Full Paper

Molecular mechanisms of Mycoredoxin-1 in resistance to oxidative stress in *Corynebacterium glutamicum*

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Glutaredoxins (Grxs) with Cys-Pro-Phe (Tyr)-Cys motif and a thioredoxin fold structure play an important role in the anti-oxidant system of bacteria by catalyzing a variety of thiol-disulfide exchange reactions with a 2-Cys mechanism or a 1-Cys mechanism. However, the catalytic and physiological mechanism of *Corynebacterium glutamicum* Mycoredoxin 1 (Mrx1) that shares a high amino acid sequence similarity to Grxs has not been fully elucidated. Here, we report that Mrx1 has a protective function against various adverse conditions, and the decrease of cell viability to various stress conditions by deletion of the Mrx1 in *C. glutamicum* was confirmed in the *mrx1* mutant. The physiological roles of Mrx1 in defence to oxidative stress were corroborated by its induced expression under various stresses, regulated directly by the stress-responsive extracytoplasmic function-sigma (ECF-σ) factor SigH. As well as reducing mycothiol (MSH) mixed disulfide bonds via a 1-Cys mechanism, *C. glutamicum* Mrx1 catalytically reduced the disulfides in the Ib RNR, insulin and 5,5′′-dithiobis-(2-nitrobenzoic acid) (DTNB) by exclusively linking the MSH/Mtr (mycothiol disulfide reductase)/NADPH electron pathway via a 2-Cys mechanism. Thus, we present the first evidence that the Mrx1 is able to protect against the damaging effects of various exogenous stresses by acting as a disulfide oxidoreductase, thereby giving a new insight in how *C. glutamicum* survives oxidative stressful conditions.

**Key Words:** *Corynebacterium glutamicum*; Mycoredoxin-1; mycothiol; oxidative stress

Introduction

Reactive oxygen species (ROS) are normal by-products produced by oxygen metabolism. When bacteria are exposed to environmental stresses, such as heat, acid, antibiotics, high salt concentrations, and toxic agents, the electron transfer chains are perturbed, which results in the formation of ROS. The excessive production or accumulation of ROS is harmful to biological systems (Valko et al., 2004). ROS in excess of physiological concentrations induces oxidative stress, leading to reversible thiol-oxidation, including inter- or intra-protein disulfides and mixed disulfides with low-molecular-weight (LMW) thiols and irreversible carbonylation of proteins, ultimately resulting in cellular damage (Poli et al., 2004). In order to defend against oxidative damage by ROS, organisms engage a diverse variety of defence systems against oxidative stress to maintain normal cell structures and functions, including antioxidiant enzymatic systems, such as catalase (CAT), glutathione S-transferase (GST), glutathione reductase (GR), glutaredoxin (Grx), thioredoxin (Trx), and superoxide dismutase (SOD) (Dalle-Donne et al., 2008), and non-enzymatic systems, such as the tripeptide glutathione (GSH; γ-L-glutamyl-L-cysteinylglycine), β-carotene, and vitamins (vitamins C and E) (Si et al., 2014).

Grxs, small thiol-disulfide oxidoreductases (about 10 kDa), which contain a ‘thioredoxin fold’ protein structure, were first discovered in *Escherichia coli*. Further study found that Grxs are widespread among Gram-negative bacteria and eukaryotes, playing an important role in reducing disulfide bonds of target proteins and maintaining intracellular redox homoeostasis (Daily, 2001; Rouhier et al., 2003). According to the difference in active site structures, Grxs are mainly divided into two categories: dithiol (Cys-Pro-Phe (Tyr)-Cys (CPF/YC) motif) and monothiol (Cys-Gly-Phe-Cys (CGFS) motif). Dithiol Grxs display...
the reduction activity of dithiol groups, including protein disulfides, such as ribonucleotide reductase (Holmgren, 1979; Laurent et al., 1964), phosphoadenosine-phosphosulfate reductase (Lillig et al., 1999, 2003), or mixed disulfides between a protein and a GSH molecule, such as methionine sulfoxide reductase (Couturier et al., 2012; Tarrago et al., 2009), and arsenate reductase (Ordóñez et al., 2009). 2-Cys and 1-Cys mechanisms are employed by dithiol Grxs for reducing disulfide bridges. The 2-Cys mechanism requires cysteine residues of the active site on both sides of the CXXC motif (Bushweller et al., 1992). The N-terminal cysteine forms an intermediate dithiol bridge between the Grx protein and the target protein, which is solved by the second cysteine in Grx to form oxidized Grx (Grx-S$_2$), and then Grx-S$_2$ is reduced back to the reduced active state of Grx by GSH. The mechanism of 1-Cys was relatively simple, only requiring N-terminal cysteine (Bushweller et al., 1992), and is employed for reducing the mixed disulfide formed between GSH and the target protein. During the catalytic process, the disulfide bond of S-glutathionylated protein is attacked by the thiolate of nucleophilic cysteine at the active site of Grx, releasing the protein thiol in a reduced form while becoming itself glutathionylated (Biswas et al., 2006). Then GSH attacks glutathionylated Grx, releasing reduced Grx and GSSG to maintain redox homeostasis.

In Actinomycetes, including Mycobacterium smegmatis, Mycobacterium tuberculosis, Corynebacterium glutamicum, Corynebacterium diphtheria, a novel Grx-like mycothiol (MSH)-dependent reductase (mycoredoxin-1, Mrx1) was identified by the homology identification to mycothiol (MSH)-dependent reductase (mycoredoxin-1), glutamicum, Mycobacterium tuberculosis, C. glutamicum, which is solved by the second cysteine in Grx to form oxidized Grx (Grx-S$_2$), and then Grx-S$_2$ is reduced back to the reduced active state of Grx by GSH. The mechanism of 1-Cys was relatively simple, only requiring N-terminal cysteine (Bushweller et al., 1992), and is employed for reducing the mixed disulfide formed between GSH and the target protein. During the catalytic process, the disulfide bond of S-glutathionylated protein is attacked by the thiolate of nucleophilic cysteine at the active site of Grx, releasing the protein thiol in a reduced form while becoming itself glutathionylated (Biswas et al., 2006). Then GSH attacks glutathionylated Grx, releasing reduced Grx and GSSG to maintain redox homeostasis.

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**Materials and Methods**

**Bacterial strains and culture conditions.** Bacterial strains and plasmids used in this study are listed in Table S1 (Jakoby et al., 1999; Schäfer et al., 1994; Si et al., 2014, 2015; Tauch et al., 2002). C. glutamicum and E. coli strains were grown at 30°C and 37°C, respectively, in Luria-Bertani (LB) medium with vigorous shaking (220 rpm). To generate and maintain mutants, a 0.5 M sorbitol-containing brain-heart broth (BHIS) medium was used. In-frame deletions were constructed by means of the method described previously (Shen et al., 2005). For complementation, the pXMJ19-mrx1 derivatives were transformed into an Δmrx1 in-frame deletion mutant by electroporation and expression in C. glutamicum was induced by the addition of 0.5 mM isopropyl β-D-thiogalactopyranoside (IPTG) into cultures (Si et al., 2018a). The antibiotics were added at the following concentrations: Kanamycin, 50 µg ml$^{-1}$ for E. coli and 25 µg ml$^{-1}$ for C. glutamicum; Nalidixic acid, 40 µg ml$^{-1}$ for C. glutamicum.

Glutathione dithioreductase (GR) (g3664-500un) and glutathione were purchased from Sigma.

**Cloning, expression and recombinant protein purification.** An open reading frame mutant of the mrx1 gene was constructed by overlapping (overlap) PCR, and pK18mobsacB-Δmrx1 was constructed by utilizing the suicide plasmid pK18mobsacB carrying kanamycin resistance gene and sacB gene (Si et al., 2018b; Su et al., 2018). According to DNA sequences of the mrx1 gene and its adjacent regions, two oligonucleotide primer pairs (Dmrx1 F1/Dmrx1-R1 and Dmrx1-F2/Dmrx1-R2) were formed. Then, the upstream 752 bp fragment and downstream 806 bp fragment of the mrx1 gene were amplified correspondingly using the primer pair Dmrx1 F1/Dmrx1-R1 and Dmrx1-F2/Dmrx1-R2, respectively. Next, the upstream and downstream fragments were fused together by overlapping PCR with the primer pair Dmrx1-F1/Dmrx1-R2. The resulting PCR products and the plasmid pK18mobsacB were cut with BamHI and SalI, respectively. Then, PCR products were cloned into pK18mobsacB with similar sites; subsequently, we obtained the recombinant plasmid pK18mobsacB-Δmrx1.

To produce pXMJ19-mrx1, a primer pair CMrx1-F1/CMrx1-R was designed to amplify the DNA fragments of the open reading frames region of the mrx1 gene from C. glutamicum genomic DNA. The amplified DNA fragments were cut with BamHI and EcoRI and then cloned into a pXMJ19 vector between BamHI and EcoRI sites. For getting the lacZ reporter vector pK18mobsacB-P$_{mrx1}$-::lacZ, the fusion of mrx1 promoter to the lacZ reporter gene by overlap PCR was created. First, two oligonucleotide primer pairs, namely P$_{mrx1}$ F$_{mrx1}$ and lacZ-F/lacZ-R, were designed in the first round of PCR to amplify the 200 bp mrx1 promoter DNA fragments (corresponding to nucleotides +12 to –188 relative to the translational start codon (ATG) of the mrx1 gene) and the lacZ DNA fragments, respectively. Second, P$_{mrx1}$-F/lacZ-R as primers and the first round PCR products as templates were used to carry out the second round of PCR, and the resulting fragments, cut with SmaI and BamHI, were inserted into pK18mobsacB between SmaI and BamHI sites to obtain the pK18mobsacB-P$_{mrx1}$-::lacZ fusion construct (Si et al., 2018b; Su et al., 2018). The fidelity of the construct was confirmed by DNA sequencing (Sangon Biotech, Shanghai, China).

In order to achieve the purpose of expressing and purifying His$_6$-tagged recombinant protein, the pET28a-mrx1,
pET28a-mrx1-C12S, pET28a-mrx1-C15S, pET28a-trx, pET28a-trxR, pET28a-mrx, and pET28a-sigH were transformed into *E. coli* BL21 (DE3). Recombinant proteins were purified according to the previously described method (Su et al., 2018). Cleavage of the His$_6$ tag was performed by adding 10 units of Enterokinase-Max (Invitrogen, Karlsruhe, Germany) and incubated at 4°C overnight to conduct subsequent enzyme activity analysis. Cleaved tag and uncleaved proteins were removed from the tag-free protein using Ni-NTA agarose. All enzymes were purchased from Sigma-Aldrich (St. Louis, MO). The proteins without His$_6$ tag were dialyzed by PBS at 4°C and concentrated for further experiments (>95% purity as estimated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)).

**Oxidants, alkylating agents, heavy metals and antibiotics sensitivity test.** According to the experimental method of Rawat et al. (2002), we performed the Disk diffusion assays for oxidative agents, alkylating agents, and heavy metals. Briefly, cells were grown to the mid-log phase and a lawn of cells was plated onto LB plates. The paper disks with various amounts of agents (10 μl) were placed into the plates and were allowed to dry and the plates were incubated at 30°C for 2 to 3 days. An antibiotics experiment was performed according to Liu et al. (2013). We offered the same cultivation conditions for the *C. glutamicum* RES167 parental strain containing the high copy number of empty plasmid pXMJ19 (the strains were named WT), ∆mrx1 (strains lacking mrx1 gene containing empty pXMJ19) and ∆mrx1* (Δmrx1 was complemented with plasmids carrying the wild-type mrx1 gene). Overnight cultures of WT, ∆mrx1, and ∆mrx1* strains were diluted in 1:100 in fresh LB medium with an indicated concentration of various antibiotics and cultivated for over 24 h with shaking at 30°C (Liu et al., 2013). We monitored the level of cell growth by measuring the optical density at 600 nm. All assays were performed in triplicate.

**Construction of chromosomal fusion reporter strains and β-galactosidase assay.** The lacZY fusion reporter plasmid pK18mobsacB-P$_{mrx1}$::lacZY was transformed into a *C. glutamicum* parental strain containing the high copy number of empty plasmid pXMJ19 (the strains were named WT), ΔsigH (strains lacking sigH gene containing empty pXMJ19) and ΔsigH* (ΔsigH was complemented with plasmids carrying the wild-type sigH gene) by electroporation. The introduced pK18mobsacB derivatives were integrated into the chromosome using fusion promoter regions homologous to the genome of *C. glutamicum* by single crossover, and then the chromosomal pK18mobsacB-P$_{mrx1}$::lacZY fusion reporter strain was selected by plating on LB agar plates containing 40 μg ml$^{-1}$ nalidixic acid, 25 μg ml$^{-1}$ kanamycin, and 10 μg ml$^{-1}$ chloramphenicol (Shen et al., 2005). The resulting strains were grown in LB medium to an optical density at 600 nm of 0.6–0.7 and then treated with different reagents of various concentrations at 30°C for 30 min. β-galactosidase activities were assayed with o-Nitrophenyl β-D-galactopyranoside (ONPG) as the substrate (Griffith and Wolf, 2002; Miller, 1992). The standard assay for quantitating the amount of β-galactosidase activity in cells, originally described by Miller (1992) for assay of bacterial cultures, involves spectrophotometric measurement of the formation of the yellow chromophore o-nitrophenol (ONP) as the hydrolytic product of the action of β-galactosidase on the colorless substrate o-Nitrophenyl β-D-galactopyranoside (ONPG). The β-galactosidase data represent the means of one representative assay performed in triplicate, and the error bars represent the standard deviation.

**Electrophoretic Mobility Shift Assay (EMSA).** EMSA was performed according to the method of Si et al. (2018a). A DNA promoter (about 150 bp) containing the predicted SigH binding site was amplified from the mrx1 promoter region using the primer pair Emrx1-F/Emrx1-R, named P$_{mrx1}$. The binding reaction mixture (20 μl) contained 10 mM Tris-HCl (pH 7.4), 5 mM MgCl$_2$, 50 mM KCl, 5% glycerol, 0.1% Nonidet P 40 (NP40), 1 μg poly (dIdC), 20 ng mrx1 DNA promoter, and 0–5.0 μg SigH. A DNA fragment (20 ng) amplified from the mrx1 open reading frame, but not the mrx1 DNA promoter, was used as a negative control. Then, the reaction mixture was incubated at room temperature for 30 min, followed by electrophoresis on a 10% non-denatured polyacrylamide gel and full staining for 30 min with a 10,000-fold diluted SYBR gold nucleic acid staining solution (molecular probe). The DNA bands were observed with UV light at 254 nm. As negative controls, a 150 bp DNA fragment (20 ng) amplified with primers Control-F and Control-R and full staining for 30 min with a 10,000-fold diluted SYBR gold nucleic acid staining solution were included in the binding assays.

**Detection of S-mycythiolated Mrx1:C15S and Mrx1-S$_2$ in vitro.** S-mycythiolated Mrx1:C15S (Mrx1:C15S-SSM, the mixed disulfide between MSH and Mrx1:C15S-SSH) was produced according to the method of Chi et al. (2014). MSH was purified from *C. glutamicum* RES167 as described previously (Si et al., 2018b). First, Mrx1:C15S (50 μM) was incubated together with excessive MSH (6 M). Then, 1 mM H$_2$O$_2$ was added into it for 30 min, and the excess MSH and H$_2$O$_2$ were removed by ultrafiltration. The sample was loaded on Ni-NTA His-Bind resin-containing column (Novagen, Wisconsin, USA) and S-mycythiolated Mrx1:C15S that was not combined with resin was directly collected. The collected solution containing S-mycythiolated Mrx1:C15S was purified on a superdex75HR column (equilibrated with 50 mM HEPES/NaOH pH 8.0, 150 mM NaCl). Prx-SSM (the mixed disulfide between MSH and peroxiredoxin (Prx)) were produced using a similar procedure as described above. Mrx1-S$_2$ and Trx-S$_2$ were produced according to the method of Van Laer et al. (2012). After Mrx1-S$_2$ and Trx-S$_2$ were oxidized by excess 10-molar diamide for 30 min at room temperature, excess diamide was removed by ultrafiltration. The sample was loaded on Ni-NTA His-Bind resin-containing column (Novagen, Wisconsin, USA) and Mrx1-S$_2$ and Trx-S$_2$ that were not combined with resin was directly collected. The collected solution containing Mrx1-S$_2$ and Trx-S$_2$ were purified on a superdex75HR column (equilibrated with 50 mM HEPES/NaOH pH 8.0,
150 mM NaCl). Pure Mrx1: C15S-SSM and Mrx1-S2 were confirmed by Matrix-Assisted Laser Desorption/Ionization Time of Flight Mass Spectrometry (MALDI-TOF MS).

The MSH/mycothiol disulfide reductase (Mtr), GSH/GR, and Trx/thioredoxin reductase (TrxR) electron transfer assays. The reduction of Mrx1: C15S-SSM and Mrx1-S2 was performed by monitoring the decrease of absorbance at 340 nm arising from NADPH oxidation. The assay mixtures contained 50 mM HEPE/NaOH pH 8.0 buffer, 500 µM NADPH, 50 µM Mrx1 (Mrx1::C15S-SSM or Mrx1-S2), and a reduced Trx-generating system (4 µM TrxR and 40 µM Trx), or MSH system (4 µM Mtr and 500 µM MSH) as the possible electron donors. The GR assay mixture consisting of 4 µM GR, 500 µM GSH and 500 µM NADPH was used as the control. We calculated all concentrations taking into account the subsequent addition of Mrx1::C15S-SSM or Mrx1-S2. After the mixtures were incubated for 30 min at 37°C, the reactions were started by adding Mrx1::C15S-SSM or Mrx1-S2 with different concentrations. For each reaction mixture with a control well, Mrx1::C15S-SSM or Mrx1-S2 deleted from the reaction mixture was added. Three independent experiments were performed at each concentration.

Enzymatic activity assay. Ribonucleotide reductase (RNR) activity, insulin disulfide reduction, DTNB (5,5′-dithiobis-(2-nitrobenzoic acid)) assay, and peroxidase activity were performed based on the method described by Si et al. (2014). Dehydroascorbic acid (DHA) and hydroxyethyl disulfide (HED) were utilized to measure DHA reductase activity or HED activity (Board and Si et al. (2014). Dehydroascorbic acid (DHA) and hydroxyethyl disulfide (HED) were utilized to measure DHA reductase activity or HED activity (Board and Menon, 2016; Si et al., 2014). Briefly, the kinetic parameters were determined in the presence of a varying concentration of DHA or HED (0–20 mM) with a saturating concentration of 50 mM MSH. The enzyme reactions were measured in 50 mM potassium phosphate buffer (pH 7.6), 250 µM NADPH, 1 µM Mrx1, and 5 µM Mtr. The assay was performed at 25°C for 10 min and the absorption monitored at 340 nm was recorded. The activity was determined after subtracting the spontaneous reduction rate observed in the absence of Mrx1, and the number of micromoles of NADPH oxidized per second per micromole of enzyme (i.e. turnover number, s⁻¹) was calculated using the molar absorption coefficient of NADPH at 340 nm (ε₃₄₀) of 6220 M⁻¹ cm⁻¹. Three independent experiments were performed at each substrate concentration. The kₗ and kₚvalues of Mrx1 for HED or DHA substrates were obtained from a nonlinear fit with the Michaelis-Menten equation using the program GraphPad Prism 5. GST activities were spectrophotometrically determined for different substrates (Han et al., 2019).

Peroxidase activity was also detected by using the ferox xylenol orange (FOX) assay (Wolff, 1994).

Quantitative RT-PCR analysis. Total RNA was isolated from exponentially growing WT, ΔsigH and ΔsigH* strains exposed to different toxic agents of indicated concentrations for 30 min using the RNeasy Mini Kit (Qiagen, Hilden, Germany) along with the DNase I Kit (Sigma-Aldrich, Taufkirchen, Germany). Purified RNA was reverse-transcribed with random 9-mer primers and MLV reverse transcriptase (TaKaRa, Dalian, China). Quantitative RT-PCR analysis (7500 Fast Real-Time PCR; Applied Biosystems, Foster City, CA) was performed as described previously (Su et al., 2018). The primers used are listed in Table S1. To obtain standardization of results, the relative abundance of 16S rRNA was used as the internal standard.

Statistical analysis. GraphPad Prism Software was used to carry out statistical analyses (GraphPad Software, San Diego California USA).

Results and Discussion

Roles of Mrx1 in oxidative stress response in C. glutamicum

As is known, Grx participates in defending against external oxidants such as hydroperoxides or the superoxide generator menadione to maintain cellular homeostasis (Luikenhuis et al., 1998). Thus, to assess the role of C. glutamicum Mrx1 in defending against condition stress, we tested the growth curve of C. glutamicum RES167 parental strain containing the high copy number of empty plasmid pXMJ19 (the strains were named WT), Δmrx1 (strains lacking mrx1 gene containing empty pXMJ19) and Δmrx1⁺ (Δmrx1 was complemented with plasmids carrying the wild-type mrx1 gene) caused by the paper disks (r = 5 mm) with several oxidative stress inducing agents. The dot plot shows the mean and standard error of the 10 samples sets for each agent. H–I. The growth (OD₆₀₀) of WT and Δmrx1⁺ strains after 24 h at 30°C in LB medium containing various antibiotics was recorded. Mean values with standard deviations (error bars) from at least three repeats are shown. **P ≤ 0.01; *P ≤ 0.05.
mutant than for the WT strain, in agreement with the results of Van Laer et al. (2012) reported for *Mycobacterium smegmatis* Mrx1. To confirm that the sensitivity to xenobiotics may occur when lacking Mrx1, complementation experiments were carried out in ∆mrx1 null mutants. As shown in Fig. 1, ∆mrx1+, corresponding complementary strains containing the *C. glutamicum* mrx1 genes provided by plasmid pXMJ19 in trans, almost fully complemented the growth defect of the mrx1 null mutants under various stresses.

Inducing the formation of ROS was a general mechanism that antibiotics induced cellular oxidative conditions (Van Acker and Coenye, 2017). To examine the impact of Mrx1 on the resistance to antibiotics, the strain growth in LB broth media supplemented with different antibiotics was detected. Although WT strain and the ∆mrx1 mutant had nearly identical levels of growth in the LB broth media, all the tested antibiotics (neomycin, vancomycin, gentamycin, and azithromycin) markedly inhibited the growth of a mutant-lacking mrx1 gene relative to WT. Together, these results showed the limited sensitivity of Mrx1, suggesting that Mrx1 might play an important role in the activation of MSH protected proteins after oxidative stress.

Oxidized Mrx1-SSM and Mrx1-S2 were specifically reduced with electrons from the MSH/Mtr/NADPH pathway

As previously reported, when dithiol Grx reduced protein disulfides to reduced thiols, the formation of intramolecular bridges or mixed disulfides between Grx and the GSH molecule occurs in oxidized Grx (Herrero and de la Torre-Ruiz, 2007). In the GSH-utilizing Gram-negative bacterium *E. coli*, proteins with intra- or inter-molecular disulfides were commonly reduced by Trx, and thioldisulfide were reduced by Grx, respectively. Oxidized Trx was reduced by using NADPH as an electron source. The oxidized Grx was reduced by the GSH reduction system, and the GSSG was recovered by glutathione reductase (GR) at the expense of NADPH (Van Laer et al., 2012). The sulfhydryl group on the active site Cys of Grx was regenerated by reducing systems. To achieve the purpose of catalytic circulation *in vivo*, Mrx1, belonging to a new class of dithiol Grx-like proteins was also oxidized to Mrx1-SSM and Mrx1-S2. Similarly, to regain reduced Mrx1, oxidized Mrx1 must be linked to the electron transfer pathway. Besides the TrxR/NADPH system, *C. glutamicum* had an alternative MSH reduction system (MSH/Mtr/NADPH) that was unique to MSH-producing high G+C Gram-positive Actinobacteria, and functionally equivalent to the GR/GSH system in most gram-negative bacteria (Villadangos et al., 2011). The efficiency of both TrxR/NADPH and MSH/Mtr/NADPH systems to reduce oxidized Mrx1-SSM and Mrx1-S2 was investigated. *C. glutamicum* Mrx1 was characterized with the active site sequence motif (i.e. C12PYC15). Moreover, *C. glutamicum* Mrx1 got S-mycothiolated on the N-terminal peptide of the N-terminal peptide of the C12XXC15 active site. In other words, oxidized Mrx1-SSM was actually a mixed disulfide intermediate that was formed between the first reactive cysteine (Cys12) of Mrx1 and MSH (Pedre et al., 2015; Si et al., 2015). Thus, to make it clear that S-mycothiolation was formed at the N-
terminal Cys of Mrx1 at the CXXC active sites, a single variant of Mrx1 protein, namely, Mrx1:C15S, was used instead of Mrx1. As shown in Fig. 2, Mrx1:C15S-SSM and Mrx1-S2 were added as substrates for two electron transfer pathways, respectively. By monitoring the reduction in absorption of NADPH consumption at 340 nm, we found that oxidized Mrx1:C15S-SSM and Mrx1-S2 obtained electrons transferred by the MSH/Mtr/NADPH pathway, while no electron transfer was observed when the TrxR/NADPH electron transfer pathway was used (Figs. 2A, 2B, 2C, and 2D). Further, we tested the possibility that oxidized Mrx1:C15S-SSM and Mrx1-S2 obtained electrons from the TrxR/NADPH pathway when an electron transfer was observed when the TrxR/NADPH electron transfer pathway was used (Figs. 2A, 2B, 2C, and 2D). We tested the possibility that oxidized Mrx1:C15S-SSM and Mrx1-S2 obtained electrons transferred by the MSH/Mtr/NADPH pathway, while no electron transfer was observed when the TrxR/NADPH electron transfer pathway was used (Fig. 3A, 3B, 3C, and 3D). We tested the possibility that oxidized Mrx1:C15S-SSM and Mrx1-S2 obtained electrons transferred by the MSH/Mtr/NADPH pathway, while no electron transfer was observed when the TrxR/NADPH electron transfer pathway was used (Fig. 3A, 3B, 3C, and 3D). We tested the possibility that oxidized Mrx1:C15S-SSM and Mrx1-S2 obtained electrons transferred by the MSH/Mtr/NADPH pathway, while no electron transfer was observed when the TrxR/NADPH electron transfer pathway was used (Fig. 3A, 3B, 3C, and 3D).

Although not present in C. glutamicum, we also tested the reduction ability of GSH for reducing oxidized Mrx1:C15S-SSM. As shown in Figs. 2G and 2H, oxidized Mrx1:C15S-SSM was not reduced by GSH in a coupled enzyme assay with an NADPH regeneration pathway. Additionally, we found that oxidized Mrx1:C15S-SSM and Mrx1-S2 obtained electrons transferred by the MSH/Mtr/NADPH pathway in C. glutamicum.

Fig. 3. Mrx1 reduced mycothiol mixed disulfide and disulfide bonds by the MSH/Mtr/NADPH pathway.

A–D. Reduction of RNR (A), insulin (B), DTNB (C), and Trx1-S2 (D) by Mrx1 (10 µM) coupled to the MSH/Mtr/NADPH regeneration system. The reduction of RNR (A), insulin (B), and Trx1-S2 (D) 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 (C). E–F. The mixed disulfide between TrxR and MSH was tested as substrate in the respective pathways and the consumption of NADPH due to the reduction of HED-MSH was monitored by A340 decrease.

Fig. 4. Peroxidase activity of Mrx1.

A–C. The activity of Mrx1 was calculated by measuring NADPH oxidation at 340 nm in the presence of the MSH/Mtr/NADPH system. D–F. Peroxidase activities of Mrx1 examined by the Fox assay. Similar results were obtained in three independent experiments, and data shown are from one representative experiment.

Mrx1 reduced MSH mixed disulfide bond and disulfide bond using monothiol and dithiol mechanism

Dithiol Grxs with the CPY/FC sequence at the active site employed two different mechanisms for reducing disulfide bridges: the 2-Cys mechanism and the 1-Cys mechanism. The 2-Cys mechanism required both cysteine residues of the active site of CXXC motif in Grxs (Bushweller et al., 1992). The most N-terminal cysteine formed a mixed disulfide between the Grx protein and the target protein, and the C-terminal cysteine was required for solving this intermediate dithiol bridge. GSH molecules restored the reduced active state of Grx. The 1-Cys mechanism of action only required N-terminal cysteine of the active site of the CXXC motif in Grxs (Bushweller et al., 1992) and was employed to reduce the mixed disulfide between GSH and the target protein. That is to say, dithiol Grxs could not only reduce dithiol groups by the 2-Cys mechanism, but also reduced mixed disulfides between a protein and a glutathione molecule by the 1-Cys mechanism. Thus, it was interesting to explore whether C. glutamicum Mrx1, the protein which shared a high similarity to dithiol Grxs, also exhibited the equivalent function of dithiol Grxs. To detect this, we first investigated the capability of the dithio-disulfide exchange reaction by using RNR, insulin, and DTNB as substrates, and the coupled NADPH oxidation by MSH/Mtr was monitored at 340 nm. As shown in Fig. 3A, Mrx1 could function as a reductant for the class Iβ RNR with MSH/Mtr/NADPH as the electronic donor system. To assess whether C. glutamicum Mrx1 was specifically involved in the reduction of class Iβ RNRs or possessed general thiol-disulfide redox activity as Trx1, we further examined the capacity of Mrx1 to catalyze the reduction of insulin and the small molecule artificial disulfide compound DTNB. Insulin reduction provided the basis for a classical assay used to test the dithiol-disulfide exchange activity (Holmgren, 1979). As shown in Fig. 3B, Mrx1 was shown to reduce insulin disulfides in the presence of MSH/Mtr/NADPH. Moreover, we found that Mrx1 had an almost identical efficiency as Trx1. We further explored the capacity of C. glutamicum Mrx1 to reduce an artificial disulfide substrate DTNB used as an exposed...
disulfide bond. As shown in Fig. 3C, the Mrx1 caused a catalytic relevant reduction of DTNB in the presence of MSH/Mtr/NADPH.

The Grx system acted as a backup of TrxR to reduce Trx in cells (Du et al., 2012). Therefore, to investigate the generality of this phenomenon, the enzyme assays were performed with oxidized Trx1-S2 as substrates, and the coupled NADPH oxidation by Mrx1/MSH/Mtr was monitored at 340 nm. As shown in Fig. 3D, Mrx1 could reduce Trx1-S2 in the presence of MSH/Mtr/NADPH.

To determine whether the conserved cysteines in the CXXC motif of Mrx1 were essential for its function of reducing disulfide bonds, we created two single substitution mutations of Cys to Ser in the C12XXC15 motif (Mrx1:C12S and Mrx1:C15S). We performed the aforementioned insulin, DTNB, RNR and Trx1-S2 reduction assays by using the MSH/Mtr/NADPH regeneration system to examine whether these mutations would alter the Mrx1 function. As shown in Figs. 3A–D, the activities of the Mrx1:C12S and Mrx1:C15S variants were significantly less efficient than that observed in the wild type Mrx1. These results indicated that both conserved cysteines in the CXXC domain are extremely important for the Mrx1 function. The results further indicated that Mrx1 was functioning as a disulfide reductase with a 2-Cys mechanism.

Next, to determine whether Mrx1 had the capacity to reduce mixed disulfides as Grx, HED-SSM (the mixed disulfide formed between HED and MSH) and Prx-SSM were first used in the MSH/Mtr/NADPH-coupled assay. As shown in Figs. 3E and F, Mrx1 WT displayed an effective reduction capacity for HED-SSM and Prx-SSM, and it was determined by the consumption of NADPH monitored spectrophotometry at 340 nm. The results supported the previous research report of Ordóñez et al. (2009) and Chi et al. (2014) about Mrx1. Moreover, the very high activity of variant Mrx1:C15S was comparable to that of Mrx1 WT, which has been shown not to reduce the disulfide of RNR, insulin, and DTNB.

However, the levels of electron transfer decrease sharply in the presence of Mrx1:C12S. The conclusion was that Mrx1 regarded the N-terminal nucleophilic cysteine of the CXXC motif as the necessary active site and performed the function as a monothiol mixed disulfide reductase. In addition, previous research had shown that dithiol Grxs displayed dehydroascorbic acid reductase (DHAR) activity in vitro assays (Bushweller et al., 1992). To determine
whether Mrx1 had DHAR activity become more interesting. As shown in Table S2, Mrx1 proteins did display the activity of DHA reductase when a fixed concentration of MSH and different concentrations of DHA was used. Similarly, no MSH-transferase activity was detected for the recombinant Mrx1 proteins, when CDNB and monobromobimane (mBBr) were used as substrates.

**Mrx1 had no peroxide reductase activity**

Dithiol Grxs that possessed GSH-dependent peroxidase activity had been previously described (Collinson et al., 2002; Lee et al., 2002). Thus, we next tested whether C. glutamicum Mrx1 had the peroxide reductase activity. To do so, the absorbance of the MSH/Mtr/NADPH system at 340 nm was monitored under H₂O₂, CHP and t-BHP circumstances.

As shown in Figs. 4A–C, the addition of Mrx1 to the MSH/Mtr/NADPH system did not result in a reduction of the absorption at 340 nm compared with the MSH/Mtr/NADPH system alone. With the FOX assay, we tested the residual peroxide concentration after the reaction. When we added Mrx1 to the MSH/Mtr/NADPH system, there was no additional degradation for H₂O₂, CHP, and t-BHP compared with the MSH/Mtr/NADPH system alone (Figs. 4D, 4E, and 4F). Thus, C. glutamicum Mrx1 did not directly eliminate peroxide. However, we believed that it possibly maintained an indirect association with the oxidative stress response by keeping the necessary protein cysteine in the reduced state.

**The regulation of Mrx1 by SigH in C. glutamicum**

*C. glutamicum* mrx1 mutants had been shown to exhibit a degree of sensitivity in the circumstances of various agents, and qRT-PCR and *lacZY* activity profiling were employed to examine whether *mrx1* expression responded to these toxic stress inducers at the transcriptional level. The *lacZY* activity of a *P*ₘᵢᵣₓ₁::*lacZY* chromosomal promoter fusion reporter in the RES167 parental strain was determined in bacterial cells, which were unexposed or exposed to different concentrations of reagents (Fig. 5A).

The level of *mrx1* expression was significantly increased in the WT (*P*ₘᵢᵣₓ₁::*lacZY*) reporter strains exposed to various agents, respectively, as compared with untreated samples (Fig. 5A). Furthermore, expression of the *P*ₘᵢᵣₓ₁::*lacZY* fusion displayed a dose-dependent increase under adverse environmental conditions (Fig. 5A). These results clearly demonstrated that environmental stresses induced the expression of *mrx1* and directly improved the tolerance of *C. glutamicum* to stress conditions with the increase of expression quantity. A similar dose-dependent pattern of the *mrx1* expression in response to different agents was also observed in qRT-PCR analysis (Fig. 5B).

The stress-responsive extracytoplasmic function-sigma (ECF-σ) factor (SigH), has been reported to respond to thiol-oxidative stress and regulate the expression of multiple resistance genes (Zhang et al., 2013). Previously, we found that SigH had a positive regulation function for Mrx1 by the transcriptomic analysis based on the RNA sequencing (RNA-seq). To further confirm that *mrx1* expression was subjected to SigH regulation, the transcription of chromosomal *P*ₘᵢᵣₓ₁::*lacZY* fusions and qRT-PCR analysis were performed, by constructing the Δ*sigH* mutant and the complementary strain Δ*sigH*⁺ tested. Significant decrease of *lacZY* activity was observed in exponentially grown Δ*sigH* mutant exposed to stress conditions, as compared with WT (Fig. 5A). Under agent-inducible or non-inducible conditions, the reduced *mrx1* expression was fully recovered in the complementary strain Δ*sigH*⁺, compared with the Δ*sigH* mutant (Fig. 5A). SigH-dependent *mrx1* activation was also confirmed by qRT-PCR analysis (Fig. 5B). These data suggested that SigH positively regulated the expression of *mrx1*.

To examine the direct interaction between His₅₆-SigH and *mrx1* promoter DNA, a promoter region DNA fragment containing a putative SigH binding site amplified by PCR with a *P*ₘᵢᵣₓ₁-F/*P*ₘᵣₓ₁-R primer pair was incubated with purified His₅₆-SigH protein of various concentrations in vitro. The reaction mixture was then analyzed by an electrophoretic mobility shift assay (EMSA). As shown in Fig. 5C, His₅₆-SigH directly bound to the *mrx1* promoter region DNA fragment, whereas a negative control DNA amplified from the *mrx1* coding region showed no interaction with His₅₆-SigH. These data provided direct evidence that His₅₆-SigH bound to the promoter region of *mrx1* to regulate its expression as a regulator.

**Conclusions**

In this work, we have demonstrated that *C. glutamicum* mycoredoxin-1 (Mrx1) with a CGYC catalytic motif belonged to a new class of Grx-like proteins. *C. glutamicum* Mrx1 acted as an oxidoreductase exclusively coupled to the MSH/Mtr/NADPH electron pathway. Not only could it reduce MSH mixed disulfides by a monothiol mechanism, but it could also reduce dithiol groups by a dithiol mechanism, similar to the reduction mechanism of Fernandes et al. (2005), reported for dithiol Grxs. The *mrx1* deletion mutant of *C. glutamicum* showed an increased, but limited, sensitivity to H₂O₂, MEN, diamide, IAM, CdCl₂, and antibiotics. Taken together, based on the results of the present study, new avenues in the general understanding of the oxidative stress defence mechanisms of *C. glutamicum* Mrx1 have been opened.

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**Supplementary Materials**

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

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