A novel mycothiol-dependent thiol–disulfide reductase in Corynebacterium glutamicum involving oxidative stress resistance

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Abstract
ncgl2478 gene from Corynebacterium glutamicum encodes a thiol–disulfide oxidoreductase enzyme annotated as dithiol–disulfide isomerase DsbA. It preserves a Cys–Pro–Phe–Cys active-site motif, which is presumed to be an exclusive characteristic of the novel DsbA–mycoredoxin 1 (Mrx1) cluster. However, the real mode of action, the nature of the electron donor pathway and biological functions of NCgl2478 in C. glutamicum have remained enigmatic so far. Herein, we report that NCgl2478 plays an important role in stress resistance. Deletion of the ncgl2478 gene increases the size of growth inhibition zones. The ncgl2478 expression is induced in the stress-responsive extra-cytoplasmic function-sigma (ECF-σ) factor SigH-dependent manner by stress. It receives electrons preferentially from the mycothiol (MSH)/mycothione reductase (Mtr)/NADPH pathway. Further, NCgl2478 reduces S-mycothiolated mixed disulfides and intramolecular disulfides via a monothiol–disulfide and a dithiol–disulfide exchange mechanism, respectively. NCgl2478 lacks oxidase activity; kinetic properties of its demycothiolation are different from those of Mrx1. Site-directed mutagenesis confirms Cys24 is the resolving Cys residue, while Cys21 is the nucleophilic cysteine that is oxidized to a sulfenic acid and then forms an intramolecular disulfide bond with Cys24 or a mixed disulfide with MSH under oxidative stress. In conclusion, our study presents the first evidence that NCgl2478 protects against various stresses by acting as an MSH-dependent thiol–disulfide reductase, belonging to a novel DsbA–Mrx1 cluster.

Keywords Thiol–disulfide interchange protein (DsbA) · Corynebacterium glutamicum · Oxidative stress · SigH

Introduction
Corynebacterium glutamicum, a well-known various L-amino acid producer in industrial applications and a model organism in systems biology, unavoidably generates or encounters a series of unfavorable circumstances in the fermenting process (Bröer et al. 1993). Various environmental insults, including oxidants, heavy metals, antibiotics, alkylating agents, and acids, induce accumulation of excessive reactive oxygen species (ROS) (Halliwell and Gutteridge 1984). These excessive ROS are highly reactive molecules that are not only capable of damaging cellular constituents such as DNA, RNA, lipids, and proteins, but also destroying intracellular redox homeostasis and provoking oxidative stress (Jiang et al. 2019; Storz et al. 1987). Thus, to survive within the diverse fermentation environment, C. glutamicum acquires a variety of mechanisms to protect its cellular constituents from ROS and maintain its redox equilibrium (Dalle-Donne et al. 2008). One of these mechanisms is the use of a low molecular weight (LMW) defense mechanism. In C. glutamicum, millimolar concentrations of MSH (mycothiol; chemically 1D-myco-inositol-2-[N-acetyl-l-cysteiny] amido-2-deoxy-α-D-glucopyranoside), a pseudodisaccharide containing a cysteine moiety as a reactive thiol, is the main LMW thiol involved in detoxification and maintaining redox homeostasis (Liu et al. 2013; Newton and Fahey 2008; Van Laer et al. 2012). The redox-active sulfhydryl group of MSH protects cells from ROS by directly scavenging free radicals and by serving as a cofactor for antioxidant enzymes such as mycothiol peroxidase (MPx) and methionine...
sulfoxide reductase A (MsrA), in conjunction with mycero-
doxin 1 (Mrx1) (Si et al. 2015a, b). Another mechanism is
to use various antioxidant enzymes, including directly
ROS-scavenging terminal enzymes such as catalase (KatG),
superoxide dismutases (SOD), and peroxidase, oxidized pro-
teins-repairing oxidoreductases, as well as regulatory proteins
(Dalle-Donne et al. 2008).

Oxidized proteins-repairing oxidoreductases, such as
thioredoxin (Trx), Mrx1, dithiol–disulfide isomerase (DsbA),
DsbA-like Mrx1, and alkyl hydroperoxide reductase subunit D
(AhpD), are believed to play a pivotal role in protecting against
oxidative stress, maintaining intracellular thiol homeostasis,
enuring the proper folding of proteins and facilitating appro-
priate functioning of proteins in bacteria (Dalle-Donne et
al. 2008; Su et al. 2019; Van Laer et al. 2012; Rosado et
al. 2017). Mrx1 with the Cys–Pro–Tyr–Cys (C–P–Y–C) motif, an
MSH-dependent disulfide oxidoreductase with a glutaredoxin-like
Mrx1 with the Cys–Pro–Trp–Cys (C–P–W–C) motif had the activ-
ity of demycothiolating and reducing a mycothiol arsenate
adduct. NCgl2339 and Rv2466c have no oxidase properties
like classic DsbA. Moreover, their kinetic properties were
different from those of classic Mrx1. Therefore, Rosado et
al. believed NCgl2339 and Rv2466c were novel oxidoreductases,
belonging to DsbA-like Mrx1. However, some aspects of this
new type of oxidoreductase are poorly understood so far, such
as their mode of action, actual enzymatic functioning, and
biological function. Thus, an in-depth analysis of the physi-
ological and biochemical characteristics and catalytic mecha-
nisms is vital. Bioinformati cal analysis reveals that C. glutami-
cum NCgl2478 preserves the C–P–F–C active-site sequence
motif, similar to that of NCgl2339. The phenomenon indicated
NCgl2478 may be a potentially new redox enzyme and protect
C. glutamicum from oxidative stress. In this study, we selected
ORF NCgl2478 and sought to explore the physiological and
biochemical functions of NCgl2478 in C. glutamicum, paving
the way for correctly classifying similar enzymes from other
organisms.

Material and methods

Bacterial strains and growth conditions

The bacterial strains and plasmids used in this study were
listed in Table 1. C. glutamicum RES167 strains used in
this study were derived from the sequenced strain ATCC
13032. Escherichia coli JM109 (Stratagene, United States)
and C. glutamicum RES167 strains were grown on either
Luria–Bertani (LB) broth or LB agar plates at 37 and
30 °C under vigorous agitation (220 rpm) as previously
reported, respectively (Shen et al. 2005). To produce and
maintain mutant of a gene in C. glutamicum, brain–heart
broth medium containing 0.5 M sorbitol (BHIS) was used
(Shen et al. 2005). ncgl2478 gene in-frame deletion was
generated using the method described (Shen et al. 2005).
For complementation, the pXMJ19-ncgl2478 derivatives
were transformed into Δncgl2478 mutant by electroporation.
The transformant was selected on nalidixic acid and
chloramphenicol-containing LB plates and its expression in
C. glutamicum was induced by adding 0.5 mM isopropyl
β-d-1-thiogalactopyranoside (IPTG) (Sigma-Aldrich) (Shen
et al. 2005). Antibiotics were added at the following concen-
trations: kanamycin, 50 µg ml−1 for E. coli and 25 µg ml−1
for C. glutamicum; nalidixic acid, 40 µg ml−1 for C. glutami-
cum; chloramphenicol, 20 µg ml−1 for E. coli and 10 µg ml−1
for C. glutamicum.

Plasmid construction

Primers used in this study were listed in Table 2. The
ncgl2478 gene region of C. glutamicum was amplified
with primer pair ONCgl2478-F and ONCgl2478-R from
geno mic DNA of C. glutamicum RES167 by PCR, and
then the resulting fragments cut with BamHI and HindIII
enzymes were cloned into appropriately digested pET28a
to give plasmids pET28a-ncgl2478. The suicide plasmid
pK18mobsacB-Δncgl2478 was obtained by two-step recombina-
tion as described previously (Su et al. 2018). To prepare
pXMJ19-ncgl2478, primer pair CNCgl2478-F/CNCgl2478-
R was used to amplify the ncgl2478 gene DNA fragments
from C. glutamicum genomic DNA. The resulting DNA
fragments were cut and then cloned into similar digested
pXMJ19. To generate pET28a-ncgl2478:C21S or pET28a-
ncgl2478:C24S, site-directed mutagenesis was performed
by two rounds of PCR as described (Si et al. 2015a, 2018a).
To obtain the lacZY fusion reporter vector pK18mobsacB-
Pncgl2478::lacZY, overlap PCR was performed to fuse the
ncgl2478 promoter to the lacZY reporter gene (Su et al.
2018). For obtaining pK18mobsacB-Pncgl2478::lacZY,
258-bp ncgl2478 promoter DNA containing the muta-
genized sequence of the predicted stress-responsive
extra-cytoplasmic function-sigma (ECF-σ) factor SigH binding site (Pncgl2478M) was first directly synthesized by Shanghai Biotechnology Co., Ltd. Start and stop sites of Pncgl2478M were the same as those of Pncgl2478 in Pncgl2478::lacZY. Then, the resulting 258-bp Pncgl2478M was fused to a lacZY reporter gene. Finally, Pncgl2478M::lacZ was inserted into similarly digested pK18mobsacB. The fidelity of all constructs was confirmed by DNA sequencing (Sangon Biotech, Shanghai, China).

**Overexpression and purification of recombinant protein**

pET28a derivatives were transformed into E. coli BL21(DE3) cells and recombinant proteins were purified as described previously (Si et al. 2018b). Eluted recombinant His6-NCgl2478 proteins were concentrated and loaded onto a Superdex-75 10/300 gel filtration column (GE Healthcare, Piscataway, NJ) with a running condition of 10 mM Tris (pH 7.4), 100 mM NaCl, and 5 mM β-mercaptoethanol. For conducting subsequent enzyme activity experiments, the His6 tag in protein was cut in the presence of 10 units of Enterokinase-Max (Invitrogen, Karlsruhe, Germany) at 4 °C overnight. To remove the cleaved tag and uncleaved protein, Ni–NTA agarose was used. All enzymes were purchased from Sigma-Aldrich (St. Louis, MO). Resulting His6-tag-free protein was dia-lyzed against PBS at 4 °C and concentrated 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 alkylating agents and oxidative agents according to Rawat et al. (2002). Briefly, bacterial strains were grown to the mid-log phase and 100 μl of culture containing about 10^7 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 50 mM hydrogen peroxide (H_2O_2), 5.5 mM cumene hydroperoxide (CHP),

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**Table 1 Bacterial strains and plasmids used in this study**

| Strains or plasmids | Relevant genotype description | Source or references |
|---------------------|------------------------------|----------------------|
| **Strains**<br>Corynebacterium glutamicum |  |
| RES167 | Restriction-deficient mutant of ATCC13032, Δ(cglIM–cglIR–cglIIR) | Tauch et al. (2002) |
| Δncgl2478 | nclg2478 deleted in RES167 | This study |
| ΔsigH |  |
| E. coli |  |
| BL21(DE3) | E. coli expression host, hsdS gal (lacI857 ind-1 Sam7 nin-5 lac UV5-T7 gene 1) | Novagen |
| JM109 | recA1 supE44 endA1 hsdR17 gyrA96 relA1 thi Δ(lac-proAB)F(traD36 proABlacF lacΔZM15) | Stratagene (catalog no. 200235) |
| **Plasmids** |  |
| pK18mobsacB | Suicide plasmid carrying sacB for selecting double crossover in C. glutamicum, Km<sup>+</sup> | Schäfer et al. (1994) |
| pK18mobsacB-Δncgl2478 | Construct used for in-frame deletion of ncgl2478 | This study |
| pK18mobsacB-P<sub>ncgl2478</sub>·lacZY | P<sub>ncgl2478</sub>·lacZY fusion in pK18mobsacB | This study |
| pXM19 | Shuttle vector (P<sub>lac</sub> lac<sup>P</sup> pBL1 oriV<sub>C. glutamicum</sub> PK18 oriV<sub>E. coli</sub>) | Jakoby et al. (1999) |
| pXM19-ncgl2478 | ncgl2478 cloned into pXM19 for complementation | This study |
| pET28a | Expression vector with N-terminal hexahistidine affinity tag | Novagen |
| pET28a-ncgl2478 | ncgl2478 in pET28a | This study |
| pET28a-ncgl2478:C21S | ncgl2478:C21S in pET28a | This study |
| pET28a-ncgl2478:C24S | ncgl2478:C24S in pET28a | This study |
| pET28a-mrx1 |  | Si et al. (2014b) |
| pET28a-mrx1:C15S |  | Si et al. (2014b) |
| pET28a-mtr |  | Si et al. (2014b) |
| pET28a-trx |  | Si et al. (2014b) |
| pET28a-trxR |  | Si et al. (2014b) |
| pET28a-sigH |  | Si et al. (2014a) |
0.2 mM sodium hypochlorite (NaClO), 5 mM diamide, 50 mM 2, 4-dinitrochlorobenzene (DNCB), and 0.6 mM iodoacetamide (IAM). The disks were allowed to dry and the plates were incubated for 2–3 days at 30 °C. The diameter of the inhibition zones was measured. Experiments were performed in triplicate.

**Electrophoretic mobility shift assay (EMSA)**

EMSA was performed according to the method described previously (Su et al. 2019).

**Preparation of oxidized NCgl2478-S₂ in vitro**

Oxidized NCgl2478-S₂ was prepared according to previously described (Van Laer et al. 2012; Pedre et al. 2015) protocol. First, NCgl2478 or Mrx1 from *C. glutamicum* was reduced by incubation with 50 mM DTT for 30 min at room temperature. Second, excess DTT was removed by ultrafiltration. Third, pre-reduced NCgl2478 or Mrx1 was oxidized with a fivefold molar excess of diamide and incubated for 30 min at room temperature. Finally, oxidized NCgl2478-S₂ or Mrx1-S₂ was purified on a Superdex 75 10/300 GL column (GE Healthcare) equilibrated with 20 mM Tris, 150 mM NaCl, pH 7.6 for further experiments. Pure NCgl2478-S₂ and Mrx1-S₂ were confirmed by Matrix-Assisted Laser Desorption/Ionization Time of Flight Mass Spectrometry (MALDI-TOF MS).

**Steady-state kinetics of oxidized NCgl2478-S₂ by Trx/TrxR/NADPH and MSH/Mtr/NADPH pathway**

Oxidized NCgl2478-S₂-dependent oxidation of NADPH in the Trx/TrxR/NADPH or MSH/Mtr/NADPH pathway was
continuously monitored at 340 nm ($\epsilon_{280} = 6220 \, \text{M}^{-1} \text{cm}^{-1}$) in reaction mixture. In the Trx/TrxR/NADPH pathway, the reaction mixture contained 50 mM Tris–HCl buffer (pH 7.5), 1 mM EDTA, 5 $\mu$M C. glutamicum TrxR, 300 $\mu$M NADPH, 5 $\mu$M C. glutamicum Trx, and varying concentrations of oxidized NCgl2478-S$_{2}$. In the MSH/Mtr/NADPH pathway, reaction mixture contained 50 mM Tris–HCl buffer (pH 7.5), 1 mM EDTA, 5 $\mu$M C. glutamicum Mtr, 300 $\mu$M NADPH, 500 $\mu$M MSH, and varying concentrations of oxidized NCgl2478-S$_{2}$. All reactions were carried out at 37 °C and started with the addition of oxidized NCgl2478-S$_{2}$ in a reaction mixture previously incubated for 3 min at 37 °C. Control measurements were performed in the absence of NCgl2478-S$_{2}$. Reactions were performed in duplicate. Mrx1-S$_{2}$ was used as a positive control. The $k_{\text{cat}}$ and $K_{m}$ values were obtained from a non-linear fit with the Michaelis–Menten equation using the program GraphPad Prism 5.

Quantitative analysis of sulfhydryl groups

Free sulfhydryl groups in wild-type NCgl2478 (NCgl2478 WT) and its variants were measured using 5, 5′-dithio–bis (2-nitrobenzoic acid) (DTNB) (Ellman 1959). The amounts of reactive sulfhydryl groups were measured using the molar absorption coefficient of TNB at 412 nm ($\epsilon_{412}$) of 13,600 M$^{-1}$·cm$^{-1}$ (Gething and Davidson 1972).

NBD–Cl analysis of the sulfenic acid state

To study the formation of cysteine sulfenic acid (Cys–SOH) as a reaction intermediate, NCgl2478:C21S and NCgl2478:C24S labeled with 4-chloro-7-nitrobenzofurazan (NBD–Cl) were assayed as described previously (Selles et al. 2012).

Enzymatic activity assay

Insulin disulfide reduction was performed based on the method described by Rosado et al. (2017). 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 of $A_{600}$ ranging from 2000 to 2500 s (Holmgren 1979).

Hydroxyethyl disulfide (HED) was utilized to prepare and measure the mixed disulfide between MSH and 2-hydroxyethyl disulfide (HED) (HED-SSM) reduction activity (Si et al. 2019). HED-SSM was formed by incubating 1000 mM HED with 50 mM MSH at 30 °C for 3 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 50 mM potassium phosphate buffer (pH 7.6), 250 $\mu$M NADPH, 5 $\mu$M Mtr, 500 $\mu$M MSH, and 1 $\mu$M NCgl2478 (WT, C21S, or C24S). The assay was performed at 25 °C. Absorption was monitored at 340 nm. The activity was determined after subtracting the spontaneous reduction rate observed in the absence of NCgl2478 and the number of micromoles of NADPH oxidized per second per micromole of enzyme (i.e. the turnover number, s$^{-1}$) was calculated using the molar absorption coefficient of NADPH at 340 nm ($\epsilon_{340}$) of 6220 M$^{-1}$·cm$^{-1}$. Three independent experiments were performed at each substrate concentration. The $k_{\text{cat}}$ and $K_{m}$ values of NCgl2478 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 unionized counterpart (R-S$^{-}$) in the same wavelength (Roos et al. 2013). To cover a broad pH range, a reaction mixture containing a poly-buffer solution composed of 10 mM sodium acetate, 10 mM sodium phosphate, 10 mM sodium borate, and 10 mM sodium citrate, pH 9.4, was used. For the oxidation of cysteine mutants, a tenfold excess of H$_2$O$_2$ was used. Excess H$_2$O$_2$ was removed by ultrafiltration. A final reaction mixture of 20 $\mu$M NCgl2478 (reduced or oxidized) was titrated with 100 mM HCl. The pKa of NCgl2478:C22S and NCgl2478:C24S variants were determined in the same conditions as described for NCgl2478 WT. The measurements were performed in a Carry UV spectrophotometer (Agilent Technologies) precooled to 10 °C. The sigmoidal pH-dependent saturation curve was fitted to the Henderson–Hasselbalch equation (Roos et al. 2007), where $A_{\exp}$ was the experimental value of $A_{280}$ and $A_{\text{SH}}$ was the $A_{280}/A_{280}$ value for the protonated form, and $A_{\text{SH}^{-}}$ is the $A_{280}/A_{280}$ for the deprotonated form. The data were fitted to the following equation using GraphPad Prism version 5 (San Diego California USA).

$$A_{\exp} = A_{\text{SH}} + \frac{(A_{\text{SH}^{-}} - A_{\text{SH}})}{1 + 10^{(pK_{a} - \text{pH})}}$$

RNase I activity assay

Oxidase activity was measured as described previously (Roos et al. 2007).

Construction of chromosomal fusion reporter strains and β-Galactosidase assay

The lacZY fusion reporter plasmid pK18mobsacB-P$_{ncgl2478}$::lacZY was transformed into the corresponding
C. glutamicum RES 167 strain by electroporation and the chromosomal fusion reporter strains were selected on LB agar plates supplemented with kanamycin (Si et al. 2019). 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 activity was assayed with O-nitrophenyl-β-D-galactopyranoside (ONPG) as the substrate (Miller 1992). The β-Galactosidase data presented were the averages of three independent biological experiments and error bars indicated the SDs from three independent experiments.

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

Isolation of the total RNA and transcript levels analysis was performed as described previously (Si et al. 2019). To obtain standardized results, the relative abundance of the 16S rRNA gene was used as the internal standard.

Statistical analysis

The results shown represented the average of three independent experiments; error bars indicated the standard deviation (SD) from three independent experiments. Statistical analyses of survival rate, transcription level, and protein level were determined with paired two-tailed Student’s t test. GraphPad Prism Software was used to carry out statistical analyses (GraphPad Software).

Results and discussion

**C. glutamicum NCgl2478 null mutant was sensitive to oxidative stress**

*C. glutamicum* NCgl2478 containing the Cys–Pro–Phe–Cys (C–P–F–C) motif is annotated as a dithiol-disulfide isomerase DsbA. Recently, a demonstration indicated that Rv2466c from *M. tuberculosis* and NCgl2339 from *C. glutamicum*, having high sequence similarity to DsbA, belong to a novel DsbA–Mrx1 cluster (Rosado et al. 2017). The novel DsbA–Mrx1 cluster has the special Cys–Pro–Trp/Phe–Cys (C–P–W/F–C) active-site sequence motif and exhibits different enzymatic features and substrate preferences from Mrx1 cluster with the catalytic motif Cys–Pro–Tyrc–Cys (C–P–Y–C) or classical DsbA cluster with the catalytic motif Cys–Pro–His–Cys (C–P–H–C) (Rosado et al. 2017). Although amino acid sequence comparison revealed NCgl2478 is only 29.8% identical to Rv2466c and 26.2% identical to NCgl2339 (Fig. 1a), we presumed it might be also a member of the DsbA-Mrx1 cluster involved in stress response as it shared the C–P–F–C signature motif presumed to be an exclusive characteristic of the DsbA–Mrx1 cluster (Fig. 1b). *M. tuberculosis* Rv2466c is validated to promote mycobacterial resistance to oxidative stress (Rosado et al. 2017). Thus, to assess the role of NCgl2478 in protecting cells against oxidative stress, we constructed an *ncgl2478* null mutant in *C. glutamicum* and tested the sensitive phenotype of *ncgl2478* mutant to various oxidizing and H₂O₂-inducing agents [hydrogen peroxide (H₂O₂), cumene hydroperoxide (CHP), sodium hypochlorite (NaClO), diamide, 2, 4-dinitrochlorobenzene (DNCB), and iodoacetamide (IAM)] by an agar-based disc diffusion assay. As shown in Table 3, Δncgl2478 strain diffused.
(the mutant lacking ncgl2478 with the empty plasmid pXMJ19) showed decreased resistance to all chemical reagents tested challenge compared to the WT strain (the C. glutamicum RES167 strain with the empty plasmid pXMJ19), giving a significantly larger zone of inhibition than WT strain. To confirm that the sensitivity to reagents may occur when lacking ncgl2478, complementary strain Δncgl2478+ was constructed by the introduction of plasmid pXMJ19 in trans containing the wild-type C. glutamicum ncgl2478 gene into ncgl2478 null mutant. As shown in Table 3, sensitive phenotypes were almost fully restored in Δncgl2478+. Although deletion of ncgl2478 did not affect C. glutamicum growth under normal condition, NCgl2478 was important for survival under various oxidative stress conditions.

Table 3 Sensitivity of C. glutamicum strains to oxidizing agents tested by disk diffusion assay

| Agents          | Size of growth inhibition zone (cm) of various strainsa |
|-----------------|--------------------------------------------------------|
|                 | WT          | Δncgl2478   | Δncgl2478*   |
| H2O2            | 1.5 ± 0.3  | 2.7 ± 0.5** | 1.6 ± 0.2    |
| CHP             | 1.8 ± 0.3  | 2.3 ± 0.4*  | 1.7 ± 0.4    |
| NaClO           | 2.7 ± 0.2  | 3.2 ± 0.3*  | 2.8 ± 0.4    |
| Diamide         | 1.4 ± 0.3  | 1.8 ± 0.2*  | 1.5 ± 0.2    |
| DNCB            | 1.6 ± 0.2  | 2.1 ± 0.3*  | 1.7 ± 0.4    |
| IAM             | 1.7 ± 0.4  | 2.2 ± 0.3*  | 1.7 ± 0.2    |

H2O2 hydrogen peroxide, CHP cumene hydroperoxide, NaClO sodium hypochlorite, DNCB 2,4-dinitrochlorobenzene, IAM iodoacetamide

*P ≤ 0.05 or **P ≤ 0.01 versus WT for the Δncgl2478 mutant

aThe values were mean ± SD for three independent determinations

Formation of an intramolecular disulfide bond Cys21–Cys24 under oxidative stress

The Cys in the Cys19–Pro–Trp–Cys22 (C19–P–W–C22) active-site motif of M. tuberculosis Rv2466c could form Cys19–Cys22 disulfide and Cys19–MSH mixed disulfide under oxidative stress (Rosado et al. 2017). Moreover, Cys19 and Cys22 of M. tuberculosis Rv2466c were the nucleophilic and resolving cysteines, respectively. Amino acid sequence comparison showed that Cys21 of NCgl2478 might be the nucleophilic and Cys24 might be the resolving Cys residues in NCgl2478. During catalysis, the labile peroxidatic Cys–SOH is easily attacked by C_R to form the redox-active disulfide. To trap Cys–SOH, the C-terminal cysteine of the active-site disulfide pair must be removed. Therefore, to investigate whether Cys21 and Cys24 have the above speculative function and test if these two cysteine residues in NCgl2478 could undergo disulfide after oxidation, we mutated the first and the second cysteine of the CXXC motif to serine, and these two mutated proteins NCgl2478:C21S and NCgl2478:C24S were purified by the Ni–NTA His·Bind Resin. NCgl2478 WT, NCgl2478:C21S and NCgl2478:C24S with and without previous exposure to H2O2 were used to carry out the free thiol content analysis. As shown in Fig. 2a, the DTT-treated NCgl2478 WT contained 1.85 ± 0.47 thiol groups per monomer, but the thiol content decreased to 0.22 ± 0.06 when NCgl2478 WT was treated with H2O2. The difference of 1.63 thiol groups between the two preparations was linked to the full oxidation of NCgl2478 WT after H2O2 treatment. These data indicated that NCgl2478 WT was fully oxidized by H2O2 to form a disulfide bond between Cys21 and Cys24.

Fig. 2 The thiol content of DTT- or H2O2-treated NCgl2478. a Free sulfhydryl groups in NCgl2478 WT and its variants were determined using 5, 5′-dithio-bis (2-nitrobenzoic acid) (DTNB). b, c Spectrophotometric analysis of NBD-labelled NCgl2478:C21S and NCgl2478:C24S. Reduced proteins treated with and without H2O2 or H2O2 and MSH were modified with NBD–Cl for 30 min. The resulting proteins were analyzed spectrophotometrically at 200–600 nm.
NBD-Cl can exclusively react with free thiol groups in protein and protein sulfenic acids (P-SOH), but not with protein sulfinic (P-SO$_2$H) and sulfonic (P-SO$_3$H) acid. The covalent attachment of NBD-Cl generated an absorption peak at $\sim$ 420 nm upon reaction with thiol groups, whereas it peaked at $\sim$ 347 nm upon reaction with sulfenic acids (Baker and Poole 2003). Following reaction with NBD–Cl, the absorption spectra of the NCgl2478:C21S variants remained unchanged before and after exposure to H$_2$O$_2$ or H$_2$O$_2$ and MSH, exhibiting only the 420 nm peak (Fig. 2b). DTNB assay for free thiol contents also showed one thiol per monomer before and after H$_2$O$_2$ or H$_2$O$_2$ and MSH treatment, implying no SOH is formed on Cys24, and Cys24 was still in thiol form under exposure to H$_2$O$_2$ or H$_2$O$_2$ and MSH (Fig. 2a). However, NCgl2478:C24S under H$_2$O$_2$ treatment lost one thiol group, compared to the thiol content of DTT-treated state, indicating that Cys21 did not exist as a thiol in H$_2$O$_2$-treated NCgl2478:C24S variant (Fig. 2a). Consistently, H$_2$O$_2$-treated and NBD-labeled NCgl2478:C24S had an absorbance maximum ($\lambda_{max}$) of 347 nm, representing the NBD-modified product Cys–S(O)–NBD (Ellis and Poole 1997), which clearly signified the detection and trapping of SOH at Cys21, the only Cys in the NCgl2478:C24S variant (Fig. 2c). Interestingly, no NBD–Cl labeling in H$_2$O$_2$-treated NCgl2478:C24S or MSH treatment occurred in the presence of MSH, indicating MSH reacted with NCgl2478:C24S–SOH to form NCgl2478:C24S–SSM (the mixed disulfide between MSH and NCgl2478:C24S). This result showed that Cys21 was sensitive to oxidant, forming a sulfenic acid (Cys21–SOH). In the presence of MSH, MSH directly interacted with Cys21–SOH to form Cys21–MSH mixed disulfide. Further, Cys24 resolved the Cys21–MSH mixed disulfide or Cys21–SOH, leading to the formation of a Cys21–Cys24 disulfide. This result agrees with what Rosado et al. reported for M. tuberculosis Rv2466c (Rosado et al. 2017).

Oxidized NCgl2478 preferred the MSH/Mtr/NADPH pathway as an electron source

To identify possible electron donor pathways coupled to NCgl2478 reduction, two of the most important electron transfer pathways in C. glutamicum responsible for keeping the redox potential in balance were reproduced in vitro, that of the MSH/Mtr/NADPH and Trx/TrxR/NADPH. To do so, NCgl2478 was first oxidized with a fivefold molar excess of diamide to obtain NCgl2478–S$_2$ (NCgl2478ox). NCgl2478–S$_2$ with a single disulfide bond between its active site cysteines was added as a substrate for the two electron transfer pathways mentioned above to measure steady-state kinetics. As shown in Fig. 3a, b, the $K_m$ value, $k_{cat}$ value, and catalytic coefficient of NCgl2478–S$_2$ for the MSH/Mtr/NADPH or the Trx/TrxR/NADPH electron donor pathway were calculated to be 1.01 ± 0.07 μM, 3.19 ± 0.05 s$^{-1}$, and 3.15 ± 0.07 × 10$^6$ M$^{-1}$ s$^{-1}$, or 20.05 ± 4.69 μM, 0.52 ± 0.04 s$^{-1}$, and 2.57 ± 0.38 × 10$^4$ M$^{-1}$ s$^{-1}$, respectively. It is worth noting that reduction of the oxidized form of Mrx1 (Mrx1ox) through the MSH/Mtr/NADPH pathway resulted in a catalytic coefficient of 2.43 × 10$^7$ M$^{-1}$ s$^{-1}$ (Fig. 3c), 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 NCgl2478ox could be reduced by both electron pathways, the enzyme was reduced about 120-times faster with the

![Fig. 3](image-url) Oxidized NCgl2478–S$_2$ was reduced preferably by the MSH/Mtr/NADPH pathway. The reduction of the oxidized NCgl2478–S$_2$ (a) and Mrx1–S$_2$ (c) by the MSH/Mtr/NADPH, or the reduction of the oxidized NCgl2478–S$_2$ (b) by the Trx/TrxR/NADPH pathway was evaluated via Michaelis–Menten steady-state kinetics using the program GraphPad Prism 5. The data were represented as mean±SD of three independent experiments. Different concentrations of oxidized NCgl2478–S$_2$ or Mrx1–S$_2$ were mixed with a pre-incubated mixture of the MSH, Mtr, and NADPH, or Trx, TrxR, and NADPH.
MSH/Mtr/NADPH pathway compared with the Trx/TrxR/NADPH pathway, indicating NCgl2478ox prefers the MSH/Mtr/MSH pathway. Mrx1ox catalyzed an approximately eightfold-faster reaction coupled to the MSH/Mtr/NADPH pathway compared with NCgl2478ox, indicating a different specificity of electron donor pathway for both enzymes (Fig. 3a, c).

The pKa of the cysteine residues

Since the thiolate ion has a higher absorption at 240 nm wavelength than the thiol group, the pKa of active site residues in NCgl2478 was therefore determined by recording the absorption at 240 nm during a pH titration (Roos et al. 2013). As shown in Fig. 4a, the pKa values of the nucleophilic Cys21 and the resolving Cys24 were less than 6 and 8.09, respectively. The result indicated that the low pKa value made Cys21 function as the nucleophilic Cys. In addition, the pKa value of nucleophilic Cys of NCgl2478 lied between the pKa values of the respective cysteines of Mrx1 (6.8) and DsbA (~3.5). Moreover, the pKa of the Cys24 (8.09) was already lower than the pKa of the MSH sulfur (8.76) (Sharma et al. 2016), which made Cys24 more attack Cys21–MSH mixed disulfide, leading to the formation of a Cys21–Cys24 disulfide.

NCgl2478 has no oxidase properties

To test 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 (Mes-sens 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 NCgl2478 and Mrx1 (Greiner-Stoeffele et al. 1996). NCgl2478 did not catalyze the oxidative refolding of RNase (Fig. 4b). Reduced RNase I (unfolded) demonstrated 15.5% of activity relative to folded RNase I (100%). In contrast, in the presence of Mrx1, 38.2% of activity was recovered. Thus, NCgl2478 did not function as an oxidase.

NCgl2478 reduced mycothiolated mixed disulfides preferably via a monothiol mechanism

M. tuberculosis Rv2466c reduced mycothiolated mixed disulfides and intramolecular disulfide bonds coupled to the MSH/Mtr/NADPH pathway via a different mechanism (Rosado et al. 2017). This led us to survey what mechanism NCgl2478 used to reduce mycothiolated mixed disulfides and intramolecular disulfide bonds coupled to the MSH/Mtr/NADPH pathway. The functionalities of NCgl2478 WT, NCgl2478:C21S, and NCgl2478:C24S to reduce insulin and the mixed disulfide between 2-hydroxyethyl disulfide (HED) and MSH (HED–SSM) were tested by following

Fig. 4 The cysteine of NCgl2478. a Cys21 was the nucleophilic cysteine of NCgl2478. The pKa measurement of the active site cysteines indicates that Cys21 has an unusual pKa value lower than 6 and that Cys24 was the resolving cysteine with a pKa of 8.09. I onized thiol groups (R-SH) extinction coefficient at 240 nm was utilized to measure pKa values of cysteines of NCgl2478 WT (○) and the NCgl2478:C24S (□) and NCgl2478:C24S (◊). The ratio composed by 240/280red and 240/280ox in a pH range of 5 to 10 as fitted with the Henderson–Hasselbalch equation. b NCgl2478 was not a DsbA-oxidoreductase. The methylene blue RNA intercalating assay was utilized to quantify the activity of RNase I (0.5 μM). E. coli RNase I was reduced, and the recovering of activity was monitored in the presence of several enzymes (5 μM NCgl2478 WT or Mrx1). NCgl2478 was unable to catalyze the disulfide bond formation in a previously reduced RNase I. Reactions were performed in duplicate.
the oxidation of NADPH in the presence of the MSH/Mtr/NADPH system.

To test the ability of NCgl2478 to reduce mixed disulfides, we compared its reactivity with C. glutamicum Mrx1 using HED–SSM as substrates (Fig. 5).

We followed NADPH consumption coupled to MSH and Mtr at 340 nm. The $K_m$ value, $k_{cat}$ value, and catalytic coefficient of NCgl2478 or Mrx1 for HED–SSM were calculated to be $0.51 \pm 0.05$ mM, $8.55 \pm 0.19$ s$^{-1}$, and $1.68 \pm 0.14 \times 10^4$ M$^{-1}$ s$^{-1}$, or $0.54 \pm 0.11$ mM, $103.81 \pm 3.14$ s$^{-1}$, and $19.22 \pm 0.31 \times 10^4$ M$^{-1}$ s$^{-1}$, respectively (Fig. 5a, b). NCgl2478:C24S has a slightly higher initial velocity of $11.37 \pm 0.31$ s$^{-1}$ toward HED-SSM, whereas, the NCgl2478:C21S was inactive. This cysteine substitution experiment indicated that NCgl2478 was functioning under a monothiol mode of action where the nucleophilic Cys21 was required to catalyze mixed disulfide bond reduction. It is worth noting that the activity of Mrx1 was about 11-fold faster in reducing HED-SSM compared with the NCgl2478 enzyme when the MSH/Mtr/NADPH pathway was introduced as an electron donor.

**NCgl2478 reduced intramolecular disulfide bonds via a dithiol mechanism**

We further checked the ability of NCgl2478 to reduce intramolecular disulfide bonds in an insulin assay (Table 4). When the MSH/Mtr/NADPH pathway was used as an electron donor, the activity of Mrx1 was 2.1-fold faster in reducing insulin compared with NCgl2478. However, NCgl2478 demonstrated no activity upon mutation of the nucleophilic Cys21 or the resolving Cys24 to serine (Table 4). This led

| Substrates            | MSH/Mtr/NADPH |
|-----------------------|---------------|
|                       | Controla | NCgl2478 | NCgl2478:C21S | NCgl2478:C24S | Mrx1    |
| Rate of precipitation |            |          |              |              |         |
| (A$_{600}$ 10$^{-5}$ s$^{-1}$) | 2.58 ± 0.1 | 3.14 ± 0.3 | 2.63 ± 0.2    | 2.93 ± 0.2  | 25.74 ± 4.1 |
| Starting point (s)    |     2876   | 1598     | 2743          | 2584          | 1215    |

*aControl, reaction without catalyst*
us to conclude that the reduction of insulin occurred via a dithiol mechanism.

**SigH positively regulated NCgl2478 expression in C. glutamicum**

Because *C. glutamicum ncgl2478* mutants exhibited sensitivity in the circumstances of various reagents, qRT-PCR, and *lacZY* activity profiling were employed to examine whether *ncgl2478* expression responded to these toxic stress inducers at the transcriptional level. However, no putative promoter was identified upstream of the *ncgl2478* gene. Interestingly, further upstream from *ncgl2478* was *ncgl2479* gene, which is identically orientated as *ncgl2478* (Fig. 6a). Thus, we speculated that *ncgl2478* was organized in a putative operon with *ncgl2479*, which were confirmed to be co-transcribed by reverse transcription PCR (Fig. 6b). Based on the basis of the information, a putative−10 region (GAGAAAAAT) and a putative−35 region (TTTCTCT) were identified, which localized within the upstream open reading frame of *ncgl2479* (Fig. 6a). Thus, we named the promoter DNA fragment of the *ncgl2478-ncgl2479* ORF region with His6-SigH led to the formation of DNA−protein complexes, and the abundance of such complexes depended on the amount of SigH (Fig. 6e left panel). However, both BSA instead of His6-SigH and a 211-bp control DNA fragment amplified from the *ncgl2478* ORF region showed no detectable binding (Fig. 6e, lanes 6 and 7). To further verify the predicted SigH binding site, the 211-bp promoter DNA containing the mutagenesis sequence of the predicted SigH-binding site (P<sub>ncgl2478M</sub>) showed no detectable binding (Fig. 6e, lanes 6 and 7). Moreover, promoter DNA mutations in the predicted SigH-binding site disrupted the formation of such complexes. Moreover, promoter DNA mutations in the predicted SigH-binding site caused the extremely low transcription activity of *ncgl2478* in WT and ∆*sigH* strains, similar to that in the Δ*sigH* mutant (Fig. 6f). Thus, SigH directly activated the expression of *ncgl2478* by specifically recognizing an operator within the *ncgl2478* promoter region.

**Conclusion**

In this study, we revealed a novel MSH-dependent oxidoreductase NCgl2478 by physiological and biochemical analysis. NCgl2478 promoted *C. glutamicum* resistance to oxidative stress. Its physiological roles in resistance to oxidative stresses were corroborated by its induced expression under various stresses, regulated directly by SigH. Despite its high sequence similarity to DsbA, NCgl2478 did not act as an oxidase. NCgl2478 was less specific in receiving electrons, because both the MSH/Mtr/NADPH and the Trx/TrxR/NADPH pathways regenerated oxidized NCgl2478. NCgl2478 preferentially linked to the MSH/Mtr/NADPH electron pathway via monothiol mechanism to reduce S-mycotiolated mixed disulfides. NCgl2478 had
a lower initial velocity toward HED-SSM. Thus, NCgl2478 had a similar mode of action, including enzymatic rate, substrate preference, and cell survival under stress, as the previously characterized DsbA–Mrx1 proteins (M. tuberculosis Rv2466c and C. glutamicum NCgl2339). Together, our work has uncovered NCgl2478 as a member of the novel DsbA–Mrx1 cluster promoted C. glutamicum resistance to oxidative stress.

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Author contributions Meiru Si, Yang Liu and Can Chen designed the research. Yang Liu, Xiaona Li, Jiaxin Luo, Tao Su, and Can Chen performed the research and analyzed the data. Can Chen and Meiru Si supervised the research. Yang Liu and Xiaona Li wrote the paper. Can Chen and Meiru Si revised the paper. All authors read and approved the final manuscript.

Data availability The study investigators have full access to the article datasets.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval and consent to participate Not applicable.
P ≤ 0.05. 

≤ 0.01; * represent the mean results from three independent cultivations, with the value obtained from WT cells without treatment. The values of expression were ncgl2478 concentrations for 30 min. The levels of cells were exposed to different toxic agents at indicated ncgl2478 to analyze the expression of C. glutamicum RES167 parental strain containing empty pXMJ19 (WT). 100 μl of exponentially growing C. glutamicum cells treated with different toxic agents at indicated concentrations for 30 min was added to the enzyme reaction system. The values represent the mean results from three independent cultivations, with standard errors. **P ≤ 0.01; *P ≤ 0.05. d qRT-PCR assay was performed to analyze the expression of ncgl2478. Exponentially growing C. glutamicum cells were exposed to different toxic agents at indicated concentrations for 30 min. The levels of ncgl2478 expression were determined by qRT-PCR. The mRNA levels were related to the value obtained from WT cells without treatment. The values represent the mean results from three independent cultivations, with standard errors. **P ≤ 0.01; *P ≤ 0.05. e EMSA was performed to analyze the interactions between Hissfg-HsigH and the ncgl2478 promoter (Pngl2478) or the promoter mutating the identified SigH binding region (Pvoid2478). A 211-bp fragment amplified from the ncgl2478 coding region instead of the 211-bp ncgl2478 promoter (lane 7) and BSA instead of SigH (lane 6) in the binding assays were used as negative controls to determine the binding specificity of SigH. f Mutations in the predicted SigH-binding site did not activate the ncgl2478 expression. Relative levels of transcripts were presented as the mean values ± SD calculated from three sets of independent experiments.

Consent for publication Not applicable.

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