MsR is a thiol-based oxidation-sensing regulator of the XRE family that modulates C. glutamicum oxidative stress resistance

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Abstract
Background: Corynebacterium glutamicum thrives under oxidative stress caused by the inevitably extreme environment during fermentation as it harbors antioxidative stress genes. Antioxidant genes are controlled by pathway-specific sensors that act in response to growth conditions. Although many families of oxidation-sensing regulators in C. glutamicum have been well described, members of the xenobiotic-response element (XRE) family, involved in oxidative stress, remain elusive.

Results: In this study, we report a novel redox-sensitive member of the XER family, MsR (multiple stress resistance regulator). MsR is encoded as part of the msrR-3-mst (3-mercaptopyruvate sulfurtransferase) operon; msrR-3-mst is divergent from multidrug efflux protein MFS. MsR was demonstrated to bind to the intergenic region between msrR-3-mst and mfs. This binding was prevented by an MsR oxidation-mediated increase in MsR dimerization. MsR was shown to use Cys62 oxidation to sense oxidative stress, resulting in its dissociation from the promoter. Elevated expression of msrR-3-mst and mfs was observed under stress. Furthermore, a ΔmsrR mutant strain displayed significantly enhanced growth, while the growth of strains lacking either 3-mst or mfs was significantly inhibited under stress.

Conclusion: This report is the first to demonstrate the critical role of MsR-3-MST-MFS in bacterial stress resistance.

Keywords: Oxidative stress, MsR, Transcription regulation, Corynebacterium glutamicum
act in response to specific ROS and coordinate the appropriate oxidative stress-associated genetic response. Thus, the regulation of antioxidant expression is an important issue. The constant sensing of ROS can be mediated by oxidation of one or more thiols in regulators [10].

Many of the best characterized bacterial sensors of ROS, such as the LysR (DNA-binding transcriptional dual-lysine regulator) family regulator OxyR (the thiol-based redox sensor for peroxides) [11, 12], zinc-associated extracytoplasmic function (ECF)-type sigma factor H (SigH) [13, 14], the ferric uptake regulator (Fur) family regulator PerR (a peroxide regulon repressor) [15], the MarR (multiple antibiotics resistance regulators) family regulator OhrR (an organic hydroperoxide resistance regulator) [16], the TetR (a tetracycline repressor protein) family regulator NemR (a N-ethylmaleimide regulator) [17], and the AraC (cytosine β-d-arabinofuranoside) family regulator RclR (a regulator of hypochlorous acid (HOCl)-specific resistance) [18], have been shown to contribute to or to modulate antioxidant gene expression [11–18]. These sensors specifically sense ROS via a thiol-based mechanism [11–18]. Upon exposure to oxidative stress, these regulators are activated or inhibited by morphological changes caused by cysteine oxidation, after which they are released from or bind to the promoters of target genes, leading to the upregulation of these target genes. Interestingly, more recently, Hu et al. found that the xenobiotic response element (XRE) family transcriptional regulator SrtR (stress response transcriptional regulator) in Streptococcus suis is also involved in oxidative stress tolerance, the only report of stress resistance in a member of the XRE family thus far [19]. Unfortunately, its exact molecular mechanism related to oxidant sensing, its target genes, and its interplay with other regulators have not yet been described. XREs, which are widely distributed in living organisms, control the expression of virulence factors, antibiotic synthesis and resistance genes, and stress response genes [20]. Although the XRE family is the second most common family of regulators in bacteria, XRE family members have been reported in only a limited number of bacteria, such as Staphylococcus aureus [21], Rhizobium etli [22], S. suis [19], and Chloroflexus aurantiacus [23]. Until now, research on XREs has mainly focused on XREs in eukaryotes. In eukaryotes, the regulatory mechanism of XREs is well known but different from that of ROS-sensing regulators; many xenobiotics acting as inducers, such as oxidants, heavy metals, antibiotics, and toxins, bind aromatic hydrocarbon (Ah) receptors in the cytoplasm to form an Ah receptor-ligand complex, which then interacts with XREs in the nucleus, finally stimulating the transcription of the target genes [24, 25]. However, the functions of XREs in eu-karyotes were not reported to be related to oxidative stress or other tolerance to other stresses. Thus, much research about XREs remains to be carried out, especially on the functions and mechanisms of XREs related to oxidative stress and tolerance to other stresses in bacteria.

Corynebacterium glutamicum, a nonpathogenic, GC-rich, and gram-positive bacterium, is not only an important industrial strain for the production of amino acids, nucleic acids, organic acids, alcohols, and biopolymers but also a key model organism for the study of the evolution of pathogens [26]. During the fermentation process, C. glutamicum inevitably encounters a series of unfavorable conditions [27, 28]. However, C. glutamicum thrives under the adverse stresses of the fermentation process using several antioxidant defenses, such as millimolar concentrations of mycothiol (MSH) and antioxidant enzymes [29–32]. Although many thiol-based redox-sensing regulators from different transcription factor families, including LysR (OxyR), MarR [RosR (regulator of oxidative stress response)/OhsR (organic hydroperoxides stress regulator)/CosR (C. glutamicum oxidant-sensing regulator)/QorR (quinone oxidoreductase regulator)], TetR [OsrR(Oxidative stress response regulator)], ArsR [CyeR (Corynebacterium yellow enzyme regulator)], and SigH, have been well studied [14, 29–31, 33–35], whether the XRE proteins of C. glutamicum play a role in protecting against oxidative stress by directly regulating antioxidant genes remains obscure. The putative XRE family transcriptional regulator NCgl2679, named MsrR (multiple stress resistance regulator) due to the results of this study, is not only located immediately downstream and in the opposite direction of the multidrug efflux protein NCgl2680 (MFS) but also organized in an operon with 3-Mercaptopyruvate sulfurtransferase (NCgl2678, 3-MST) and the putative protein NCgl2677. This genetic organization allowed us to investigate the function of C. glutamicum MsrR in response to environmental stresses. In the present study, MsrR was found to directly control expression of the msrR-3-mst-ncg2677 operon and the mfs gene as a thiol-based redox-sensing transcriptional repressor. The expression of msrR, 3-mst and mfs was induced by oxidative stress. MsrR contains only one cysteine residue at position 62 (Cys62). Upon oxidative stress induced by various xenobiotics, MsrR underwent dimerization and lost its DNA-binding activity through the formation of an intermolecular disulfide bond between the Cys62 residue of each subunit. These findings suggest that MsrR is a redox-sensing transcriptional regulator involved in the oxidative stress response of C. glutamicum by its regulation of 3-mst and mfs expression.
Methods

Strains and culture conditions

Bacterial strains and plasmids used in this study were listed in Additional file 1: Table S1. Escherichia coli and C. glutamicum were cultured in Luria–Bertani (LB) broth aerobically or on LB agar plates as previously reported [36]. ΔmsrR, Δ3-mst and Δmfs in-frame deletion mutants were produced as described [37]. Briefly, the pK18mobsacB-ΔmsrR plasmid was transformed into C. glutamicum wild type (WT) through electroporation to carry out single crossover. The transconjugants were selected on LB agar medium containing 40 µg/ml nalidixic acid and 25 µg/ml kanamycin. Counter-selection for markerless in-frame deletion was performed on LB agar plates with 40 µg/ml nalidixic acid and 20% sucrose [37]. Strains growing on this plate were tested for kanamycin sensitivity (KAN5) by parallel picking on 40 µg/ml nalidixic acid-containing LB plate supplemented with either 25 µg/ml kanamycin or 20% sucrose. Sucrose-resistant and kanamycin-sensitive strains were tested for deletion by PCR using the DMsrR-F1/DMsR-R2 primer pair (Additional file 1: Table S2) and confirmed by DNA sequencing. The Δ3-mst and Δmfs in-frame deletion mutants were constructed in similar manners by plasmid pK18mobsacB-Δ3-mst and pK18mobsacB-Δmfs using primers listed in Additional file 1: Table S2. For performing sensitivity assays, bacteria growth in LB broth containing 0.3 mM cumene hydroperoxide (CHP), 0.9 mM menadione (MEN), 45 mM H₂O₂, 0.4 mM HOCI, 1.5 mM tert-butyl hydroperoxide (t-BHP), 5 mM iodoacetamide (IAM), 0.1 µg/ml gentamicin, or 17 µM cadmium chloride (CdCl₂) was measured according to Helbig et al. [38].

Cloning, expression, and recombinant protein purification

The genes encoding C. glutamicum MsrR (NCgl2679), 3-MST (NCgl2678), MFS (NCgl2680) were amplified using primers listed in Additional file 1: Table S2 by PCR. The amplified DNA fragments were digested and subcloned into similar digested pET28a, pXMJ19, or pXMJ19-His₆ vectors, obtaining pET28a-msrR, pXMJ19-msrR, pXMJ19-His₆-msrR, pXMJ19-3-mst, and pXMJ19-mfs, respectively.

The plasmids pK18mobsacB-ΔmsrR, pK18mobsacB-Δ3-mst, and pK18mobsacB-Δmfs were constructed by overlap-PCR [39]. Briefly, primer pairs DMsrR-F1/ DMsR-R1 and DMsR-F2/DMsR-R2 listed in Additional file 1: Table S2 were used to amplify the 806-bp upstream fragment and the 820-bp downstream fragment of msrR, respectively. The primer pair DMsrR-F1/ DMsR-R2 was used to fuse the upstream and downstream fragments together by overlap extension PCR [39]. The obtained PCR products were digested with EcoRI and BamH1, and cloned into similar digested pK18mobsacB to produce pK18mobsacB-ΔmsrR. The knock-out plasmid pK18mobsacB-Δ3-mst and pK18mobsacB-Δmfs were constructed in a similar manner by using the primers listed in Additional file 1: Table S2.

The lacZY fusion reporter vectors pK18mobsacB-P₉₅M::lacZY and pK18mobsacB-P₉₄M::lacZY and lacZY reporter gene via overlap-PCR [40]. Firstly, the primers P₉₅M₃-F/P₉₅M₃-R and lacZY-F/lacZY-R were used in the first round of PCR to amplify the 232-bp mfsR promoter DNA fragments (corresponding to nucleotides +12 to −220 relative to the translational start codon (ATG) of mfsR gene) and the lacZY DNA fragments, respectively. Secondly, P₉₅M₃-F/lacZY-R as primers and the first round PCR products as templates were used to perform the second round of PCR, and the resulting fragments were digested with SmaI and PstI, and inserted into similar digested pK18mobsacB to obtain the pK18mobsacB-P₉₅M::lacZY fusion construct [29]. A similar process was used to construct pK18mobsacB-P₉₄M::lacZY. Briefly, the 235-bp mfs promoter DNA fragments (corresponding to nucleotides +15 to −220 relative to the translational start codon (ATG) of mfs gene) was amplified with the primers listed in Additional file 1: Table S2 and fused to the lacZY reporter genes. The resulting P₉₄M::lacZY was inserted into similar digested pK18mobsacB.

For obtaining pK18mobsacB-P₉₅M::lacZY, 232-bp mfsR promoter DNA containing mutagenesis sequence of the predicted MsrR binding site (P₉₅M₃) was first directly synthesized by Shanghai Biotechnology Co., Ltd.. Start and stop sites of P₉₅M₃ were the same as those of P₉₅M₃ in P₉₅M₃::lacZY. Then, the resulting 232-bp P₉₅M₃ was fused to a lacZY reporter gene. Finally, P₉₅M₃::lacZY was inserted into similar digested pK18mobsacB. A similar process was used to construct pK18mobsacB-P₉₄M::lacZY. Briefly, 235-bp mfs promoter DNA containing a mutagenesis sequence of the predicted MsrR binding site (P₉₄M₃) was directly synthesized and its start and stop sites were the same as those of P₉₄M₃ in P₉₄M₃::lacZY. Then, 235-bp P₉₄M₃ was fused to a lacZY reporter gene to obtain P₉₄M₃::lacZY. Finally, P₉₄M₃::lacZY was inserted into similar digested pK18mobsacB.

For complementation or overexpression in C. glutamicum strains, pXMJ19 or pXMJ19-His₆ derivatives were transformed into the corresponding C. glutamicum strains by electroporation, and the transformants were selected on 10 µg/ml chloramphenicol and 40 µg/ml nalidixic acid-containing LB agar plates. The transformant's expression was induced by adding 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) into medium [40].

To make the cysteine residue at position 62 of MsrR into a serine residue (MsrR:C62S), site-directed mutagenesis was made by two rounds of PCR [41]. In brief, in the
first round of PCR, primer pairs DMsrR-F1/MsrR-C62S-R and MsrR-C62S-F/DMSrR-R2 were used to amplify segments 1 and 2, respectively. The second round of PCR was performed by using CMsrR-F/CMsrR-R or OMsrR-F/OMsrR-R as primers and fragment 1 and fragment 2 as templates to produce the mssR:C62S DNA segment. The mssR:C62S segment was digested and subcloned into digested pET28a, pXMJ19 or pXMJ19-His6 plasmid, obtaining the corresponding plasmids. To express and purify His6-tagged recombinant proteins, the pET28a derivatives were transformed into E. coli BL21(DE3). Recombinant proteins were purified according to previously described method [40]. Primers used in this study were listed in Additional file 1: Table S2.

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

Construction of chromosomal fusion reporter strains and β-galactosidase assay
The lacZY fusion reporter plasmids pK18mobsacB-PmsrR::lacZY, pK18mobsacB-PMF::lacZY, pK18mobsacB-PMsR::lacZY, and pK18mobsacB-Pmfs::lacZY were transformed into C. glutamicum parental strain containing the high copy number of empty plasmid pXMJ19 (the strains were named WT), ΔmsrR (strains lacking mssR gene containing empty pXMJ19) and ΔmsrR+ (ΔmsrR was complemented with pXMJ19 plasmids carrying the wild-type mssR gene) by electroporation, respectively. 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 WT(PmsrR::lacZY), ΔmsrR(PmsrR::lacZY), ΔmsrR+ (PmsrR::lacZY), WT(PmsrR::lacZY), ΔmsrR(Pmfs::lacZY), ΔmsrR+ (Pmfs::lacZY), WT(PMfs::lacZY), ΔmsrR(Pmfs::lacZY), ΔmsrR+ (Pmfs::lacZY), fusion reporter strains were selected by plating on LB agar plates containing 40 µg/ml nalidixic acid, 25 µg/ml kanamycin, and 10 µg/ml 1-chloramphenicol [37]. The resulting strains were grown in LB medium to an optical density of 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 [39]. The standard assay for quantitating the amount of β-galactosidase activity in cells, originally described by Miller for assay of bacterial cultures, involves spectrophotometric measurement of the formation of the yellow chromophore o-nitrophenol (ONP) as the hydrolytic product of the action of β-galactosidase on the colorless substrate o-Nitrophenyl β-D-galactopyranoside (ONPG) [42]. 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, ΔmsrR and ΔmsrR+ 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 [40]. The primers used were listed in Additional file 1: Table S2. To obtain standardization of results, the relative abundance of 16S rRNA was used as the internal standard.

H2O2-dependent structural change of MsrR in vivo
The H2O2-dependent structural change of MsrR and its variant in vivo were determined by a previously reported method [39]. ΔmsrR (pXMJ19-His6-msrR) and ΔmsrR (pXMJ19-His6-msrR:C62S) strains were cultured in LB containing 0.5 mM IPTG, 10 µg/ml chloramphenicol, and 40 µg/ml nalidixic acid at 30 °C. Cells were grown to mid-exponential phase and split into 100 ml aliquots for H2O2 treatment (0–30 mM, 60 min). The treated samples were harvested immediately by centrifugation, broken through ultrasound on ice, and then crude cell lysates were centrifuged. Obtained supernatants were subjected to nonreducing sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) or reducing SDS-PAGE, and the structural properties of MsrR and its variant were visualized by immunoblotting using the anti-His antibody.

Electrophoretic mobility shift assay (EMSA)
EMSA was performed using the method of Si et al. [30]. Briefly, a 162-bp mssR promoter sequence [PmsrR] corresponding to nucleotides −154 to +8 relative to the translational start codon (GTG) of the cssS ORF containing the predicted MsrR binding site was amplified using primer pair EMsrR-F/EMsrR-R (Additional file 1: Table S2). The binding reaction mixture (20 µl) contained 10 mM Tris–HCl (pH 7.4), 5 mM MgCl₂, 50 mM KCl, 5% glycerol, 0.1% Nonidet P 40 (NP40), 1 µg poly(dI:dC), 0–60 nM of MsrR, and 40 ng PmsrR. 162-bp DNA fragments amplified from MsrR ORF (40 ng) instead of PmsrR were used as a negative control. A 162-bp EMSA promoter DNA containing the mutated sequence of the predicted MsrR-binding site and having the same start and stop sites as PmsrR (PmsrRM) was directly synthesized.
by Shanghai Biotechnology Co., Ltd. After the binding reaction mixture was incubated at room temperature for 30 min, the mixture was subjected to electrophoresis on 8% nondenaturing polyacrylamide gel made with 10 mM Tris buffer containing 50 mM KCl, 5 mM MgCl₂, and 10% glycerol in 0.5× TBE electrophoresis buffer [50 mM Tris, 41.5 mM borate (pH 8.0), 10 mM Na₂EDTA.H₂O], and stained either with a 10,000-fold diluted Synergy Brand (SYBR) Gold nucleic acid staining solution (Molecular Probes) or GelRed™ and photographed. The DNA bands were visualized with UV light at 254 nm.

The reversibility of the loss of binding due to oxidation was tested as follows. H₂O₂ was added to MsrR solution to a final concentration of 10 mM, immediately aliquots were taken and incubated with 40 ng PmsrR for EMSA. In the next step, dithiothreitol (DTT) was added to the H₂O₂-treated MsrR solutions to a final concentration of 50 mM, and again aliquots were taken for EMSA. All aliquots were incubated in binding buffer with 40 ng PmsrR for 30 min at room temperature and separated on an 8% nondenaturing polyacrylamide gel and the gel was stained using SYBR Gold nucleic acid staining solution.

For the determination of apparent K_D values, increasing concentrations of the MsrR (0–100 nM) were incubated for 30 min at room temperature with 40 ng PmsrR. The samples were applied onto an 8% native polyacrylamide gel and the gel was stained using GelRed™ and photographed were quantified using ImageQuant software (GE Healthcare), and the percentage of shifted DNA was calculated. These values were plotted against the MsrR concentration on a log₁₀ scale, and a sigmoidal fit was performed using GraphPad Prism software (GraphPad Software, San Diego California USA), considering the error bars as well as 0 and 100% shifted DNA as asymptotes, the turning point of the curve was defined as the apparent K_D value. All determinations were performed in triplicate.

Western blot analysis
Western blot analysis was conducted as previously described [29]. The cytosolic RNA polymerase β (RNA polβ) was used as a loading control as in our previous study [29].

Statistical analysis
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, San Diego California USA).

Results and discussion

The ΔmsrR C. glutamicum strain showed reduced sensitivity to challenge by oxidants, antibiotics, heavy metals, and alkylating agents

The 723-bp C. glutamicum nclg2679 gene is located from bp 2,960,466 to 2,961,188 (Fig. 1a, upper panel) and encodes a hypothetical transcriptional regulator consisting of 240 amino acid residues with a molecular mass of 26.2 kDa. The putative protein product, which contains a helix-turn-helix motif, shares similarity with XRE (xenobiotic response element) family transcription factors from Corynebacterium crusii, Corynebacterium efficiens, Corynebacterium callunae, Corynebacterium epidemidicanis, and Corynebacterium minutissimum (80%, 68%, 64%, 42%, and 40% amino acid sequence identity, respectively) (Additional file 1: Figure S1). A recent study showed that the transcriptional regulator SrtR, an XRE family member, is involved in oxidative and high temperature stress tolerance [19]. This finding prompted us to examine whether NCg2679 plays a role in protecting the soil bacterium C. glutamicum from various stresses. The functions of NCg2679 were identified by gene disruption and complementation (Fig. 1a, lower panel). Growth analysis of different C. glutamicum strains on LB medium in the absence of stress revealed that the wild-type C. glutamicum strain (WT, C. glutamicum transformed with the empty plasmid pXMJ19), the Δncg2679 mutant strain (the nclg2679 deletion mutant expressing pXMJ19) and the Δncg2679+ strain (the nclg2679 deletion mutant expressing the wild-type nclg2679 gene in the shuttle vector pXMJ19) showed almost the same growth rates (Fig. 1b). However, the growth of the WT strain in LB...
a. Genome of *Corynebacterium glutamicum* ATCC13032

b. Normal condition

c. 0.3 mM CHP

d. 0.9 mM MEN

e. 45 mM H$_2$O$_2$

f. 0.4 mM HCl

g. 1.5 mM t-BHP

h. 5 mM IAM

i. 0.1 μg/ml Gentamicin

j. 17 μM CdCl$_2$
medium containing oxidants, alkylation agents, antibiotics, or heavy metals was markedly inhibited relative to the growth of the Δncgl2679 mutant strain (Fig. 1c–j). The complementary strain Δncgl2679+ exhibited a growth rate equivalent to that of the wild-type strain under various stresses, consistent with a previous evaluation of XREs under stress [19]. These results indicated that NCgl2679 is involved in the resistance of C. glutamicum to various stresses. Thus, we named NCgl2679 multiple stress response regulator (MsrR).

MsrR negatively regulates expression of the divergently oriented genes mfs and msrR-3-mst
In the C. glutamicum genome, msrR (ncgl2679) is organized in a putative operon with ncg2678 and ncg2677, which were shown to be co-transcribed by reverse transcription PCR (Additional file 1: Figure S2). Further downstream from ncg2679 is the ncg2680 gene, which was annotated as the multidrug efflux protein MFS. The mfs and msrR genes are oriented in opposite directions. By bioinformatics molecular analysis, two putative overlapping and divergent promoter sequences in the intergenic region between the start codons of mfs and msrR were found (Additional file 1: Figure S3), and one of these promoter sequences was found to be located upstream of the msrR gene. Neighboring mfs is a putative −10 and −35 promoter sequence, which was found to be the mfs promoter.

On the basis of bioinformatics analysis, a putative MsrR-binding site in the putative overlapping, divergent promoters of the msrR-ncg2678 locus and mfs gene was found (Additional file 1: Figure S3). Thus, we speculated that MsrR negatively regulates the msrR-ncg2678-ncg2677 locus and represses transcription of the adjacent, oppositely oriented mfs gene. To verify this speculation, msrR, ncg2678 and mfs transcription levels in the WT, ΔmsrR mutant, and ΔmsrR+ strains were analyzed by qRT-PCR and determination of the lacZY activity of the chromosomal promoter fusion reporter. Notably, to study the expression of msrR in the ΔmsrR mutant strain by qRT-PCR, a 104-bp msrR transcript (corresponding to nucleotides +1 to +104 relative to the translational start codon (GTG) of the msrR gene) was amplified from the remaining msrR ORF in the ΔmsrR mutant strain with the primers QmsrR-F and QmsrR-R (Additional file 1: Figure S4). As expected, msrR, ncg2678 and mfs transcription levels in the ΔmsrR mutant strain were obviously higher than those in the WT and ΔmsrR+ strains (Fig. 2 and Additional file 1: Figure S5). These results indicated that MsrR negatively controls the expression of NCgl2678, MFS, and its structural gene.

ncg2678, which was annotated as 3-mercaptopropyl sulfurretransferase (3-MST), is mainly responsible for hydrogen sulfide (H2S) production [43]. Previous studies found that H2S made by nonsulphur bacteria alleviates oxidative stress imposed by diverse stresses through increasing levels of intracellular antioxidants, including glutathione (GSH); antioxidant enzymes; and glutamate uptake [44, 45]. This finding suggests that the absence of 3-mst probably cause the decrease of H2S content, which in turn reduction of the antioxidant capacity of C. glutamicum strains. In addition, many reports have revealed that cells expressing MFS can excrete various poisons [46, 47], suggesting that C. glutamicum MFS is also important for resistance to diverse stresses. Thus, the functions of 3-mst and mfs were identified by gene disruption and complementation with C. glutamicum (Fig. 1a, lower panel). As shown in Fig. 3, while deletion of 3-mst or mfs did not affect bacterial growth under normal conditions, compared to the WT strain, the Δ3-mst and Δmfs mutant strains devoid of 3-mst or mfs, respectively, exhibited obvious growth inhibition under challenge with various diverse stresses. The growth of 3-mst or mfs deletion mutant strains under diverse stresses was restored to a level similar to that of the WT strain by transformation with the plasmid-encoded wild type 3-mst or mfs gene (Δ3-mst+ or Δmfs+), in agreement with the results of Li et al. regarding MST [48] (Fig. 3).

Expression of msrR, 3-mst and mfs was induced by oxidative stress via MsrR
Previous studies revealed that the transcriptional activation of target genes controlled by XREs is mediated by xenobiotics, which act as inducers [49, 50]. The mechanism by which various xenobiotics act as inducers and affect the conformation of XREs is a key feature for induction activity. Thus, these studies, combined with the above finding that MsrR is involved in tolerance to various stresses, led us to investigate whether MsrR participates in the induction of its own gene and the 3-mst and mfs genes by xenobiotics. For simplicity, we used H2O2 and CdCl2 as inducers in the following experiments. As shown in Fig. 2a and Additional file 1: Figure S5c, in the absence of H2O2, the ΔmsrR strain had significantly higher msrR and mfs expression levels than the WT and ΔmsrR+ strains, whereas the lacZY activities of msrR and mfs in the WT strain exposed to H2O2 were obviously higher than those in the untreated-H2O2 WT strain. The addition of H2O2 did not change the lacZY activities of msrR or mfs in the ΔmsrR strain, which were maintained at the same levels observed in the ΔmsrR strain without H2O2 treatment. Moreover, analysis of the lacZY activities showed a dose-dependent change in expression in the WT and ΔmsrR+ strains in response to H2O2 (Fig. 2a and Additional file 1: Figure S5c). A similar regulatory pattern
of msrR, 3-mst or mfs by MsrR was also observed at the mRNA transcriptional level by qRT-PCR analysis (Fig. 2c, e and Additional file 1: Figure S5a). These results clearly demonstrated that msrR, 3-mst and mfs were upregulated in response to increasing H₂O₂ concentration, indicating that oxidation inhibited the DNA binding of MsrR, inducing the expression of its own gene and the 3-mst and mfs genes. This derepression of msrR, 3-mst and mfs transcription by CdCl₂ was mediated via MsrR in a matter similar to that of H₂O₂ (Fig. 2b, d, f and Additional file 1: Figure S5b, d).
The ability of MsrR to bind the intergenic region between msrR and mfs was reversibly inhibited by ROS

To determine whether MsrR directly regulates its own transcription and the transcription of 3-MST and MFS, we examined the interaction between purified MsrR and a DNA promoter fragment in the intergenic region between msrR and mfs (named PmsrR) using EMSA. Incubation of PmsrR with His6-MsrR caused a clear delay in promoter DNA migration, and PmsrR migrated in a manner dependent on the concentration of His6-MsrR (Fig. 4b and Additional file 1: Figure S6b). The apparent KD value for PmsrR was about 17 nM MsrR (Additional file 1: Figure S7a), which is within the range found for other transcriptional regulators [33]. Moreover, this effect was specific because the combination of His6-MsrR and DNA fragments amplified from the MsrR ORF did not delay migration (Fig. 4a and Additional file 1: Figure S6a). However, the binding of His6-MsrR to PmsrR was prevented by the addition of 10 mM H2O2 (Fig. 4c and Additional file 1: Figure S6c). Importantly, the impaired DNA-binding activity of His6-MsrR by H2O2 could be restored via the addition of an excess of the reducing agent DTT (50 mM), indicating that the effects of oxidation and reduction on the DNA-binding activity of MsrR were reversible. (Fig. 4c and Additional file 1: Figure S6c). Mutations in the predicted MsrR-binding site (a 162-bp EMSA promoter DNA contained the mutated sequence of the predicted MsrR-binding site (PmsrRM), which had the same start and stop sites as PmsrR) (Additional file 1: Figure S3) disrupted the formation of DNA–protein complexes (Fig. 4d and Additional file 1: Figure S6d), and promoter DNA mutations in the predicted MsrR-binding site (a 232-bp DNA fragment contained the mutated sequence of the predicted MsrR-binding site for lacZY activity, which had the same start and stop sites as a 232-bp DNA fragment on Pmsr::lacZY; a 235-bp DNA fragment contained the mutated sequence of the predicted MsrR-binding site for lacZY activity, which had the same start and stop sites as a 235-bp DNA fragment on Pmsr::lacZY; a 235-bp DNA fragment contained the mutated sequence of the predicted MsrR-binding site for lacZY activity, which had the same start and stop
that the formation of dimeric MsrR occurs via a disulfide bond between MsrR proteins.

To further examine whether the formation of MsrR dimers can be induced under H$_2$O$_2$ treatment in vivo, we treated cells of the ΔmsrR (pXMJ19-His$_6$-msrR) and ΔmsrR (pXMJ19-His$_6$-msrR:C62S) strains with H$_2$O$_2$ at various concentrations and probed the forms of MsrR by immunoblotting with anti-His antibody after non-reducing SDS-PAGE separation (Fig. 5c, d; Additional file 1: Figure S9). Under normal conditions (no stress), MsrR in the ΔmsrR (pXMJ19-His$_6$-msrR) strain existed as monomers, but upon exposure to different concentration of H$_2$O$_2$, the monomeric form changed into an intermolecular disulfide bond-containing dimeric form (Fig. 5c, upper panel and Additional file 1: Figure S9a, upper panel). The dimeric form completely disappeared on reducing SDS-PAGE, indicating that dimeric MsrR in vivo could be also reversed, which was consistent with the results in vitro (Fig. 5c, lower panel and Additional file 1: Figure S9a, lower panel). However, whether under H$_2$O$_2$ treatment or not, MsrR in the ΔmsrR (pXMJ19-His$_6$-msrR:C62S) strain existed in a monomeric form.
Inactivation of the DNA binding of MsrR by ROS is dependent on the oxidation state of Cys62

The reduction and oxidation of cysteine residues is involved in the control of ROS-sensing sensor activity [10]. It would be interesting to know whether Cys62 of MsrR plays an important role in the H₂O₂-sensing and transcription mechanisms of MsrR. Thus, the ability of the MsrR:C62S variant to suppress msrR, 3-mst and mfs expression in response to H₂O₂ was evaluated in the ΔmsrR strain using promoter lacZ activity and qRT-PCR analysis. Analysis of the transcriptional levels revealed that ΔmsrR+C62S (the ΔmsrR strain containing the pXMJ19-msrR:C62S plasmid) inhibited msrR, 3-mst and mfs expression under H₂O₂ treatment conditions to equal degrees, similar to that in the untreated-H₂O₂ WT strain, indicating that Cys62 plays a role in the dissociation of MsrR from the promoter under H₂O₂ treatment conditions (Fig. 2 and Additional file 1: Figure S5).

To further probe whether Cys62 is responsible for the observed dissociation of MsrR under oxidation, MsrR:C62S was used instead of WT MsrR to perform the EMSA experiment. As shown in Fig. 4f and Additional file 1: Figure S6f, in the presence or absence of 10 mM H₂O₂, MsrR:C62S still exhibited obviously retarded mobility. Although its affinity constant for Pₘₚ (Kₕ = 23.08) was slightly high than that of MsrR, MsrR:C62S behaved high similarly to MsrR without H₂O₂ condition (Additional file 1: Figure S7b). These results mean that oxidation of Cys62 was important for inhibition of DNA binding by H₂O₂. The above results further showed that the inhibition of DNA binding by H₂O₂ was caused by the oxidation of cystein residue.
Conclusions
Thiol-based redox-sensing regulators are recognized as an efficient way to combat diverse ROS-inducing stress conditions and enhance the survival of bacteria under oxidative stress. The XRE family is involved in the control of the response to environmental stress, but the functions of XREs related to oxidative stress tolerance, especially their antioxidative molecular mechanisms, are very rarely reported. In this study, we found a MsrR-binding site in the intergenic region between two divergent gene clusters, msrR-3-mst and mfs. β-galactosidase activity assay and qRT-PCR analysis showed that MsrR is indeed negatively autoregulated and also negatively controls the adjacent 3-mst and mfs. In vivo, expression of msrR is induced by H₂O₂ and CdCl₂, and the msrR-deleted (ΔmsrR) mutant displays increased resistance to H₂O₂ and CdCl₂. However, EMSA experiment shows the ability of MsrR to bind the promoter DNA is inhibited by H₂O₂ but not CdCl₂. Many studies reported that the most potent xenobiotics, including oxidants, alkylating agents, antibiotics, or heavy metals, are capable of generating ROS by redox-cycling to produce oxidative stress inside bacteria [51–56]. Thus, CdCl₂ might contribute indirectly to ROS production, thereby leading to the derepression of the MsrR operon. Considering the regulation of the stress response mechanisms of these species. On the contrary, the regulatory mechanism of MsrR is similar to those of the ROS sensors OxyR, PerR, and OhrR, which are activated or inhibited by changes in conformation caused by cysteine oxidation.

The XRE family is the second most common family of regulators in bacteria, only four members of which have been reported in previous researches, including S. suis SrtR [19], S. aureus XdrA (XRE-like DNA-binding regulator, A) [21], R. etli RHE-CH00371 [22], and C. aurantiacus MltR (MmyB-like transcription regulator) [23]. Except for SrtR, no obvious effect on oxidative stress resistance for any of the previously studied examples has been reported so far. S. aureus XdrA is shown to play an important role in the β-lactam stress response. Expression of R. etli RHE-CH00371 is reported to be down-regulated in an H₂O₂-sensitive R. etli mutant. C. aurantiacus MltR is described as being involved in the regulation of antibiotic biosynthesis and thus represents an example for a rather specialized XRE-type regulator. Sequence analysis clearly indicates that the similarity between MsrR and the XREs of bacteria mentioned above is very low, and Cys62 of MsrR is not very conserved (Figure S1b–e), which only appears in position 66 of S. suis SrtR and 55 of S. aureus XdrA. The result is consistent with the previous report that the XRE family contains more than 35,000 proteins and more than 70 structures are available [23]. We suggested that differences in structure may cause versatile features and regulatory mechanisms. It is important to point out, despite their low sequence similarity to MsrR (about 30% identity), we thought S. suis SrtR and S. aureus XdrA might share an oxidation-sensing mechanism as they not only contain the cysteine presumed to serve for oxidation sensing in a relatively conserved position, but they confer resistance to oxidant and β-lactam, respectively, which is similar to MsrR. Combining a phenomenon that β-lactam antibiotics, such as penicillin, can also generate ROS by redox-cycling to produce oxidative stress inside bacteria [55], we speculate that S. suis SrtR and S. aureus XdrA act as a transcriptional sensor via cysteine oxidation-based thiol modifications. Thus, our results provided, for the first time, insight into a new regulatory mechanism adopted by an XRE protein in which DNA-binding ability is regulated by the oxidation of a cysteine residue in the MsrR protein in response to oxidants but not directly bound ligands, such as antibiotics, heavy metals, and alkylating agents. Our data further confirmed the results of Hu et al. showing a member of the XRE family of transcriptional regulators responsible for oxidant tolerance in bacteria [19], facilitating understanding of oxidant mechanisms in bacteria and providing initial insight into the molecular mechanisms of XREs involved in oxidative stress tolerance. In addition, MsrR is found to be widely distributed in several species of the genera Corynebacterium, such as C. crudilactis, C. efficiens, C. callunae, C. epidermidicans, and C. minutissimum. Therefore, our study on the regulatory mechanism of MsrR may lead to a better understanding of the stress response mechanisms of these species. Together, our data show that C. glutamicum MsrR acts as a thiol-based redox sensor and, with 3-MST and MFS, comprises an important pathway for protection against oxidative stress.

Supplementary information
Supplementary information accompanies this paper at https://doi.org/10.1186/s12934-020-01444-8.
Additional file 1: Table S1. Bacterial strains and plasmids used in this study. Table S2. Primers used in this study. Figure S1. Multiple sequence alignment of MsrB with XRs from other organisms. Figure S2. Assays for the nong2678-nong2678-nong2677 co-transcription by reverse transcription-PCR. Figure S3. Detailed genetic maps of the regulatory region of MsrR. Figure S4. 104-bp msrR transcript (from the translational start codon [GTC] of msrR gene to 104th nucleotide) was amplified from the remaining msrR ORF (Open Reading Frame) in ΔmsrR mutant with primers QmsrR1-F and QmsrR-R. Figure S5. Negative regulation of mrsR and mfs by MsrR. Figure S6. Reversible inhibition of the DNA binding activity of MsrR by H$_2$O$_2$ and role of Cysteine residue. Figure S7. Determination of the apparent $K_v$ values of MsrR and MsrR-C62S for $P_{max}$ msrR expression. Mutations in the predicted MsrR binding site derepressed the msrR expression. Figure S9. Oxidative stress-dependent structural changes of relevant MsrR in vivo.

Abbreviations
ROS: Reactive oxygen species; CHP: Cumene hydroperoxide; t-BHP: t-Butyl hydroperoxide; H$_2$O$_2$: Hydrogen peroxide; MSH: Mycothiol; GSH: Tripeptide glutathione; MEN: Menadione; DMPP: N,N-dimethyl-p-phenylenediamine; ONPG: α-Nitrophenyl β-D-galactopyranoside; DTT: Dithiothreitol; 3-MST: 3 Mercaptopyruvate sulfurtransferase; XRE: Xenobiotic response element; HOCI: Hypochlorous acid.

Acknowledgements
Not applicable.

Authors’ contributions
MS and CC designed the research. MS, CC, JZ, XL, YL, and TS performed the research and analyzed the data. GY supervised the research. MS and GY wrote the paper. All authors read and approved the final manuscript.

Funding
This work was supported by the National Natural Science Foundation of China (31970034), the Key Scientific and Technological Project of Henan Province, China (201202310493), Doctoral Start Fund of Zhoukou Normal University (ZKNUC2018013), and Qufu Normal University Young Teacher Ability Enhancement Program Country (Overseas) Overseas Study (1 year).

Availability of data and materials
All the data generated or analyzed during this study are included in the manuscript and its additional file.

Ethics approval and consent to participate
Not applicable.

Consent for publication
All authors have agreed to submit this manuscript to microbial cell factories.

Competing interests
The authors declare that they have no competing interests.

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Received: 19 May 2020 Accepted: 25 September 2020 Published online: 02 October 2020

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