Characterization of Xi-class mycothiol S-transferase from Corynebacterium glutamicum and its protective effects in oxidative stress

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Abstract

Background: Oxidative stress caused by inevitable hostile conditions during fermentative process was the most serious threat to the survival of the well-known industrial microorganism Corynebacterium glutamicum. To survive, C. glutamicum developed several antioxidant defenses including millimolar concentrations of mycothiol (MSH) and protective enzymes. Glutathione (GSH) S-transferases (GSTs) with essentially defensive role in oxidative stress have been well defined in numerous microorganisms, while their physiological and biochemical functions remained elusive in C. glutamicum thus far.

Results: In the present study, we described protein NCgl1216 belonging to a novel MSH S-transferase Xi class (MstX), considered as the equivalent of GST Xi class (GSTX). MstX had a characteristic conserved catalytic motif (Cys-Pro-Trp-Ala, C-P-W-A). MstX was active as thiol transferase, dehydroascorbate reductase, mycothiolyl-hydroquinone reductase and MSH peroxidase, while it showed null activity toward canonical GSTs substrate as 1-chloro-2,4-dinitrobenzene (CDNB) and GST Omega's specific substance glutathionyl-acetophenones, indicating MstX had some biochemical characteristics related with mycoredoxin (Mrx). Site-directed mutagenesis showed that, among the two cysteine residues of the molecule, only the residue at position 67 was required for the activity. Moreover, the residues adjacent to the active Cys67 were also important for activity. These results indicated that the thiol transferase of MstX operated through a monothiol mechanism. In addition, we found MstX played important role in various stress resistance. The lack of C. glutamicum mstX gene resulted in significant growth inhibition and increased sensitivity under adverse stress condition. The mstX expression was induced by stress.

Conclusion: Corynebacterium glutamicum MstX might be critically involved in response to oxidative conditions, thereby giving new insight in how C. glutamicum survived oxidative stressful conditions.

Keywords: Glutathione transferase Xi, Mycothiolyl-hydroquinone reductase, Mycothiolyl-acetophenone reductase, Redox regulation, Corynebacterium glutamicum, Oxidative stress
or tyrosine residue at the G-site in N-terminus [3]. The Ser or Tyr residue was responsible for the nucleophilic attack on substrate, making GSTs conjugate GSH to the electrophilic groups of a variety of the hydrophobic molecules. Thus, Ser/Tyr-GSTs displayed glutathionylation activity [3]. However, Cys-GSTs contained a catalytic cysteine residue at the G-site in N-terminus, catalyzing GSH removal from specific substrates and was simultaneously oxidized through the formation of an enzyme-GSH mixed disulfide with the concomitant reduction of a co-substrate. Thus, Cys-GSTs mainly exhibited deglutathionylation activity [4]. Ubiquitous glutathionylation function of Ser/Tyr-GSTs not only could neutralize free radicals and active components, but also cause the formation of GSH conjugates. Many studies showed that GSH conjugates were more soluble, easier to be excreted from cells, and less toxic than the original compounds [5]. The deglutathionylation function of Cys-GSTs facilitated the proper folding of proteins. Ser/Tyr-GSTs were usually dimeric, while Cys-GSTs exhibited more structural diversity, including monomer, the canonical dimer and atypical dimer. Although Cys-GSTs contained less members than Ser/Tyr-GSTs, Cys-GSTs also represented important GST class. Cys-GSTs were further classified into about 6 subclasses on biochemical characterization so far, including Xi [6], Omega [7], Beta [8], CLIC [9], dehydroascorbate reductases [10], and Lambda [4].

The GSTs Xi class (GSTXs) had been well studied in bacteria [11, 12], fungi [13], P. chrysosporium [6], and plants [14], and constituted a new phylogenetic group of GSTs [6]. In fact, GSTXs were initially identified as the GST omegas subclass I (GSTOIs), once named S-glutathionyl-(chboro) hydroquinone reductase (GHR). Hereafter, Meux and co-worker believed, on the basis of sequence analysis and biochemical and structural differences from canonical GSTOs, that GHRs of the GSTOIs should be considered as a new class of GSTs, reclassifying them as GSTXs [6]. In summary, the activities associated to GSTXs were: (i) GSH-dependent thiol transferase activity, being attributable to its structural and functional similarity to glutaredoxins (Grxs), but low or lacking activity toward classical GSTs substrate as 1-chloro-2,4-dinitrobenzene (CDNB) [15], (ii) Catalyses of several reductive reactions in cellular biochemistry, including dehydroascorbate (DHA) and monomethylarsonic acid (MMA) [15]. As a DHA reductase, GSTXs reduced DHA for ascorbic acid (AA). AA played a major role in scavenging free radical and ROS [16]. Thus, GSTXs played an important protective role in the response to oxidative stress. The above two catalytic functions of GSTXs, usually also existing in GSTOs, rarely existed in members of the Ser/Tyr-GST. (iii) Specific reduction of 5-glutathionyl-(chloro)hydroquinone (GH), and such activity was not detected for the other GSTOs [6]. However, unlike GSTOs, GSTXs did not have the activity of reducing glutathionyl-acetophenones (which was once thought to be specific to GSTOs), although more recently Schwartz et al. [15] found that the GSTX3 from the white rot Trametes versicolor (TvGSTX3) had glutathionyl-acetophenone and GH reductase activities. That is to say that TvGSTX3 reduced both Xi and Omega substrates [15]. However, nothing was known about the universality and exact molecular mechanism of the catalytic duality of GSTXs. Thus, an in-depth analysis of the catalytic mechanism of GSTXs was a subject of active investigation. Corynebacterium glutamicum not only was a well-known industrial microorganism widely used for the amino acids and nucleotides production, but also was an excellent model organism to study the physiology of its pathogenic relatives such as Mycobacterium tuberculosis, C. jeikeium and C. diphtheriae [17]. During fermentative process, C. glutamicum inevitably encountered a series of unfavorable circumstances including high osmotic pressure, antibiotics, nutrient starvation, low pH, and/or oxidation [18]. However, C. glutamicum robustly survived the adverse stresses of the fermentative process using several antioxidant defenses, such as millimolar concentrations of low molecular weight (LMW) thiol mycothiol (MSH) and antioxidant enzymes [19, 20]. Sufficient evidences showed that MSH had the equivalent functions with GSH in bacteria [19, 20]. MSH played an important role in the defence against several external stresses including oxidative stress, alkylating agents and antibiotics [19, 20]. Also, our previous studies showed that many antibiotics and electrophilic compounds were conjugated to MSH and then were detoxified by MSH S-conjugate amidase (Mca) [21]; many antioxidant proteins, including MSH peroxidase MPx, methionine sulfoxide reductase MsrA, and peroxiredoxin Prx, were discovered as involving in mycothiolation/demycothiolation when detoxifying various peroxides [22–24]. These studies suggested that the putative MSH S-transferase (MST) catalyzing MSH-dependent detoxification reactions existed in C. glutamicum. Newton et al. identified MSMEG_0887 from M. smegmatis and Rv0443 from M. tuberculosis, the member of the DinB superfamily, as MSTs, while an MSH-dependent protein of the DinB superfamily that was homology with M. smegmatis MSMEG_0887 showed maleylpyruvate isomerase activity in C. glutamicum [25, 26]. Interestingly, previous study identified a putative GSH S-transferase (GST) (NCGL1216) negatively regulated by a hydrogen peroxide-sensitive MarR (multiple antibiotic resistance regulator)-type sensor RosR (regulator of oxidative stress response) using DNA
microarrays in C. glutamicum [27]. This study suggested that NCgI216 might be the MSH-dependent MST. Therefore, we sought to explore the physiological and biochemical function of NCgI216 of C. glutamicum.

Methods

Strains and culture conditions
The bacterial strains and plasmids that were in this study listed in Additional file 1: Table S1. Luria–Bertani (LB) broth and LB agar plates were used for growing Escherichia coli or C. glutamicum. E. coli and C. glutamicum were cultivated at 37 °C and 30 °C under vigorous agitation (220 rpm) as previously reported, respectively [26]. 0.5 M sorbitol-containing brain–heart broth medium (BHI) was used for producing and maintaining mutant of a gene in C. glutamicum [26]. To create a mstX (ncgl1216) gene deletion in C. glutamicum RES167 parental strain, the pK18mobsacB–ΔmstX plasmids were transformed into C. glutamicum WT through electroporation according to the method of Shen et al. and then integrated into the chromosome of C. glutamicum through homologous recombination to execute single crossover [26]. The transconjugants were selected on LB agar plate in the presence of 40 µg ml⁻¹ nalidixic acid (NAL) and 25 µg ml⁻¹ kanamycin (KAN). Counter-selection for markerless in-frame deletion was carried out on 40 µg ml⁻¹ NAL and 20% (W/V) sucrose-containing LB agar plates [28]. Strains growing on this plate were detected for KAN sensitivity (KAN²) by parallel picking on LB plates in the presence of 40 µg ml⁻¹ NAL and 25 µg ml⁻¹ KAN or 40 µg ml⁻¹ NAL and 20% sucrose. Sucrose-resistant and KAN-sensitive strains were detected for in-frame deletion by PCR using the DmstX-F1/DmstX-R2. The resulting stream fragments were fused together by overlap PCR to produce pXMJ19-mstX (Additional file 1: Table S2) and verified by DNA sequencing. To create a ΔmstX in-frame deletion mutant, the suicide plasmid pK18mobsacB–ΔmstX was constructed by overlap PCR [28, 30]. First, based on DNA sequences of mstX gene and its adjacent regions, two oligonucleotide primer pairs namely DmstX-F1/DmstX-R1 and DmstX-F2/DmstX-R2 were listed in Additional file 1: Table S2 were made (Sangon Biotech Co., Ltd., Shanghai, China). Primer pair DmstX-F1/DmstX-R1 was used to amplify the mstX's upstream 852 bp fragment; while primer pair DmstX-F2/DmstX-R2 was used to amplify the mstX's downstream 816 bp fragment. The upstream and downstream fragments were fused together by overlap PCR with the primer pair DmstX-F1/DmstX-R2. The resulting PCR products were cut with EcoRI and BamHI and then cloned into similar sites of pK18mobsacB vector to generate plasmid pK18mobsacB–ΔmstX.

To produce pXMJ19-mstX, primer pair CmstX-F/CmstX-R was designed to amplify the DNA fragments of open reading frames region of mstX gene from C. glutamicum genomic DNA. The amplified DNA fragments were cut with BamHI and EcoRI and then cloned into pXMJ19 vector between BamHI and EcoRI sites.

To obtain the DNA fragment of mutant mstX:C67S, site-directed mutagenesis was carried out by two rounds of PCR [31]. Briefly, two oligonucleotide primer pairs namely DmstX-F1/mstX-C67S-R and mstX-C67S-F/DmstX-R2 listed in Additional file 1: Table S2 were designed and synthesized. In the first round of PCR, primer pair DmstX-F1/mstX-C67S-R was used to amplify the 5’ prime region of mstX (Fragment I); while primer pair mstX-C67S-F/DmstX-R2 was used to amplify the 3’ prime region of mstX (Fragment II). The second round of PCR was performed by using OmstX-F/OmstX-R as primers and fragment I and fragment II as templates to get the mstX:C67S fragment. The BamHI and HindIII cut mstX:C67S DNA fragments were cloned in pET28a plasmid digested with similar enzymes to create plasmid pET28a-mstX:C67S. The mstX:C67S, mstX:C67A, mstX:C67Y, mstX:P68G, mstX:W69F, mstX:A70S, mstX:V192A, mstX:N193A, mstX:G235L, mstX:J238A,
mstX::T239A, mstX::D242G, mstX::I243S, mstX::T248A, mstX::R251A, mstX::C262G, and mstX::C262S fragments were obtained using a similar procedure as described above and cloned into pET28a to result in corresponding plasmid derivatives.

For getting the lacZY fusion reporter vector pK18mob sacB-P_{mstX::lacZY}, the fusion of mstX promoter to the lacZY reporter gene by overlap PCR was created. First, two oligonucleotide primer pairs namely P_{mstX::F}/P_{mstX::R} and lacZY-F/lacZY-R were designed in the first round of PCR to amplify the 200-bp mstX promoter DNA fragments (corresponding to nucleotides +12 to −188 relative to the translational start codon (ATG) of mstX gene) and the lacZY DNA fragments, respectively. Second, P_{mstX::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 Smal and BamHI were inserted into pK18mob sacB between Smal and BamHI sites to get the pK18mob sacB-P_{mstX::lacZY} fusion construct [28, 29]. The fidelity of all constructs was confirmed by DNA sequencing (Sangon Biotech, Shanghai, China).

**Plasmid construction overexpression and purification of recombinant protein**

The overexpression of His_{6}-MstX protein in *E. coli* BL21 (DE3) cells harboring pET28a derivatives plasmids and purification of the overexpressed protein with the His · Bind Ni^{2+}-nitrioltriacetate (Ni-NTA) resin (Novagen, Madison, WI) were carried out as described previously [29]. Eluted recombinant His_{6}-MstX 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. Cleavage of the His_{6} tag was performed by adding 10 units of Enterokinase-Max (Invitrogen, Karlsruhe, Germany) and incubation at 4 °C overnight to conduct subsequent enzyme activity analysis. Ni-NTA agarose was used to remove the cleaved tag and uncleaved protein from the tag-free protein. All enzymes were purchased from Sigma-Aldrich (St. Louis, MO). The resulting His_{6}-tag-free proteins were dialyzed against PBS at 4 °C and concentrated for further experiments (>95% purity as estimated by sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE)).

**Sensitivity assays for oxidants, antibiotics, alkylating agents, and heavy metals**

The minimum inhibitory concentration (MIC) assay was performed for oxidants according to Rawat et al. [32]. Briefly, after oxidants were serially diluted (0.5×) in LB medium (1 ml), cells grown to the stationary phase were inoculated with 1% inoculum. After 1 to 2 days of cultivation at 30 °C, the tubes were checked for growth. The bacteriostasis growth curve assay was used to determine the sensitivity of *C. glutamicum* to heavy metal stress. LB-grown strains (50 μl) were transferred to LB broth (5 ml), with and without addition of various concentrations of heavy metals (Cd^{2+}, Ni^{2+} and Cu^{2+}) and incubated at 30 °C. The cellular growth was monitored turbidimetrically after 24 h. Antibiotics and alkylating agents experiments were performed according to Liu et al. [20] with minor modifications as follows: Overnight cultures of *C. glutamicum* RES167 strain were diluted in 1:100 in fresh LB medium with indicated concentration of various antibiotics or alkylating agents and cultivated for over 24 h with shaking at 30 °C [20]. Cellular growth was monitored by determining optical density at 600 nm at indicated time points. All assays were performed in triplicate.

**Quantitative analysis of sulphhydryl groups**

Free sulphhydril groups in MstX WT and its variants were measured using 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB) [33]. After 10 μM proteins were treated with 200 μM H_{2}O_{2} and 50 mM dithiothreitol (DTT) at room temperature for 30 min, respectively, followed by removing residual DTT or H_{2}O_{2} 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 (pH8.0) and the absorbance at 412 nm was measured against a 2 mM DTNB solution as the reference. The amounts of reactive sulphhydril groups were measured using the molar absorption coefficient of TNB at 412 nm (ε_{412}) of 13,600 M⁻¹ cm⁻¹ [34].

**In vitro state of MstX under stress**

The redox states of MstX WT and its variants (10 μM) were analyzed by incubating the proteins with 200 μM H_{2}O_{2} and 50 mM DTT, or 600 μM MSH and 200 μM H_{2}O_{2} + 50 mM DTT, or 200 μM H_{2}O_{2} + 600 μM MSH + 50 mM DTT for 30 min before separating on nonreducing 15% SDS-PAGE. For nonreducing conditions, DTT and β-mercaptoethanol were omitted from the loading buffer. All the samples were boiled for 5 min prior to electrophoresis.

**Detection of S-mycothiolated MstX:C262S in vitro**

For in vitro test, S-mycothiolated MstX:C262S (MstX::C262S-SSM, the mixed disulphide between MSH and MstX::C262S-H) was produced according to the method of Chi et al. [35]. MSH was purified from *C. glutamicum* RES167 as described previously [29]. MstX::C262S (50 μM) was incubated with excess of reduced MSH (6 M) prior to the addition of 2 mM H_{2}O_{2}. After 30 min incubation, excess MSH and H_{2}O_{2} were removed by ultrafiltration. The sample was loaded...
on Ni-NTA His-Bind resin-containing column (Novagen, Wisconsin, USA) and S-mycobiotlated MstX:C262S that could not combine with resin was directly collected. To obtain a pure sample, collected solution containing S-mycobiotlated MstX:C262S was purified on a superdex75HR column equilibrated with 50 mM HEPES/NaOH pH 8.0, 150 mM NaCl. Pure S-mycobiotlated MstX:C262S were confirmed by Matrix-Assisted Laser Desorption/Ionization Time of Flight Mass Spectrometry (MALDI-TOF MS).

The thioredoxin (Trx)/thioredoxin reductase (TrxR) and mycothione reductase (Mtr)/MSH electron transfer assays
The reduction of MstX:C262S-SSM was performed by monitoring the decrease in absorbance at 340 nm arising from NADPH oxidation. The assay mixtures contained 50 mM HEPES/NaOH pH 8.0 buffer, 500 ìM NADPH, 50 ìM MstX:C262S-SSM, and a reduced Trx-generating system [4 ìM TrXR and 40 ìM Trx], or MSH system [4 ìM Mtr and 500 ìM MSH] as the possible electron donors. All concentrations were calculated taking into account the subsequent addition of MstX:C262S-SSM. After the mixtures were incubated for 30 min at 37 °C, the reactions were started by adding different concentration of MstX:C262S-SSM. For each reaction mixture with a control well, MstX:C262S-SSM deleted from the reaction mixture was added. Three independent experiments were performed at each concentration. The kcat and km values of MstX:C262S-SSM for MSH were obtained from a non-linear fit with the Michaelis–Menten equation using the program GraphPad Prism 5.

Enzymatic activity assay and enzyme kinetics
Activities with hydroxyethyl disulphide (HED) and DHA as substrates were determined through the reduction of the mixed disulphide formed between HED and MSH or DHA and MSH, as described previously [36]. The HED and DHA assay for GSH was modified for MSH [37]. The kinetic parameters were determined in the presence of varying concentration of DHA or HED (0.05–10 mM) with a saturating concentration of 100 mM MSH, while that for MSH (0.1–10 mM) was determined in the presence of a saturating concentration of 100 mM HED or DHA. The enzyme reactions were measured in 50 mM potassium phosphate buffer (pH 7.6), 300 ìM NADPH, 1 ìM His tag-free MstX, and 5 ìM Mtr. 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 MstX, 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 MstX for MSH, HED or DHA substrates were obtained from a non-linear fit with the Michaelis–Menten equation using the program GraphPad Prism 5.

The reductase activities for mycothiolyl-phenylacetophenone (MS-PAP) and mycothiolyl-menadione (MS-MEN), were tested as described previously [38]. The appearance of the expected products (phenylacetophenone and menadione) was followed by reverse phase chromatography (4.6 × 250 mm Ecosil C18-AQ/C18-AQ PLUS column No. AJ1205-1546, Japan, Tokyo). The assays were carried out as developed previously [4]. Catalytic parameters were determined using varying substrate concentrations (0.05–10 mM MS-PAP, or 0.5–10 mM MS-MEN) at saturating MSH concentration (100 mM), respectively, while that for MSH (0–20 mM) was determined in the presence of a saturating concentration of 50 mM MS-MEN. The kcat and km values were obtained from a non-linear fit with the Michaelis–Menten equation using the program GraphPad Prism 5.

GST activities were spectrophotometrically determined for different substrates [39]. Peroxidase activity assays were performed by monitoring the decrease in absorbance at 340 nm arising from NADPH oxidation [40]. The catalytic properties of MstX were determined using a reduced system (Mtr and 500 ìM MSH) as the possible electron donors. These assays were carried out in a total volume of 500 ìl containing 50 mM Tris–HCl buffer (pH 7.5), 1 mM EDTA, 250 ìM NADPH, 1 ìM MstX, 500 ìM MSH, 4 ìM Mtr. The reactions were started by addition of peroxide substrates following 5 min of preincubation. The catalytic parameters of MstX for peroxides have been obtained by varying peroxide concentration at saturating concentrations of the other substrate (between 0 and 2000 ìM for peroxides). NADPH oxidation was monitored as A340. The activity was determined after subtracting the spontaneous reduction rate observed in the absence of MstX, 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 were obtained from a non-linear fit with the Michaelis–Menten equation using the program GraphPad Prism 5.

The Ferrous Oxidation of Xylenol orange (FOX) assay was performed as described [41].
**β-galactosidase assay**

β-Galactosidase activities were detected using o-nitrophenyl-β-d-galactopyranoside (ONPG) as the substrate [42].

**Quantitative RT-PCR analysis**

Quantitative RT-PCR analysis (7500 Fast Real-Time PCR; Applied Biosystems, Foster City, CA) was performed as described previously [30]. 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.

**Statistical analysis**

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

**Results and discussion**

**Involvement of MstX in oxidative resistance**

The ngcl1216 gene of C. glutamicum RES167 encoded a protein of 359 amino acids (mass, 40,619 Da), which has been annotated as a putative protein of GSH S-transferase (GST) family. Amino acid sequence alignment showed that NCgl1216 had very high similarity with representative Archaea and Gram-positive GSTs Xi class containing a Cys-Pro-Trp-Ala (C-P-W-A) typical active site motif, the hydrophobic co-substrate, the N-capping box (TXXD), and the hydrophobic stable motif (Additional file 1: Figure S1). Previous works suggested the GSTs Xi class were functionally related to Grxs [15], and Grx provided defense against oxidants and other stresses [43]. However, the role of NCgl1216 protein has not been studied so far. To get clues on the function of NCgl1216 in defending against condition stress, we determined the phenotype of a knockout in C. glutamicum to various agents by the MIC and growth curve inhibition assay. As shown in Table 1, the MIC of ncgl1216 knockout in C. glutamicum strains to various oxidants was detected. All the strains tested had nearly identical levels of growth in the LB broth media, while WT and the complementary strain Δncgl1216+ (Δncgl1216 mutant was complemented with plasmids carrying the wild-type ncgl1216 gene) obviously grew better than the Δncgl1216 mutant under neomycin, vancomycin, gentamycin, lincomycin, azithromycin, iodoacetamide (IAM), 2,4-dinitrochlorobenzene (CDNB), methylglyoxal (MG), CdCl2, NiSO4, and CuSO4 challenge (Figs. 1, 2). In short, these results confirmed that NCgl1216 had an influence on the C. glutamicum oxidative stress response. Thus, we named NCgl1216 as MstX (mycothiol S-transferase Xi class).

**In vitro MstX state upon oxidation**

To investigate the molecular and biochemical property of MstX, we cloned the open-reading frame of MstX into the pET28a plasmid and expressed the recombinant proteins in E. coli (BL21). Recombinant MstX was produced as a fusion with a 6×His tag and purified with a Ni2+ affinity resin. The molecular mass of recombinant His6-MstX was estimated to be ~45 kDa by nonreducing SDS-PAGE gel (Additional file 1: Figure S2a), in close agreement with the sum of its native molecular weight of 39.5 kDa and His6 (about 5 kDa). The result showed that MstX was monomeric, different from the reported GSTXs, mainly in the form of dimer [15].

Amino acid sequence alignment showed that MstX had two cysteine residues (at positions 67 and 262, as shown in Additional file 1: Figure S1). To reveal the state of Cys in MstX under oxidant treatment, the two variants, C67S and C262S, with each Cys mutated to Ser, respectively, were prepared, and the analytical methods of nonreducing SDS-PAGE gel and DTNB were used. H2O2-treated MstX WT still appeared as monomer on nonreducing SDS-PAGE gel, suggesting that MstX WT was functionally monomeric and did not form disulfide bond-containing dimer (Additional file 1: Figure S2b). But what’s interesting was that MstX WT treated with H2O2 and

| Oxidants | MIC (mM) | ΔmstX | ΔmstX+ |
|----------|---------|-------|--------|
| H2O2     | 120–100 | 60    | 100–80 |
| Diamide  | 6.4–8   | 3.2–1.6 | 6.4   |
| CHP      | 1.1     | 0.55–0.275 | 1.1   |
| MEN      | 3.6     | 1.8–0.9 | 3.6   |

**Table 1 The minimum inhibitory concentrations (MICs) of various oxidants for C. glutamicum strains**
MSH at the same time migrated more slowly than the forms treated only with MSH or H₂O₂. Importantly, MstX WT treated with H₂O₂ and MSH at the same time could be reduced by adding excessive DTT, indicating that these bands were reversed (Additional file 1: Figure S2b). Similarly, MstX:C262S treated with H₂O₂ and MSH at the same time migrated more slowly than the forms treated only with MSH or H₂O₂. However, the migration status of MstX:C67S treated with H₂O₂ and MSH simultaneously was similar to that of MstX:C67S under DTT treatment. Since MSH can reversibly modify sulfhydryl groups of some protein by combination, the apparent delay of electrophoretic mobility would occur in proportion to the number of free thiol groups in proteins. Therefore, according to our results, we speculated that S-mycothiolation was generated on Cys67 of MstX. DTNB assay also showed the reduced MstX WT had two thiols per monomer, as expected (Additional file 1: Figure S2c). After treating with 3 equivalents (per MstX WT) of H₂O₂, only one thiol per monomer was observed. However, MstX:C262S variant showed no thiol per monomer after H₂O₂ treatment, indicating that Cys67 was susceptible to oxidation. DTT-treated MstX:C67S proteins contained 0.78±0.2 thiol groups per monomer (Additional file 1: Figure S2c), equal to the thiol content measured in the H₂O₂-treated MstX:C67S proteins, indicating Cys262 in MstX:C67S proteins still existed in thiol state.

**MstX:C262S-SSM was specially reduced with electrons from the MSH/Mtr/NADPH pathway**

First, we investigated the electron transfer pathway coupled to MstX. To test this, the MSH/Mtr/NADPH electron transfer pathway, an important physiological reducing system in *C. glutamicum*, was used. We employed an in vitro assay system started by adding oxidized MstX:C262S-SSM as substrate for the electron transfer pathway, and the reduction in the absorption at 340 nm due to NADPH consumption was monitored. Only the sample with oxidized MstX:C262S-SSM by linking the MSH/Mtr/NADPH electron transfer pathway showed consumption of NADPH (Additional file 1: Figure S3a). Pedre et al. showed that Trx could reduce a cysteine-MSH mixed disulfide [47]. Thus, we tested whether Trx reduced oxidized MstX:C262S-SSM coupled with TrxR/NADPH pathway. Interestingly, although the reduction ability of Trx for S-mycothiolated MPx (MPx-SSM) linked to TrxR/NADPH pathway was about ×10² in *C. glutamicum* [47], Trx did not
show reproducible activity for MstX:C262S-SSM (Additional file 1: Figure S3b). After determining the ability of reduction, we investigated the reduction rate of oxidized MstX:C262S-SSM in a coupled enzyme assay, in which different concentration of oxidized MstX:C262S-SSM was used as substrate linked to C. glutamicum MSH/Mtr/NADPH pathway (Additional file 1: Figure S3c). From the Michaelis–Menten kinetic plot, a $k_{\text{cat}}$ value of $0.725 \pm 0.014 \text{ s}^{-1}$ and a $k_m$ of $0.811 \pm 0.054 \text{ μM}$ were obtained, resulting in a specificity constant ($k_{\text{cat}}/k_m$) of $8.93 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$.

**MstX had significant thiol transferase (mycoredoxin) and reductase activities but not mycothiol transferase activity**

According to previous study, GSTX exhibited higher Grx activity [15]. Moreover, Grxs had been known for a long time as GSH-dependent disulfide oxidoreductases that reduced S-glutathiolated mixed disulphides [48]. Thus, the thiol transferase activity of MstX was first determined using HED and MSH as co-substrates in vitro. The kinetic properties of MstX at a fixed concentration of MSH and different concentrations of HED could be measured (Additional file 1: Table S3). The $k_m$ value, $k_{\text{cat}}$ value, and catalytic coefficient of HED for MstX were calculated to be $0.192 \pm 0.03 \text{ mM}$, $116.73 \pm 3.048 \text{ s}^{-1}$, and $6.08 \pm 0.18 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ (Additional file 1: Table S3). Specific activity of the recombinant MstX proteins as a function of MSH concentration at a fixed concentration of HED was also determined. As shown in Fig. 3a, $k_m$ value, $k_{\text{cat}}$ value, and catalytic coefficient of MSH for MstX at 100 mM HED as substrate were $0.643 \pm 0.068 \text{ mM}$, $110.9 \pm 3.048 \text{ s}^{-1}$, and $1.72 \pm 0.2 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$. The above results indicated that MstX had considerably high Mrx activity, similar to that of MSH-dependent reductase (mycoredoxin 1, Mrx1), acting as an oxidoreductase of reducing S-mycothiolated mixed disulphides linked to the MSH electron transfer pathway [49].
MstX proteins also displayed quite high activity of DHA reductase when a fixed concentration of MSH and different concentrations of DHA were used (Additional file 1: Table S3). Moreover, specific activity of the recombinant MstX proteins as a function of MSH concentration at a fixed concentration of DHA was also determined. As shown in Fig. 3b, the $k_m$ value, $k_{cat}$ value, and catalytic coefficient for MSH were 0.461 ± 0.07 mM, 129.3 ± 4.471 s$^{-1}$, 2.8 ± 0.3 × 10$^5$ M$^{-1}$ s$^{-1}$, respectively. In addition, we found that MstX displayed slightly higher activity as a DHA reductase than that as HED thiol transferase.

GSTXs were specialized in the reduction of GH. Thus, the recombinant MstX proteins were tested with different concentration of mycothiolyl-menadione (MS-MEN) as substrates to assess its reductase activity. As showed in Additional file 1: Table S4, MstX proteins had mycothiolyl-menadione reductase (MHR) activity with the concentration of MS-MEN (Additional file 1: Table S3). Moreover, specific activity of the recombinant MstX proteins as a function of MSH concentration at a fixed concentration of substrates (100 mM HED and DHA, or 50 mM MS-MEN). The Michaelis–Menten plots of MstX activity versus HED (a), DHA (b), and MS-MEN (c) were calculated using the program GraphPad Prism 5. The data were represented as mean ± SD of three independent experiments.

Next, we investigated whether MstX had the ability of conjugating MSH to a variety of exogenous and endogenous electrophilic compounds, which was a typical trait for Ser/Tyr-GSTs class. However, no MSH-transferase activity was detected for the recombinant MstX proteins, when CDNB and monobromobimane (mBBr) were used as substrates (Additional file 1: Table S3). We speculated that the active Cys in MstX could not perform the nucleophilic attack on substrate CDNB and mBBr. MstX therefore had different characteristic from those of most other GSTs, and functionally resembled Grx activity.

MstX had low activity as MSH peroxidase
Many members of GSTs family exhibited GSH peroxidase activity against peroxides [50–52]. Moreover, as MstX showed a role in the defence against oxidants (Figs. 1, 2), we then investigated whether MstX proteins were also active as MSH peroxidases. H$_2$O$_2$, CHP, and t-BHP were added to the MSH/Mtr/NADPH electron transfer pathway in the presence and absence of MstX. Addition of MstX to the MSH/Mtr/NADPH system resulted in a slight reduction of the absorption at 340 nm compared to only the MSH/Mtr/NADPH system (Fig. 4a–c). With the FOX assay, we tested the residual peroxide concentration after the reaction. Addition of MstX also caused a slight decrease in H$_2$O$_2$, CHP, and t-BHP content compared to that in only the MSH/Mtr/NADPH system (Fig. 4d–f). The kinetic parameters of MstX were determined with a fixed concentration of MstX (1 μM) and different concentrations of H$_2$O$_2$, CHP, and t-BHP (0–2000 μM). The $k_m$ value, $k_{cat}$ value, and catalytic coefficient of MstX were calculated to be 154.9 ± 26.80 μM, 0.171 ± 0.006 s$^{-1}$, and 11.0 ± 0.6 × 10$^5$ M$^{-1}$ s$^{-1}$ for H$_2$O$_2$; 328.1 ± 33.27 μM, 0.153 ± 0.005 s$^{-1}$, and 4.7 ± 0.2 × 10$^5$ M$^{-1}$ s$^{-1}$ for CHP; 301.3 ± 46.62 μM, 0.135 ± 0.006 s$^{-1}$, and 4.5 ± 0.6 × 10$^5$ M$^{-1}$ s$^{-1}$ for t-BHP (Fig. 4g–i). These
results indicated that the peroxide scavenging role of MstX was at a relatively low rate of $10^2 \text{ M}^{-1} \text{s}^{-1}$ (using 500 µM MSH), which were several orders of magnitudes lower than the reaction rate of the known peroxidases of *C. glutamicum* ($10^5$ to $10^7 \text{ M}^{-1} \text{s}^{-1}$) [22–24]. Together, MstX proteins showed low but reproducible activity against these three substrates.

**Cys67 was essential for MstX's thiol transferase and peroxidase activity**

The secondary structure prediction showed Cys67 in MstX was located between a β-strand and an α-helix region in the model of Fig. 5a. Previous studies showed the Cys residue, completely conserved in all reference GSTX, was essential for thiol transferase, indicating Cys67 was required for the activity of MstX as a thiol transferase. Another cysteine residue at position 262 of MstX did not have an equivalent in other reference GSTXs. In order to investigate the role of Cys in function of MstX, we constructed the MstX:C67G, MstX:C67S, MstX:C67A, MstX:C67Y, MstX:C262S, and MstX:C262G variants via site-directed mutation. The activity of these variants towards HED and MSH was measured and the steady-state kinetic constants were compared with that of MstX WT. As shown in Fig. 5b, MstX:C67G, MstX:C67S, MstX:C67A, and MstX:C67Y reduced the thiol transferase activity of MstX to the same undetectable levels as background. Another cysteine residue at position 262 of MstX did not have an equivalent in other reference GSTXs. In order to investigate the role of Cys in function of MstX, we constructed the MstX:C67G, MstX:C67S, MstX:C67A, MstX:C67Y, MstX:C262S, and MstX:C262G variants via site-directed mutation. The activity of these variants towards HED and MSH was measured and the steady-state kinetic constants were compared with that of MstX WT. As shown in Fig. 5b, MstX:C67G, MstX:C67S, MstX:C67A, and MstX:C67Y reduced the thiol transferase activity of MstX to the same undetectable levels as background.
value from the reactive mixture in the absence of MstX (Fig. 5b). However, the MstX:C262G and MstX:C262S variants, in which Cys67 was left unchanged, had enzyme activity levels comparable with that of the MstX WT, indicating that the thiol transferase activity of MstX was strictly related with a single cysteine residue at position 67, and operated through using a monothiol mechanism.

Next, we tested whether Cys67 and Cys262 of MstX for peroxidase activity also showed the behavior. As shown in Fig. 5b, MstX peroxidase activity depended on the presence of a cysteine residue at position 67, no peroxidase activity was observed in the MstX:C67G, MstX:C67S, MstX:C67A, and MstX:C67Y variants, suggesting involvement of the Cys in the reaction mechanism. However, the MstX:C262G and MstX:C262S variant showed comparable activity with MstX WT.

The three residues adjacent to Cys67 and C-terminal domain of MstX were also required for the activity
The three residues adjacent to the cysteine at position 67, consisting of the proline, tryptophan, and asparagine (Pro-Trp-Ala, P-W-A), were also completely conserved in all reference GSTXs (Additional file 1: Figure S1). Thus, we tested whether replacing Cys67 plus the adjacent residues Pro68, Trp69 and Ala70 in MstX with the conserved Cys-Gly-Phe-Ser (CGFS) sequence of monothiol Grxs active site had some effect on the activity of the protein. Although CGFS motif could interact with GSH, monothiol Grxs was unable to deglutathionylate the small mixed disulfide between β-mercaptoethanol and a GSH moiety [53]. To test this, MstX: P68GW69FA70S three variant was constructed. The MstX: P68GW69FA70S variant protein with the CGFS motif of Grx5 showed no detectable activity as a thiol transferase with HED (Fig. 5b). This confirmed that, although Cys67 was required for MstX activity, the adjacent residues were also important. Similarly, the three residues adjacent to Cys67 were also very vital for MstX peroxidase activity.

Previous study showed that the C-terminal domain of GST was also required for thiol transferase activity in addition to the thioredoxin fold domain containing C-P-W-A motif [13]. Nine residues (Val192, Asn193, Gly235, Ile238, Thr239, Asp242, Ile243, Thr248, and Arg251) in the C-terminal region of MstX were conserved at equivalent positions of GSTX homologues from other species,
belonging to the N-capping box, the hydrophobic staple motif and the hydrophobic co-substrate site, respectively (Additional file 1: Figure S1). Thus, we constructed MstX:V192AN193AT248AR251A and MstX: G235LI238AT-239AD242GI243S variants separately and studied their effects on activity. These changes seriously affected the enzyme activity (Fig. 5b), indicating C-terminal domain of MstX were also important for maintaining enzyme activity.

**mstX expression**

Since *C. glutamicum* mstX provided resistance to various agents, we performed the lacZY activity profiling and qRT-PCR analysis to determine the expression pattern of mstX in response to stress conditions. The lacZY activity of P_mstX::lacZY chromosomal promoter fusion reporter in *C. glutamicum* RES167 parental strain was quantitatively measured in bacterial cells either untreated or treated with different agents (Fig. 6a). The level of mstX expression was increased by approximately 3.1-, 2.1-, or 2.51-fold in the *C. glutamicum* RES167 parental reporter strain treated with 10 mM H2O2, 1 mM MEN, or 7.5 mM IAM, respectively, as compared to untreated samples (Fig. 6a). Further, expression of the P_mstX::lacZY fusion displayed a dose-dependent increase in response to these adverse environmental conditions (Fig. 6a). These results clearly demonstrated that environmental stress induced mstX expression, which in turn directly contributed to tolerance of *C. glutamicum* to these stress conditions. A similar pattern of mstX expression in response to environmental conditions was also observed by qRT-PCR analysis (Fig. 6b).

**Conclusions**

Glutathione transferases (GSTs) from the Xi classes had a catalytic cysteine residue, giving them reductase and thiol transferase activities. GSTX can protect against irreversible oxidation and act as a redox switch regulating metabolic pathways. Here, we presented the biochemical and structural analysis of *C. glutamicum* MSH S-transferase Xi class (MstX). Our data demonstrated that *C. glutamicum* MstX not only had multiple reductase activities, including dehydroascorbate reductase, mycothiolyl-hydroquinone reductase and MSH peroxidase, but also could take over the role of Mrx and reduce the protein-MSH mixed disulfide using a monothiol mechanism, thereby contributing to better understanding of MSH-dependent reactions. However, MstX had no glutathionyl-acetophenones reductase activity so far thought to be specific to GSTOs. In addition, *C. glutamicum* MstX was involved in the cellular defense against various hostile environments. Thus, the experiments reported here first revealed the previously unrecognized contribution of the members of MST family to the demycothionylation of proteins, substrate reduction, and cell resistance. Moreover, these detailed understanding on the bioactive MstX including its biochemical feature and action mechanism might be helpful to establish control strategies during ferment process, where a series of unfavorable circumstances inevitably were generated.

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**Fig. 6** Stress response of mstX in *C. glutamicum*. **a** β-Galactosidase analysis of the mstX promoter activity was performed using the transcriptional P_mstX::lacZY chromosomal fusion reporter expressed in *C. glutamicum* RES167 parental strain. 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. β-Galactosidase activity was assayed as described in "Methods". Mean values with standard deviations (error bar) from at least three repeats were shown. **b** qRT-PCR assay was performed to analyze the expression of mstX. Exponentially growing *C. glutamicum* RES167 parental strains were exposed to different toxic agents at indicated concentrations for 30 min. The levels of mstX expression were determined by quantitative RT-PCR. The mRNA levels were presented relative to the value obtained from cells without treatment. The values represent the mean results from three independent cultivations, with standard errors.
Additional file 1: Table S1. Bacterial strains and plasmids used in this study. Table S2. Primers used in this study. Table S3. Activity of recombinant MstX of C. glutamicum with CDNB, mBBr, HED, and DHA. Table S4. Activity of recombinant MtxX of C. glutamicum with MS-MEN and MS-PAP. Figure S1. Multiple sequences alignment of C. glutamicum NCg1216 with representative Archaea and Gram-positive GST Xi class from H. lacusprofundi (YP_002565306), N. magadii (YP_003479316), S. agalactiae (NP_687815), and E. coli YgG (NP_417573) and Saccharomyces cerevisiae glutathione transferase 2 Omega-like Gto2 (YKR076W). Figure S2. Redox response of MstX in vitro. Figure S3. Oxidized MstX:C262SS-SSM was mainly reduced via the Mtr/MSH/NADPH pathway.

Abbreviations
ROS: reactive oxygen species; GST: glutathione transferase; GSTX: GST Xi class; GSTO: GST omega class; MtxX: mycothiol S-transferase Xi class; GHR: glutathionyl-hydroquinone reductase; GSH: glutathionyl-hydroquinone; GSH: glutathione; HED: β-hydroxyethyl disulfide; MstX:C262SS-55M: the mixed disulfide between MSH and MstX:C262SS-SSM. CpxA: mycothiol; MEN: menadione; Mrx: mycoredoxin; M: mycothione reductase; MSH: mycothiol; MEN: menadione; MtxX: mycothione reductase; CHP: cumene hydroperoxide; LMW: low molecular weight; Gto2: glutathione transferase 2 omega-like.

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Authors’ contributions
MS, CC (second author), GL, and XL performed the experiments. MS, CC (second author), GL, XL, ZG, JL, CC (seventh author), and GY analyzed the data. MS and GY wrote the paper. All authors read and approved the final manuscript.

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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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