Mycothiol protects *Corynebacterium glutamicum* against acid stress via maintaining intracellular pH homeostasis, scavenging ROS, and S-mycothiolating MetE

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Mycothiol (MSH) plays a major role in protecting cells against oxidative stress and detoxification from a broad range of exogenous toxic agents. In the present study, we reveal that intracellular MSH contributes significantly to the adaptation to acidic conditions in the model organism *Corynebacterium glutamicum*. We present evidence that MSH confers *C. glutamicum* with the ability to adapt to acidic conditions by maintaining pH$_i$ homeostasis, scavenging reactive oxygen species (ROS), and protecting methionine synthesis by the S-mycothiolation modification of methionine synthase (MetE). The role of MSH in acid adaptation was further confirmed by improving the acid tolerance of *C. glutamicum* by overexpressing the key MSH synthesis gene *mshA*. Hence, our work provides insights into a previously unknown, but important, aspect of the *C. glutamicum* cellular response to acid stress. The results reported here may help to understand acid tolerance mechanisms in acid sensitive bacteria and may open a new avenue for improving acid resistance in industry strains for the production of bio-based chemicals from renewable biomass.

Key Words: acid stress; *Corynebacterium glutamicum*; mycothiol; pH homeostasis; S-mycothiolation

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Roles of mycothiol in acid resistance

Diphtheriae, C. jeikeium or mycobacteria (Follmann et al., 2009), and for the disclosing of catabolism mechanisms of aromatic compounds in high GC content Gram-positive bacteria (Shen et al., 2012). C. glutamicum is an acid sensitive bacterium as the minimal pH it tolerated was found to be 5.5 (Jakob et al., 2007). To gain a better understanding of molecular adaptation induced by acid stress, the global gene expression profile of C. glutamicum adapted to pH 5.7 was characterized by using a microarray (Jakob et al., 2007), or proteomic analysis (Barriuso-Iglesias et al., 2008), revealing the up-regulation of genes encoding transcriptional regulators, proteins responsible for transportation and metabolism, and several proteins of unknown function. Interestingly, the atp gene cluster encoding the F$_1$F$_0$-ATPase, whose roles in proton pump-of unknown function. Interestingly, the for transportation and metabolism, and several proteins (Iglesias et al., 2008), revealing the up-regulation of genes (Jakob et al., 2007), or proteomic analysis (Barriuso-Iglesias et al., 2008; Zhang et al., 2007). Besides, the genes encoding glutamate dehydrogenase, arginine decarboxylase and arginine deiminase, which represent another widely-distributed acid resistance mechanism resulting in the alkalization of the cytoplasm, are missing in C. glutamicum (Follmann et al., 2009). Therefore, there are some novel acid adaptation mechanisms in the acid sensitive C. glutamicum that are worthy of investigation.

Further studies on acid stress response in C. glutamicum, based on transcriptome, proteome, and metabolome, unraveled a functional link between pH acclimatization, oxidative stress, iron homeostasis, and metabolic alterations (Follmann et al., 2009). The occurrence of oxidative stress under acid stress was also observed in Bacillus cereus, accompanied by the formation of reactive oxygen species (ROS), and the activation of oxidative stress associated genes such as thioredoxins, catalases and superoxide dismutase (Mols and Abee, 2011b; Mols et al., 2010). However, although elimination of acid induced H$_2$O$_2$ by the addition of external catalase facilitates the growth of C. glutamicum at a neutral pH, the addition of catalase had no significant beneficial effect on growth under acidic pH conditions (Follmann et al., 2009). This observation raises the question as whether, and how, other non-enzymatic antioxidants, such as mycothiol, function in the adaptation of C. glutamicum to acidic pH conditions.

Mycothiol (MSH), the dominant low-molecular-weight thiol (LMWT) restricted to the high-(G+C)-content Gram-positive Actinobacteria, has been regarded as a functional equivalent of glutathione (GSH) in these species and plays an important role in maintaining cytosolic redox homeostasis and in adapting to ROS (Newton et al., 2006, 2008). The MSH biosynthetic pathway consists of four steps: UDP-GlcNAc (Uridine diphosphate-N-acetylglucosamine) and Ins (1L-myo-inositol-1-phosphate) are linked by MshA to form GlcNAc-Ins(1-O-(2-acetamido-2-deoxy-α-d-glucopyranosyl)-d-myo-inositol-3-phosphate), which is then deacetylated by MshB to generate GlcN-Ins, the latter is ligated to L-cysteine through MshC, and finally MshD transacylated the cysteinyl residue to produce MSH with the presence of CoASAc (Newton and Fahey, 2002). So far, MSH has been reported to be involved in the detoxification of a broad range of poisons such as oxidants, electrophiles, antibiotics, aromatic compounds, heavy metals and ethanol (Buchmeier et al., 2006; Liu et al., 2013; Rawat et al., 2002). In Streptomyces coelicolor, MSH appears to detoxify endogenously generated antibiotics and reactive intermediates by converting them to S-conjugates of mycothiol (Carney et al., 1997). MSH is also an essential cofactor for maleylpuruvate isomerase (Feng et al., 2006; Shen et al., 2005), nitrosoymycocothiol reductase (Vogt et al., 2003) and arsenate reductase (Ordóñez et al., 2009). Recently, protein S-myothiolation was discovered as an important thiol protection and redox switch mechanism in response to hypochlorite stress in C. glutamicum (Chi et al., 2014). Interestingly, although none of the MSH related genes has been identified to be induced by acid stress in previous transcriptomics analysis, it was reported that a Mycobacterium tuberculosis MSH null mutant did show restricted growth in an acidic medium (Buchmeier et al., 2006). However, the mechanisms underlying MSH protection in acid adaptation still remain unknown.

Recently, it has been reported that acid stress can induce an oxidative stress response in Bacillus subtilis (Mols et al., 2010) and C. glutamicum (Follmann et al., 2009). Interestingly, it is well known that MSH plays an important role in the resistance to oxidative stress and in scavenging ROS in the high-(G+C)-content Gram-positive Actinobacteria (Liu et al., 2013; Rawat and Av-Gay, 2007; Rawat et al., 2002). These findings prompted us to examine whether MSH protects C. glutamicum against acid stress by reducing the levels of deleterious ROS induced by acid stress.

In this study, we found that intracellular MSH contributes significantly to the adaptation of acid conditions in the model organism C. glutamicum. In addition, we present evidence that MSH protects C. glutamicum by scavenging ROS, S-myothiolation protecting MetE and maintaining pH homeostasis. Moreover, overexpression of mshA, the key MSH synthesis gene, significantly improve the acid tolerance activity of C. glutamicum. Our insights into the protective effects of MSH in C. glutamicum provide a further understanding in the C. glutamicum cellular response to acid stress, and may also have a reference value for industrial fermentation, e.g., to maintain a higher viability of C. glutamicum in the process of the production of amino acids and organic acids from lignocellulosic biomass.

Materials and Methods

Bacterial strains and culture conditions. Bacterial strains and plasmids used in this study are listed in Table 1. E. coli strains were grown aerobically on a rotary shaker (180 rpm) at 37°C in Luria-Bertani (LB) broth or on LB agar plates. The C. glutamicum strain RES167 was the parent of all derivatives used in this study. C. glutamicum strains were routinely grown in LB medium or in mineral salts medium supplemented with 2 mM glucose as a carbon source on a rotary shaker (180 rpm) at 30°C (Shen et al., 2005). For the generation of mutants and the maintenance of C. glutamicum, BHIS medium (brain heart broth with 0.5 M sorbitol) was used. Cellular growth was monitored.
by determining the optical density at 600 nm. When needed, antibiotics were used at the following concentrations: chloramphenicol, 20 μg ml⁻¹ for *E. coli* and 10 μg ml⁻¹ for *C. glutamicum*; nalidixic acid, 30 μg ml⁻¹ for *C. glutamicum*.

**DNA manipulations.** General DNA manipulations, transformations and agarose gel electrophoresis were carried out by applying standard molecular techniques (Sambrook and Russell, 2001). Restriction enzyme digestion, ligation, and plasmid purification were done in accordance with the manufacturer’s instructions (Feng et al., 2006). Restriction enzyme digestion, ligation, and plasmid purification were done in accordance with the manufacturer’s instructions (Feng et al., 2006). Restriction enzyme digestion, ligation, and plasmid purification were done in accordance with the manufacturer’s instructions (Feng et al., 2006).

Plasmids were introduced into *C. glutamicum* strains by electroporation as described (Tauch et al., 2002) and induced by the addition of 0.4 mM isopropyl-β-D-thiogalactopyranoside (IPTG) to the culture broth. The genes encoding for *C. glutamicum* mycoredoxin 1 (*mrx1*, nclg10808) and mycothione reductase (*mtr*, nclg11928) were amplified by PCR using genomic DNA of *C. glutamicum* RES167 as a template with primer pairs *mrx1F/mrx1R* and *mtrF/mtrR*. These DNA fragments were digested and afterwards subcloned into similar digested PET28a vectors, obtaining plasmids pET28a-*mrx1* and pET28a-*mtr*, respectively. The fidelity of all constructs was confirmed by DNA sequencing (Sangon Biotech, Shanghai, China).

**Overexpression and purification of recombinant proteins.** To express and purify His₆-tagged Mrx1 and Mtr proteins, the pET28a-*mrx1* and pET28a-*mtr* plasmids were transformed into *E. coli* BL21(DE3) host strains, respectively. For protein production, bacteria were grown at 37°C in LB medium to an OD₆₀₀ of 0.4, shifted to 22°C and then induced with 0.4 mM IPTG, and cultivated for an additional 12 h at 22°C. Harvested cells were disrupted by sonication and purified with the His-Bind Ni-NTA resin (Novagen, Madison, WI, USA) according to manufacturer’s instructions. Purified recombinant proteins were dialyzed against PBS overnight at 4°C and stored at –80°C until use. Protein concentrations were determined using the Bradford assay according to the manufacturer’s instructions. Purified recombinant proteins were dialyzed against PBS overnight at 4°C and stored at –80°C until use. Protein concentrations were determined using the Bradford assay according to the manufacturer’s instructions.

**Plasmid construction.** Primers used in this study are listed in Table 1. To overexpress *mshA* (nclg10389) and *metE* (nclg2194) in *C. glutamicum*, primer pairs *mshAF/mshAR* and *metEF/metER* were used to amplify intact gene fragments from *C. glutamicum* genome. Both DNA fragments were digested and afterwards subcloned into similar digested pXMJ19 vectors to produce the plasmid pXMJ19-*mshA* and pXMJ19-*metE*, respectively. The pXMJ19-*mshA* and pXMJ19-*metE* plasmids were introduced into *C. glutamicum* strains by electroporation as described (Tauch et al., 2002) and induced by the addition of 0.4 mM isopropyl-β-D-thiogalactopyranoside (IPTG) to the culture broth. The genes encoding for *C. glutamicum* mycoredoxin 1 (*mrx1*, nclg10808) and mycothione reductase (*mtr*, nclg11928) were amplified by PCR using genomic DNA of *C. glutamicum* RES167 as a template with primer pairs *mrx1F/mrx1R* and *mtrF/mtrR*. These DNA fragments were digested and afterwards subcloned into similar digested PET28a vectors, obtaining plasmids pET28a-*mrx1* and pET28a-*mtr*, respectively. The fidelity of all constructs was confirmed by DNA sequencing (Sangon Biotech, Shanghai, China).

<table>
<thead>
<tr>
<th>Strains or plasmids</th>
<th>Relevant characteristic(s)</th>
<th>Source or reference</th>
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<tr>
<td><em>E. coli</em> IM109</td>
<td>recA1 supE44 endA1 hsdR17 gyrA96 relA1 thi Δ(lac-proAB)F’(traD36 proABlacF’ lacZM15)</td>
<td>Stratagene</td>
</tr>
<tr>
<td>BL21(DE3)</td>
<td>Host for expression vector pET28a</td>
<td>Novagen</td>
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<td><em>C. glutamicum</em></td>
<td></td>
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<tr>
<td>RES167(pXMJ19)</td>
<td>Restriction-deficient mutant of ATCC13032, Δ(cglIM-cglIR-cglIR)</td>
<td>Tauch et al. (2002)</td>
</tr>
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<td>ΔmshA/CgXMJ19</td>
<td>mshC deleted in RES167</td>
<td>Feng et al. (2006)</td>
</tr>
<tr>
<td>ΔmshDmXMJ19</td>
<td>mshD deleted in RES167</td>
<td>Feng et al. (2006)</td>
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<td>ΔmshC</td>
<td>mshC containing pXMJ19-mshC</td>
<td>Liu et al. (2013)</td>
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<tr>
<td>ΔmshD</td>
<td>mshD containing pXMJ19-mshD</td>
<td>Liu et al. (2013)</td>
</tr>
<tr>
<td>Plasmids</td>
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<td>pXMJ19</td>
<td>Shuttle vector (Ptac lacI*) pBL1 oriV_C. glutamicum pK18oriV_C. glutamicum</td>
<td>Jakob et al. (2007)</td>
</tr>
<tr>
<td>pXMJ19-metE</td>
<td>metE cloned into pXMJ19 for S-mycoredoxin assay</td>
<td>This study</td>
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<tr>
<td>pXMJ19-mshA</td>
<td>mshA cloned into pXMJ19 for overexpression</td>
<td>This study</td>
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<tr>
<td>pET28a-mrx1</td>
<td>mrx1 cloned into pET28a for protein purification</td>
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<tr>
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<td>mtr cloned into pET28a for protein purification</td>
<td>This study</td>
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<td><strong>Primers</strong></td>
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<td>metEF</td>
<td>CGGTCGACAAGAGGACACACATGCACACACACACACACATCGTCCACTCGACATTCT</td>
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<tr>
<td>metER</td>
<td>CAAGACTCTTTAGATGTGGTCCGATTTTT(EcoRI)</td>
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</tr>
<tr>
<td>mshAF</td>
<td>ACAGAAGCTTTAGAGGACACACATGCACACACACACACATCGTCCACTCGACATTCT</td>
<td>This study</td>
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<tr>
<td>mshAR</td>
<td>ACTCTCGAGTTACCCGTCGTACGTACGTACGTTACAC (Khol)</td>
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<td>mrx1F</td>
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<td>mrx1R</td>
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<tr>
<td>mtrR</td>
<td>AGGCTCGACCTAAACTCTTAGGGACACAAAG</td>
<td>This study</td>
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Underlined sites indicate restriction enzyme cutting sites added for cloning. Letters in italics denote ribosome binding sites. The His₆ tag is given in boldface.
according to Yin et al. (2010). In brief, the cells (50–200 mg of wet weight) were suspended in 100–600 µl aqueous perchloric acid (3%) and were incubated for 30 min at room temperature. Cellular debris was pelleted by centrifugation at 15,000 × g for 5 min at 4°C. The supernatant from the perchloric acid treatment, diluted 400 times, was used for MSH determination. The substrate for MSH-dependent maleylpyruvate isomerase (MDMPI) was freshly prepared by the reaction of 120 µM 1-1-gentisate and purified gentisate 1,2-dioxygenase (G12D) in 50 mM Tris-HCl (pH 8.0) at room temperature until the absorbance at 330 nm (A330) did not change. This mixture, containing maleylpyruvate, was used as a substrate for maleylpyruvate isomerase. MDMPI and FPH (fumarylpyruvate hydrodolase) were added to 5 µl supernatant for the MSH determination. The reaction proceeded at room temperature until no further change in A330.

**Acid survival assays.** Acid survival assays were performed according to Zhang et al. (2013) with a minor modification as follows: The overnight cultures of *C. glutamicum* strains in LB were appropriately diluted into LB or mineral salts medium (pH 4.0), and incubated at 30°C for 1 h. After acid stress, the cultures were serially diluted and plated onto LB agar plates, and colonies were counted after 24 h growth at 30°C. The percentage survival was calculated as follows: [(C.F.U. ml⁻¹ after acid challenge)/(C.F.U. ml⁻¹ without acid challenge)] × 100. Survival values were the averages of three independent experiments.

**Measurement of intracellular ROS level.** Intracellular ROS was detected by using the ROS-sensitive probe 2′,7′-dichlorofluorescein diacetate (H₂DCFDA)-based assay described by Wang et al. (2015). After this procedure, H₂DCFDA was added from a fresh 5 mM stock (prepared in ethanol) to a final concentration of 10 µM in 1 ml of acid-challenged *C. glutamicum* cells and then incubated at 28°C for 20 min. Finally, cells were cooled on ice, harvested by centrifugation, and washed twice with distilled water. The fluorescence was measured by means of a spectro-max spectrophotometer (RF-5301PC, USA) with excitation at 502 nm and emission at 521 nm.

**Measurement of intracellular pH (pHi).** The internal pH was determined by using the pH-sensitive fluorescent probe 2,7-bis-(2-carboxylethyl)-5-6-carboxyfluorescein (BCECF) as described previously (Jakob et al., 2007). In brief, 1 ml of *C. glutamicum* culture (OD₆₀₀ = 0.6) was centrifuged at 10,000 g for 2 min, re-suspended in 1 ml PBS (pH 7.0), and incubated with BCECF-AM (final concentration 1.2 µM) for 30 min at 30°C in the dark. After BCECF-AM was removed by washing, cells were pelleted again and re-suspended in 1 ml acidic LB medium (pH 4.0) for 10 min, acid stress terminated by centrifugation and re-suspended in 1 ml PBS. BCECF fluorescence was measured with the spectro-max spectrophotometer (RF-5301PC, USA) at 535 nm after excitation at 450 nm (pH insensitive) or 490 nm (pH sensitive). Calibration was performed by incubation of cells at external pH values in the range pH 3.5, 4.5, 5.5, 6.5 and 7.5 in the presence of a mixture of CCCP, valinomycin, and nigericin (final concentrations of 50, 20 and 5 µM, respectively) in order to equilibrate internal and external pH values.

**S-mycothiolation of MetE.** A biotin switch assay was applied to detect MetE S-mycothiolation. NEM-biotin-tagged, demycothiolated proteins were obtained as described previously with minor modifications (Si et al., 2015). Briefly, WT(pXMJ19-metE), ΔmshC(pXMJ19-metE) and ΔmshD(pXMJ19-metE) strains grown aerobically to OD₆₀₀ = 0.6 were divided into two parts, one part exposed to acid stress (pH 4.0) for 3 h at 30°C, and another part without acid stress used as a negative control. Cells were harvested by centrifugation at 10,000 g, 4°C for 10 min and resuspended in the urea/chaps alkylation buffer (100 mM Tris-HCl, pH 8.0; 1 mM EDTA; 8 M urea; 1% CHAPS; 100 mM NEM) for 30 min in the dark prior to sonication on ice. After sonication, the resulting mixtures continued to be alkylated for 30 min in the dark followed by centrifugation at 10,000 g for 1 h. His₅-MetE was enriched with His-Bind Ni-NTA resin (Novagen, Madison, WI, USA) according to manufacturer’s instructions. The resulting His₅-MetE was dissolved in a Tris-HCl buffer (pH 8.0) and demycothiolated using 20 µM purified Mrx1 in the presence of 1 mM NADPH and 20 µM Mtr for 30 min at room temperature. The demycothiolated His₅-MetE was then treated with 5 mM biotin-maleimide dissolved in dimethyl sulfoxide for 30 min. Unreacted biotin-maleimide was removed by an ice-cold acetone precipitation for 1 h followed by centrifugation at 10,000 × g for 30 min. This precipitation was repeated 3 times. The pellet was dissolved in Tris-HCl (pH 8.0) buffer, resolved by non-reducing SDS-PAGE, and transferred onto nitrocellulose membranes (Millipore, Bedford, MA, USA). After being blocked with 1% BSA for 4 h at room temperature, membranes were probed with a 1:300 dilution of the stabilized Streptavidin-Horseradish Peroxidase (Thermo Scientific, IL, USA) or 1:1,000 dilution of the anti-His antibody (Millipore) for 2 h at room temperature. After washing, the signals were visualized by using the ECL plus kit (GE Healthcare, Piscataway, NJ, USA) based on the manufacturer’s specified protocol.

**Overexpression of mshA in C. glutamicum under acid stress.** Overnight cultures of *C. glutamicum* WT(pXMJ19) and WT(pXMJ19-mshA) strains were diluted in 1:100 in fresh LB medium (pH 7.5). Cultures were incubated at 30°C with agitation at 200 rpm. Appropriate volumes of the culture were aseptically withdrawn for the quantitative detection of MSH, and the assay of MSH was performed as described previously (Yin et al., 2010).

**MALDI-TOF MS-MS analyses.** The His₅-MetE from WT(pXMJ19-metE) and ΔmshD(pXMJ19-metE) strains treated with or without acid (pH 4.0) for 3 h at 30°C were subjected to non-reducing SDS-PAGE, and Coomassie brilliant blue stained bands were excised, then in-gel digested with trypsin, and analyzed by MALDI-TOF MS-MS (Voyager-DE STR; Applied Biosystems, Waltham, MA, USA).

**Statistical analysis.** All experiments were performed at least in triplicate and repeated on two different occasions. The ANOVA analysis was used to investigate statistical differences, and pairwise comparisons were performed among groups. Samples with P-values < 0.05 were considered to be statistically different.
Results

**MSH protects C. glutamicum cells against acid stress**

To address the question of whether MSH can protect *C. glutamicum* cells against acid stress, late-exponential phase *C. glutamicum* strains were challenged at pH 4.0 for 1 h. As shown in Fig. 1, the survival rates of the Δ*mshC* and Δ*mshD* mutants decreased significantly compared to that of the wild-type cells (*P* < 0.05), and MSH cannot be detected in the Δ*mshC* and Δ*mshD* mutants (Table 2). However, the acid sensitivity phenotype of the mutants was completely rescued in the complementary strains Δ*mshC*+ and Δ*mshD*+ (Figs. 1A and B). These data suggest that MSH contributes to the survival of *C. glutamicum* cells under acid stress conditions.

To further confirm the effect of MSH on the resistance of *C. glutamicum* cells to acid stress, we examined whether exogenously supplemented MSH can protect the MSH-deficient mutants against acid stress. As predicted, exogenously added MSH (5 μM) resulted in a substantial recovery of the survival rates of both Δ*mshC* and Δ*mshD* to a level similar to that of the wild-type strain (Fig. 1C). These data suggest that MSH contributes to the survival of *C. glutamicum* cells under acid stress conditions. Collectively, these data unambiguously demonstrate that either intracellular produced, or exogenously supplemented, MSH played a protective role against acid stress in *C. glutamicum*.

**MSH regulates intracellular pH (pHᵢ) of *C. glutamicum* under acid stress**

Most bacteria are capable of maintaining a neutral or slightly alkaline intracellular pH when subjected to acidic or alkaline stress (Booth, 1985; Follmann et al., 2009). To explore whether MSH plays a role in maintaining steady pHᵢ under acid stress, we measured the pHᵢ in *C. glutamicum* cells after treatment at pH 4.0 and 7.0 for 10 min, respectively. Deletion of *mshC* and *mshD* had no effect on pHᵢ when the external pH (pHₑₓ) was 7.0 (Fig. 2A). In contrast, when the external pH was lowered to 4.0, the pHᵢ in the *mshC* and *mshD* mutants abruptly decreased to 5.37 and 5.44, respectively, which is significantly lower than the pHᵢ in the wild-type (5.72 ± 0.05, *P* ≤ 0.05) (Fig. 2B). Consistently, the MSH content can hardly be detected in Δ*mshC* and Δ*mshD* strains (Table 2). Interestingly, the pHᵢ in the complementary strains Δ*mshC*+ and Δ*mshD*+ were restored to that of the wild-type strain after being challenged at pH 4.0, further supporting the conclusion that MSH played an important role for maintaining intracellular pH homeostasis under acid stress conditions.

**MSH is able to reduce intracellular levels of ROS in *C. glutamicum* under acid stress**

To determine whether MSH protects *C. glutamicum* against acid stress by reducing the levels of deleterious ROS induced by acid stress, we measured the intracellular ROS levels after acid stress treatment by using the ROS-sensitive fluorescent probe 2′,7′-dichlorofluorescein diacetate. The data revealed that, as expected, MSH-deficient mutants have a markedly higher ROS level than that of the wild-type strain at pH 4.0. Accordingly, the ROS levels in the complementary strains Δ*mshC*+ and Δ*mshD*+ were completely restored to the level of the wild-type (Fig. 3), indicating that the lack of *mshC/mshD* is strongly linked to the function in the ROS scavenging of the mutant. These data suggest that MSH protects *C. glutamicum* against acid stress via scavenging deleterious ROS.

**Acid stress leads to methionine limiting which could involve S-mycothiolation of MetE**

Previous studies on acid stress response based on transcriptome, proteome, and metabolome, have revealed the methionine synthesis impairment in *C. glutamicum*...
Effects of MSH on intracellular ROS accumulation in C. glutamicum wild-type, MSH-deficient mutants (ΔmshC and ΔmshD) and complementary strains (ΔmshC<sup>+</sup> and ΔmshD<sup>+</sup>) treated at pH 7.0 (A), and pH 4.0 (B), for 10 min. Then cells were pelleted and prepared for pH measurement. Error bars indicate standard deviations (n = 3). Statistically significant differences (P < 0.05) were determined by ANOVA analysis and are indicated with an asterisk.

Changes in the pH of C. glutamicum strains upon acid challenge. Logarithmically growing cells (OD<sub>500</sub> = 0.6) of C. glutamicum wild-type, MSH-deficient mutants (ΔmshC and ΔmshD) and complementary strains (ΔmshC<sup>+</sup> and ΔmshD<sup>+</sup>) treated at pH 7.0 and pH 4.0 for 1 h. Error bars indicate standard deviations (n = 3). Statistically significant differences (P < 0.05) were determined by ANOVA analysis and are indicated with an asterisk.

Roles of mycothiol in acid resistance

(A) C. glutamicum wild-type and MSH-deficient mutants (ΔmshC and ΔmshD) were cultured in 5 ml LB broth, 30°C at 220 rpm overnight. Then bacterial suspension was amplified by 1:100 with glucose-minimal medium (MMG) (pH 7.5) and grown till OD<sub>500</sub> = 0.6. Cells were collected by centrifugation and re-suspended with acid MMG (pH 5.7) in the presence and absence of L-methionine (1 mM). And the A<sub>500</sub> was monitored. (B) S-mycothiolation of MetE under acid stress. S-mycothiolation of MetE was monitored by biotin switch assay. The protein extracts of WT(pXMJ19-metE), ΔmshC(pXMJ19-metE) and ΔmshD(pXMJ19-metE) were harvested after being challenged at pH 4.0 for 3 h and subjected to the His-Bind Ni-NTA resin to enrich His<sub>6</sub>-MetE. Enriched His<sub>6</sub>-MetE was de-S-mycothiolated using the Mrx1/MSH/Mtr system and the free protein thiol was tagged with biotin-maleimide followed by resolving on 12% non-reducing SDS-PAGE, and blotted onto nitrocellulose membranes for Western blot analysis. The signal for S-mycothiolation of MetE was detected with the HRP-conjugated streptavidin, and the same amounts of His<sub>6</sub>-MetE used for de-S-mycothiolation analysis were detected with the anti-His antibody. (C) MALDI-TOF MS analysis of His<sub>6</sub>-MetE from WT(pXMJ19-metE) and ΔmshD(pXMJ19-metE) exposed, and unexposed, to acid stress. An increase of 483.2 Da was observed for His<sub>6</sub>-MetE from WT(pXMJ19-metE) and ΔmshD(pXMJ19-metE) after treatment with acid. (D), (E) Logarithmically growing C. glutamicum strains (OD<sub>500</sub> = 0.6) as in (A) were collected by centrifugation and challenged at pH 5.5 for 2 hours, then collected by centrifugation and re-suspended in neutral MMG (pH 7.5) and the resumption of their growth was monitored in the presence, and absence, of L-methionine (1 mM).

Protection of MetE by S-mycothiolation under acid stress.

Fig. 2. Changes in the pH<sub>5</sub> of C. glutamicum strains upon acid challenge.

Fig. 3. Effects of MSH on intracellular ROS accumulation in C. glutamicum.

The methionine starvation phenotype has been well studied in oxidative stressed E. coli and B. subtilis cells, resulting from the S-thiolation modification of MetE, one of the methionine synthesis enzymes vulnerable to oxidation (Chi et al., 2011; Hondoorp and Matthews, 2004). Recently, S-mycothiolation of MetE was also observed as an important protection mechanism under oxidative stress in C. glutamicum (Chi et al., 2014). To examine whether MetE was modified by S-mycothiolation under acid stress,
as this is known to induce a secondary oxidative stress, a biotin switch assay was performed in *C. glutamicum*. WT(pXMJ19-\textit{metE}), \textit{\textit{mshD}}(pXMJ19-\textit{metE}) and \textit{\textit{mshD}}(pXMJ19-\textit{metE}) strains overexpressing His\textsubscript{8}-MetE were allowed to grow to an OD\textsubscript{650} of 0.8, and exposed to acid stress (pH 4.0) for 3 h at 30°C. Then, the enriched His\textsubscript{8}-MetE protein from different cell extracts treated by the biotin switch assay was visualized by Western blotting. As shown in Fig. 4B, His\textsubscript{8}-MetE overexpressed in the wild-type pretreated with acid challenge showed a strong band of S-mycothiolation signal, but no band was shown for the unchallenged sample (Fig. 4B). Interestingly, a weak S-mycothiolation signal was observed for His\textsubscript{8}-MetE overexpressed in the \textit{\textit{mshD}} mutant subjected to acid challenge, indicating that the \textit{\textit{mshD}} mutant may still produce trace amounts of MSH (Fig. 4B). Consistent with our results, the \textit{M. smegmatis} \textit{\textit{mshD}} mutant was also reported to produce trace amounts of MSH along with two novel thiols, N-formyl-Cys-GlcN-Ins and N-succinyl-Cys-GlcN-Ins (Newton et al., 2005). Thus, the production of this biotechnologically important microorganism in its natural habitat and biotechnological production processes, which are subject to severe environmental fluctuations.

Overexpression of \textit{\textit{mshA}} enhanced the ability of \textit{\textit{C. glutamicum}} against acid stress

The above data demonstrates that MSH endowed \textit{\textit{C. glutamicum}} with the functions of scavenging free radicals, maintaining pH\textsubscript{i} and protecting key enzymes of methionine synthesis by posttranslational modification under acid stress. Thus, we infer that overproduction of MSH by genetic engineering should increase the resistance of \textit{\textit{C. glutamicum}} to acid stress. Overexpression of \textit{\textit{mshA}}, a key gene catalyzing MSH biosynthesis, has been reported to significantly enhance the intracellular content of MSH in \textit{\textit{C. glutamicum}} (Liu et al., 2014). The \textit{\textit{mshA}} overexpressing in \textit{\textit{C. glutamicum}} through pXMJ19-\textit{mshA} significantly increased the MSH contents (Table 3). Also, as expected, the wild-type strain with pXMJ19-\textit{mshA} had a significantly higher survival rate than the vector only control WT(pXMJ19) (Fig. 5A), suggesting that overproduction of MSH can enhance the survival of \textit{\textit{C. glutamicum}} under acid stress. Accordingly, the ROS level in the WT(pXMJ19-\textit{mshA}) cells markedly decreased compared with the vector only control (Fig. 5B), indicating that the higher the MSH production, the stronger the ability of the scavenging ROS. These data demonstrate that overexpression of \textit{\textit{mshA}} is a simple, economic, and effective way to enhance the tolerance of \textit{\textit{C. glutamicum}} to acid stress.

**Discussion**

\textit{\textit{C. glutamicum}}, a workhorse in biotechnology for the production of amino acids and nucleotides, is an acid sensitive moderate alkaliphile which grows optimally at pH 7–9 (Barriuso-Iglesias et al., 2008; Jakob et al., 2007). Thus, acid response is central to the growth and survival of this biologically important microorganism in its habituation and biotechnological production processes, which are subject to severe environmental fluctuations. Although acid stress responses have been well studied in multiple highly acid resistant bacteria (Baker-Austin and Dorposon, 2007; Cotter and Hill, 2003; Foster, 2001, 2004; Kruwich et al., 2011), they are poorly understood in acid sensitive bacteria, especially those of an ecological and biotechnological importance.

Recently, functional genomics analysis has revealed for the first time the occurrence of oxidative stress in \textit{\textit{C. glutamicum}} cells at low pH conditions accompanied by iron starvation response activation and metabolic alterations (Follmann et al., 2009). Further studies in \textit{Bacillus cereus} have confirmed not only increased expression of antioxidant enzymes, such as catalase and thioredoxin, but also increased production of reactive oxygen species.
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(ROS) at low pH conditions (Mols and Abee, 2011b; Mols et al., 2010). The non-enzymatic antioxidant MSH is well-known in playing a vital role in cellular redox homeostasis, and in protecting cells from oxidative stress. Interestingly, previously it has also been reported that MSH protects *Mycobacterium tuberculosis* against acid stress, although the underlying mechanism remains unknown (Buchmeier et al., 2006). Here, we have investigated the physiological roles and underlying mechanisms of MSH in *C. glutamicum* under acid stress. We have presented evidence that MSH confers *C. glutamicum* with the ability to adapt to acidic conditions by maintaining pHi homeostasis, scavenging ROS, and protecting methionine synthesis by S-mycothiolation of MetE.

The ability to maintain a neutral intracellular pH (pHi) is essential for bacterial viability when subjected to acid stress (Booth, 1985; Follmann et al., 2009). Our observation that MSH-deficient mutants exhibited markedly lower pHi values under acidic environments suggests that MSH may confer to cells a higher capability to maintain physiological activities and combat against acid stress. As the main low-molecular-weight thiol in *C. glutamicum*, we speculated that the protective role of MSH against acid stress is correlated to its ability to scavenge reactive oxygen species (ROS). The formation of ROS upon acid stress has previous been experimentally verified in *B. subtilis* (Chi et al., 2011). In addition, data is accumulating to demonstrate that the exposure of microorganisms to various stresses, such as heavy metals, antibiotics, xenobiotics, heat and salt stress, can also increase the production of ROS and induce secondary oxidative stress (Kohanski et al., 2007; Mols and Abee, 2011a). Interestingly, previous studies have indicated that ROS can regulate pHi, at least in eukaryotic cells, via inhibiting proteins and biochemical pathways that affect pHi (Mulkey et al., 2004; Tsai et al., 1997). Our results demonstrate that acid stress does induce the generation of ROS in vivo in *C. glutamicum*, and that MSH-deficient mutants show a significantly higher ROS level than that of the wild-type due to losing the ability to synthesis MSH (Fig. 3). The ROS scavenging role of MSH in acid adaptation was also confirmed by the overexpression of mshA, which resulted in decreased levels of ROS correlated with an increased survival rate under acidic conditions (Fig. 5). Thus, MSH functions to assist in the scavenging of deleterious ROS, which is known to damage a wide range of biological molecules including those involved in pHi maintenance (Mulkey et al., 2004; Tsai et al., 1997). Consistently, it has been reported previously that GSH, the main low-molecular-weight thiol in eukaryotes and Gram-negative bacteria, has the capability of maintaining a significantly higher pHi value under acid stress in *Lactococcus lactis* (Zhang et al., 2007).

Another protective strategy of MSH against acid stress is the protection of methionine synthesis by the S-mycothiolation of MetE. Protein S-thiolation is a reversible post-translational thiol-modification that protects active site cysteine residues of key enzymes against irreversible oxidation to sulfonic acids (Dalle-Donne et al., 2009; Shenton and Grant, 2003). S-glutathionylation, a well-documented protein S-thiolation modification formed between cysteine residues and low-molecular-weight thiols such as glutathione, is induced in response to oxidative stress in eukaryotic and most GSH containing Gram-negative bacteria cells, and plays important roles in various biological processes, including cell signaling, metabolism and energy, redox homeostasis and protein degradation (Dalle-Donne et al., 2009; Mieyal and Chock, 2012; Shenton and Grant, 2003). Recently, six S-bacillithiolated proteins and 25 S-mycothiolated proteins, formed between cysteine residues and low-molecular-weight thiols BSH (S-bacillithiolation) or MSH (S-mycothiolation), respectively, were identified upon oxidative stress in *B. subtilis* and *C. glutamicum* (Chi et al., 2011, 2014). MetE, one of the proteins most susceptible to oxidation, is subjected to all 3 kinds of S-thiolation modification, and plays a key role in linking oxidative stress and methionine availability (Chi et al., 2011, 2013, 2014; Hondorp and Matthews, 2004). Both S-glutathionylation and S-bacillithiolation lead to MetE inactivation and Met auxotrophy in oxidative stressed *E. coli* and *B. subtilis* cells (Hondorp and Matthews, 2004; Chi et al., 2011). The methionine auxotrophy phenotype upon oxidative stress was confirmed by the resumption of bacteria growth immediately after the supplementation of methionine (Hondorp and Matthews, 2004). Thus, glutathionylation/bacillithiolation of MetE provides a strategy to modulate its activity, while protecting the active site from further oxidation, in an easily reversible manner. Unfortunately, to date, no studies have been performed to determine the role of protein S-thiolation in environmental stresses other than oxidative stress.

To the best of our knowledge, it is shown here, for the first time, that MetE is protected by S-mycothiolation under acid stress. The S-mycothiolation protection of MetE in *C. glutamicum* under acidic conditions is supported by several lines of evidence shown in the present study. First, our biotin switch assay directly revealed the incorporation of the MSH moiety to MetE in the wild-type. In contrast, no S-mycothiolation modification of MetE was observed in the ΔmshC mutant that did not produce MSH, irrespective of whether this was, with, or without, acid challenge (Fig. 4B). Interestingly, a weak S-mycothiolation signal was observed for Hisγ-MetE overexpressed in the ΔmshD mutant subjected to acid challenge, indicating that the ΔmshD mutant may still produce trace amounts of MSH (Fig. 4B). Consistent with our results, the *M. smegmatis* ΔmshD mutant has also been reported to produce trace amounts of MSH along with two novel thiols, N-formyl-Cys-GlcN-Ins and N-succinyl-Cys-GlcN-Ins (Newton et al., 2005). Thus, the production of thiols in the *C. glutamicum* ΔmshD mutant needs to be further investigated. In addition, the reversible protection of MetE was confirmed by the growth resumption experiment. After neutralization of the acid treated medium, the MSH-deficient mutants displayed a delayed recovery of growth compared with the wild-type, and their growth immediately resumed to the wild-type level after the addition of methionine to the growth medium (Figs. 4D and E). Moreover, upon acid stress, a methionine starvation phenotype was revealed for *C. glutamicum*, with the observation that bacterial growth at pH 5.7 was significantly improved af-
ter methionine addition (Fig. 4A). All these results are consistent with a previous report that oxidative stress was induced in *C. glutamicum* at acid conditions accompanied by methionine synthesis impairment (Follmann et al., 2009). Thus, the reversible S-mycythiolation of MetE plays a key role in the adaptation of *C. glutamicum* to a low pH.

In summary, we have demonstrated the protective role of MSH on the acid tolerance of *C. glutamicum* in this study. Our results show that MSH acts by maintaining intracellular pH, scavenging ROS, and protecting methionine synthesis by S-mycythiolation of MetE under acid stress. Hence, this work provides insights into a previously unknown, but important, aspect of the *C. glutamicum* cellular response to acid stress.

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Supplementary Materials

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

References


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