxR2, pET28a-pdi2, and pET28a-pdi3, respectively.

Site-directed mutagenesis was carried out by Overlap PCR to make the cysteine residue at position 30 of TrxR1 into a serine residue (TrxR1:SXXC) (Higuchi et al., 1988; Ho et al., 1989). In brief, the mutant trxR1:SXXC DNA segment was amplified by two rounds of PCR. Primer pairs TrxR1-F1/TrxR1-C142S-R and TrxR1-C142S-F/TrxR1-R were used to amplify segments 1 and 2, respectively. The second round of PCR was carried out by using TrxR1-F/TrxR1-R as a primer pair while fragment 1 and fragment 2 as templates to get the trxR1:SXX segment, which contained a mutation in the C₁₄² site of TrxR1. The trxR1:SXXC DNA fragment was digested and cloned into similarly digested pET28a, obtaining plasmid pET28a-trxR1:SXXC. The trxR1:CXXS, trxR1:SXXS, trxR2:CXXS, trxR2:SXXC, trxR2:SXXS, pdi1:SXXC, pdi1:CXXS, pdi1:SXXS, pdi2:CXXS, pdi2:SXXC, pdi2:SXXS, pdi3:CXXS, pdi3:SXXS, and pdi3:SXXS DNA fragments were obtained using the same procedure as described above with primers listed in Table S2. These DNA fragments were also cloned into pET28a, obtaining corresponding derivatives.

To get the pK18mobSacB-P_ptrx1::lacZY fusion construct, fusion of the ptrx1 promoter to the lacZY reporter genes was made by Overlap PCR, and the resulting PCR fragment was inserted into the suicide vector pK18mobSacB (Si et al., 2018). In brief, to make sure that the whole trxl...
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promoter was included, 175 bp trxl promoter DNA sequences (corresponding to nucleotides +15 to −160 relative to the translational start codon (ATG) of trxl gene) was amplified with the primer pair Ptrxl-F and Ptrxl-R. In the second round of overlap PCR, the promoter fragment was fused with the lacZY fragment (amplified with lacZY-F1/lacZYT-R) by using primers P ptrxl-F and lacZYT-R. The resulting PCR fragments were digested with SmallII/PstI and inserted into the SmallII/PstI site of the suicide vector pK18mobsacB to get the pK18mobsacB-P trxl::lacZY fusion construct. Similarly, 235 bp trx2 promoter DNA sequences (corresponding to nucleotides +15 to −220 relative to the translational start codon (ATG) of txs2 gene), 261 bp pdi1 promoter DNA sequences (corresponding to nucleotides −1282 to −1021 relative to the translational start codon (ATG) of pdi1 gene), 145 bp pdi2 promoter DNA sequences (corresponding to nucleotides +15 to −130 relative to the translational start codon (ATG) of pdi2 gene), and 255 bp pdi3 promoter DNA sequences (corresponding to nucleotides −745 to −1000 relative to the translational start codon (ATG) of pdi3 gene) were amplified with the primers listed in Table S2, respectively. After the resulting fragments were fused with the lacZY fragments, the P ptrxl::lacZY, P trx2::lacZY, P pdi1::lacZY, and P pdi3::lacZY were digested and inserted into similarly digested suicide vector pK18mobsacB.

The fidelity of all constructs was confirmed by DNA sequencing (Sangon Biotech, Shanghai, China).

Recombinant proteins expression and purification. Each expression plasmid was transformed into E. coli strain BL21 (DE3). The transformed cells were grown at 37°C in LB medium to an OD600 of 0.5. Expression was induced by the addition of 0.6 mM IPTG followed by further culture at 22°C for 12 h. The cultures were centrifuged and then cells were harvested. After the harvested cells were disrupted by sonification, the supernatant was obtained by centrifugation (100,000 × g for 60 min) to purify the protein of interest with the His(Bind Ni-NTA resin (Novagen, Madison, USA) according to the manufacturer’s instructions. Imidazole was removed from the purified recombinant proteins by dialysis against PBS overnight at 4°C and stored at −80°C until use. All the purification procedures were performed at 4°C. The protein concentration was determined with the Bradford assay and the protein concentration of the sample was calculated from the A280 of the standard curve. As a positive control, Trx1 (10 µM) was used in place of other Trxs or PDIs under the same conditions.

Insulin reduction assay. The ability of proteins to reduce the insulin disulfide was measured as described previously (Du et al., 2012). Briefly, 0.32 mM insulin was added to the assay mixture containing 0.5 ml of 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 200 µM NADPH, and 10 µM TrxR. The addition of 10 µM different Trxs or PDIs to the mixtures was to start the reaction. The change in turbidity was monitored, during the first hour of reaction every 1 min, by measuring the decrease in absorbance at 340 nm. As a positive control, Trx1 (10 µM) was used in place of other Trxs or PDIs under the same conditions.

DTNB assay. DTNB [5,5-dithiobis-(2-nitrobenzoic acid)] assays were used to measure the reduction of DTNB disulfides (Holmgren, 1979). The assay mixture contained 50 nM Tris-HCl, pH 7.5, 1 mM EDTA, 200 µM NADPH, 1 nM DTNB, 10 µM TrxR, and either 10 µM different Trxs or PDIs. The activity was monitored by following the increase of absorbance at 412 nm during the first 10 min due to production of 3-carboxy-4-nitrobenzenethiol (NBT). Maintaining all the reactions under the same conditions, Trx1 (10 µM) was used in place of Trx or PDIs as a positive control.

Reductase activity of protein toward mycotoxin (MSH) mixed disulfides. The ability of protein to catalyze the reduction of the mixed disulphide between hydroxyethyl disulphide (HED) and MSH (HED-SSM) was measured according to Kim et al. (2012). The HED-SSM was formed by incubating 0.7 mM HED with 1 mM MSH at 30°C for 5 min. 0.25 mM HED-SSM as the substrate was added to the electron transfer mixtures in a final volume of 200 µl.
with 5 \( \mu M \) TrxR, 200 mM NADPH and 10 \( \mu M \) of either different Trxs or PDIs. The reactions were carried out at 25°C for 10 min and the absorption of NADPH at 340 nm was recorded. As a positive control, Trx1 was used in place of other Trxs or PDIs in the reaction mixture.

**MSH purification.** MSH was purified from *C. glutamicum* RES167 with tripropyl sepharose 6B followed by Sephadex LH-20 chromatography as described (Feng et al., 2006; Newton et al., 1996). The concentration of purified MSH was measured by using the thiol-specific fluorescent-labeling HPLC method (16) with commercial glutathione (GSH) as the thiol standard reference. The HPLC used in this study was equipped with an Extend-C18 column (ZORBAX, 250 × 4.6 mm) and was operated with aqueous acetic acid-methanol gradient elution (eluant flow rate of 0.9 ml/min). The bimane derivative of MSH was eluted about after 15 min in this system.

**Enzyme assays.** The catalytic properties of MPx were determined using a reduced Trx-generating system (4 \( \mu M \) TrxR and 40 \( \mu M \) Trx (Trx1 or Trx2) or PDI (PDI1, PDI2, or PDI3)) as the possible electron donors (Si et al., 2015a). These assays were carried out in a total volume of 500 \( \mu l \) containing 50 mM Tris-HCl buffer (pH 8.0), 2 mM EDTA, 250 \( \mu M \) NADPH, 0.5 \( \mu M \) MPx, and the electron donor (Trx or PDI system). The reactions were started by addition of 200 \( \mu M \) peroxide substrates following 5 min of preincubation. The catalytic parameters for one substrate were obtained by varying its concentration at saturating concentrations of the other substrate (between 0 and 150 \( \mu M \) for Trx or PDI). NADPH oxidation was monitored as absorption coefficient of NADPH at 340 nm (\( \epsilon_{340} \)) of 6220 \( M^{-1} \cdot cm^{-1} \). Three independent experiments were performed at each substrate concentration. The \( k_{cat} \) and \( K_m \) values of MPx, PrxQ, or Prx for Trx or PDI were obtained from a non-linear fit with the Michaelis-Menten equation using the program GraphPad Prism 5.

**Total RNA isolation and real-time RT-PCR for quantification of trx or pdi transcription.** The total RNA was isolated from *C. glutamicum* cells grown to the mid-exponential phase by using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer’s protocol and was treated with DNase I (Qiagen, Hilden, Germany) to completely remove the chromosomal DNA. The purity and concentration of the RNA were determined by gel electrophoresis and a spectrophotometer (NanoDrop, Thermo Scientific). First-strand cDNA was reverse transcribed from 1 \( \mu g \) of total RNA with the Transcript First-Strand cDNA Synthesis SuperMix (TransGen Biotech, Beijing, China). Quantitative real-time PCR (qRT-PCR) was performed in CFX96 Real-Time PCR Detection System (Bio-Rad, USA) with TransStart Green qPCR SuperMix (TransGen Biotech, Beijing, China). For all primer sets (Table S2), the following cycling parameters were used: 95°C for 30 s followed by 40 cycles of 94°C for 15 s, and 50°C for 30 s. For standardization of the results, the relative abundance of 16S rRNA was used as the internal standard.

**Determination of the redox state of thiol-dependent enzymes in vitro.** The redox state was performed based on Motohashi et al. (2001). Briefly, MPx or PrxQ proteins (30 \( \mu M \)) were reduced by incubation with 50 mM dithiothreitol (DTT) at room temperature for 30 min, and excess DTT was removed by ultrafiltration. The reduced MPx or PrxQ (15 \( \mu M \)) was treated with 0.1 mM H\(_2\)O\(_2\) at room temperature for 30 min. The H\(_2\)O\(_2\)- and DTT-treated proteins (5 \( \mu M \)) were precipitated in 5% (v/v) trichloroacetic acid (TCA), washed with acetone, and then redissolved in 50 mM Tris-HCl (pH 7.5) containing 1% SDS and 15 mM thiol-reactive probe 4-acetamido-4′maleimidylstilbene-2,2′-disulfonic acid (AMS; Molecular Probes, Eugene, OR). After incubation for 30 min in the dark, the obtained MPx and PrxQ (2 \( \mu M \) each) were incubated with 3 \( \mu M \) Trx, 3 \( \mu M \) PDI, or 5 mM DTT in a mixture containing 50 mM Tris-HCl (pH 7.5) and 50 mM NaCl, respectively. After incubation for 30 min at 25°C, the proteins were subjected to 20% nonreducing SDS-PAGE.

**Formation and separation of heterodimers.** The assays of heterodimers were performed based on the method described by Su et al. with minor modifications (Su et al., 2018). Peroxidase (25 \( \mu M \) MPx or PrxQ) and 15 \( \mu M \) Trx variants (Trx1:CXXS, Trx2:CXXS, PDI1:CXXS, PDI2:CXXS, or PDI3:CXXS) were mixed in a TE buffer (30 mM Tris-HCl, pH 8.0, 1 mM EDTA) to a final volume of 20 \( \mu l \). This reaction mixture was incubated at room temperature for 15 min before the addition of 50 \( \mu M \) H\(_2\)O\(_2\). The mixture was incubated at room temperature for another 30 min and then subjected to 20% nonreducing SDS-PAGE.
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Results and Discussion

Identification of trx and pdi genes from C. glutamicum genome

Based on BLAST Search and genome sequence analysis, we found that C. glutamicum contained two cytoplasmic thioredoxins encoded by trx1 (ncgl2985) and trx2 (ncgl2874); two cytoplasmic thioredoxin reductases encoded by trxR1 (ncgl2984) and trxR2 (ncgl0663); and three thiol-disulfide isomerases encoded by ncgl0289, ncgl2339, ncgl2478, respectively. Multiple alignment analysis showed that Trx2, NCgl0289, NCgl2339, and NCgl2478 shared 41.8%, 31%, 29% and 23% amino acid sequence identity with the Trx1, respectively (Fig. S1A); TrxR2 shared a high homology of amino acid sequence with TrxR1 (e.g. TrxR2 and TrxR1, 72%) (Fig. S1B). Moreover, Trx2, NCgl0289, NCgl2339, and NCgl2478 contained the Cys-X-X-Cys signature motif near the N terminus (Trx2_C30XXC33, NCgl0289_C84XXC87, NCgl2339_C13XXC16, and NCgl2478_C21XXC24) that was homologous to the redox center of Trx1; and TrxR2 shared the Cys-X-X-Cys signature motif (TrxR2_C134XXC137) with TrxR1 (Figs. S1A and B). Lyles et al. (1991) found that the conserved Cys-X-X-Cys active site at the N-terminal region of thiol-disulfide isomerases, claimed to be involved in the catalyzing reduction of disulfide bonds in many Trxs, contributed more to catalysis at saturating concentrations of substrate. We deduced that PDI represented a variety of thioredoxin-like molecules involved in the reduction of substrates that contained a disulfide, such as insulin, RNR, DTNB, and thiol-dependent peroxidases.

Therefore, we named the three thiol-disulfide isomerases NCgl0289, NCgl2339, and NCgl2478 as PDI1, PDI2, and PDI3, respectively.

Trx and PDI for redox regulation of RNR, insulin, and DTNB in vitro

To determine whether Trx2, PDI1, PDI2, and PDI3 had the same capability of dithiol-disulfide exchange reaction as Trx1, we employed a series of in vitro assays (see Materials and Methods) by using RNR, insulin, and DTNB as substrates, and the coupled NADPH oxidation by TrxR was monitored at 340 nm. As shown in Fig. 1A, Trx2, PDI1, PDI2, and PDI3 could function as a reductant for the class Ib RNR with TrxR1 and NADPH as the electronic donor system, consistent with a previous finding that E. coli PDI was capable of reducing class Ib RNR linked to TrxR (Wilkinson et al., 2005). Trx2 showed a comparable activity towards class Ib RNR with that of Trx1, while the reduction activities of PDI1, PDI2, and PDI3 were much lower than that of Trx1 (NCgl2985) in transferring electrons from NADPH via the TrxR1 pathway, as the reduction rates measured by the ∆∆A340/min were 0.376, 0.338, 0.305, and 0.601 for PDI1, PDI2, and PDI3, respectively (Fig. 1A). Similarly, TrxR2 also showed a similar ability to TrxR1 in severing as the reducing equivalents of different Trxs (Fig. 1D). To assess whether C. glutamicum Trx2, PDI1, PDI2, and PDI3 were specially involved in the reduction of class Ib RNRs, or possessed general thiol-disulfide redox activity as Trx1, we further examined the capacity of Trx2, PDI1, PDI2, and PDI3 to catalyze the reduction of the classic disulfide-

Fig. 1. Trx and PDI reduced disulfide bonds by the TrxR/NADPH pathway.

Reduction of RNR (A and D), insulin (B and E) and DTNB (C and F) by Trxs or PDIs (10 µM) coupled to the TrxR (TrxR1 or TrxR2)/NADPH regeneration system. Trx1 was used as a positive control. Negative control was the omission of Trxs in the presence of TrxR. The reduction of RNR and insulin were recorded by measuring the decrease of NADPH oxidation at 340 nm. The reduction of DTNB was recorded as an increase in absorption at 412 nm.
containing insulin and small molecule artificial disulfide compound DTNB. As shown in Fig. 1B, the addition of Trx2 to the reaction mixture led to an obviously reduced absorbance at 340 nm in the presence of TrxR1/NADPH, similar to that of Trx1, while PDI1, PDI2, and PDI3 showed a lower efficiency. Moreover, the addition of \textit{C. glutamicum} TrxR2 to the reaction mixture also caused a comparable absorption at 340 nm with that of TrxR1 (Fig. 1E). The results demonstrated that Trx2 was almost as effective as Trx1 in reducing insulin disulfides by using the TrxR/NADPH regeneration system; and that PDI1, PDI2, and PDI3 had the capacity of \textit{C. glutamicum} Trx2, PDI1, PDI2, and PDI3 to reduce an artificial disulfide substrate DTNB used as an exposed disulfide bond. As shown in Fig. 1C, F, the Trx2, PDI1, PDI2, and PDI3 caused a catalytic relevant reduction of DTNB in the presence of TrxR and NADPH. Although the activity of PDI1, PDI2, and PDI3 was much less effective than that of \textit{C. glutamicum} Trx1, Trx2 showed an almost equal affinity to Trx1 \textit{in vitro}.

**The efficiency of Trx and PDI in reducing thiol-dependent peroxidases**

Thiol-dependent peroxidases, including MPx, PrxQ, and Prx, played a vital role in the detoxification of ROS and was accordingly known as an important component of the antioxidant system in \textit{C. glutamicum} (Si et al., 2015a, 2017; Su et al., 2018). During the process of clearing ROS,

| Table 1. Reducing substrate specificities of MPx in catalyzed reduction of H$_2$O$_2$. |
|------------------------------------------|
| $K_v$ (µM) | $k_{cat}$ (s$^{-1}$) | $k_{cat}/K_v \times 10^3$ (M$^{-1}$ s$^{-1}$) | $K_v$ (µM) | $k_{cat}$ (s$^{-1}$) | $k_{cat}/K_v \times 10^3$ (M$^{-1}$ s$^{-1}$) |
| Trx1/TrxR1 | 15.8±1.4 | 5.1±0.2 | 322.8 | Trx1/TrxR2 | 16.2±2.3 | 5.6±0.4 | 333.3 |
| Trx2/TrxR1 | 14.6±0.7 | 4.5±0.1 | 308.2 | Trx2/TrxR2 | 20.3±1.9 | 6.1±2.4 | 300.5 |
| PDI1/TrxR1 | ND | ND | ND | PDI1/TrxR2 | ND | ND | ND |
| PDI2/TrxR1 | ND | ND | ND | PDI2/TrxR2 | ND | ND | ND |
| PDI3/TrxR1 | ND | ND | ND | PDI3/TrxR2 | ND | ND | ND |

Peroxidase assays were performed as described in the experimental procedures with a fixed concentration of peroxides (200 µM), MPx (0.5 µM), TrxR (4 µM) and different concentration of various Trx or PDI (0–120 µM). The data were presented as means of values obtained from three independent assays and analyzed by non-linear regression using the program GraphPad Prism 5.

ND: not detected under the conditions used.

| Table 2. Reducing substrate specificities of PrxQ in catalyzed reduction of H$_2$O$_2$. |
|------------------------------------------|
| $K_v$ (µM) | $k_{cat}$ (s$^{-1}$) | $k_{cat}/K_v \times 10^3$ (M$^{-1}$ s$^{-1}$) | $K_v$ (µM) | $k_{cat}$ (s$^{-1}$) | $k_{cat}/K_v \times 10^3$ (M$^{-1}$ s$^{-1}$) |
| Trx1/TrxR1 | 7.09±0.6 | 1.34±0.3 | 189.9 | Trx1/TrxR2 | 7.18±1.5 | 1.16±0.6 | 161.6 |
| Trx2/TrxR1 | 8.28±1.2 | 1.09±0.2 | 131.6 | Trx2/TrxR2 | 7.99±1.3 | 0.95±0.2 | 118.9 |
| PDI1/TrxR1 | ND | ND | ND | PDI1/TrxR2 | ND | ND | ND |
| PDI2/TrxR1 | ND | ND | ND | PDI2/TrxR2 | ND | ND | ND |
| PDI3/TrxR1 | ND | ND | ND | PDI3/TrxR2 | ND | ND | ND |

Peroxidase assays were performed as described in the experimental procedures with a fixed concentration of peroxides (1 mM), PrxQ (1 µM) and TrxR (5 µM) using different concentrations of various Trx or PDI (0–120 µM). The data were presented as means of values obtained from three independent assays and analyzed by non-linear regression using the program GraphPad Prism 5.

ND: not detected under the conditions used.

| Table 3. Reducing substrate specificities of Prx in catalyzed reduction of H$_2$O$_2$. |
|------------------------------------------|
| $K_v$ (µM) | $k_{cat}$ (s$^{-1}$) | $k_{cat}/K_v \times 10^3$ (M$^{-1}$ s$^{-1}$) | $K_v$ (µM) | $k_{cat}$ (s$^{-1}$) | $k_{cat}/K_v \times 10^3$ (M$^{-1}$ s$^{-1}$) |
| Trx1/TrxR1 | 32.3±2.7 | 78.8±6.1 | 244.0 | Trx1/TrxR2 | 29.1±1.7 | 72.6±4.1 | 249.5 |
| Trx2/TrxR1 | 55.6±1.8 | 80.2±9.2 | 225.3 | Trx2/TrxR2 | 31.1±2.4 | 75.6±6.3 | 243.1 |
| PDI1/TrxR1 | ND | ND | ND | PDI1/TrxR2 | ND | ND | ND |
| PDI2/TrxR1 | ND | ND | ND | PDI2/TrxR2 | ND | ND | ND |
| PDI3/TrxR1 | ND | ND | ND | PDI3/TrxR2 | ND | ND | ND |

Peroxidase assays were performed as described in the experimental procedures with a fixed concentration of peroxides (0.1 mM), Prx (1 µM) and TrxR (5 µM) using different concentrations of various Trx or PDI (0–120 µM). The data were presented as means of values obtained from three independent assays and analyzed by non-linear regression using the program GraphPad Prism 5.

ND: not detected under the conditions used.
reduced thiol-dependent peroxidase directly attacked peroxide substrate to form the active site disulfide bond-containing oxidized peroxidase. To carry on another catalytic cycle and reform the active reduced sulfhydryl groups in peroxidase, the oxidized peroxidase must receive a donor of reducing equivalents from Trx (or other reductants). Therefore, we further identified the ability of Trx2, PDI1, PDI2, and PDI3 to provide electrons for thiol-dependent peroxidases under steady-state conditions at saturating concentrations of peroxides (1 mM or 0.1 mM) and different concentrations of various Trxs or PDIs (0 to 150 µM). As shown in Tables 1–3, MPx showed a very high activity when the Trx2/TrxR1/NADPH system, or the Trx2/TrxR2/NADPH system, were used as the electron transfer pathway. The $k_{cat}$ values of MPx to H$_2$O$_2$ with the Trx2/TrxR1/NADPH and Trx2/TrxR2/NADPH systems were 4.5 ± 0.1 s$^{-1}$ and 6.1 ± 2.4 s$^{-1}$, respectively, while the respective $K_{m}$ values were 14.6 ± 0.7 µM and 20.3 ± 1.9 µM, respectively. This corresponded to a catalytic efficiency of 308.2 × 10$^3$ M$^{-1}$ s$^{-1}$ and 300.5 × 10$^3$ M$^{-1}$ s$^{-1}$, respectively. Similar results were also observed in PrxQ or Prx with the Trx2/TrxR1/NADPH system and Trx2/TrxR2/NADPH system as terminal electron acceptors for H$_2$O$_2$ elimination. The activities of the Trx2/TrxR/NADPH system in reducing MPx, PrxQ, and Prx were similar to that of the Trx1/TrxR/NADPH system. However, MPx, PrxQ and Prx showed no activities in the presence of the three reducing reductants PDI1, PDI2, or PDI3, when H$_2$O$_2$ was used as substrate. These data indicated that PDI1, PDI2, and PDI3, could not promote MPx, PrxQ and Prx reduction.

Previously, it was reported that C. glutamicum Trx1 could also take over the role of Mrx1 and reduce MSH mixed disulphide bonds using electrons from the Trx/R/NADPH pathway via a dithiol mechanism (Pedre et al., 2015). Thus, it was of interest to probe whether Trx2, PDI1, PDI2, or PDI3 also supported MSH-disulphide oxidoreductase activity. To prove this, we utilized the standard mycothiol coupled hydroxethyl disulphide (HED) as substrate and Trx1 and Mrx1 as a positive control. As shown in Fig. S2, although Trx2 displayed a certain reduction ability toward the mixed disulfide formed between HED and MSH via TrxR/NADPH pathway, as judged by the consumption of NADPH monitored spectrophotometrically at 340 nm, similar to that of Trx1, it showed a lower efficiency than that of Mrx1 via Mtr/MSH/NADPH. Moreover, no MSH-disulfide oxidoreductase activity could be detected for PDI1, PDI2, or PDI3. These results indicated that not all dithiol oxidoreductases containing a CXXC motif could catalyze the reduction of mixed disulfide bonds.

**Trxs formed a mixed disulfide with thiol-dependent peroxidase but not PDIs**

To further investigate whether Trx facilitated thiol-dependent peroxidase activity by direct interaction, the redox state of thiol-dependent peroxidases was first determined by discriminating the thiol status. For simplicity, we used MPx and PrxQ as the target objects in the following experiments. Previous reports showed that H$_2$O$_2$-treated MPx and PrxQ showed two bands, the lower one of which corresponded to the intramolecular disulfide bonds containing oxidized states, but the upper one of which corresponded to reduced states (Si et al., 2015a; Su et al., 2018). This phenomenon was explained by the fact that the formation of intramolecular disulfide bonds led to a more compact configuration and a more rapid migration than the reduced state on non-reducing SDS-PAGE gels. Consistent with the previous reports (Si et al., 2015a; Su et al., 2018), two bands-containing MPx and PrxQ exposed to H$_2$O$_2$ were also confirmed by our study (Figs. 2A and 2B). Furthermore, DTT-treated MPx and PrxQ only showed a single band, which corresponded to the upper band in H$_2$O$_2$-treated MPx or PrxQ (Fig. S3). When 5 mM DTT was added to the H$_2$O$_2$-treated MPx and PrxQ, the increase of the upper band and the decrease of the lower band were observed, suggesting that the oxidized form MPx and PrxQ became reduced (Fig. S3). When Trx2, PDI1, PDI2, or PDI3 was incubated with H$_2$O$_2$-treated MPx and PrxQ, the only Trx2 assisted in the shift of MPx and PrxQ from an oxidized to a reduced form (oxidized MPx and PrxQ (lower band) almost disappeared and accompanied by an increase in reduced MPx and PrxQ (upper band)) was similar to that of Trx1 (Si et al., 2015a; Su et al., 2018). The phenomenon that the intramolecular disulfide bonds-containing oxidized MPx and PrxQ disappeared indicated that Trx2 could reduce oxidized MPx and PrxQ. In contrast, PDI1, PDI2, or PDI3 did not cause a decrease in oxidized MPx and PrxQ (lower band) (Figs. 2A and 2B).

To prove the truth of the Trx2-peroxidase interaction seen in the above thiol status assay, the formation and separation of heterodimers were performed. MPx and PrxQ were incubated with Trx2:C33S, PDI1:C87S, PDI2:C16S, and PDI3:C21XXS24 in the presence of various Trxs or PDIs (0 to 150 µM). As shown in Tables 1–3, MPx showed a very high activity when the Trx2/TrxR1/NADPH system, or the Trx2/TrxR2/NADPH system, were used as the electron transfer pathway. The $k_{cat}$ values of MPx to H$_2$O$_2$ with the Trx2/TrxR1/NADPH and Trx2/TrxR2/NADPH systems were 4.5 ± 0.1 s$^{-1}$ and 6.1 ± 2.4 s$^{-1}$, respectively, while the respective $K_{m}$ values were 14.6 ± 0.7 µM and 20.3 ± 1.9 µM, respectively. This corresponded to a catalytic efficiency of 308.2 × 10$^3$ M$^{-1}$ s$^{-1}$ and 300.5 × 10$^3$ M$^{-1}$ s$^{-1}$, respectively. Similar results were also observed in PrxQ or Prx with the Trx2/TrxR1/NADPH system and Trx2/TrxR2/NADPH system as terminal electron acceptors for H$_2$O$_2$ elimination. The activities of the Trx2/TrxR/NADPH system in reducing MPx, PrxQ, and Prx were similar to that of the Trx1/TrxR/NADPH system. However, MPx, PrxQ and Prx showed no activities in the presence of the three reducing reductants PDI1, PDI2, or PDI3, when H$_2$O$_2$ was used as substrate. These data indicated that PDI1, PDI2, and PDI3, could not promote MPx, PrxQ and Prx reduction.

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or PDI3:C24S in the presence of H\textsubscript{2}O\textsubscript{2}. These mixtures were separated on non-reducing SDS-PAGE and evaluated based on molecular mass determinations. As shown in Fig. 2B, about 35 KDa of additional polypeptides occurred in the mixture of Trx2:C33S, MPx and H\textsubscript{2}O\textsubscript{2}, or Trx2:C33S, PrxQ and H\textsubscript{2}O\textsubscript{2}, which corresponded to the MPx-Trx2:C33S, or PrxQ-Trx2:C33S, heterodimer, respectively. However, the above polypeptides did not exist in only Trx2:C33S, MPx, or PrxQ under H\textsubscript{2}O\textsubscript{2} treatment. We speculated that Trx2:C33S and its target proteins MPx or PrxQ may form a mixed-disulphide intermediate at the first reactive cysteine of the N-terminus cysteinyl residue, in line with the result of Su et al. (2018) reported for \textit{C. glutamicum} Trx1. Taken together, these data clearly demonstrated that Trx2 could support the thiol-dependent peroxidase in clearing H\textsubscript{2}O\textsubscript{2}, by serving as a reducing reductant of thiol-dependent peroxidase.

The conserved Cys-X-X-Cys active site in Trx played a crucial role

To check whether the conserved cysteines in the CXXC motif of Trx and TrxR were essential for its function, we mutated the first, the second, and both cysteines of the CXXC motif to Ser (Trx:CXXS, Trx:SXXC, and Trx:SXXS; PDI:CXXS, PDI:SXXC, and PDI:SXXS; TrxR:CXXS, TrxR:SXXC, and TrxR:SXXS). Their functionality was tested by progress curves with the aforementioned insulin reduction assays in the presence of the TrxR1/NADPH regeneration system, or the Trx1(Trx2)/NADPH reducing reductants, respectively, and compared with wild type protein (Figs. 3 and 4). As shown in Figs. 3 and 4, electron transfers were drastically reduced to background levels when Trx:CXXS, Trx:SXXC, Trx:SXXS, PDI:CXXS, PDI:SXXC, PDI:SXXS, TrxR:CXXS, TrxR:SXXC, and TrxR:SXXS were present, which were significantly less efficient than that observed for the wild type protein. These results indicated that both conserved cysteines in the CXXC domain were extraordinary important for Trx, PDI and TrxR function.

\textit{trx} and \textit{pdi} expressions were regulated by SigH

As Trxs and PDIs had been identified as effective activators of repair enzyme RNR and/or thiol-dependent peroxidase, \textit{trx} (\textit{trx1} and \textit{trx2}), \textit{pdi} (\textit{pdi1}, \textit{pdi2}, or \textit{pdi3}) and \textit{trxR} expressions in response to H\textsubscript{2}O\textsubscript{2} were examined by
LacZY activity and qRT-PCR analysis. As shown in Fig. 5, compared to the untreated samples, the promoter activities and expression of different *trx*, *pdi* and *trxR2* were not significantly increased in the wild type strain treated with H$_2$O$_2$, which was in agreement with the result of Pedre et al. (2015) reported for Trx.

As SigH, the stress-responsive extracytoplasmic function-sigma (ECF-σ) factor, was reported to respond to thiol-oxidative stress and regulate the expression of multiple resistance genes (Busche et al., 2012), we examined whether their expressions were subjected to SigH regulation by measuring the transcription of chromosomal promoter lacZ fusions. Whether exposure to H$_2$O$_2$ or not, marked decrease of *trx* (*trx1* and *trx2*), *pdi* (*pdi1*, *pdi2*, or *pdi3*) and *trxR2* promoter activity were detected in the ∆*sigH* mutant compared with the wild-type strain (Fig.
5). However, the trxl (trxl1 and trxl2), pdi (pdi1, pdi2, or pdi3) and trxlR2 expression in the ΔsigH mutant was almost fully recovered when the regulatory protein was complemented, either under H2O2-treated or H2O2-untreated conditions (Fig. 5). A similar patterns of trxl (trxl1 and trxl2), pdi (pdi1, pdi2, or pdi3) and trxlR2 expression in response to H2O2 were observed in the qRT-PCR analysis (Fig. 5).

Conclusions

In this study, we have characterized the functional specificity and redundancy of two Trxs and three PDIs in the redox regulation. The two Trxs and three PDIs had a role in the cleavage of the classic disulfide bond-containing insulin and RNR, and the small molecule artificial disulfide compound DTNB, whereas pdi1, pdi2, and pdi3 had a lower efficiency than Trxl1 and Trxl2. Moreover, Trxl1 and Trxl2 had equivalent efficiencies in reducing thiol-dependent peroxidases, but PDI1, PDI2, or PDI3 were inefficient, suggesting that Trxl1 and Trxl2 could be regarded as compatible partners in antioxidant systems for providing reducing powers. Although pdi1, pdi2, and pdi3 shared the Cys-X-X-Cys signature with Trxl1, they had overlapping and distinct functions. To our knowledge, this was the first report clarifying overall the functional diversity in the redox regulation of C. glutamicum Trxs and PDIs. Hence, our results are provide a useful insight into a previously unknown, but important, aspect of C. glutamicum Trxs and PDIs that acted as a thiol-dependent peroxidase reducing power to facilitate the degradation of peroxides.

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Supplementary Materials

Supplementary figures and tables are available in our J-STAGE site (http://www.jstage.jst.go.jp/browse/jgam).

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