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ORIGINAL RESEARCH PAPER

# Mycothiol peroxidase MPx protects *Corynebacterium glutamicum* against acid stress by scavenging ROS

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**Abstract**
*Objectives* To investigate mycothiol peroxidase (MPx) of *Corynebacterium glutamicum* that is a novel CysGPx family peroxidase using both the mycoredoxin and thioredoxin reducing systems as proton donors for peroxide detoxification and may be involved in the relief of acid stress.
*Results* A Δ*mpx* mutant exhibited significantly decreased resistance to acid stress and markedly increased accumulation of reactive oxygen species (ROS) and protein carbonylation levels in vivo. Over-expression of *mpx* increased the resistance of *C. glutamicum* to acid stress by reducing ROS accumulation. The stress-responsive extracytoplasmic function-sigma (ECF-σ) factor, SigH, mediated acid-induced expression of *mpx* in the wild-type under acid conditions, which in turn directly contributed to tolerance to acid stress.
*Conclusion* MPx is essential for combating acid stress by reducing intracellular ROS levels induced by acid stress in *C. glutamicum*, which adds a new dimension to the general physiological functions of CysGPx.

**Keywords** Acid stress · *Corynebacterium glutamicum* · Mycothiol peroxidase · Reactive oxygen species · Sigma factor (SigH)

## Introduction

Microbial production of biological products, such as biofuels, bio-based chemicals and biological

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materials, has attracted attention. However, most industrial bacteria are inevitably exposed to acid stress during production of acidic compounds and raw material pretreatment (Nicolaou et al. 2010; Ibraheem and Ndimba 2013; Lee et al. 2013). To survive acid stress, bacteria adopt a variety of acid-resistant mechanisms (Zhang et al. 2007). However, acid stress responses have mainly been studied in highly acid-resistant Gram-negative enteric pathogens (Richard and Foster 2003) and in a select number of Gram-positive bacteria (van de Guchte et al. 2002; Ryan et al. 2008). Acid adaptation mechanisms central to the growth and survival of acid-sensitive bacteria of ecological and biotechnological importance are not well-understood.

*Corynebacterium glutamicum* is widely used for industrial production of amino acids and nucleotides (Kelle et al. 2005; Kimura 2005) but also other products from renewable and eco-efficient lignocellulosic biomass, such as, γ-aminobutyric acid, polyhydroxybutyrate, and organic acids (Mimitsuka et al. 2007; Schneider and Wendisch 2010; Smith et al. 2010; Blombach et al. 2011; Kind and Wittmann 2011). However, the mechanism of pH homeostasis and the components participating in the acclimatization process are not well known in this acid-sensitive bacterium. Acid stress can lead to the formation of ROS and the activation of various oxidative stress-related genes in *Bacillus cereus* (Mols et al. 2010; Mols and Abee 2011). This raises the question of whether ROS-scavenging enzymatic antioxidants, such as mycothiol peroxidase (MPx), function in the adaptation of *C. glutamicum* to acidic pH conditions.

MPx is a novel CysGPx family peroxidase that degrades $H_2O_2$ and alkyl hydroperoxides in the presence of either the thioredoxin/thioredoxin reductase (Trx/TrxR) or mycoredoxin 1/mycothione reductase/mycothiol (Mrx1/Mtr/MSH) reducing systems (Pedre et al. 2015; Si et al. 2015). MPx protects against the damaging effects of ROS induced by multiple stressors, and the over-expression of *mpx* significantly enhanced the resistance of *C. glutamicum* to various peroxides by decreasing protein carbonylation and intracellular ROS accumulation (Pedre et al. 2015; Si et al. 2015). In this study, we reveal for the first time that MPx is also crucial for cellular survival under low pH conditions, acting by scavenging ROS induced by acid stress. Our work provides insight into a previously unknown, but important aspect of the *C. glutamicum* cellular response to acid stress. Our results will aid in the understanding of acid tolerance mechanisms in acid-sensitive bacteria and open a new avenue to improve acid resistance in industrial strains for the production of bio-based chemicals from renewable biomass.

## Materials and methods

### Bacterial strains and culture conditions

Bacterial strains and plasmids used in this study are listed in Supplementary Table 1. *C. glutamicum* and *E. coli* strains were aerobically cultured in lysogeny broth (LB) on a rotary shaker (220 rpm) or on LB plates at 30 and 37 °C, respectively. When needed, antibiotics were added: chloramphenicol, 20 μg $ml^{-1}$ for *E. coli* and 10 μg $ml^{-1}$ for *C. glutamicum*; kanamycin, 50 μg $ml^{-1}$ for *E. coli* and 25 μg $ml^{-1}$ for *C. glutamicum*; nalidixic acid, 40 μg $ml^{-1}$ for *C. glutamicum*.

### Acid survival assays

Acid survival assays were performed as previously described (Zhang et al. 2013; Liu et al. 2014). Overnight cultures of *C. glutamicum* strains in LB were diluted 100-fold into LB (different pH values) and incubated at 30 °C with shaking at 100 rpm for 1 h. After acid stress, the cultures were serially diluted and plated onto LB agar plates, and colonies were counted after 36 h growth at 30 °C. Percentage survival was calculated as follows: [(cfu $ml^{-1}$ after acid challenge)/(cfu $ml^{-1}$ without acid challenge)] × 100 %.

### Measurement of intracellular ROS levels and protein carbonylation

To detect intracellular ROS, the fluorescent reporter dyes, 3′-(*p*-hydroxyphenyl) fluorescein (HPF) and 2′, 7′-dichlorodihydrofluorescein diacetate ($H_2$DCFDA), were used as previously described (Wang et al. 2015). Fluorescence was measured using a plate reader with excitation/emission wavelengths of 490/515 nm (HPF) and 495/520 nm ($H_2$DCFDA). Protein carbonylation assays were performed based on the method described by Vinckx et al. (2011).

### Construction of chromosomal fusion reporter strains and β-galactosidase activity assay

The *lac*Z fusion reporter plasmid, pK18*mobsacB*-$P_{mpx}$::*lac*Z was transformed into *C. glutamicum* WT(pXMJ19), Δ*sigH*(pXMJ19) and Δ*sigH*(pXMJ19-*sigH*) by electroporation, and the chromosomal pK18*mobsacB*-$P_{mpx}$::*lac*Z fusion reporter strain was selected by plating onto LB-kanamycin plates (Si et al. 2014). The resulting strains were grown in LB medium to an $OD_{600}$ of 0.9–1, and then treated under different pH conditions at 30 °C for 30 min. β-Galactosidase activity was assayed with *o*-nitrophenyl-β-galactoside (ONPG) as substrate.

### Quantitative RT-PCR analysis

Total RNA was isolated from rapidly growing WT(pXMJ19), Δ*sigH*(pXMJ19) and Δ*sigH*(pXMJ19-*sigH*) strains exposed to different pH conditions for 30 min using the RNeasy Mini Kit (Qiagen, Hilden, Germany) along with the DNase I Kit (Sigma-Aldrich). Purified RNA was reverse-transcribed with random 9-mer primers and MLV reverse transcriptase (TaKaRa, Dalian, China). The primers used for qRT-PCR analysis are listed in Supplementary Table 1. To standardize the results, the relative abundance of 16S rRNA was used as an internal standard.

### Statistical analysis

The results shown represent the average of three independent experiments; error bars indicate SD from three independent experiments. Statistical analysis was carried out using Student's *t* test. Statistical analyses were performed using GraphPad Prism Software (GraphPad Software, San Diego California USA).

## Results and discussion

### Survival response of *C. glutamicum* to acid stress

The survival response of *C. glutamicum* to acid stress was studied using the wild-type strain treated with pH values ranging from pH 6 to pH 3 adjusted with HCl (Fig. 1). Upon exposure to the different acid shocks, the survival rate of stationary phase wild-type *C. glutamicum* showed significant differences. At pH 6, the *C. glutamicum* survival rate was 100 %. Upon exposure to pH 5.5, pH 5, pH 4.5, and pH 4, the *C. glutamicum* wild-type strain had relative survival rates of approx. 90, 80, 70 and 50 %, respectively. This strain was unable to survive at a pH lower than pH 3.5, as shown by the inability to form colonies on LB plates incubated at 30 °C for 36 h. This response is hereafter referred to as the inactivation phenotype and the condition as bactericidal.

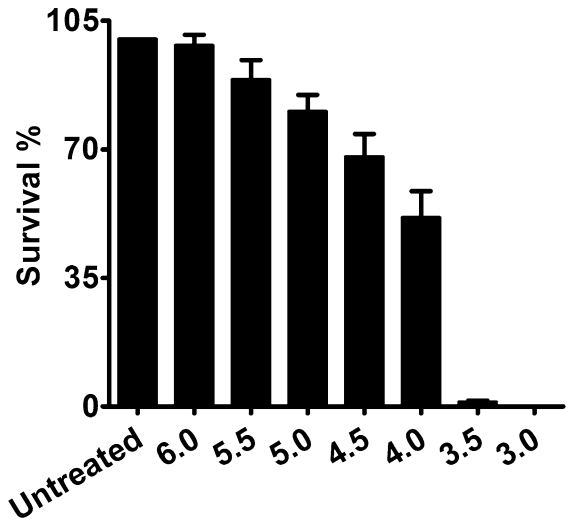


**Fig. 1** Physiological response of *C. glutamicum* upon exposure to different acidic pH levels. Wild-type *C. glutamicum* was grown in LB medium to an $OD_{600}$ of 1.6–1.7 and exposed to acidic pH at 30 °C for 1 h. After treatment, the cultures were serially diluted, spread onto LB plates and incubated at 30 °C for 36 h. Survival percentages were calculated as follows: [(CFU $ml^{-1}$ with stress)/(CFU $ml^{-1}$ without stress)] × 100. Mean values with standard deviations (*error bars*) from at least three repeats are shown

### Acid stress-induced ROS formation in *C. glutamicum*

In *Bacillus cereus*, acid stress induces the production of deleterious reactive oxygen species (ROS), including the highly destructive hydroxyl radicals (OH·), which are generated via the Fenton reactiony (Mols et al. 2010). This finding prompted us to investigate whether ROS are produced in *C. glutamicum* held at a low pH. Formation of ROS in *C. glutamicum* wild-type cells was examined after exposure to selected pH levels (pH 6, pH 5.5, pH 5, pH 4.5, pH 4, and pH 3.5) using the ROS-sensitive fluorescent probe $H_2$DCFDA (Fig. 2a). *C. glutamicum* showed excess ROS formation

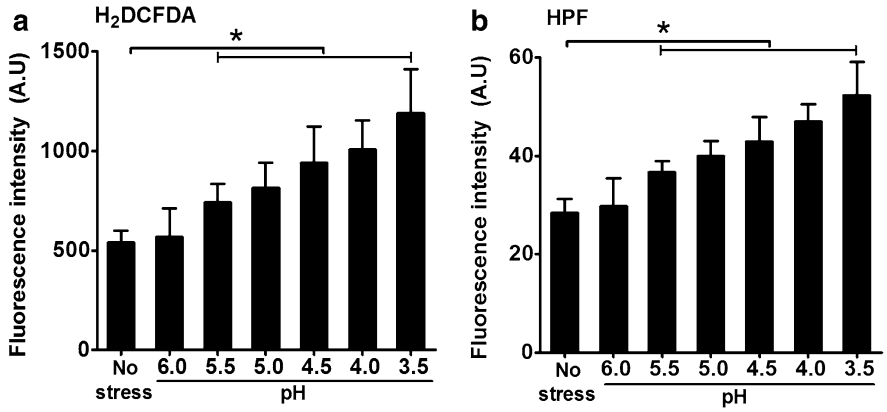


**Fig. 2** ROS formation in *C. glutamicum* upon exposure to low pH. Quantification of intracellular ROS and OH· levels in *C. glutamicum* wild-type cultures after treatment at different acidic pH for 1 h with fluorescent probes $H_2$DCFDA and HPF, respectively. Mean values with standard deviations (*error bars*) from at least three repeats are shown. *: $P \leq 0.05$

corresponding to the survival rate observed at all pH values. The strain was unaffected at pH 6 and no excess ROS formation was measured. The intracellular ROS levels, however, increased as the pH decreased (Fig. 2a).

We monitored the generation of hydroxyl radicals (OH·), the most toxic ROS produced upon acidic pH exposure, using the OH·-specific fluorescent probe HPF (Fig. 2b). Significantly higher amounts of OH· were observed in *C. glutamicum* wild-type cells treated with acid, and OH· production increased in response to the decrease in pH. Taken together, these data provide evidence that acid stress induced excess formation of ROS in *C. glutamicum*, which contributed to cell toxicity upon acid stress.

## MPx protects *C. glutamicum* cells against acid stress

To address whether MPx can protect *C. glutamicum* cells against acid stress, wild-type, Δ*mpx* mutant and complemented strains were challenged at pH 4 and 6 for 1 h, and survival rates were assessed using a cell viability assay (Fig. 3). pH 4 treatment reduced the survival rate of the wild-type and led to a mortality rate of about 50 % (Fig. 1). As shown in Fig. 3, while the survival rate of the Δ*mpx* mutant was nearly identical to that of the wild-type at pH 6, the survival rate of the Δ*mpx* mutant decreased by 38 % compared with that of the wild-type under pH 4 treatment. However, the acid sensitivity phenotype of the Δ*mpx* mutant was completely rescued in the complemented strain Δ*mpx*(pXMJ19-*mpx*). Moreover, *mpx* overexpression increased the resistance of the wild-type strain to acid stress (Fig. 3). In addition, deletion of the *mpx* gene

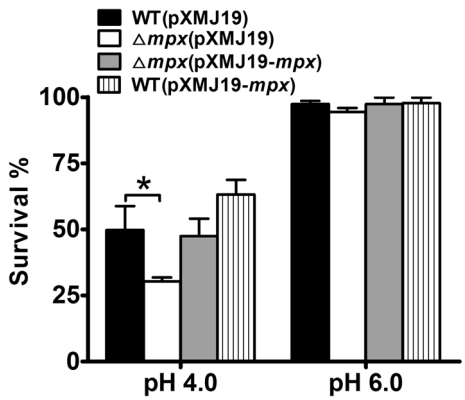


**Fig. 3** MPx was required for cellular resistance to acid stress in *C. glutamicum*. Indicated *C. glutamicum* strains were exposed to acid stress (pH 4 and 6) for 1 h at 30 °C. Cell survival rate was measured by a viability assay. Mean values with standard deviations (*error bars*) from at least three repeats are shown. *: $P \leq 0.05$

did not affect bacterial growth under normal conditions without acid stress (Supplementary Fig. 1), further supporting the conclusion that MPx plays a protective role against acid stress in *C. glutamicum*.

## MPx is able to reduce intracellular ROS levels produced under acid stress

Acid stress induces oxidative stress and leads to ROS production in *C. glutamicum*. As MPx plays an important role in resistance to oxidative stress generated by multiple stressors and in scavenging ROS in *C. glutamicum* (Pedre et al. 2015; Si et al. 2015), this prompted us to examine whether the acid stress tolerance in *C. glutamicum* conferred by MPx was associated with a reduction in the levels of deleterious

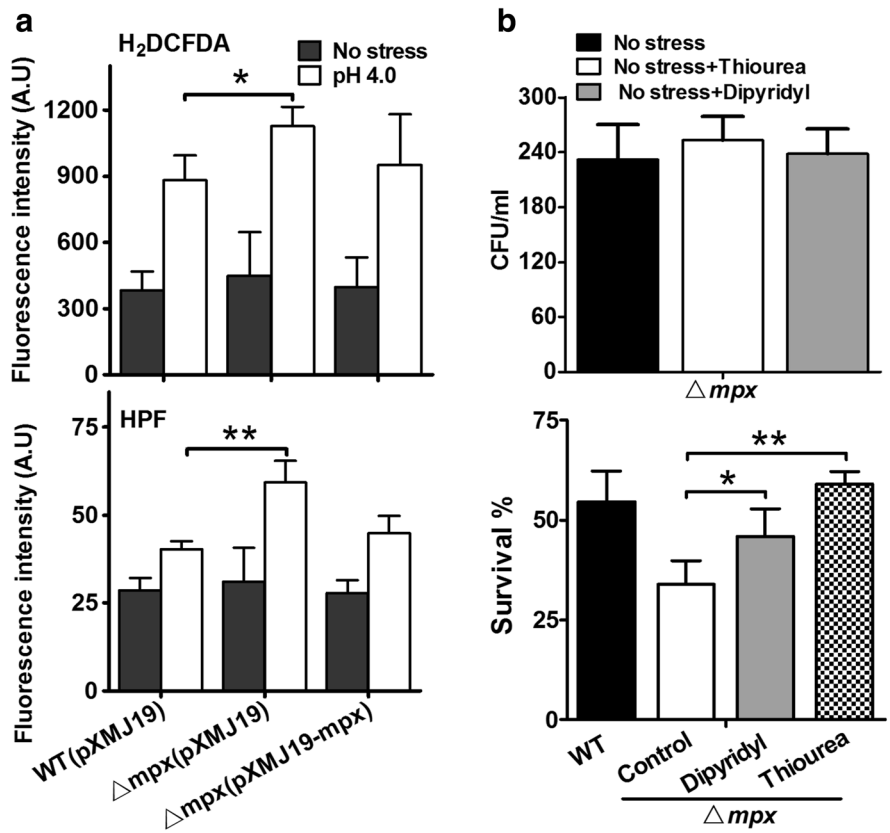


**Fig. 4** Mutants lacking MPx had increased ROS production under acid stress. **a** Intracellular ROS and OH· levels of WT(pXMJ19), Δ*mpx*(pXMJ19) and Δ*mpx*(pXMJ19-*mpx*) strains exposed to pH 4.0 were measured using $H_2$DCFDA and HPF, respectively. **b** Effects of hydroxyl radical mitigation agents on the killing efficiency of acid stress. 20 μM 2,2′-dipyridyl or 6.5 mM thiourea has no effect on the survival of the Δ*mpx* mutant (upper panel). After the addition of 20 μM 2,2′-dipyridyl or 6.5 mM thiourea to the Δ*mpx* mutant exposed to pH 4.0, the survival rate of the Δ*mpx* mutant was nearly recovered to that of wild-type (*lower panel*). Mean values with standard deviations (*error bars*) from at least three repeats are shown. **: $P \leq 0.01$; *: $P \leq 0.05$

ROS induced by acid stress. We thus examined intracellular ROS and OH· levels after acid stress treatment with $H_2$DCFDA and HPF, respectively. The data revealed that, as expected, the Δ*mpx* mutant had markedly higher ROS and OH· levels than the wild-type strain at pH 4 (Fig. 4a). ROS and OH· levels in the complemented strain, Δ*mpx*(pXMJ19-*mpx*), were almost completely reduced to the level of the wild-type strain (Fig. 4a), indicating that *mpx* is strongly linked to ROS scavenging in the mutant. These data suggest that MPx protects *C. glutamicum* against acid stress by scavenging ROS, especially the highly toxic OH·, produced via Fenton chemistry under acid stress. Thus, we speculate that the survival rate of Δ*mpx* mutants is restored to the wild-type level under acid stress by blockage of the Fenton reaction-mediated hydroxyl radical formation by 2,2′-dipyridyl or thiourea, agents known to effectively mitigate the damaging effects of hydroxyl radicals (Kohanski et al. 2007). As expected, when added to bacterial cultures challenged by acid stress, each of these two chemicals was able to increase the survival rates of the Δ*mpx* mutant to levels almost comparable to those of the wild-type (Fig. 4b), further validating the notion that MPx is critical in removing deleterious ROS accumulated in *C. glutamicum* under acid stress conditions.

ROS escaping from the antioxidant defense system can react with the cysteine thiol groups of proteins, resulting in irreversible sulfoxidation products, inter- or intra-protein disulfides, and mixed disulfides with low molecular weight thiol, and eventually lead to protein carbonylation (Nystrom 2005; Ying et al. 2007). Given that MPx removes ROS in *C. glutamicum*, we hypothesized that MPx may also function in protecting against protein carbonylation under acid stress conditions. To test this hypothesis, we applied a well-established method to measure protein carbonylation using the OxyBlot assay. Total proteins of the wild-type and Δ*mpx* mutant cells treated at pH 4 were extracted and subjected to SDS-PAGE before and after OxyBlot treatment (Fig. 5). A high number of proteins were found to harbor carbonyl groups in *C. glutamicum* protein extracts of wild-type and Δ*mpx* mutant cells treated at pH 4. Furthermore, the carbonylation

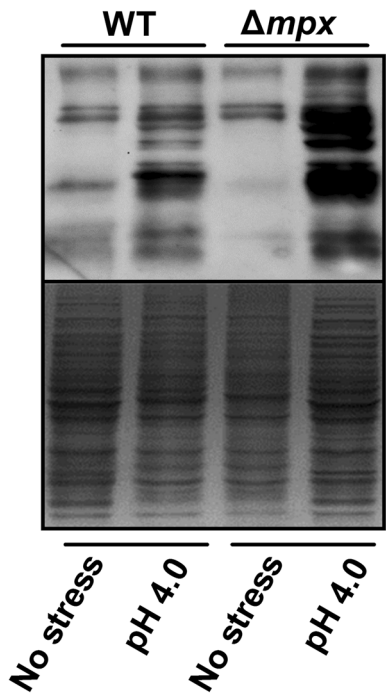


**Fig. 5** Mutants lacking MPx had increased protein carbonyl content under acid stress. Protein carbonyl content was analyzed by Western blotting with an anti-dinitrophenyl antibody (*upper panel*). A parallel run was stained with Coomassie Brilliant Blue (*lower panel*). Total proteins were extracted from wild-type and Δ*mpx* mutant cells

level of protein extracts was significantly lower in wild-type compared to the Δ*mpx* mutant after 1 h of exposure to pH 4 (Fig. 5). Taken together, these data provide evidence that MPx plays protective roles against acid stress in *C. glutamicum* via removal of acid stress-induced ROS production.

## Acid-induced *mpx* expression is mediated by SigH

As MPx is involved in scavenging cellular ROS induced by acid stress, we next performed RT-PCR and LacZ activity profiling to examine whether *mpx* expression responds to acid stress inducers at the transcriptional level. The LacZ activity of the $P_{mpx}$::*lacZ* chromosomal promoter fusion reporter in the *C. glutamicum* wild-type strain was quantitatively measured in bacterial cells either untreated or treated with pH 5 and pH 5.5 (Fig. 6a). The level of *mpx* expression was increased by approximately 46 and 23 % in the wild-type strain treated with pH 5 and pH 5.5, respectively, compared to untreated samples (Fig. 6a). Further, expression of the $P_{mpx}$::*lacZ* fusion displayed $H^+$ concentration-dependent increase in response to acidic environmental conditions (Fig. 6a). A similar $H^+$ concentration-dependent pattern of *mpx* expression in response to acid stress was also observed in qRT-PCR analysis (Fig. 6b). These results clearly demonstrate that acid stress induces *mpx* expression, which in turn directly contributes to the tolerance of *C. glutamicum* to acidic stress conditions.

As SigH, the stress-responsive extracytoplasmic function-sigma (ECF-σ) factor, responds to thiol-oxidative stress and regulates the expression of

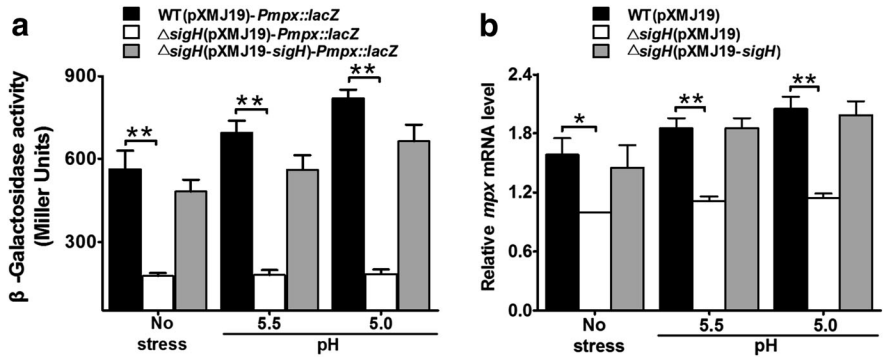


**Fig. 6** Positive regulation of *C. glutamicum mpx* expression by SigH under acid stress. **a** β-Galactosidase analysis of *mpx* promoter activities using the transcriptional $P_{mpx}$::*lacZ* chromosomal fusion reporter expressed in the indicated *C. glutamicum* strains. Mean values with standard deviations (*error bars*) from at least three repeats are shown. **: $P \leq 0.01$. **b** qRT-PCR assay revealed that expression of *mpx* was under positive regulation of SigH under acid stress. Exponentially growing *C. glutamicum* cells were exposed to pH 5.0 and pH 5.5 for 30 min. The levels of *mpx* expression were determined by quantitative RT-PCR. The mRNA levels are presented relative to the value obtained from wild-type cells without treatment. Mean values with standard deviations (*error bars*) from at least three repeats are shown. **: $P \leq 0.01$

multiple resistance genes (Kim et al. 2005; Ehira et al. 2009), we examined whether *mpx* expression was subjected to SigH regulation by measuring the transcription of chromosomal $P_{mpx}$::*lacZ* fusions. A significant decrease in LacZ activity was observed for the rapidly growing Δ*sigH* mutant under both acid stress and unstressed conditions compared with that in the wild-type, and the reduced promoter activity in the Δ*sigH* mutant was fully recovered in the complemented strain Δ*sigH*(pXMJ19-*sigH*) (Fig. 6a), indicating a crucial role for SigH in the regulation of *mpx* expression. SigH-dependent *mpx* activation was also confirmed by qRT-PCR analysis (Fig. 6b). Strikingly, the acid-induced *mpx* expression was completely abolished in the Δ*sigH* mutant (Fig. 6), further supporting the role of SigH in mediation of the acid-induced *mpx* expression. Together, these findings clearly demonstrate that SigH positively regulates the expression of *mpx*.

## Conclusion

As *C. glutamicum* is susceptible to acid stress, enhancing its acid resistance is a key parameter during its growth. Although the mechanisms of acid resistance have been well-described in multiple highly acid-resistant bacteria, including the roles of proton pumps, regulators, altered metabolism, protein and DNA repair, cell envelope alterations, and alkali production, most of these important general mechanisms are missing or ineffective in acid-sensitive *C. glutamicum* (Kalinowski et al. 2003; Follmann et al. 2009). In this paper, we describe the role of MPx in the protection of *C. glutamicum* against acid stress through the reduction of intracellular ROS levels induced under acid treatment. Acid stress induced the formation of ROS in *C. glutamicum*, and MPx-deficient mutants showed a significantly lower cell viability rate and more damaged protein carbonylation than the wild-type, owing to the loss of the ability to scavenge ROS (Fig. 3). The physiological role of MPx in resistance to acid stress was also corroborated by the induced expression of MPx in *C. glutamicum* under acid stress, regulated directly by the stress-responsive ECF-sigma factor, SigH (Fig. 6). Thus MPx expression is important for growth under acid stress. To our knowledge, this is the first report describing the acid-resistant role of a mycothiol peroxidase. Hence, our work provides insight into a previously unknown, but important, aspect of cellular response to acid stress that could be used to develop *C. glutamicum* as an efficient bio-based chemical production strain in the future.

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**Supporting Information** Supplementary Table 1—Bacterial strains, plasmids and primers used in this study.

Supplementary Figure 1—Deletion of the *mpx* gene does not affect bacterial growth under normal conditions without acid stress.

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