

Full Paper

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

Introduction

Bacteria have developed different strategies to adapt to changing environmental conditions. Acid stress is one of the common conditions bacteria are exposed to in nature, which generally affects the solubility of nutrients and trace elements, and the physiology of the cell (Follmann et al., 2009). To survive acid stress, bacteria adopt a variety of acid-resistant mechanisms, including the restriction of proton entry, the expelling of intracellular protons, the production of macromolecular protection proteins and chaperones, and the neutralization of the cytoplasm (Baker-Austin and Dopson, 2007; Foster, 2004; Krulwich et al., 2011). However, acid stress responses have mainly been studied in highly acid resistant Gram-negative enteric pathogens such as *Escherichia coli* and *Salmonella* that encounter an extremely low pH of the stomach during ingestion (Foster, 2001, 2004; Stincone et al., 2011), and in a select number of Gram-positive bacteria, such as lactic acid bacteria and *Listeria monocytogenes*, that usually persist in an acid environment (Cotter and Hill, 2003; Zhang et al., 2007). Acid adaptation mechanisms central to the growth and survival of acid sensitive bacteria of ecological and biotechnological importance are little understood.

Corynebacterium glutamicum, a fast growing soil bacterium widely used for the industrial production of amino acids and nucleotides, serves also as a laboratory model for the investigation of its pathogenic relatives *C.*

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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 F₁F₀-ATPase, whose roles in proton pumping under acid stress are well documented, was found to be down-regulated under acidic conditions (Barriuso-Iglesias et al., 2008; Zhang et al., 2007). Besides, the genes encoding glutamate decarboxylase, 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 H₂O₂ 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 transacetylated 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 poi-

sonous chemicals 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), nitrosomycothiol reductase (Vogt et al., 2003) and arsenate reductase (Ordóñez et al., 2009). Recently, protein *S*-mycothiolation 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*-mycothiolation 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

Table 1. Bacterial strains, plasmids and primers used in this study.

Strains or plasmids	Relevant characteristic(s)	Source or reference
<i>E. coli</i>		
JM109	<i>recA1 supE44 endA1 hsdR17 gyrA96 relA1 thi Δ(lac-proAB)F'(traD36 proABlacI^q lacΔZM15)</i>	Stratagene
BL21(DE3)	Host for expression vector pET28a	Novagen
<i>C. glutamicum</i>		
RES167(pXMJ19)	Restriction-deficient mutant of ATCC13032, $Δ(cglIM-cglIIR-cglIIR)$	Tauch et al. (2002)
$ΔmshC$ (pXMJ19)	<i>mshC</i> deleted in RES167	Feng et al. (2006)
$ΔmshD$ (pXMJ19)	<i>mshD</i> deleted in RES167	Feng et al. (2006)
$ΔmshC^+$	$ΔmshC$ containing pXMJ19- <i>mshC</i>	Liu et al. (2013)
$ΔmshD^+$	$ΔmshD$ containing pXMJ19- <i>mshD</i>	Liu et al. (2013)
Plasmids		
pXMJ19	Shuttle vector (<i>Ptac lacI^q pBL1 oriV_{C. glutamicum} pK18oriV_{E. coli}</i>)	Jakob et al. (2007)
pXMJ19- <i>metE</i>	<i>metE</i> cloned into pXMJ19 for <i>S</i> -mycothiolation assay	This study
pXMJ19- <i>mshA</i>	<i>mshA</i> cloned into pXMJ19 for overexpression	This study
pET28a- <i>mrx1</i>	<i>mrx1</i> cloned into pET28a for protein purification	This study
pET28a- <i>mtr</i>	<i>mtr</i> cloned into pET28a for protein purification	This study
Primers		
metEF	<u>CGCGTCGACAAAGGAGGACAACCATGCACCACCACCACCACATGACTTCCAACCTTTCTTC</u> (<i>Sall</i>)	This study
metER	<u>CAAGAATTCCTTAGATAGTTGCTCCGATTTTC</u> (<i>EcoRI</i>)	This study
mshAF	<u>ACGAAGCTTAAAGGAGGACAACCATGCGTGTAGCTATGATTTTC</u> (<i>HindIII</i>)	This study
mshAR	<u>ACTCTCGAG</u> TTAGCCGTGATGCGTTTCAC (<i>XhoI</i>)	This study
mrx1F	<u>CCGGAATTCATGAGCAACGTAACCATTTACGCC</u> (<i>EcoRI</i>)	This study
mrx1R	<u>CCCAGCTTTTAGCTAATGCTTCGATTTTGG</u> (<i>HindIII</i>)	This study
mtrF	<u>CGGGATCCATGTCTGAGCAGCCAGCTTC</u> (<i>BamHI</i>)	This study
mtrR	<u>ACGCGTCGACCTAAAACCTCTAGCCCCAGAAG</u> (<i>Sall</i>)	This study

Underlined sites indicate restriction enzyme cutting sites added for cloning. Letters in italics denote ribosome binding sites. The His₆ tag is given in boldface.

by determining the optical density at 600 nm. When needed, antibiotics were used at the following concentrations: chloroamphenicol, 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 manufacturer's instructions (TaKaRa, Dalian, China). PCR was performed with EasyTaq or EasyPfu DNA polymerase (TransGen Biotech, Beijing, China). Plasmid DNA was isolated with the plasmid DNA miniprep spin columns (TIANGEN, Beijing, China), and DNA fragments were purified from agarose gels by using the TIANGEN gel extraction kit (TIANGEN). DNA sequencing and primer synthesis were carried out by Sangon Biotech (Shanghai, China).

Plasmid construction. Primers used in this study are listed in Table 1. To overexpress *mshA* (*ncgl0389*) and *metE* (*ncgl2194*) 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*, *ncgl0808*) and mycothione reductase (*mtr*, *ncgl1928*) 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 (Bio-Rad, Hercules, CA, USA) with bovine serum albumin as standard.

MSH purification and determination. Purification of MSH from *C. glutamicum* RES167 was carried out with thiopropyl sepharose 6B followed by Sephadex LH-20 chromatography as described previously (Feng et al., 2006; Si et al., 2014). The MSH concentration was determined

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 $15,000 \times g$ 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 $120 \mu\text{M l}^{-1}$ 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 (A_{330}) did not change. This mixture, containing maleylpyruvate, was used as a substrate for maleylpyruvate isomerase. MDMPI and FPH (fumarylpyruvate hydrolase) were added to 5 μl supernatant for the MSH determination. The reaction proceeded at room temperature until no further change in A_{330} .

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: $[(\text{C.F.U. ml}^{-1} \text{ after acid challenge}) / (\text{C.F.U. ml}^{-1} \text{ without acid challenge})] \times 100$. Survival values reported are 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 (H_2DCFDA)-based assay described by Wang et al. (2015). After this procedure, H_2DCFDA was added from a fresh 5 mM stock (prepared in ethanol) to a final concentration of $10 \mu\text{M}$ 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 spectrofluorimeter (RF-5301PC, USA) with excitation at 502 nm and emission at 521 nm.

Measurement of intracellular pH (pH_i). The internal pH was determined by using the pH-sensitive fluorescent probe 2,7-bis-(2-carboxyethyl)-5-6-carboxyfluorescein (BCECF) as described previously (Jakob et al., 2007). In brief, 1 ml of *C. glutamicum* culture ($\text{OD}_{600} = 0.6$) was centrifuged at $10,000 \text{ g}$ for 2 min, re-suspended in 1 ml PBS (pH 7.0), and incubated with BCECF-AM (final concentration $1.2 \mu\text{M}$) 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 spectrofluorimeter (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 $5 \mu\text{M}$, 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 $\text{OD}_{600} = 0.6$ 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 $10,000 \text{ g}$, 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 $10,000 \text{ g}$ for 1 h. His₆-MetE was enriched with His-Bind Ni-NTA resin (Novagen, Madison, WI, USA) according to manufacturer's instructions. The resulting His₆-MetE was dissolved in a Tris-HCl buffer (pH 8.0) and demycothiolated using $20 \mu\text{M}$ purified Mrx1 in the presence of 1 mM NADPH and $20 \mu\text{M}$ Mtr for 30 min at room temperature. The demycothiolated His₆-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 $10,000 \times g$ 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 analysis. The His₆-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.

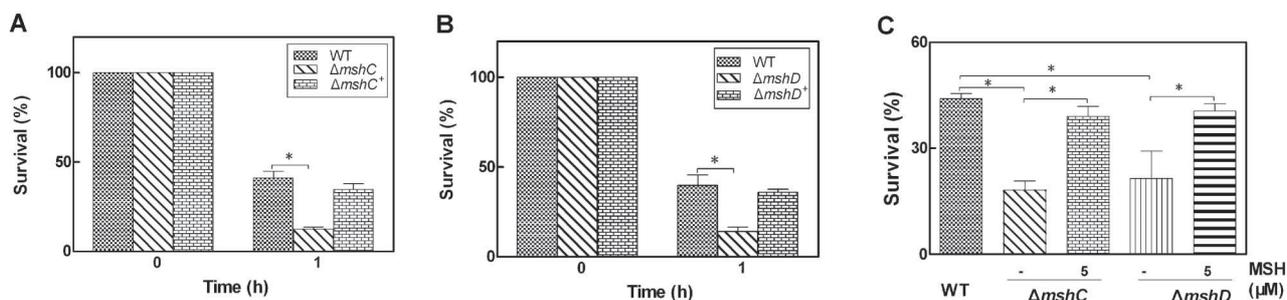


Fig. 1. Effects of MSH on the survival of *C. glutamicum* under acid stress.

Survival rates of $\Delta mshC$ (pXMJ19) (A), $\Delta mshD$ (pXMJ19) (B), and corresponding complementary strains ($\Delta mshC^+$ and $\Delta mshD^+$), after acid challenge (pH 4.0) for 1 hour. (C) Survival rates of MSH-deficient mutants ($\Delta mshC$ and $\Delta mshD$ with pXMJ19) in the presence of 5 μM MSH after acid challenge (pH 4.0) for 1 hour. Error bars indicate standard deviations ($n = 3$). Statistically significant differences ($P < 0.05$) were determined by ANOVA analysis and are indicated with an asterisk.

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 $\Delta mshC$ and $\Delta mshD$ mutants decreased significantly compared to that of the wild-type cells ($P < 0.05$), and MSH cannot be detected in the $\Delta mshC$ and $\Delta mshD$ mutants (Table 2). However, the acid sensitivity phenotype of the mutants was completely rescued in the complementary strains $\Delta mshC^+$ and $\Delta mshD^+$ (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 $\Delta mshC$ and $\Delta mshD$ 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 (pH_i) 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 pH_i under acid stress, we measured the pH_i 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 pH_i when the external pH (pH_{ex}) was 7.0 (Fig. 2A). In contrast, when the external pH was lowered to 4.0, the pH_i in the *mshC* and *mshD* mutants abruptly decreased to 5.37 and 5.44, respectively, which is significantly lower than the pH_i in the wild-type (5.72 ± 0.05 , $P \leq 0.05$) (Fig.

Table 2. Contents of MSH in five *C. glutamicum* strains.

<i>C. glutamicum</i> Strains	MSH ($\mu\text{mol/g}$ residual dry weight)
WT(pXMJ19)	3.29 ± 0.63
$\Delta mshC$ (pXMJ19)	—
$\Delta mshC^+$	3.88 ± 0.80
$\Delta mshD$ (pXMJ19)	—
$\Delta mshD^+$	5.04 ± 0.44

The bacteria were cultured in LB broth for 20 h until $\text{OD}_{600} = 1.8$. “—” means ΔA (absorbance) $< 0.05/\text{min}$, the MSH amount was negative.

2B). Consistently, the MSH content can hardly be detected in $\Delta mshC$ and $\Delta mshD$ strains (Table 2). Interestingly, the pH_i in the complementary strains $\Delta mshC^+$ and $\Delta mshD^+$ 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 $\Delta mshC^+$ and $\Delta mshD^+$ 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*

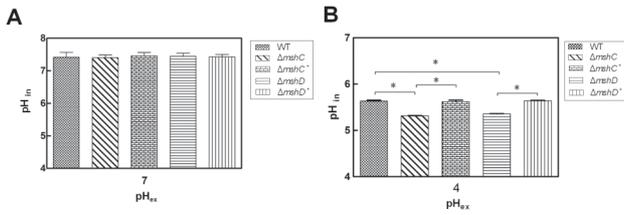


Fig. 2. Changes in the pH_i s of *C. glutamicum* strains upon acid challenge.

Logarithmically growing cells ($OD_{600} = 0.6$) of *C. glutamicum* wild-type, MSH-deficient mutants ($\Delta mshC$ and $\Delta mshD$) and complementary strains ($\Delta mshC^+$ and $\Delta mshD^+$) treated at pH 7.0 (A), and pH 4.0 (B), for 10 min. Then cells were pelleted and prepared for pH_i determination. Error bars indicate standard deviations ($n = 3$). Statistically significant differences ($P < 0.05$) were determined by ANOVA analysis and are indicated with an asterisk.

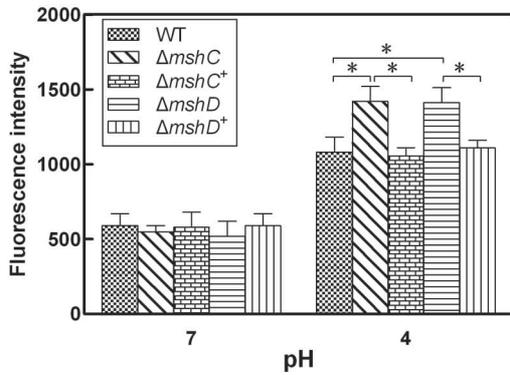


Fig. 3. Effects of MSH on intracellular ROS accumulation in *C. glutamicum*.

Quantification of intracellular ROS in cultures of *C. glutamicum* wild-type, MSH-deficient mutants ($\Delta mshC$ and $\Delta mshD$) and complementary strains ($\Delta mshC^+$ and $\Delta mshD^+$) after treatment 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.

(Follmann et al., 2009), suggesting a growth requirement for methionine under acid stress. To determine the methionine starvation phenotype induced by acid stress, we investigated the growth of *C. glutamicum* strains growing in glucose-minimal medium (pH 5.7) containing or lacking methionine. As shown in Fig. 4A, while all *C. glutamicum* strains (wild-type, $\Delta mshC$ and $\Delta mshD$) logarithmically growing in glucose-minimal medium experienced an inhibition in growth upon acidification of the medium from pH 7.0 to pH 5.7, the growth of MSH-deficient mutants were inhibited more severely. However, the decreased growth of *C. glutamicum* strains at pH 5.7 was significantly improved by the extracellular addition of 1 mM methionine (Fig. 4A). Without acid stress, all *C. glutamicum* strains had an identical growth in neutral glucose-minimal medium with or without the presence of 1 mM methionine (Fig. S1). These data indicate that methionine becomes limiting under acid stress due to an impairment of the methionine synthesis pathway in *C. glutamicum*.

The methionine starvation phenotype has been well stud-

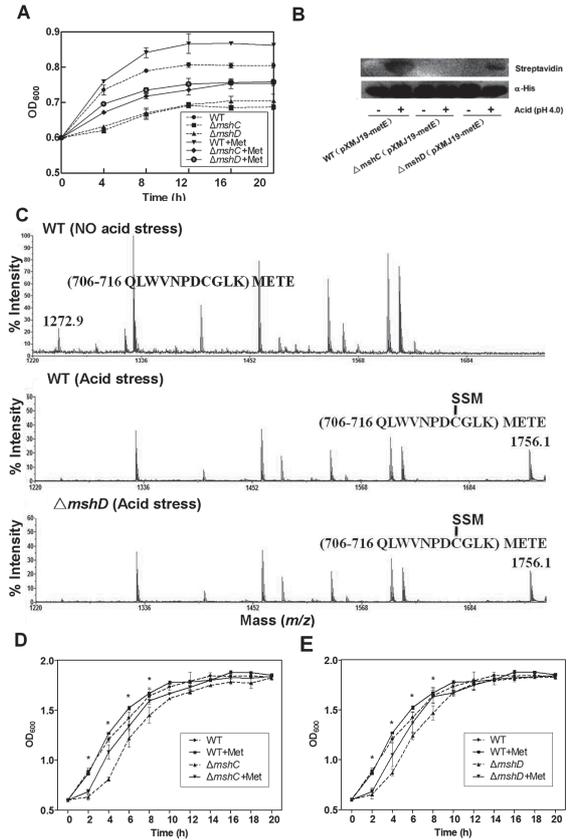


Fig. 4. Protection of MetE by S-mycothiolation under acid stress.

(A) *C. glutamicum* wild-type and MSH-deficient mutants ($\Delta mshC$ and $\Delta mshD$) 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 $OD_{600} = 0.6$. 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_{600} 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*), $\Delta mshC$ (pXMJ19-*metE*) and $\Delta mshD$ (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 His₆-MetE. Enriched His₆-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 His₆-MetE used for de-S-mycothiolation analysis were detected with the anti-His antibody. (C) MALDI-TOF MS analysis of His₆-MetE from WT (pXMJ19-*metE*) and $\Delta mshD$ (pXMJ19-*metE*) exposed, and unexposed, to acid stress. An increase of 483.2 Da was observed for His₆-MetE from WT (pXMJ19-*metE*) and $\Delta mshD$ (pXMJ19-*metE*) after treatment with acid. (D), (E) Logarithmically growing *C. glutamicum* strains ($OD_{600} = 0.6$) 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).

ied 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; Hondorp 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,

Table 3. Contents of MSH in *mshA* overexpressing *C. glutamicum*.

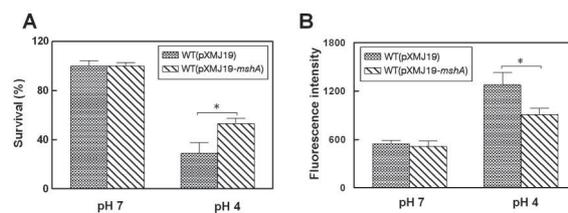
Cultivation time (h)	MSH ($\mu\text{mol/g}$ residual dry weight)	
	WT(pXMJ19)	WT(pXMJ19- <i>mshA</i>)
5	0.24 \pm 0.2	0.42 \pm 0.1
10	0.65 \pm 0.1	1.19 \pm 0.05
15	2.33 \pm 0.08	4.39 \pm 0.2
20	3.03 \pm 0.05	6.48 \pm 0.1

as this is known to induce a secondary oxidative stress, a biotin switch assay was performed in *C. glutamicum*. WT(pXMJ19-*metE*), ΔmshC (pXMJ19-*metE*) and ΔmshD (pXMJ19-*metE*) strains overexpressing His₆-MetE were allowed to grow to an OD₆₀₀ of 0.8, and exposed to acid stress (pH 4.0) for 3 h at 30°C. Then, the enriched His₆-MetE protein from different cell extracts treated by the biotin switch assay was visualized by Western blotting. As shown in Fig. 4B, His₆-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 His₆-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 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 thiols in the *C. glutamicum* ΔmshD mutant needs to be further investigated in the future. Moreover, a mass of 1756.1 Da was identified in His₆-MetE from WT(pXMJ19-*metE*) and ΔmshD (pXMJ19-*metE*) exposed to acid stress by peptide mass spectroscopy, which was 483.2 Da higher than Cys713-containing 706–716 peptide of the His₆-MetE from WT(pXMJ19-*metE*) unexposed to acid stress (calculated and observed mass 1272.9 Da), consistent with results from the addition of MSH (Fig. 4C).

Protein *S*-thiolation is a reversible post-translational thiol-modification that protects the cysteine residues of key enzymes against irreversible overoxidation to sulfonic acids (Lindahl et al., 2011). To investigate whether *S*-mycothiolation of MetE provides reversible protection to methionine synthesis upon acid stress, we performed the growth resumption experiment. Logarithmically growing *C. glutamicum* strains were challenged at pH 5.5 for 2 hours, neutralized to pH 7.5, and the resumption of their growth was monitored (Figs. 4D and E). While the MSH-deficient mutants displayed a delayed resumption of growth compared to the wild-type, their growth was immediately resumed to the wild-type level after the addition of methionine to the growth medium. Taken together, our data suggests that the reversible *S*-mycothiolation protection of MetE plays a key role in the methionine synthesis, and also the adaptation of *C. glutamicum* to acid stress.

Overexpression of *mshA* enhanced the ability of *C. glutamicum* against acid stress

The above data demonstrates that MSH endowed *C. glutamicum* with the functions of scavenging free radi-

**Fig. 5.** Overexpression of *mshA* enhanced the acid resistance activity of *C. glutamicum*.

Survival (A), and intracellular ROS accumulation (B), in *C. glutamicum* WT(pXMJ19) and WT(pXMJ19-*mshA*) strains determined after being challenged at pH 4.0 for 1 h.

cals, maintaining pH_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 *C. glutamicum* to acid stress. Overexpression of *mshA*, a key gene catalyzing MSH biosynthesis, has been reported to significantly enhance the intracellular content of MSH in *C. glutamicum* (Liu et al., 2014). The *mshA* overexpressing in *C. glutamicum* through pXMJ19-*mshA* significantly increased the MSH contents (Table 3). Also, as expected, the wild-type strain with pXMJ19-*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 *C. glutamicum* under acid stress. Accordingly, the ROS level in the WT(pXMJ19-*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 *mshA* is a simple, economic, and effective way to enhance the tolerance of *C. glutamicum* to acid stress.

Discussion

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 biotechnologically important microorganism in its habitation 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 Dopson, 2007; Cotter and Hill, 2003; Foster, 2001, 2004; Krulwich 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 *C. glutamicum* cells at low pH conditions accompanied by iron starvation response activation and metabolic alterations (Follmann et al., 2009). Further studies in *Bacillus cereus* have confirmed not only increased expression of antioxidant enzymes, such as catalase and thioredoxin, but also increased production of reactive oxygen species

(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 pH_i homeostasis, scavenging ROS, and protecting methionine synthesis by *S*-mycothiolation of MetE.

The ability to maintain a neutral intracellular pH (pH_i) 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 pH_i 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 previously 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 pH_i, at least in eukaryotic cells, via inhibiting proteins and biochemical pathways that affect pH_i (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 synthesize MSH (Fig. 3). The ROS scavenging role of MSH in acid adaptation was also confirmed by the overexpressing 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 pH_i 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 pH_i 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 overoxidation 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 $\Delta mshC$ 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 His₆-MetE overexpressed in the $\Delta mshD$ mutant subjected to acid challenge, indicating that the $\Delta mshD$ mutant may still produce trace amounts of MSH (Fig. 4B). Consistent with our results, the *M. smegmatis* $\Delta mshD$ 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* $\Delta mshD$ 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-mycothiolation 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-mycothiolation 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>).

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