Involvement of a mycothiol-dependent reductase NCgl0018 in oxidative stress response of Corynebacterium glutamicum

(Received November 18, 2020; Accepted March 16, 2021; J-STAGE Advance publication date: September 6, 2021)

Keyan Chen #, Xiaoyang Yu #, Xinyu Zhang, Xiaona Li, Yang Liu, Meiru Si, Tao Su *

College of Life Sciences, Qufu Normal University, Qufu, Shandong 273165, China

Running title: Function of NCgl0018 in stress

# These authors contributed equally to this work.

* Corresponding author:

Tao Su

E-mail vincenttao2@163.com

Tel: 86-15563718633
Abstract

*Corynebacterium glutamicum* is an important industrial strain for amino acids and a key model organism for human pathogens. The study of *C. glutamicum* oxidoreductases, such as mycoredoxin 1 (Mrx1), dithiol-disulfide isomerase DsbA, and DsbA-like Mrx1, is helpful for understanding the survival, pathogenic infection, and stress resistance of its homologous species. However, the action mode and enzymatic function of *C. glutamicum* NCgl0018 preserving the Cys-Pro-Phe-Cys motif, annotated as a putative DsbA, have remained enigmatic. Here, we report that the NCgl0018-deleted strain increased sensitivity to various oxidative stresses. The ncgl0018 expression was induced in the stress-responsive extracytoplasmic function-sigma (ECF-σ) factor SigH- and organic peroxide- and antibiotic- sensing regulator (OasR)-dependent manner by stress. NCgl0018 reduced S-mycocthiolated mixed disulfides and intramolecular disulfides via a monothiol-disulfide mechanism preferentially linking the mycothiol/mycothione reductase/NADPH electron pathway. Site-directed mutagenesis confirmed Cys107 was the resolving Cys residue, while Cys104 was the nucleophilic cysteine that was oxidized to a sulfenic acid and then could form an intramolecular disulfide bond with Cys107 or a mixed disulfide with mycothiol under stress. Biochemical analyses indicated that NCgl0018 lacked oxidase properties like the classical DsbA. Further, enzymatic rates and substrate preferences of NCgl0018 were highly similar to those of DsbA-like Mrx1. Collectively, our study presented the first evidence that NCgl0018 protected against stresses by functioning as a novel DsbA-like Mrx1 but not DsbA and Mrx1.

**Keywords:** Dithiol-disulfide isomerase DsbA, oxidative stress, Mrx1, *Corynebacterium glutamicum*.

Introduction

*Corynebacterium glutamicum* is a nonpathogenic gram-positive bacterium used as an important industrial strain for amino acid, nucleic acid, organic acid, alcohol, and biopolymer production and is a key model organism for studying pathogen evolution (Portevin et al., 2005). During fermentation, *C. glutamicum* inevitably encounters an adverse stress environment, causing excessive reactive oxygen species (ROS) generation (Lee et al., 2013; Oide et al., 2015). The ROS
are not only highly reactive molecules that are capable of damaging cellular constituents, such as DNA, RNA, lipids and proteins, resulting in irreversible sulfidation products, inter- or intra-protein disulfides (Protein-S-S-Protein, Protein-S$_2$), and mixed disulfides with low-molecular-weight (LMW) thiol, but also, at the same time, results in cells constantly being exposed to oxidative stress (Dalle-Donne et al., 2008). Oxidative stress can reduce the survival of C. glutamicum and affect the yield and quality of bio-based products. As such, C. glutamicum’s defenses against oxidative stress has resulted in much scientific attention, and they have been analyzed in this strain. In fact, in adaptation to oxidative stress, C. glutamicum has evolved multiple enzymatic and non-enzymatic antioxidant defenses to cope with endogenous ROS from metabolic processes, and exogenous ROS from adverse stress environment, and to maintain their cellular redox state, including the production of mycothiol (MSH) and protective enzymes such oxidoreductases (Lee et al., 2013; Oide et al., 2015; Liu et al., 2013; Milse et al., 2014; Si et al., 2018a; Si et al., 2018b; Imlay, 2015; Hillion and Antelmann, 2015; Antelmann and Helmann, 2011).

MSH, the most abundant LMW thiol, constitutes a redox buffer in the cytoplasm and is considered to be the main nonenzymatic antioxidant in the high-G+C-content gram-positive Actinobacteria, including a group of bacteria of medical, industrial, and environmental significance, such as members of Corynebacterium, Mycobacterium, Rhodococcus, and Streptomyces (Newton et al., 1995; Newton et al., 1996). The cysteine thiol of MSH can protect cells against ROS by directly clearing free radicals, and also by functioning to reduce disulfide bonds for antioxidant enzymes such as MSH peroxidase and methionine sulfoxide reductase (Couturier et al., 2012; Pedrajas et al., 2010; Pérez-Pérez et al., 2006). Protein S-mycothiolation, which is a post-translational modification that protects sensitive cysteines from overoxidation, is probably the link between stress resistance and MSH (Chi et al., 2014). Post-translational mycothiolation can be reversed with mycoredoxin-1 (Mrx1, NCgl0808) by a monothiolic or a dithiolic mechanism (Van Laer et al., 2012). Mrx1, small thiol-disulfide oxidoreductases containing a conserved catalytic site Cys-Pro-Tyr-Cys (C-P-Y-C), is exclusively linked to the MSH electron transfer pathway and its main function is to reduce protein-MSH mixed disulfides (Van Laer et al., 2012). Besides Mrx1, thioredoxin (Trx) and dithiol-disulfide isomerase (DsbA) are also the major oxidoreductases containing a conserved catalytic site (CXXC) with a
redox-active disulfide (Holmgren, 1985; Sutoh et al., 2019). Trx with the Cys-Gly-Pro-Cys (C-G-P-C) catalytic motif is essential for reducing disulfide bonds of target proteins and maintaining intracellular redox homoeostasis (Holmgren, 1985). *Escherichia coli* DsbA with the Cys-Pro-His-Cys (C-P-H-C) catalytic motif is an oxidase that can catalyze the oxidative refolding of RNase I (Sutoh et al., 2019). Interestingly, Rosado et al. (2017) found that *M. tuberculosis* Rv2466c with the Cys-Pro-Trp-Cys (C-P-W-C) catalytic motif and *C. glutamicum* NCgl2339 with the Cys-Pro-Phe-Cys (C-P-F-C) catalytic motif, annotated as DsbA, are novel MSH-dependent reductases. Rv2466c and NCgl2339 provide electrons to S-mycobothiolated mixed disulfides and intramolecular disulfides-containing substrate with a thiol-disulfide exchange mechanism preferably by linking the MSH/mycothione reductase (Mtr)/NADPH pathway. NCgl2339 displays very high demycothiolating activity toward an As (V) MSH thiol-arseno adduct compared with *C. glutamicum* Mrx1; disruption of the ncgl2339 gene resulted in lowered resistance to As (V) but mrx1 did not (Rosado et al. 2017). *M. tuberculosis* Rv2466c exhibits low demycothiolating activity toward HED-SSM compared with Mrx1 (Rosado et al. 2017). These results indicated that despite sharing a common feature of demycothiolation with Mrx1, Rv2466c and NCgl2339 have different enzymatic rates and substrate preferences from Mrx1. Moreover, Rv2466c and NCgl2339, exhibiting a high sequence similarity to DsbA, have no oxidase properties such as the canonical DsbA, indicating that they clearly are not DsbA oxidoreductase. Thus, according to the catalytic CXXC motif and physiological and biochemical differences, *M. tuberculosis* Rv2466c and *C. glutamicum* NCgl2339 were classified into a new DsbA-like Mrx1 cluster, preserving the C-P-W-C or C-P-F-C active-site sequence motif, which was different from *E. coli* oxidase and *C. glutamicum* NCgl0808 embedded in the DsbA (C-P-H-C) and Mrx1(C-P-Y-C) cluster, respectively (Fig. S1A). However, nothing is known about the universality and exact molecular mechanism of the DsbA-like Mrx1 cluster. Therefore, an in-depth analysis of the real mode of action, the nature of the electron donor pathway, as well as the biological function of DsbA has been the subject of active investigation, which can help us correctly classifying similar enzymes from other organisms.

Recently, Si et al. (2020), reported that *C. glutamicum* OasR (organic peroxide- and antibiotic-sensing regulator), a MarR family regulatory protein, negatively controlled the
expression of ncgl0018 gene encoding an oxidoreductase enzyme annotated as the putative DsbA of *C. glutamicum*. Although NCgl0018 was supposed to be another candidate in the antioxidant system of bacteria, its physiological functions have not been experimentally confirmed in *C. glutamicum*. Thus, in this study, we have chosen NCgl0018 and analyzed it further to gain insight into its role in developing stress resistance in *C. glutamicum*. By employing genetic, physiological and biochemical approaches, we have demonstrated that NCgl0018 plays an important role in developing oxidative stress resistance. We have also provided evidence for the mechanism of NCgl0018 protein in reducing disulfides by acting as a DsbA-like Mrx1.

**Material and methods**

**Bacterial strains and growth conditions**

The bacterial strains and plasmids used in this study are listed in Table S1. Luria-Bertani (LB) broth and LB agar plates were used for growing *E. coli* or *C. glutamicum* RES167. *E. coli* and *C. glutamicum* were cultivated at 37°C and 30°C, respectively, under vigorous agitation (220 rpm) as previously reported (Shen et al., 2005). Brain-heart broth medium containing 0.5 M sorbitol (BHIS) was used for producing and maintaining a mutant of a gene in *C. glutamicum* RES167 strain (Shen et al., 2005). For creating a ncgl0018 gene in-frame deletion in *C. glutamicum* RES167 strain, the pK18mobsacB-Δncgl0018 plasmids were introduced into *C. glutamicum* RES167 strain through electroporation according to the method of Shen et al. (2005), and then integrated into the chromosome of *C. glutamicum* RES167 strain through homologous recombination to execute single crossover. The transconjugants were selectively isolated on LB medium agar plates supplemented with 40 µg ml\(^{-1}\) nalidixic acid and 25 µg ml\(^{-1}\) kanamycin and controlled by PCR to verify the first crossover event. The transferred plasmids appeared as suicide in *C. glutamicum*; therefore, only transconjugant clones with the chromosomally integrated plasmid should be selected under the pressure of kanamycin (25 µg ml\(^{-1}\)). PCR amplification analysis and further fragment sequencing validated the correct chromosomal plasmid integration (Shen et al., 2005). Colonies with the correct integration were grown in LB broth until the stationary phase and then plated on LB medium agar plates containing 20% (w/v) sucrose and 40 µg ml\(^{-1}\) nalidixic acid at 30°C for selecting the second crossover (Shen et al., 2005). Colonies growing on 20% (w/v) sucrose and 40 µg ml\(^{-1}\) nalidixic acid were detected for kanamycin (25 µg ml\(^{-1}\)).
by parallel picking on LB plates in the presence of 40 µg ml$^{-1}$ nalidixic acid and 25 µg ml$^{-1}$ kanamycin or 40 µg ml$^{-1}$ nalidixic acid and 20% sucrose. Sucrose-resistant and kanamycin-sensitive strains were verified for in-frame deletion by PCR using the DNCgl0018-F1/DNCgl0018-R2 primer pair (Table S2). Complementation in Δncgl0018 mutants was carried out using the pXMJ19-ncgl0018 derivatives, which were transformed into Δncgl0018 mutants by electroporation [Su et al., 2019]. 0.5 mM isopropyl β-D-thiogalactopyranoside (IPTG) (Sigma-Aldrich) was added into the medium to induce the expression of ncgl0018 gene on the pXMJ19-ncgl0018 derivatives in complementary strains. For creating chromosomal fusion reporter strains, the plasmid pK18mobsacB-P_ncgl0018::lacZY was transformed into relevant C. glutamicum strains by electroporation. The chromosomal pK18mobsacB-P_ncgl0018::lacZY fusion reporter strain was selected on LB agar plates with 25 µg ml$^{-1}$ kanamycin and 40 µg ml$^{-1}$ nalidixic acid. All chemicals were of Analytical Reagent Grade purity or higher. 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; chloramphenicol, 20 µg ml$^{-1}$ for E. coli and 10 µg ml$^{-1}$ for C. glutamicum.

Plasmid Construction

Primers used in this study are listed in Table S2. Primers for the amplification of genes and quantitative real-time transcription-PCR (qRT-PCR) were synthesized at Sangon Biotech Co., Ltd. (Shanghai, China). pET28a vector (Novagen) was used for expression in this study. The ncgl0018 gene region of C. glutamicum was amplified by PCR with primer pair ONCgl0018-F and ONCgl0018-R from genomic DNA of C. glutamicum RES167 and subcloned into pET28a vector between EcoRI and XhoI sites, yielding pET28a-ncgl0018.

The suicide plasmid pK18mobsacB-Δncgl0018 was obtained by introducing a 600 bp in-frame deletion by two-step recombination as described previously (Si et al., 2018a; Su et al., 2018). First, two DNA fragments flanking and overlapping the distal parts of ncgl0018 were amplified from genomic DNA of C. glutamicum RES167. Two oligonucleotide primer pairs, namely DNCgl0018-F1/DNCgl0018-R1 and DNCgl0018-F2/DNCgl0018-R2 listed in Table S2, were made and used for the amplification (Sangon Biotech Co., Ltd., Shanghai, China). Primer pair DNCgl0018-F1/DNCgl0018-R1 was used to amplify the ncgl0018's upstream 720 bp
fragment; while primer pair DNCgl0018-F2/DNCgl0018-R2 was used to amplify the ncg0018's downstream 780 bp fragment. The upstream and downstream fragments were ligated together using overlap PCR with the primer pair DNCgl0018-F1/DNCgl0018-R2. The resulting PCR products were cut with BamHI and HindIII and subsequently subcloned into pK18mobsacB vector between BamHI and HindIII sites to produce plasmid pK18mobsacB-Δncg0018.

For obtaining pXMJ19-ncg0018, the primer pair CNCgl0018-F/CNCgl0018-R was used to amplify DNA fragments of open reading frames region of ncg0018 gene from C. glutamicum genomic DNA. The resulting DNA fragments were cut with SalI and BamHI and then subcloned into pXMJ19 vector between SalI and BamHI sites.

To create the ncg0018:C104S mutation construct, site-directed mutagenesis was carried out by two rounds of PCR (Si et al., 2018b). Briefly, two oligonucleotide primer pairs, namely DNCgl0018-F1/NCgl0018-C104S-R and NCgl0018-C104S-F/DNCgl0018-R2 listed in Table S2, were designed and synthesized. In the first round of PCR, primer pair DNCgl0018-F1/NCgl0018-C104S-R was used to amplify the 5′ prime region of ncg0018 (Fragment I); while primer pair NCgl0018-C104S-F/D NCgl0018-R2 was used to amplify the 3′ prime region of ncg0018 (Fragment II). The second round of PCR was performed by using ONCgl0018-F/ONCgl0018-R as primer pair and fragment I and fragment II as templates to get the ncg0018:C104S fragment. The EcoRI and Xhol cut ncg0018:C104S DNA fragments were cloned in pET28a plasmid digested with EcoRI and Xhol to create plasmid pET28a-ncg0018:C104S. The ncg0018:C107S fragments were obtained using a similar procedure as described above and cloned into pET28a to produce pET28a-ncg0018:C107S derivatives.

For obtaining the lacZY fusion reporter vector pK18mobsacB-ncg0018::lacZY, the fusion of ncg0018 promoter to the lacZY reporter gene by overlap PCR was performed. First, two oligonucleotide primer pairs namely P_{ncg0018}^F/P_{ncg0018}^R and lacZY-F/lacZY-R were designed in the first round of PCR to amplify the 352 bp ncg0018 promoter DNA fragments (corresponding to nucleotides +12 to -340 relative to the translational start codon (ATG) of ncg0018 gene) and the lacZY DNA fragments, respectively. Second, P_{ncg0018}^F/lacZY-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 PstI were inserted into pK18mobsacB between SmaI and PstI sites to
get the pK18mobsacB-P<sub>ncgl0018</sub>::lacZY fusion construct (Si et al., 2018b; Su et al., 2018).

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

**Overexpression and Purification of Recombinant Protein**

*E. coli* BL21(DE3) cells harboring the pET28a derivatives plasmid were grown in LB medium containing kanamycin (50 µg ml<sup>−1</sup>) at 37°C with shaking at 220 rpm. After cells were grown to an OD<sub>600</sub> nm of 0.6, 0.5 mM IPTG was added and then the cultures were cultivated for an additional 10 h at 22°C. Cells were harvested by centrifugation at 4°C. Cell pellets were suspended in 30 ml lysis buffer [10 mM Tris (pH 6.8), 10% glycerol and 10 mM β-Mercaptoethanol (β-ME)], sonicated, and centrifuged at 10,000 × g for 60 min. Target proteins in the supernatant were purified with the His • Bind Ni-NTA resin (Novagen, Madison, WI) according to the manufacturer’s instructions. The purified His<sub>6</sub>-tag proteins were dialyzed against PBS at 4°C and concentrated with Ultracel-10 kD membrane-containing Amicon Ultra-4 (Millipore, MA, USA) for further experiments (>95% purity as estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)).

**Agar-based disk diffusion assay**

Disk diffusion assays were performed for oxidants, antibiotics, alkylating agents, and heavy metal according to Rawat et al. (2002). Briefly, bacterial strains were grown to the stationary phase and 100 µl of culture containing about 10<sup>9</sup> cfu was spread onto 20-ml LB agar plates. Paper disks soaked with 10 µl of a stock solution of reagents were placed on top of the agar. Stock solutions were 200 mM hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), 0.5 mM hypochlorous acid (HClO), 5 mM diamide, 11 mM cumene hydroperoxide (CHP), 30 mM tert-butyl hydroperoxide (t-BHP), 70 mM 2, 4-dinitrochlorobenzene (CDNB), 1 mM iodoacetamide (IAM), 5 µg ml<sup>−1</sup> streptomycin (STR), 400 µg ml<sup>−1</sup> ciprofloxacin (CIP), 0.5 mM cadmium chloride (CdCl<sub>2</sub>), and 10 mM nickel sulfate (NiSO<sub>4</sub>). The disks were allowed to dry and the plates were incubated for 2 to 3 days at 30°C. The diameter of the inhibition zone was measured. Experiments were performed in triplicate.

**Electrophoretic mobility shift assay (EMSA)**

The binding of OasR and SigH to ncgl0018 promoters was performed using the method of Si et al. (2018a). Briefly, 211 bp ncgl0018 DNA promoter (P<sub>ncgl0018</sub>) containing the predicted His<sub>6</sub>-OasR or
His\textsubscript{6}-SigH binding site was amplified from the sequence (-993 to -782 relative to the ATG start codon of the first ORF of the \textit{ncgl0018} gene) using primer pair ENCgl0018-F/ENCgl0018-R (Supplementary Table S2). Different concentrations of purified His\textsubscript{6}-OasR or His\textsubscript{6}-SigH (0-4.0 µg) were incubated with 40 nM \textit{P_{ncgl0018}} in a total volume of 20 µl. A 211 bp fragment from the \textit{ncgl0018} coding region amplified with primers Control-F and Control-R instead of \textit{P_{ncgl0018}} and bovine serum albumin (BSA) instead of His\textsubscript{6}-OasR or His\textsubscript{6}-SigH were used as negative controls. The binding reaction buffer contained 10 mM Tris-HCl (pH 7.4), 5 mM MgCl\textsubscript{2}, 50 mM KCl, 5% glycerol, 0.1% Nonidet P 40 (NP40), 1 µg poly(dI:dC), 1 mM dithiothreitol (DTT). The binding reaction mixtures were incubated at room temperature for 30 min, and then loaded onto 8% native polyacrylamide gel made with 10 mM Tris buffer containing 50 mM KCl, 5 mM MgCl\textsubscript{2} and 10% glycerol in 0.5×TBE electrophoresis buffer [50 mM Tris, 41.5 mM borate (pH 8.0), 10 mM Na\textsubscript{2}EDTA.H\textsubscript{2}O]. Electrophoresis was performed at 4°C and 100V using 1×TBE (89 mM Tris, 89 mM borate, 2 mM EDTA) as the electrophoresis buffer. The gel was subsequently stained with a 10,000-fold diluted SYBR Gold nucleic acid staining solution (Molecular Probes) for 30 min. The DNA bands were visualized with UV light at 254 nm. Experiments were performed in triplicate.

**Preparation of S-mycothiolated NCgl0018 and NCgl0018-S\textsubscript{2} in vitro**

S-mycothiolated NCgl0018:C107S (NCgl0018:C107S-SSM, the mixed disulfide between MSH and NCgl0018:C107S) was prepared according to the method of Chi et al. (2014). MSH was purified from \textit{C. glutamicum} RES167 as described previously (Feng et al., 2006). First, NCgl0018:C107S or Mrx1:C15S was reduced by incubation with 50 mM DTT for 30 min at room temperature. Second, excess of DTT was removed by ultrafiltration. Third, 50 µM NCgl0018:C107S or Mrx1:C15S was incubated together with excessive MSH (6 mM). Fourth, 1 mM H\textsubscript{2}O\textsubscript{2} was added into it for 30 min, and the excess MSH and H\textsubscript{2}O\textsubscript{2} were removed by ultrafiltration. Finally, the sample was purified on a Superdex75 10/300 GL column (GE Healthcare), which was pre-equilibrated with 50 mM HEPES/NaOH pH 8.0, 150 mM NaCl. Oxidized NCgl0018-S\textsubscript{2} (intramolecular disulfide bond-containing NCgl0018) was produced according to the method of Van Laer et al. (2012). First, NCgl0018 or Mrx1 was reduced by incubation with DTT for 30 min at room temperature. Second, excess of DTT was removed by ultrafiltration. Third, pre-reduced NCgl0018 or Mrx1 was oxidized with a 5-fold molar excess of
diamide and incubated for 30 min at room temperature. Finally, oxidized NCgl0018-S₂ or
Mrx1-S₂ was purified on a Superdex75 10/300 GL column (GE Healthcare) equilibrated with 20
mM Tris, 150 mM NaCl, pH 7.6 for further experiments. Pure NCgl0018-SSM and NCgl0018-S₂
were confirmed by Matrix-Assisted Laser Desorption/Ionization Time of Flight Mass
Spectrometry (MALDI-TOF MS).

Steady-state kinetics of oxidized NCgl0018-S₂ and NCgl0018:C107S-SSM by
Trx/thioredoxin reductase (TrxR)/NADPH, MSH/ Mtr/NADPH, and Lpd/SucB/NADH
pathways
NCgl0018-S₂ and NCgl0018:C107S-SSM-dependent oxidation of NADPH or NADH in the
Trx/TrxR/NADPH, MSH/Mtr/NADPH, and Lpd/SucB/NADH pathways was continuously
monitored at 340 nm (ε₃₄₀=of 6220 M⁻¹·cm⁻¹) in a 300 µl reaction mixture containing 50 mM
Tris-HCl buffer (pH 7.5), 1 mM EDTA, varying concentrations of oxidized substrates
(NCgl0018-S₂ or NCgl0018:C107S-SSM), and a reduced Trx-generating system (5 µM TrxR, 5
µM Trx and 300 µM NADPH), MSH system (5 µM Mtr, 500 µM MSH and 300 µM NADPH), or
Lpd-generating system (5 µM Lpd, 5 µM SucB and 300 µM NADH). All reactions were carried
out at 37°C and started by the addition of oxidized NCgl0018-S₂ or NCgl0018:C107S-SSM in a
reaction mixture previously incubated for 5 min at 37°C. Control measurements were performed
in the absence of NCgl0018-S₂ or NCgl0018:C107S-SSM. Reactions were performed in triplicate.

The k₅ₐ₅ and Kₘ values were obtained from a non-linear fit with the Michaelis-Menten equation
using the program GraphPad Prism 5.

NBD-Cl analysis of the sulfenic acid state
To study the formation of cysteine sulfenic acid (Cys-SOH) as a reaction intermediate,
NCgl0018:C104S and NCgl0018:C107S labeled with 4-chloro-7-nitrobenzofurazan (NBD-Cl)
were assayed as described previously (Ellis and Poole, 1970) with minor modifications.
NBD-Cl is prepared by bubbling argon through the solution for 10 min. Under anaerobic conditions, NCgl0018:C104S
and NCgl0018:C107S were divided into three equal portions (final concentration of 20 µM), the
first of which was treated with 100 µM H_2O_2, the second of which was treated with 100 µM H_2O_2 and 500 µM MSH, while the last of which was directly used as an untreated sample (negative control). The H_2O_2-treated and untreated proteins were incubated with NBD-Cl (5 mM) for 30 min at 25°C in the dark. Excess NBD-Cl was removed by ultrafiltration, and the protein samples were analyzed at 200 to 600 nm (DU 7500 diode array spectrophotometer; Beckman, Fullerton, CA). Experiments were performed in triplicate.

Quantitative analysis of sulfhydryl groups

Free sulfhydryl groups in NCgl0018 WT, NCgl0018:C104S and NCgl0018:C107S were measured using 5, 5′-dithio-bis (2-nitrobenzoic acid) (DTNB) (Ellman, 1959). After 20 µM proteins were treated with 100 µM H_2O_2, 100 µM H_2O_2 + 500 µM MSH, and 50 mM DTT at room temperature for 30 min, respectively, residual DTT or H_2O_2 was removed with a PD10 desalting column (GE Healthcare, Piscataway, NJ). The resulting proteins (10 µM) were added to 2 mM DTNB in 50 mM Tris-HCl buffer (pH 8.0) and the absorbance at 412 nm was measured against a 2 mM DTNB solution as the reference. The amounts of reactive sulfhydryl groups were measured using the molar absorption coefficient of TNB at 412 nm (ε_412) of 13,600 M⁻¹·cm⁻¹ (Gething and Davidson, 1972). Experiments were performed in triplicate.

Enzymatic activity assay

Insulin disulfide reduction was performed based on the method described by Rosado et al. (2017). Briefly, NCgl0018-dependent reduction of insulin was monitored continuously at 600 nm in a 96-well plate reader at 37°C in a reaction mixture containing PBS, pH 7.4, 1 mM EDTA, 500 µM NADPH, 5 µM Mtr, 500 µM MSH, 100 µM insulin, and 5 µM NCgl0018 (WT and its variants) or Mrx1. The reaction was started by the addition of insulin, and control measurements were performed in the absence of NCgl0018 or Mrx1. The precipitation starting point was defined as an increase of 0.02 absorbance units at A_600 after a stable baseline recording, and the rate of precipitation was calculated using a linear regression composed by A_600 ranging from 2,000 to 2,500 s.

The mixed disulfide between hydroxyethyl disulphide (HED) and MSH (HED-SSM) was utilized to measure activity of NCgl0018 reducing mixed disulfide (Si et al., 2014). The HED-SSM was formed by incubating 1000 mM HED with 100 mM MSH at 30°C for 5 min.
Briefly, the kinetic parameters were determined in the presence of varying concentrations of HED-SSM (0–20 mM). The enzyme reactions were measured in 100 mM potassium phosphate buffer (pH 7.6), 300 µM NADPH, 500 µM MSH, 5 µM Mtr and 1 µM NCgl0018 (WT, NCgl0018:C104S, and NCgl0018:C107S). The assay was performed at 25°C and the absorption monitored at 340 nm. The activity was determined after subtracting the spontaneous reduction rate observed in the absence of NCgl0018, 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 kcat and Km values of NCgl0018 for HED-SSM substrates were obtained from a non-linear fit with the Michaelis-Menten equation using the program GraphPad Prism 5. Mrx1 was used as a control.

**pKₐ determination**

The extinction coefficient of thiol groups (R-SH) at 240 nm was the main readout utilized to measure pKₐ values of cysteine residues due to the lack of absorption of its un-ionized counterpart (R-S) in the same wavelength (Roos et al., 2007; Roos et al., 2013). The pKₐ of the nucleophilic cysteine (Cys104) in NCgl0018 was determined spectrophotometrically (Roos et al., 2007). To measure the pKₐ of the C-terminal cysteine (Cys107) in NCgl0018, the poly-buffer was changed to a buffer consisting of 10 mM Tris, 10 mM sodium phosphate, 10 mM sodium borate and 10 mM CAPS pH 13.0. All of the measurements were performed in a Carry UV spectrophotometer (Agilent Technologies) precooled at 10°C. The sigmoidal pH-dependent saturation curve was fitted to the Henderson–Hasselbalch equation (Roos et al., 2007), where Aexp was the experimental value A₂₄₀/A₂₈₀, Aₛ was the A₂₄₀/A₂₈₀ value for the protonated form, and Aₛ⁻ is the A₂₄₀/A₂₈₀ for the deprotonated form. The data were fitted to the following equation using GraphPad Prism version 5.

\[
A_{\text{exp}} = A_{\text{sh}} + \frac{(A_{S^{-}} - A_{\text{sh}})}{1+10^{pK_{\alpha}(\text{pH})}}
\]

**RNase I activity assay**

Oxidase activity was measured as described previously (Roos et al., 2007). Reduced RNase I was preincubated with the thiol/disulfide oxidoreductases in 50 mM Hepes, pH 7.5, 150 mM NaCl for 3 min at room temperature at a final concentration of 0.5 µM reduced RNase I before RNase
activity measurements. The RNA hydrolysis activity was measured in a methylene blue RNase assay using a buffer solution containing 1 mg of methylene blue in 200 ml of MOPS buffer solution (0.1 M MOPS, pH 7.5, 2 mM EDTA). The absorbance was followed as a function of time at 659 nm, the wavelength with a maximum difference between methylene blue intercalated with RNA and without RNA. The measured RNase activities are the initial velocities determined on the first 5% of the progress curves.

**Peroxidase activity assays**

Peroxidase activity assays were performed by monitoring the decrease in absorbance at 340 nm arising from NADPH oxidation (Si et al., 2019). The catalytic properties of NCgl0018 were determined using a reduced system (Mtr and 500 µM MSH) as the electron donors. The assay was carried out in a total volume of 300 µl containing 50 mM Tris-HCl buffer (pH 7.5), 1 mM EDTA, 250 µM NADPH, 1 µM NCgl0018, 500 µM MSH, 4 µM Mtr. The reaction was started by the addition of peroxide substrates following 5 min of preincubation. The catalytic parameters of NCgl0018 for peroxides have been obtained by varying the peroxide concentration at saturating concentrations of the other substrate (between 0 and 2000 µM for peroxides). NADPH oxidation was monitored as $A_{340}$. The activity was determined after subtracting the spontaneous reduction rate observed in the absence of NCgl0018, 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 ($e_{340}$) of 6220 M$^{-1}$·cm$^{-1}$. Three independent experiments were performed at each substrate concentration. The $k_{cat}$ and $K_m$ values were obtained from a non-linear fit with the Michaelis-Menten equation using the program GraphPad Prism 5.

**Construction of chromosomal fusion reporter strains and β-Galactosidase assay**

The lacZY fusion reporter plasmid pK18mobsacB-P$_{ncgl0018}$::lacZY was transformed into the C. glutamicum RES167 parental strains with empty plasmid pXMJ19 [WT(pXMJ19)], ΔoasR(pXMJ19) (strains lacking oasR gene contained empty pXMJ19), ΔsigH(pXMJ19) (strains lacking sigH gene contained empty pXMJ19), ΔoasR(pXMJ19-oasR) (ΔoasR was complemented with plasmids carrying the wild-type oasR gene), and ΔsigH(pXMJ19-sigH) (ΔsigH was complemented with plasmids pXMJ19 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_{ncg10018::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 (Miller, 1992). All β-Galactosidase experiments were performed with at least three independent biological replicates.

**Quantitative real-time polymerase chain reaction (qRT-PCR) analysis**

Total RNA was isolated from exponentially growing WT(pXMJ19), ΔsigH(pXMJ19), ΔoasR(pXMJ19), ΔsigH(pXMJ19-ΔsigH), and ΔoasR(pXMJ19-ΔoasR) 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 (Si et al., 2018a). The primers used were listed in Table S2. To obtain a 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**

NCg10018 was a DsbA-like Mrx1 homolog

KEGG database (Kyoto Encyclopedia of Genes and Genomes) shows that the *ncg10018* gene is located at bp 18733 to 19497, encoding a putative dithiol-disulfide isomerase DsbA of 254 amino acid residues with a molecular mass of 26.7 kDa. The NCg10018 shared amino acids sequence identities of about 50% with the Cys-Pro-Phe/Tyr-Cys (C-P-F/Y-C) catalytic motif-containing oxidoreductases of *Jonesia denitrificans*, *Flaviflexus salsibiostraticola*, *Glutamicibacter arilaitensis*, *Nocardiopsis dassonvillei*, and *Brevibacterium* (Fig. S1A). Recently, on the basis of
the catalytic CXXC motif and physiological and biochemical experiments, Rosado et al. (2017) created a new DsbA-like Mrx1 cluster, preserving the C-P-W-C or C-P-F-C active-site sequence motif. The new DsbA-Mrx1 cluster showed different enzymatic rates and substrate preference from the canonical DsbA cluster with the C-P-H-C catalytic motif and Mrx1 cluster with the C-P-Y-C catalytic motif. Although NCgl0018 has very low identity with members of the DsbA-like Mrx1 cluster M. tuberculosis Rv2466c and C. glutamicum NCgl2339, we thought it might share the same action mode and enzymatic function, as it contains a C-P-F-C active-site sequence motif presumed to be exclusive to the new DsbA-Mrx1 cluster (Fig. S1A and S1C). Therefore, we speculated that NCgl0018 behaved like DsbA-like Mrx1, but not Mrx1 and classic DsbA.

ncgl0018 null mutant was sensitive to oxidative stress

The ncgl0018 of C. glutamicum was one of the main targets of organic peroxide and antibiotic resistance regulator OasR, which is strongly linked to the oxidative stress response in C. glutamicum (Si et al., 2020). Moreover, M. tuberculosis Rv2466c was validated to promote mycobacterial resistance to oxidative stress (Rosado et al., 2017). Thus, to assess the role of NCgl0018 in protecting cells against oxidative stress, an ncgl0018 null mutant in C. glutamicum RES167 strain was first obtained by homologous recombination-based gene knock-out. Next, an agar-based disc diffusion assay was performed for examining the sensitive phenotype of ncgl0018 null mutant to various oxidizing and ROS-inducing reagents [hydrogen peroxide (H$_2$O$_2$), hypochlorous acid (HClO), diamide, cumene hydroperoxide (CHP), tert-butyl hydroperoxide (t-BHP), 2, 4-dinitrochlorobenzene (CDNB), iodoacetamide (IAM), streptomycin (STR), ciprofloxacin (CIP), cadmium chloride (CdCl$_2$), and nickel sulfate (NiSO$_4$)]. As shown in Fig. 1, deletion of NCgl0018 did not affect bacterial growth under normal conditions, while the Δncgl0018 (pXMJ19) strain (the mutant lacking ncgl0018 with the empty plasmid pXMJ19) exhibited a significant increase in sensitivity to all tested chemical reagents as compared with WT(pXMJ19) strain (the C. glutamicum RES167 parental strain with the empty plasmid pXMJ19), giving a significantly larger zone of inhibition than WT(pXMJ19) strain. For example, the inhibition zone around the paper disc was about 1.2-fold larger in the case of Δncgl0018 (pXMJ19) cells (1.9±0.2 cm for H$_2$O$_2$ and 2.2 ±0.3 cm for STR) than in the case of WT (pXMJ19) cells (1.6±
0.1 cm for H$_2$O$_2$ and 1.9 ± 0.1 mm for STR), indicating that the absence of ncgl0018 caused a significantly increased sensitivity. To confirm that the sensitivity to reagents was attributed to the absence of the ncgl0018 gene, the complementary strains Δncgl0018 (pXMJ19-ncgl0018) were constructed by the introduction of plasmid pXMJ19 in trans containing the wild-type C. glutamicum ncgl0018 gene into Δncgl0018 null mutant, and complementation experiments were performed. As shown in Fig. 1, the complementary strains Δncgl0018 (pXMJ19-ncgl0018) gave a significantly smaller (from 1.4 to 2.0 cm) inhibition zone under all tested chemical reagents again, which were the equivalent of those of the WT (pXMJ19) strains, indicated that resistant phenotypes were almost fully restored in Δncgl0018(pXMJ19-ncgl0018) strains. In addition, ncgl0018 overexpression markedly increased the resistance of wild-type strain to various stress challenges (Fig 1). The result suggested that NCgl0018 was very critical for survival under various oxidative stress conditions.

### Formation of an intramolecular disulfide bond Cys104-Cys107 under oxidative stress

*M. tuberculosis* Rv2466c forms Cys19-Cys22 disulfide in Cys$^{19}$-Pro-Trp-Cys$^{22}$ (C$^{19}$-P-W-C$^{22}$) active-site motif and Cys19-MSH mixed disulfide under oxidative stress (Rosado et al., 2017). Moreover, Cys19 and Cys22 of *M. tuberculosis* Rv2466c are the nucleophilic (C$_p$) and resolving (C$_R$) cysteines, respectively. NCgl0018 contains a conserved catalytic motif at position 104-107 consisting of Cys$^{104}$-Pro-Phe-Cys$^{107}$ (Fig. S1C). Sequence alignment indicates that Cys104 might be the nucleophilic cysteine residue that had been reported to be involved in catalysis via the transient formation of a sulfenic acid, while Cys107 might be the resolving Cys residue (Fig. S1C). Therefore, we speculated that Cys104 and Cys107 might participate in the formation of disulfide bonds. To confirm this speculation, we mutated the first and the second cysteine of the CXXC motif to serine to gain two variants of NCgl0018, namely, NCgl0018:C104S and NCgl0018:C107S. NCgl0018 WT and these two variants of NCgl0018 with and without H$_2$O$_2$ treatment were used to perform DTNB analysis and NBD-Cl modification. As shown in Fig. 2A, the DTT-treated NCgl0018 WT contained 1.78 ± 0.35 thiol groups of per monomer, but the thiol content decreased to 0.19 ± 0.02 when NCgl0018 WT was treated with H$_2$O$_2$. The difference of 1.59 thiol groups between the two preparations was linked to the complete oxidation of NCgl0018 WT after H$_2$O$_2$ treatment.
NBD-Cl can specifically react with free sulphydryl groups of cysteines and cysteine sulfenic acid, but not with cysteines that are present as sulfinic acid or sulfonic acid. The covalent attachment of NBD-Cl generated an absorption peak at ∼420 nm upon reaction with thiol groups (P-SH), whereas it peaked at ∼347 nm upon reaction with sulfinic acids (P-SOH) (Baker and Poole, 2003). Following the reaction with NBD-Cl, the absorption spectra of NCgl0018:C104S variants were unchanged before and after exposure to H₂O₂ or H₂O₂ and MSH, exhibiting only the 420 nm peak (Fig. 2B). The NCgl0018:C104S variant showed one thiol per monomer before and after H₂O₂ or H₂O₂ and MSH treatment, indicating that Cys107 was still in thiol form under exposure to H₂O₂ (Fig. 2A). However, NCgl0018:C107S under H₂O₂ treatment lost one thiol group, compared to the thiol content of DTT-treated state, indicating that Cys104 did not exist as a thiol in H₂O₂-treated NCgl0018:C107S variant (Fig. 2A). The redox state of thiol in NCgl0018:C107S was further examined using NDB-Cl modification. H₂O₂-treated and NBD-labeled NCgl0018:C107S had an absorbance maximum (λ_max) of 347 nm, representing the NBD-modified product Cys-S(O)-NBD (Ellis and Poole, 1997), which clearly signified the detection and trapping of Cys104-SOH in the NCgl0018:C107S variant (Fig. 2B). However, DTT-treated NCgl0018:C107S modified with NBD-Cl produced a new covalently attached spectral species with a λ_max of 420 nm, consistent with previously characterized thiol adducts with NBD-Cl (Cys-S-NBD). This result showed that Cys104 was sensitive to an oxidant, forming sulfenic acid at cysteine 104 (Cys104-SOH).

Surprisingly, no NBD-Cl labelling in H₂O₂-treated NCgl0018:C107S occurred in the presence of MSH (Fig. 2B), indicating that MSH reacted with NCgl0018:C107S-SOH to form NCgl0018:C107S-SSM. Therefore, according to our results, we speculated that S-mycobiotilation occurred on Cys104 of NCgl0018 in the presence of MSH and H₂O₂.

Together, MSH directly interacted with Cys104 under oxidative stress. Further, we indicated with NCgl0018 WT that Cys107 resolved the Cys104-MSH mixed disulfide or Cys104-SOH, leading to the formation of a Cys104-Cys107 disulfide. The result was consistent with the result of Rosado et al. (2017) reported for *M. tuberculosis* Rv2466c.

Oxidized NCgl0018 was preferentially reduced with electrons from the MSH/Mtr/NADPH pathway
Oxidized DsbA must be regenerated to continue to exert its antioxidant function. Rosado et al. (2017) showed that oxidized Rv2466c, namely, Cys19-Cys22 disulfide-containing Rv2466c and S-mycothiolated Rv2466c (Rv2466c-SSM, the mixed disulfide between MSH and Rv2466c), preferentially received electrons through the MSH/Mtr/NADPH pathway. Since *C. glutamicum* contains three common electron transfer pathways, i.e. the MSH/Mtr/NADPH system, Trx/TrxR/NADPH system, and Lpd/SucB/NADH system, reducing the disulfide bonds between the active site cysteine in protein or the mixed disulfides between MSH and protein, we identified possible electron donor pathways coupled to oxidized NCgl0018 reduction. To do so, NCgl0018 was first oxidized with a 5-fold molar excess of diamide to obtain oxidized NCgl0018-S2. Next, oxidized NCgl0018-S2 with a single disulfide bond between its active site cysteines was added as a substrate for the above electron transfer pathways to measure steady-state kinetics.

As shown in Figs. 3A-C, the $K_m$ value, $k_{cat}$ value, and catalytic coefficient of oxidized NCgl0018-S2 for the MSH/Mtr/NADPH, Trx/TrxR/NADPH, or Lpd/SucB/NADH electron donor pathways were calculated to be $1.06 \pm 0.17$ $\mu$M, $3.69 \pm 0.11$ s$^{-1}$, and $3.51 \pm 0.22 \times 10^6$ M$^{-1}$ s$^{-1}$, $23.66 \pm 3.01$ $\mu$M, $0.32 \pm 0.01$ s$^{-1}$, and $1.34 \pm 0.17 \times 10^4$ M$^{-1}$ s$^{-1}$, or $65.92 \pm 9.61$ $\mu$M, $0.03 \pm 0.01$ s$^{-1}$, and $4.4 \pm 0.18 \times 10^2$ M$^{-1}$ s$^{-1}$, respectively. It is worth noting that reduction of the oxidized form of Mrx1 through the MSH/Mtr/NADPH pathway resulted in catalytic coefficient of $23.3 \pm 0.23 \times 10^6$ M$^{-1}$ s$^{-1}$ (Fig. 3D), and Mrx1 has been shown previously not to use the Trx/TrxR/NADPH electron donor pathway (Van Laer et al., 2012). Here, we have clearly shown that, although oxidized NCgl0018-S2 could be reduced by the MSH/Mtr/NADPH and Trx/TrxR/NADPH electron pathways, the catalytic coefficient of the enzyme was about 260-fold higher with the MSH/Mtr/NADPH pathway compared with the Trx/TrxR/NADPH pathway, indicating oxidized NCgl0018-S2 preferred the MSH/Mtr/NADPH pathway. The above catalytic coefficient of oxidized NCgl0018-S2 obtained from the Lpd/SucB/NADH pathway was several orders of magnitude lower than that from the MSH/Mtr/NADPH pathway, indicating that it was highly unlikely for oxidized NCgl0018-S2 to receive electrons from the Lpd/SucB/NADH pathway in vivo. Interestingly, oxidized Mrx1-S2 catalyzed at about a 6.6-fold faster reaction coupled to the MSH/Mtr/NADPH pathway when compared with oxidized NCgl0018-S2, indicating a different specificity of electron donor pathways for both enzymes (Fig. 3D).
Finally, NCgl0018:C107S-SSM was used as a substrate for the above electron transfer pathways to measure steady-state kinetics instead of NCgl0018-SSM, because a part of NCgl0018-SSM will become NCgl0018-S2 when there is Cys107 in NCgl0018. Previous study showed that *C. glutamicum* Mrx1:C15S, which was created with a single substitution mutation of Cys to Ser in the C12XXC15 motif of Mrx1, could form stable Mrx1:C15S-SSM in the presence of MSH under oxidative stress and Mrx1:C15S-SSM could be reduced via the MSH/Mtr/NADPH pathway (Si et al., 2015; Li et al., 2021). Thus, Mrx1:C15S-SSM was used as a positive control. As shown in Fig. S2, the $K_m$ value, $k_{cat}$ value, and catalytic coefficient of NCgl0018:C107S-SSM for the MSH/Mtr/NADPH, Trx/TrxR/NADPH, or Lpd/SucB/NADH electron donor pathways were calculated to be 0.99±0.07 µM, 2.07±0.03 s⁻¹, and 2.09±0.06×10⁶ M⁻¹ s⁻¹, 21.12±4.11 µM, 0.61±0.04 s⁻¹, and 2.89±0.37×10⁴ M⁻¹ s⁻¹, or 93.76±15.36 µM, 0.03±0.01 s⁻¹, and 3.31±0.89×10² M⁻¹ s⁻¹, respectively. Catalytic efficiency of the MSH/Mtr/NADPH pathway for NCgl0018:C107S-SSM was lower than that for Mrx1:C15S-SSM. In addition, the Trx/TrxR/NADPH electron transfer pathway gave a very low reproducible activity for oxidized NCgl0018:C107S-SSM, and no reduction activation could be observed in the presence of the Lpd/SucB/NADH pathway. So, oxidized NCgl0018 was reduced preferentially by the Mtr/MSH/NADPH pathway in *C. glutamicum*, but not the Trx/TrxR/NADPH and Lpd/SucB/NADH reducing systems.

**The pKa of the cysteine residues**

Previous research has shown the pKa of the nucleophilic cysteine involved in the reaction was a determining factor for the rates of the thiol-disulfide exchange reactions (Jensen et al., 2014). The nucleophilic cysteine in the CXXC motif of oxidoreductases was often in the local electrostatic environment due to the influence of nearby residues (Hansen et al., 2005), leading to the phenomenon that the pKa value of the N-terminal cysteine in the CXXC motif was lower than that of cysteine (8.6) (Thurlkill et al., 2006; Lillig et al., 2008). Due to the low pKa value of the nucleophilic cysteine, the N-terminal cysteine could perform a nucleophilic attack on the substrate disulphide (Lillig et al., 2008). Thus, the pKa of active site residues in NCgl0018 was determined by recording the absorption at 240 nm during a pH titration (Roos et al., 2013), since the thiolate ion has a higher absorption at this wavelength than the thiol group. As shown in Fig. S3, the pKa
values of the nucleophilic Cys104 and the resolving Cys107 were less than 6 and 8.09, respectively. The result indicated that the low pKa value made Cys104 function as the nucleophilic Cys. In addition, the pKa value of nucleophilic Cys104 was between the pKa values of the respective cysteines of Mrx1 (6.8) and DsbA (~3.5), which was consistent with the fact that NCgl0018 was less reactive compared with Mrx1 when coupled to the MSH/Mtr/NADPH pathway (Fig. 3). Moreover, the pKa of the Cys107 (8.43) was already lower than the pKa of the MSH sulfur (8.76) (Sharma et al., 2016), which made Cys107 attack Cys104-MSH mixed disulfide more, leading to the formation of a Cys104-Cys107 disulfide.

NCgl0018 reduced mycothiolated mixed disulfides preferably via a monothiol mechanism

*M. tuberculosis* Rv2466c employed two different mechanisms for reducing disulfide bridges: the 2-Cys dithiol mechanism and the 1-Cys monothiol mechanism (Rosado et al., 2017). The 2-Cys dithiol mechanism required N-terminal and C-terminal cysteine residues of the active site of CXXC motif, but the 1-Cys monothiol mechanism only required N-terminal cysteine of the active site of CXXC motif. *M. tuberculosis* Rv2466c reduced mycothiolated mixed disulfides coupled to the MSH/Mtr/NADPH pathway preferably via a monothiol mechanism, but it reduced intramolecular disulfide bonds coupled to the MSH/Mtr/NADPH pathway via either a dithiol or monothiol mechanism. Thus, this phenomenon prompted us to investigate what mechanism NCgl0018 used to reduce mycothiolated mixed disulfides and intramolecular disulfide bonds coupled to the MSH/Mtr/NADPH pathway. To answer this question, the functionalities of NCgl0018 WT, NCgl0018:C104S, and NCgl0018:C107S to reduce mycothiolated mixed disulfides and intramolecular disulfide bonds were detected by following the oxidation of NADPH in the presence of the MSH/Mtr/NADPH system.

To assess the ability of NCgl0018 to reduce mycothiolated mixed disulfides, HED-SSM (the mixed disulfide formed between HED and MSH) was used as a substrate in the MSH/Mtr/NADPH-coupled assay. *C. glutamicum* Mrx1 was used as positive control to compare its reactivity with NCgl0018. The $K_m$ value, $k_{cat}$ value, and catalytic coefficient of NCgl0018 or Mrx1 for HED-SSM were calculated to be 0.48±0.06 mM, 10.69±0.28 s$^{-1}$, and 2.25±0.34×10$^4$ M$^{-1}$ s$^{-1}$, or 0.49±0.07 mM, 104.48±1.99 s$^{-1}$, and 21.32±0.45×10$^4$ M$^{-1}$ s$^{-1}$, respectively (Figs. 4A and 4B). These results showed that *C. glutamicum* Mrx1 had a higher reduction capacity for HED-SSM compared with the NCgl0018 WT enzyme when the MSH/Mtr/NADPH pathway was introduced.
as an electron donor. It was worth noting that NCgl0018:C107S has about a 22% faster initial velocity of 13.04±0.241 s⁻¹ toward HED-SSM compared with that of NCgl0018 WT, whereas the NCgl0018:C104S was unable to reduce HED-SSM (Figs. 4C and 4D). This cysteine substitution experiment indicated that NCgl0018 was functioning under a monothiol mode of action where the nucleophilic Cys104 was required to catalyze mixed disulfide bond reduction.

**NCgl0018 reduced intramolecular disulfide bonds preferably via a monothiol mechanism**

Insulin reduction provided the basis for a classical assay used to test dithiol-disulfide exchange activity (Holmgren, 1979). Thus, we further checked the ability of Mrx1 and NCgl0018 to reduce intramolecular disulfide bonds in an insulin assay (Table 1). When the MSH/Mtr/NADPH pathway was used as an electron donor, the activity of Mrx1 was about 2-fold faster in reducing insulin compared with NCgl0018 WT. NCgl0018:C107S exhibited a slightly higher reducing activity toward insulin compared with NCgl0018 WT (Table 1), while NCgl0018:C104S was inactive toward insulin. This led us to conclude that NCgl0018 regarded the N-terminal nucleophilic cysteine of the CXXC motif as the necessary active site and performed the function as a monothiol disulfide reductase.

**NCgl0018 has no oxidase and peroxidase properties**

To investigate its putative DsbA-oxidoreductase activity, we used *E. coli* RNase I as a substrate. RNase I was active with its four disulfide bonds correctly formed, making it an ideal model enzyme for oxidative protein folding evaluation (Messens et al., 2007). We used methylene blue intercalated RNA as a substrate to check the RNase activity at 659 nm after the incubation of reduced unfolded RNase I with NCgl0018 and Mrx1 (Greiner-Stoeffele et al., 1996). NCgl0018 did not have a capacity of catalyzing the oxidative refolding of RNase (Fig. S4A). Reduced RNase I (unfolded) demonstrated 15.6% of activity relative to folded RNase I (100%). In contrast, in the presence of Mrx1, 39.7% of activity was recovered. Thus, NCgl0018 did not act as an oxidase.

As NCgl0018 played an important role in the resistance to oxidative stresses, we therefore examined the possible role of NCgl0018 as a peroxidase. H₂O₂ and CHP were added to the MSH/Mtr/NADPH pathway in the presence and absence of NCgl0018, and the ability of NCgl0018 to reduce H₂O₂ and CHP was investigated by steady-state kinetics. As shown in Fig. S4B and S4C, the *K_m* value, *k_cat* value, and catalytic coefficient of NCgl0018 for H₂O₂ and CHP were calculated to be 222.56±43.73 µM, 0.14±0.01 s⁻¹, and 6.25±0.97×10² M⁻¹ s⁻¹, or...
\[324.84 \pm 32.85 \text{ M}, 0.11 \pm 0.01 \text{ s}^{-1}, \text{ and } 3.51 \pm 0.73 \times 10^2 \text{ M}^{-1} \text{ s}^{-1}, \text{ respectively (Figs. S4B and S4C).}

The above catalytic coefficients of NCgI0018 for \( \text{H}_2\text{O}_2 \) and CHP were several orders of magnitude lower than the reaction rate of the known peroxidases of \( C. \text{ glutamicum} \) \((10^5 \text{ to } 10^7 \text{ M}^{-1} \text{ s}^{-1})\) (Su et al., 2018; Si et al., 2015). A direct peroxide detoxification role of NCgI0018 is therefore highly unlikely \textit{in vivo}.

**NCgI0018 expression was regulated by SigH and OasR in \( C. \text{ glutamicum} \)**

Previous studies showed that the expression of Rv2466c was induced by oxidative stress (Rosado et al., 2017). Moreover, ncgl0018 mutants exhibited sensitivity to oxidative stress-inducing various reagents. Therefore, to test whether ncgl0018 expression responded to these reagents at the transcriptional level, qRT-PCR and \( \text{lacZY} \) activity profiling were performed.

The \( \text{lacZY} \) activity of \( P_{\text{ncgl0018}}::\text{lacZY} \) chromosomal promoter fusion reporter in the \( C. \text{ glutamicum} \) RES167 parental strain treated with different concentrations of reagents was determined. As shown in Fig. 5A, the ncgl0018 expression level was significantly improved in the WT(pXMJ19)(\( P_{\text{ncgl0018}}::\text{lacZY} \)) reporter strains exposed to various reagents as compared with strain without reagents treatment. Further, the activity of the \( P_{\text{ncgl0018}}::\text{lacZY} \) fusion reporter strain exhibited a dose-dependent increase under all tested reagents (Fig. 5A). These results significantly demonstrated that various reagents induced the expression of the ncgl0018 gene, thereby increasing the resistance of \( C. \text{ glutamicum} \) to stress conditions. A similar dose-dependent pattern of ncgl0018 expression in response to different reagents was also observed in qRT-PCR analysis (Fig. 5B).

Because previous study identified NCgI2339 positively regulated by the stress-responsive ECF-sigma factor SigH using DNA microarrays in \( C. \text{ glutamicum} \), we first investigated whether NCgI0018 was also regulated by SigH (Busche et al., 2012). The fortunate reality was that SigH positively controlled the expression of NCgI0018 (Fig. S5). Recently, Si et al. (2020), found that NCgI0018 was also one of the main targets of OasR by microarray analysis, which is strongly linked to the oxidative stress response in \( C. \text{ glutamicum} \). Therefore, we further detected OasR’s regulatory capacity for NCgI0018. As shown in Fig. 5A, \( \Delta \text{oasR} \) (pXMJ19) (strains lacking the \( \text{oasR} \) gene contained empty pXMJ19) significantly increased the \( \text{lacZY} \) activity of the ncgl0018 promoter, almost fully recovered by introducing a plasmid pXMJ19 expressing wild-type \( \text{oasR} \) in
deletion of oasR gene in C. glutamicum \(\Delta\)oasR(pXMJ19-oasR) strain, \(\Delta\)oasR mutant was complemented with plasmid pXMJ19 carrying the wild-type oasR gene]. These results clearly demonstrated that NCgl0018 was negatively regulated by OasR. The negative regulation of \(ncg\)l0018 by OasR was also confirmed by qRT-PCR, with the observation that the mRNA levels of \(ncg\)l0018 were reduced in the \(\Delta\)oasR(pXMJ19) mutant and restored to the wild-type level in the complemented strain \(\Delta\)oasR(pXMJ19-oasR) strain (Fig. 5B). To further determine whether OasR regulated NCgl0018 expression directly, we examined the interaction between OasR and the NCgl0018 promoter using electrophoretic mobility shift assay (EMSA). Incubation of a 211-bp DNA element containing the \(ncg\)l0018 promoter (\(P_{ncg\)l0018}) sequence (-993 to -782 relative to the ATG start codon of the first ORF of the \(ncg\)l0018 gene) with His\(_6\)-OasR led to the formation of DNA-protein complexes, and the abundance of such complexes depended on the amount of OasR (Fig. 5C left panel). However, both a 211-bp control DNA fragment amplified from the \(ncg\)l0018 coding open reading frame region and BSA instead of His\(_6\)-OasR showed no detectable binding (Fig. 5C, lane 6 and 7). Thus, OasR negatively controlled the expression of \(ncg\)l0018.

**Conclusion**

In this study, we have revealed the mode of action and actual enzymatic function of NCgl0018 by physiological and biochemical analysis. NCgl0018 was directly involved in various oxidizing and ROS-inducing reagents response of C. glutamicum. The physiological roles of NCgl0018 in the resistance to oxidative stresses were corroborated by its induced expression under various stresses, regulated directly by SigH and OasR. Unlike classic DsbA, NCgl0018 displayed no oxidase activity. Compared with Mrx1, NCgl0018 can use both the MSH/Mtr/NADPH and the Trx/TrxR/NADPH pathways as electrons, but preferred the former. NCgl0018 used a monothiol-disulfide exchange mechanism to reduce S-myclothiolated mixed disulfides, similar to C. glutamicum Mrx1. However, NCgl0018 had a lower initial velocity for HED-SSM than C. glutamicum Mrx1. Interestingly, NCgl0018 had a similar mode of action as the previously characterized DsbA-Mrx1 proteins (M. tuberculosis Rv2466c and C. glutamicum NCgl2339), displaying very high and low demycothiolating activity toward an As (V) MSH thiol-arseno adduct and HED-SSM, respectively. These phenomena indicated that members of the Mrx1 and the DsbA-like Mrx1 clusters have different sensitive properties to stress and substrate preference, indicating their biological significance for cell survival was different under different
oxidative stress conditions. We assumed that the DsbA-like Mrx1 cluster did not act as a backup for the Mrx1 cluster, but their roles seemed to be complementary to a certain degree. Based on our results, a catalytic model of NCgl0018 could be proposed (Fig. 6). In the first step, Cys104 reacted with S-mycothiolated mixed disulfide of the target protein A, or intramolecular disulfide of the target protein B, forming a mycothiolated Cys104 and reduced target protein or an intermediate dithiol bridge between Cys104 and the target protein B. For mycothiolated NCgl0018, two reduction pathways could take place. In the presence of MSH, MSH attacked mycothiolated Cys104, releasing the reduced form of Cys104 and mycothione (mycothiol disulfide; MSSM). In a parallel reaction, Cys107 nucleophilicly attacked mycothiolated Cys104, releasing MSH and a new Cys104-Cys107 disulfide. For an intermediate dithiol bridge between Cys104 and the target protein B, Cys107 solved it to form a Cys104-Cys107 disulfide. Next, Cys104-Cys107 disulfide was reduced by two molecules of MSH, accompanied by the generation of MSSM. Finally, MSSM was reduced back to MSH by an NADPH-dependent Mtr. Overall, our work showed that NCgl0018 was another DsbA-like Mrx1 besides NCgl2339 that promoted C. glutamicum resistance to oxidative stress and reacted with free MSH and mycothiolated targets.

Acknowledgments

This study was supported by the National Natural Science Foundation of China (31970034).

Supplementary Materials

Supplementary Table S1-Bacterial strains and plasmids used in this study.

Supplementary Table S2-Primers used in this study.

Supplementary Fig. S1-Multiple sequence alignment of NCgl0018 with DsbAs in other organisms.

Supplementary Fig.S2-Oxidized NCgl0018:C107S-SSM were preferably reduced by the MSH/Mtr/NADPH pathway.

Supplementary Fig. S3-Cys104 was the nucleophilic cysteine of NCgl0018.

Supplementary Fig. S4-NCgl0018 was not an oxidase and peroxidase.

Supplementary Fig. S5-Positive regulation of ncgl0018 expression by SigH in C. glutamicum.

References

Antelmann, H., and Helmann, J. D. (2011) Thiol-based redox switches and gene regulation. *Antioxid. Redox. Signal.*, **14(6)**, 1049–1063.
Baker, L. M., and Poole, L. B. (2003) Catalytic mechanism of thiol peroxidase from *Escherichia coli*. Sulfenic acid formation and overoxidation of essential CYS61. *J Biol Chem*, **278**(11), 9203–9211.

Busche, T., Silar, R., Pičmanová, M., Pátek, M., and Kalinowski, J. (2012) Transcriptional regulation of the operon encoding stress-responsive ECF sigma factor SigH and its anti-sigma factor RshA, and control of its regulatory network in *Corynebacterium glutamicum*. *BMC Genomics*, **13**, 445.

Chi, B. K., Busche, T., Van Laer, K., Bäsell, K., Becher, D., Clermont, L., Seibold, G. M., Persicke, M., Kalinowski, J., Messens, J., and Antelmann, H. (2014) Protein S-myocthiothiolation functions as redox-switch and thiol protection mechanism in *Corynebacterium glutamicum* under hypochlorite stress. *Antioxid. Redox Signal.*, **20**(4), 589–605.

Couturier, J., Vignols, F., Jacquot, J. P., and Rouhier, N. (2012) Glutathione- and glutaredoxin-dependent reduction of methionine sulfoxide reductase A. *FEBS Lett.*, **586**(21), 3894–3899.

Dalle-Donne, I., Milzani, A., Gagliano, N., Colombo, R., Giustarini, D., and Rossi, R. (2008) Molecular mechanisms and potential clinical significance of S-glutathionylation. *Antioxid. Redox Signal.*, **10**(3), 445–473.

Ellis, H. R., and Poole, L. B. (1997) Novel application of 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole to identify cysteine sulfenic acid in the AhpC component of alkyl hydroperoxide reductase. Biochemistry, **36**(48), 15013-15018.

Ellman, G. L. (1959) Tissue sulfhydryl groups. *Arch. Biochem. Biophys.*, **82**(1), 70–77.

Feng, J., Che, Y., Milse, J., Yin, Y. J., Liu, L., Rückert, C., Shen X. H., Qi, S. W., Kalinowski, J., and Liu, S. J. (2006) The gene ncl2918 encodes a novel maleylpyruvate isomerase that needs mycothiol as cofactor and links mycothiol biosynthesis and gentisate assimilation in *Corynebacterium glutamicum*. *J. Biol. Chem.*, **281**(16), 10778–10785.

Gething, M. J. H. and Davidson, B. E. (1972) Molar absorption-coefficient of reduced Ellmans reagent–3-carboxylato-4-nitro-thiophenolate. *Eur. J. Biochem.*, **30**, 352.

Greiner-Stoeffele, T., Grunow, M., and Hahn, U. (1996) A general ribonuclease assay using
methylene blue. *Anal. Biochem.*, **240**(1), 24–28.

Hansen, R. E., Østergaard, H., and Winther, J. R. (2005) Increasing the reactivity of an artificial dithiol-disulfide pair through modification of the electrostatic milieu. *Biochemistry*, **44**(15), 5899–5906.

Hillion, M. and Antelmann, H. (2015) Thiol-based redox switches in prokaryotes. *Biological Chem.*, **396**(5), 415–444.

Holmgren A. (1985) Thioredoxin. *Annu. Rev. Biochem.*, **54**, 237–271.

Holmgren, A. (1979) Glutathione-dependent synthesis of deoxyribonucleotides. Purification and characterization of glutaredoxin from *Escherichia coli*. *J. Biol. Chem.*, **254**(9), 3664-3671.

Imlay, J. A. (2015) Transcription factors that defend bacteria against reactive oxygen species. *Annual Rev. Microbiol.*, **69**, 93–108.

Jensen, K. S., Pedersen, J. T., Winther, J. R., and Teilum, K. (2014) The pKa value and accessibility of cysteine residues are key determinants for protein substrate discrimination by glutaredoxin. *Biochemistry*, **53**(15), 2533–2540.

Lee, J. Y., Seo, J., Kim, E. S., Lee, H. S., and Kim, P. (2013) Adaptive evolution of *Corynebacterium glutamicum* resistant to oxidative stress and its global gene expression profiling. *Biotechnol. Lett.*, **35**(5), 709–717.

Lillig, C. H., Berndt, C., and Holmgren, A. (2008) Glutaredoxin systems. *Biochim Biophys Acta*, **1780**(11), 1304–1317.

Li, X., Liu, Y., Zhong, J., Che, C., Gong, Z., et al. (2021) Molecular mechanisms of Mycoredoxin-1 in resistance to oxidative stress in *Corynebacterium glutamicum*. *J Gen Appl Microbiol.*, **67**, 15-23.

Liu, Y. B., Long, M. X., Yin, Y. J., Si, M. R., Zhang, L., Lu, Z. Q., Wang, Y., Shen, X. H. (2013) Physiological roles of mycothiol in detoxification and tolerance to multiple poisonous chemicals in *Corynebacterium glutamicum*. *Arch. Microbiol.*, **195**(6), 419–429.

Messens, J., Collet, J. F., Van Belle, K., Brosens, E., Loris, R., and Wyns, L. (2007) The oxidase DsbA folds a protein with a nonconsecutive disulfide. *J. Biol. Chem.*, **282**(43), 31302–31307.

Miller, J. H. (1992) A short course in bacterial genetics: a laboratory manual and handbook for
Escherichia coli and related bacteria. vol. 1. Cold spring harbor laboratory press New York.

Milse, J., Petri, K., Rückert, C., and Kalinowski, J. (2014) Transcriptional response of Corynebacterium glutamicum ATCC 13032 to hydrogen peroxide stress and characterization of the OxyR regulon. J. Biotechnol., 190, 40–54.

Newton, G. L., Arnold, K., Price, M. S., Sherrill, C., Delcardayre, S. B., Aharonowitz, Y., Cohen, G., Davies, J., Fahey, R. C., and Davis, C. (1996) Distribution of thiols in microorganisms: mycothiol is a major thiol in most actinomycetes. J. Bacteriol., 178(7), 1990–1995.

Newton, G. L., Bewley, C. A., Dwyer, T. J., Horn, R., Aharonowitz, Y., Coheng, G., Davies, J., Faulkner, D. J., and Fahey, R. C. (1995) The structure of U17 isolated from Streptomyces clavuligerus and its properties as an antioxidant thiol. Eur. J. Biochem., 230(2), 821–825.

Oide, S., Gunji, W., Moteki, Y., Yamamoto, S., Suda, M., Jojima, T., Yukawa, H., and Inui, M. (2015) Thermal and solvent stress cross-tolerance conferred to Corynebacterium glutamicum by adaptive laboratory evolution. Appl. Environ. Microbiol., 81(7), 2284–2298.

Pedrajas, J. R., Padilla, C. A., McDonagh, B., and Bárcena, J. A. (2010) Glutaredoxin participates in the reduction of peroxides by the mitochondrial 1-Cys peroxiredoxin in Saccharomyces cerevisiae. Antioxid. Redox Signal., 13(3), 249-258.

Pérez-Pérez, M. E., Florencio, F. J., and Lindahl, M. (2006) Selecting thioredoxins for disulphide proteomics: target proteomes of three thioredoxins from the cyanobacterium Synechocystis sp. PCC 6803. Proteomics., Suppl 1: S186-S195.

Portevin, D., de Sousa-D’Auria, C., Montrozier, H., Houssin, C., Stella, A., Lanéelle, M., A., Bardou, F., Guilhot, C., and Daffé, M. (2005) The acyl-AMP ligase FadD32 and AccD4-containing acyl-CoA carboxylase are required for the synthesis of mycolic acids and essential for mycobacterial growth: identification of the carboxylation product and determination of the acyl-CoA carboxylase components. J. Biol. Chem., 280(10), 8862–8874.

Rawat, M., Newton, G. L., Ko, M., Martinez, G. J., Fahey, R. C., and Av-Gay, Y. (2002) Mycothiol-deficient Mycobacterium smegmatis mutants are hypersensitive to alkylating agents, free radicals, and antibiotics. Antimicrob Agents Chemother, 46(11), 3348–3355.

Roos, G., Foloppe, N., and Messens, J. (2013) Understanding the pKa of redox cysteines: the key role of hydrogen bonding. Antioxid Redox Signal, 18(1), 94-127.
Roos, G., Garcia-Pino, A., Van Belle, K., Brosens, E., Wahni, K., Vandenbussche, G., Wyns, L., Loris, R., and Messens, J. (2007) The conserved active site proline determines the reducing power of *Staphylococcus aureus* thioredoxin. *J. Mol. Biol.*, **368**(3), 800–811.

Rosado, L. A., Wahni, K., Degiacomi, G., Pedre, B., Young, D., Rubia, A. G. de. la , Boldrin, F., Martens, E., Marcos-Pascual, L., Sancho-Vaello, E., Albesa-Jové, D., Provvedi, R., Martin, C., Makarov, V., Versées, W., Verniest, G., Guerin, M.E., Mateos, L.M., Manganelli, R., and Messens, J. (2017) The antibacterial prodrug activator Rv2466c is a mycothiol-dependent reductase in the oxidative stress response of *Mycobacterium tuberculosis*. *J Biol Chem*, **292**(32), 13097-13110.

Sharma, S. V., Van Laer, K., Messens, J., and Hamilton, C. J. (2016) Thiol redox and pKa properties of mycothiol, the predominant low-molecularweight thiol cofactor in the Actinomycetes. *Chembiochem*, **17**(18), 1689-1692.

Shen, X. H., Ji, C. Y., Huang, Y., Liu, Z. P., and Liu, S. J. (2005) Functional identification of novel genes involved in the glutathione-independent gentisate pathway in *Corynebacterium glutamicum*. *Appl. Environ. Microbiol.*, **71**(7), 3442-3452.

Si, M., Chen, C., Che, C., Li, G., Li, X., and Su, T. (2020) The thiol oxidation-based sensing and regulation mechanism for the OasR-mediated organic peroxide and antibiotic resistance in *C. glutamicum*. *Biochem J.*, **477**(19), 3709-3727.

Si, M., Che, C., Li, G., Li, X., Gong, Z. J., Liu, J. F., Yang, G., and Chen, C. (2019) Characterization of Xi-class mycothiol S-transferase from *Corynebacterium glutamicum* and its protective effects in oxidative stress. *Microb Cell Fact.*, **18**(1),182.

Si, M., Chen, C., Su, T., Che, C., Yao, S., Liang, G. J., Li, G. Z., and Yang, G. (2018a) CosR is an oxidative stress sensing a MarR-type transcriptional repressor in *Corynebacterium glutamicum*. *Biochem J.*, **475**(24), 3979-3995.

Si, M., Su, T., Chen, C., Liu, J. F., Gong, Z. J., Che, C. C., Li, G. Z., and Yang, G. (2018b) OhsR acts as an organic peroxide-sensing transcriptional activator using an S-mycothiolation mechanism in *Corynebacterium glutamicum*. *Microb Cell Fact.*, **17**(1), 200.

Si, M., Xu, Y., Wang, T., Long, M., Ding, W., Chen, C., Guan, X. M., Liu, Y. B., Wang, Y., Shen, X. H., and Liu, S. J., (2015) Functional characterization of a mycothiol peroxidase in
Corynebacterium glutamicum that uses both mycoredoxin and thioredoxin reducing systems in the response to oxidative stress. *Biochem J.*, 469(1), 45-57.

Si, M. R., Zhang, L., Yang, Z. F., Xu, Y. X., Liu, Y. B., Jiang, C. Y., Wang, Y., Shen, X. H., and Liu, S. J. (2014) NrdH Redoxin enhances resistance to multiple oxidative stresses by acting as a peroxidase cofactor in *Corynebacterium glutamicum*. *Appl Environ Microbiol.*, **80**(5), 1750-1762.

Su, T., Si, M., Zhao, Y., Liu, Y., Yao, S., Che, C. C., and Chen, C. (2018) A thioredoxin-dependent peroxiredoxin Q from *Corynebacterium glutamicum* plays an important role in defense against oxidative stress. *PLoS One*, **13**(2), e0192674.

Su, T., Si, M., Zhao, Y., Yao, S., Che, C., Liu, Y., and Chen, C. (2019) Function of alkyl hydroperoxidase AhpD in resistance to oxidative stress in *Corynebacterium glutamicum*. *J Gen Appl Microbiol*, **65**(2), 72-79.

Sutoh, S., Uemura, Y., Yamaguchi, Y., Kiyotou, A., Sugihara, R., , Nagayasu, M., Kurokawa, M., Ito, K., Tsunekawa, N., Nemoto, M., Inagaki, K., and Tamura, T. (2019) Redox-tuning of oxidizing disulfide oxidoreductase generates a potent disulfide isomerase. *Biochim Biophys Acta Proteins Proteom*, **1867**(3), 194-201.

Thurlkill, R.L., Grimsley, G.R., Scholtz, J.M., and Pace, C.N. (2006) pK values of the ionizable groups of proteins. *Protein Sci.*, **15**(5), 1214–1218.

Van Laer, K., Buts, L., Foloppe, N., Vertommen, D., Van Belle, K., Wahni, K., Roos, G., Nilsson, L., Mateos, L.M., Rawat, M., Nuland Nico, A.J.v., and Messens, J. (2012) Mycoredoxin-1 is one of the missing links in the oxidative stress defence mechanism of Mycobacteria. *Mol Microbiol.*, **86**(4), 787–804.
Table 1. Insulin reduction parameters

| Substrates                   | MSH/Mtr/NADPH |
|------------------------------|---------------|
|                              | Control\(^a\) | NCgl0018 | NCgl0018:C104S | NCgl0018:C107S | Mrx1 |
| Rate of precipitation \((A_{600} \times 10^{-5} \text{ s}^{-1})\) | 2.65±0.5      | 13.18±0.4 | 2.14±0.1       | 15.25±0.3     | 26.13±2.6 |
| Starting point (s)           | 2934          | 16929     | 2857           | 1533          | 1237  |

\(^a\) Control, reaction without catalyst.

Figure Legends

Figure 1. The \(\Delta\)ncgl0018 strains of \(C.\ glutamicum\) are more sensitive to various stress-inducing reagents.

(A) Growth curves of the \(C.\ glutamicum\) RES167 strain containing pXMJ19 vector [WT (pXMJ19)], \(\Delta\)ncgl0018(pXMJ19) (the mutant lacking ncgl0018 with the empty plasmid pXMJ19), and the complemented strain \(\Delta\)ncgl0018(pXMJ19-ncglool018) (the \(\Delta\)ncgl0018 mutant expressing the wild-type ncgl0018 gene in the shuttle vector pXMJ19) under normal condition. The growth of the indicated strains in LB was monitored by measuring \(A_{600}\) at indicated time points. (B-L) The zone of inhibition (cm) of WT(pXMJ19), \(\Delta\)ncgl0018(pXMJ19), \(\Delta\)ncgl0018(pXMJ19-ncglool018), and WT(pXMJ19-ncglool018) strains caused by the paper disks \((\phi = 5 \text{ mm})\) with oxidative stress-inducing reagents, including hydrogen peroxide (\(\text{H}_2\text{O}_2\)), hypochlorous acid (\(\text{HClO}\)), diamide, cumene hydroperoxide (CHP), tert-butyl hydroperoxide (\(\text{t-BHP}\)), 2, 4-dinitrochlorobenzene (CDNB), iodoacetamide (IAM), streptomycin (STR), ciprofloxacin (CIP), cadmium chloride (CdCl\(_2\)), and nickel sulfate (NiSO\(_4\)). The dot plot shows the mean and standard error of the 3 samples sets for each agent.

Figure 2. The form and thiol content of DTT-, \(\text{H}_2\text{O}_2\), or \(\text{H}_2\text{O}_2-\) and MSH-treated NCgl0018.

(A) Free sulphhydryl groups in NCgl0018 WT, NCgl0018:C104S, and NCgl0018:C107S were determined using 5, 5'-dithio-bis(2-nitrobenzoic acid) (DTNB). The data were represented as the mean±SD of three independent experiments. (B) Spectrophotometric analysis of NBD-labelled NCgl0018:C104S and NCgl0018:C107S. Reduced proteins treated with and without \(\text{H}_2\text{O}_2\) or \(\text{H}_2\text{O}_2\) and MSH were modified with NBD-Cl for 30 min. The resulting proteins were analyzed...
spectrophotometrically at 200-600 nm. The data are represented as the mean±SD of three independent experiments.

**Figure 3.** Oxidized NCgl0018-S₂ is preferably reduced by the MSH/Mtr/NADPH pathway.

The reduction of oxidized NCgl0018-S₂ by the MSH/Mtr/NADPH (A), the Trx/TrxR/NADPH (B), or Lpd/SucB/NADH (C) pathways was evaluated via Michaelis-Menten steady-state kinetics using the program GraphPad Prism 5. Mrx1-S₂ coupled to the MSH/Mtr/NADPH pathway (D) was used as control. The data are represented as the mean ± SD of three independent experiments.

Different concentrations of oxidized NCgl0018-S₂, or Mrx1-S₂ was mixed with a pre-incubated reaction mixture.

**Figure 4.** NCgl0018 reduced mycothiolated mixed disulfides via a monothiol mechanism. The activity of proteins were measured with the mixed disulfide between hydroxyethyl disulphide (HED) and MSH (HED-SSM) concentrations varying in the range 0-200 mM. The Michaelis-Menten plots of NCgl0018 WT (A), Mrx1(B), NCgl0018:C107S (C), and NCgl0018:C104S (D) activity were calculated using the program GraphPad Prism 5. The data are represented as the mean±SD of three independent experiments.

**Figure 5.** Negative regulation of NCgl0018 expression by OparR in C. glutamicum.

(A) β-Galactosidase analysis of the ncgl0018 promoter activity was performed using the transcriptional $P_{ncgl0018}$::lacZY chromosomal fusion reporter expressed in the indicated strain under different adverse conditions for 30 min. β-Galactosidase activity was assayed as described in “Materials and Methods”. Mean values with standard deviations (error bar) from at least three independent experiments are shown. **: $P \leq 0.01$; *: $P \leq 0.05$. (B) qRT-PCR assay was performed to analyze the expression of ncgl0018. Exponentially growing C. glutamicum cells were exposed to different reagents at the indicated concentrations for 30 min. The levels of ncgl0018 expression were determined by quantitative RT-PCR. The mRNA levels are presented relative to the value obtained from WT cells without treatment. The values represent the mean results from three independent cultivations, with standard errors. **: $P \leq 0.01$; *: $P \leq 0.05$. (C) EMSA was performed to analyze the interaction between the ncgl0018 promoter ($P_{NCgl0018}$) and His₆-OasR. As negative controls, a 211-bp fragment amplified from the ncgl0018 coding region using the primers control F and control R instead of the 211-bp ncgl0018 promoter (control A, lane 7) and
an irrelevant protein BSA instead of His₆-OasR (control B, lane 8) were included in the binding assays.

Figure 6 Proposed reaction mechanism for NCgl0018 in C. glutamicum.

(1), In the first chemical step, the nucleophilic Cys104 reacted with S-mycothiolated mixed disulfide of the target protein A, or intramolecular disulfide of the target protein B, forming S-mycothiolated Cys104 in NCgl0018 or an intermediate dithiol bridge between Cys104 of NCgl0018 and the target protein B. Subsequently, two pathways could take place. (2), In the presence of a second molecule of MSH (mycothiol), a nucleophilic attack took place at the mixed disulfide, releasing mycothione (mycothiol disulfide; MSSM) and the reduced form of Cys104. (3), In a parallel reaction, the resolving Cys107 pKa was lowered via deprotonation and performed a nucleophilic attack on the mixed disulfide, releasing one molecule of MSH and forming an intramolecular disulfide in NCgl0018, leading to the formation of a new Cys104-Cys107 disulfide. (4), For an intermediate dithiol bridge between Cys104 of NCgl0018 and the target protein B, the second cysteine Cys107 could solve it to form a Cys104-Cys107 disulfide. (5), Cys104-Cys107 disulfide was restored to the reduced, active, state of NCgl0018 by two molecules of MSH, accompanied by the generation of MSSM. (6), MSSM was reduced back to MSH by an NADPH-dependent Mtr.
Fig. 4

A. NCgl0018 WT

- Turnover (s⁻¹) vs. HED-SSM (mM)
- $K_m = 0.48 \pm 0.06$ mM
- $k_{cat} = 10.69 \pm 0.28 \text{ s}^{-1}$

B. Mrx1

- Turnover (s⁻¹) vs. HED-SSM (mM)
- $K_m = 0.49 \pm 0.07$ mM
- $k_{cat} = 104.48 \pm 1.99 \text{ s}^{-1}$

C. NCgl0018:C107S

- Turnover (s⁻¹) vs. HED-SSM (mM)
- $K_m = 0.46 \pm 0.04$ mM
- $k_{cat} = 13.04 \pm 0.24 \text{ s}^{-1}$

D. NCgl0018:C104S

- Turnover (s⁻¹) vs. HED-SSM (mM)
- $K_m = 62.81 \pm 15.01$ mM
- $k_{cat} = 0.0009 \pm 0.0001 \text{ s}^{-1}$

Fig. 5

A. Microwell analysis

- WT(pXMJ19) ($P_{ncgl0018}$:lacZY)
- ΔoasR(pXMJ19) ($P_{ncgl0018}$:lacZY)
- ΔoasR(pXMJ19-oasR) ($P_{ncgl0018}$:lacZY)

β-Galactosidase activity (Miller Units)

- No stress
- H₂O₂ (mM)
- CHP (mM)
- CDNB (mM)
- STR (µg/ml)
- CdCl₂ (µM)

B. Relative mRNA level

- No stress
- 25 mM H₂O₂
- 4.5 µM CHP
- 30 mM CDNB
- 0.5 µg/ml STR
- 100 µM CdCl₂

C. DNA-Protein Complexes

- Lane 1: No stress
- Lane 2: 0.5 µg/ml STR
- Lane 3: 1.0 µg/ml STR
- Lane 4: 1.5 µg/ml STR
- Lane 5: 2.0 µg/ml STR
- Lane 6: 2.5 µg/ml STR
- Lane 7: 3.0 µg/ml STR
- Lane 8: Free DNA

| $P_{ncgl0018}$ | Control |
|----------------|---------|
| 0              | A B     |
| 0.5            | OasR (µg) |
| 1.0            |         |
| 1.5            |         |
| 2.0            |         |
| 2.5            |         |
| 3.0            |         |
| 3.5            |         |
| 4.0            |         |
| 4.5            |         |
| 5.0            |         |
| 5.5            |         |
| 6.0            |         |
| 6.5            |         |
| 7.0            |         |
| 7.5            |         |
| 8.0            |         |
| 8.5            |         |
| 9.0            |         |
| 9.5            |         |
| 10.0           |         |
