Arch Microbiol
DOI 10.1007/s00203-016-1306-5

ORIGINAL PAPER

Global transcriptomic analysis of the response of *Corynebacterium glutamicum* to ferulic acid

Can Chen[1,2,3] · Junfeng Pan[1,2] · Xiaobing Yang[1] · He Xiao[1,2] · Yaoling Zhang[1] · Meiru Si[1,4] · Xihui Shen[1] · Yao Wang[1,2]

Received: 13 April 2016 / Revised: 24 August 2016 / Accepted: 8 October 2016

Abstract *Corynebacterium glutamicum* can survive by using ferulic acid as the sole carbon source. In this study, we assessed the response of *C. glutamicum* to ferulic acid stress by means of a global transcriptional response analysis. The transcriptional data showed that several genes involved in degradation of ferulic acid were affected. Moreover, several genes related to the stress response; protein protection or degradation and DNA repair; replication, transcription and translation; and the cell envelope were differentially expressed. Deletion of the *katA* or *sigE* gene in *C. glutamicum* resulted in a decrease in cell viability under ferulic acid stress. These insights will facilitate further engineering of model industrial strains, with enhanced tolerance to ferulic acid to enable easy production of biofuels from lignocellulose.

Communicated by Jorge Membrillo-Hernández.

Electronic supplementary material The online version of this article (doi:10.1007/s00203-016-1306-5) contains supplementary material, which is available to authorized users.

✉ Xihui Shen
xihuishen@nwsuaf.edu.cn

✉ Yao Wang
wangyao@nwsuaf.edu.cn

[1] State Key Laboratory of Crop Stress Biology for Arid Areas and College of Life Sciences, Northwest A&F University, Yangling 712100, Shaanxi, China

[2] Department of Biochemistry and Molecular Biology, College of Life Sciences, Northwest A&F University, Yangling 712100, Shaanxi, China

[3] College of Life Science and Agronomy, Zhoukou Normal University, Zhoukou 466001, Henan, China

[4] College of Life Sciences, Qufu Normal University, Qufu 273165, Shandong, China

Keywords *Corynebacterium glutamicum* · Ferulic acid · KatA · SigE · Lignocellulosic biomass

Introduction

Lignocellulose provides an abundant renewable resource for production of biofuels and bio-based chemicals (Almeida et al. 2007; Jönsson et al. 2013). However, during pretreatment of lignocellulosic biomass, a broad range of inhibitory compounds including phenolic compounds are produced (Almeida et al. 2007). These inhibitors greatly reduce microbial fermentation into desired products due to their toxicity to microbes (Mills et al. 2009; Parawira and Tekere 2011). Therefore, understanding of the tolerance mechanisms of fermentative microbes to phenolic compounds would be crucial for engineering of robust strains to toxic lignocellulosic hydrolysate-related inhibitors (Mills et al. 2009).

Corynebacterium glutamicum is one of the most important microorganisms in industrial biotechnology widely used for the production of amino acids, nucleotides, vitamins and various bio-based chemicals. Intriguingly, *C. glutamicum* was recently found to utilize a large variety of lignin-derived aromatic compounds (e.g., ferulate, vanillin, phenol, benzoate, phenylacetic acid, 4-cresol) as sole carbon and energy source for growth (Shen et al. 2005, 2012; Merkens et al. 2005; Huang et al. 2008; Chen et al. 2012; Li et al. 2014; Ding et al. 2015; Kallscheuer et al. 2016; Du et al. 2016). The remarkable capability of *C. glutamicum* in degradation of phenolic compounds as carbon source to sugars makes it a unique advantage in using hydrolysates of lignocellulose in industrial fermentation. A systematic and deeper identification of additional genes involved in the assimilation and tolerance to phenolic inhibitors and genetic engineering of strains seems to be a very promising

Published online: 20 October 2016

way to obtain efficient industrial strains in the use of renewable lignocellulosic biomass.

Due to its abundance in lignocellulosic biomass and chemical similarity to many phenolic acids, ferulic acid (a lignin-derived aromatic compound) was considered as an inhibitor model for studies on tolerance to phenolic compounds (Winkler and Kao 2011). The degradation of ferulic acid by two-carbon cleavage to produce valuable flavor compounds—such as vanillic acid, protocatechuic acid and vanillin—has been reported in both fungi (Tsujiyama and Ueno 2008; Bonnina et al. 2001) and bacteria (Plaggenborg et al. 2006; Abdelkafi et al. 2006; Civolani et al. 2000). Several gene clusters involved in degradation of ferulic acid have also been identified in *C. glutamicum*, for example, the *phd* (Kallscheuer et al. 2016), *vanABK* (Merkens et al. 2005) and *pcaHGBC* (Shen et al. 2012) gene clusters. However, although the response of *Escherichia coli* (Kot et al. 2015), *Clostridium beijerinckii* (Lee et al. 2015) and *Lactobacillus brevis* (Winkler and Kao 2011) to ferulic acid has been evaluated, the adaption and tolerance to ferulic acid in *C. glutamicum* have not been investigated. Therefore, in this study, microarray analysis of the response of *C. glutamicum* to ferulic acid was conducted.

Materials and methods

Bacterial strains and culture conditions

Bacterial strains and plasmids used in this study are listed in Supplementary Table S1. *Escherichia coli* was grown aerobically on a rotary shaker (220 rpm) at 37 °C in Luria–Bertani (LB) broth or on LB plates with 1.5 % (wt/vol) agar. *C. glutamicum* strains were routinely grown in LB medium or in mineral salts medium supplemented with 0.05 g l^{-1} of yeast extract to meet the requirement of vitamins for the strains on a rotary shaker at 30 °C (Shen et al. 2005). *C. glutamicum* RES167, a restriction-deficient strain derived from *C. glutamicum* ATCC 13032, was the parent of all derivatives used in this study. For generation of mutants and maintenance of *C. glutamicum*, BHIS (brain heart broth with 0.5 M sorbitol) medium was used. Cell growth was monitored by measuring absorbance at 600 nm (A_{600}). Antibiotics were added at the following concentrations: 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*; and chloramphenicol, 20 μg ml^{-1} for *E. coli* and 10 μg ml^{-1} for *C. glutamicum*.

DNA manipulation and plasmid construction

The genes coding for *sigE* was amplified by PCR using *C. glutamicum* genomic DNA as template with indicated primers listed in Supplementary Table S2. The amplified DNA fragments were digested and then subcloned into similar digested pXMJ19-His_6 plasmid (Liu et al. 2014), obtaining plasmids pXMJ19-His_6-*sigE*. pXMJ19-His_6-*katA* and pXMJ19-His_6-*sigB* were constructed in a similar approach as pXMJ19-His_6-*sigE*. To construct the deletion plasmid pK18*mobsacB-sigE*, a 996-bp upstream fragment and a 960-bp downstream fragment of *sigE* were amplified using primer pairs *sigE*-F1/*sigE*-R1 and *sigE*-F2/*sigE*-R2, respectively (Supplementary Table S2). In the next step, the upstream and downstream PCR fragments were fused together with the primer pair *sigE*-F1/*sigE*-R2 by overlap PCR. The resulting DNA fragments were digested with BamHI/HindIII and inserted into similar digested suicide plasmid pK18*mobsacB* to create pK18*mobsacB-sigE* (Supplementary Table S1). Plasmid pK18*mobsacB-katA* and pK18*mobsacB-sigB* were constructed in a similar approach using primer pairs *katA*-F1/*katA*-R1, *katA*-F2/*katA*-R2 and *sigB*-F1/*sigB*-R1, *sigB*-F2/*sigB*-R2, respectively (Supplementary Table S2).

Construction of deletion mutants and complemented strains

To construct the *sigE* in-frame deletion mutant, the pK18*mobsacB-sigE* plasmid was transformed into *C. glutamicum* wild type by electroporation. Integration of the introduced plasmid into *C. glutamicum* chromosome by single crossover was selected on BHIS plates containing 25 μg/ml kanamycin and 40 μg/ml nalidixic acid. The kanamycin-resistant (Km^R) colonies were grown overnight in LB allowing for a second crossover to occur. Selection for loss of the genome-integrated *sacB*-containing plasmid was performed on LB plates containing 20 % sucrose and 40 μg/ml nalidixic acid. Strains growing on this plate were tested for kanamycin sensitivity (Km^S) by parallel picking on LB plates containing either kanamycin or sucrose. Kanamycin-sensitive and sucrose-resistant strains were tested for deletion by PCR using the *sigE*-F1/*sigE*-R2 primer pair (Supplementary Table S2) and confirmed by PCR and DNA sequencing as previously described (Shen et al. 2005; Si et al. 2015). The *katA* and *sigB* deletion mutant was similarly constructed by using the pK18*mobsacB-katA* plasmid and pK18*mobsacB-sigB*, respectively. For complementation, pXMJ19-His_6-*sigE*, pXMJ19-His_6-*katA* and pXMJ19-His_6-*sigB* were transformed into *sigE*, *katA* and *sigB*, respectively, by electroporation and expression of each gene in *C. glutamicum* was induced by the direct addition of 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) to cultures (Shen et al. 2005; Liu et al. 2014).

Sensitivity assays to ferulic acid

To test the susceptibility of *C. glutamicum* strains to ferulic acid, overnight cell cultures were diluted 100-fold with fresh LB medium and exposed to 15 mM ferulic acid for 30 min at 30 °C with shaking. The cultures were serially diluted and plated onto LB agar plates, and then, the survival percentage was calculated as [(CFU ml^{-1} with stress)/(CFU ml^{-1} without stress)] × 100 (Liu et al. 2013; Si et al. 2014). All assays were performed in triplicate.

Measurement of intracellular ROS levels

In vivo ROS levels were measured using the fluorogenic probe 2′,7′-dichlorofluorescein diacetate (DCFH-DA) as described (Schurig-Briccio et al. 2009; Wang et al. 2015), with the following modifications. Briefly, cells grown aerobically (OD_{600} = 1.6) were collected, washed and resuspended in 50 mM PBS (pH 7.4) prior to preincubation with 2 μM DCFH-DA at 28 °C for 20 min. Ferulic acid at indicated (3 mM) concentrations was added to these mixtures and incubated for another 30 min. After that, cells were washed two times with PBS, centrifuged and resuspended in PBS. The fluorescence intensity was measured using a SpectroMax spectrofluorimeter (excitation, 502 nm; emission, 521 nm).

Validation of microarray data by quantitative real-time PCR (qRT-PCR)

The expression levels of 14 representative genes were examined by qRT-PCR to validate the microarray data. The primers for qRT-PCR were designed using Primer 5. The sizes of PCR products were confirmed by electrophoresis on 2 % agarose gel. The RNA extraction was conducted the same as in RNA extraction and cDNA synthesis. Moreover, the cDNA synthesis was conducted using PrimeScript RT reagent Kit with gDNA Eraser (TaKaRa, Japan). qRT-PCR was conducted on BioRad CFX96 Real-Time System using SYBR Premix Ex Taq (TaKaRa). For each gene/sample combination, three replicate reactions were carried out. In addition, the 16 S rDNA gene was chosen as a reference gene.

Microarray experiments

The *C. glutamicum* DNA microarrays were custom designed using the Agilent eArray 5.0 program according to the manufacturer's recommendations (Agilent Technologies, Santa Clara, CA, US). The chip specification was 8 × 15 K (design ID: 045822). Samples were collected during the mid-logarithmic growth phase in minimal medium with added glucose (control sample: 100 mM) or ferulic acid as the sole carbon source (3 mM), respectively (Supplementary Fig. 1). Total RNA was extracted using TRIzol Reagent (Life Technologies, Carlsbad, CA, USA). Total RNA was amplified and labeled using the Low Input Quick Amp Labeling Kit, One-Color (Agilent Technologies). Labeled cRNAs were purified using an RNeasy mini kit (Qiagen, GmBH, Germany). Each slide was hybridized with 600 ng Cy3-labeled cRNA using the Gene Expression Hybridization Kit (Agilent Technologies) in a hybridization oven (Agilent Technologies). After 17 h of hybridization, slides were washed in staining dishes (Thermo Shandon, Waltham, MA, US) with a Gene Expression Wash Buffer Kit (Agilent Technologies). Slides were scanned using an Agilent Microarray Scanner (Agilent Technologies) with the default settings: dye channel, green; scan resolution, 3 μm; 20-bit color. Data were extracted using the Feature Extraction software version 10.7 (Agilent Technologies). Raw data were normalized using the quantile algorithm in the Gene Spring Software version 11.0 (Agilent Technologies).

Results and discussions

Overview of microarray analysis

Gene expression patterns were assessed in the presence of ferulic acid and glucose as the sole carbon sources. To identify differentially expressed genes, bacteria in the mid-logarithmic growth phase were harvested for RNA extraction and further microarray experiment (hybridizations). A total of 517 genes were up-regulated and 521 down-regulated. qRT-PCR of 14 representative genes was chosen to verify the microarray data. The log_2-transformed mean values of 3 biological replicates for each gene were in good consistency of the log_2-transformed fold changes in the microarray data (Fig. 1).

Further analysis of microarray data

We next identified the functions of the differentially expressed genes by KEGG pathway analysis (Figs. 2, 3). We were interested in the following pathways: degradation of aromatic compounds, biosynthesis of amino acids, nucleotide excision repair and DNA replication.

Differentially expressed genes related to ferulic acid degradation

The ferulic acid generated by lignocellulose treatment can affect the growth and production of microbial cells. However, bacteria such as *C. glutamicum* can adapt to the presence of this compound and use it as the sole carbon and

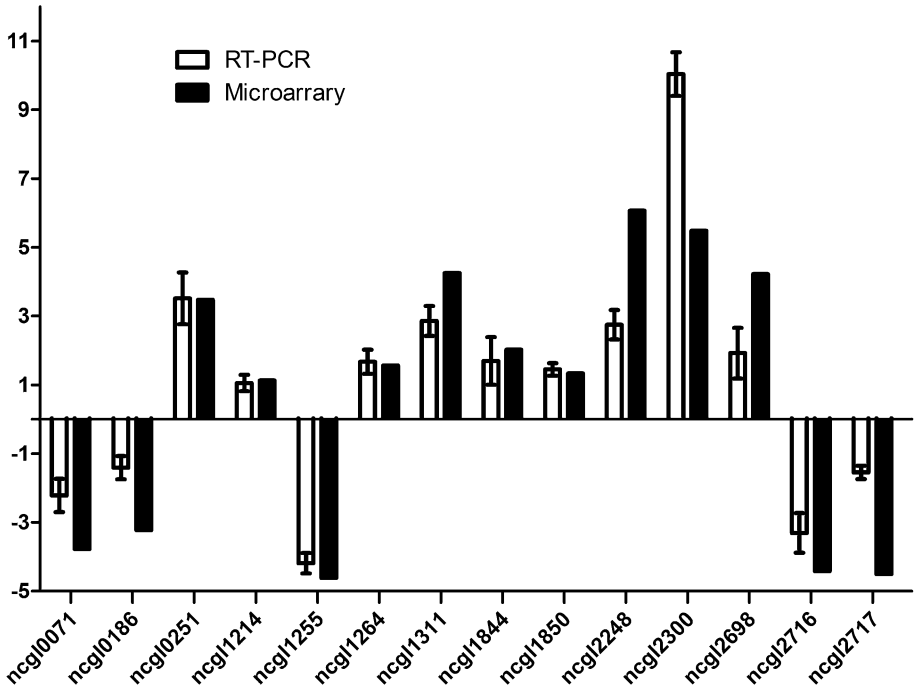

Fig. 1 Validation of microarray results by qRT-PCR. Fourteen representative genes were evaluated for validation of the microarray data using qRT-PCR. *White bars* show the $\log_2$-transformed fold changes of qRT-PCR from three biological replicates, and *error bars* indicate the standard deviations. *Black bars* represent $\log_2$-transformed fold changes of microarray data

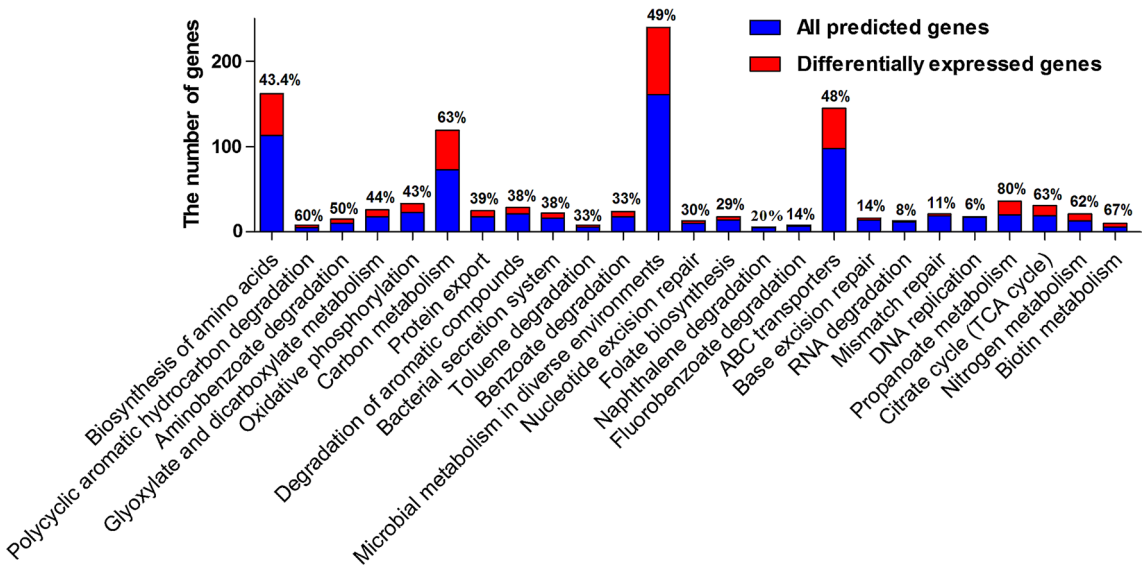

Fig. 2 KEGG pathway analysis of differentially expressed genes. Summary of the number of differentially expressed genes in each KEGG pathway. The percentage of the differentially expressed genes account for the predicted genes are shown above the *bars*

energy source for growth (Shen et al. 2012). *C. glutamicum* cells can survive by degrading ferulic acid; therefore, this degradation pathway was evaluated.

The *phdA-E* gene cluster showed increased expression in response to ferulic acid (Table 1). *phdR*, the regulator of this cluster, was up-regulated (Table 1). *phdT*, which encodes a ferulic acid transporter, was up-regulated. The enzymes encoded by this cluster catalyze the conversion of ferulic acid to vanillate (Kallscheuer et al. 2016).

Our microarray data showed that *vanA* and *vanB* were up-regulated. *vanK*, a major facilitator superfamily permease was up-regulated. *vanAB*, which encodes vanillate demethylase, catalyzes the conversion of vanillate to protocatechuate (Merkens et al. 2005).

pcaGH, which encode two subunits of protocatechuate 3,4-dioxygenase, were up-regulated. This enzyme catalyzes conversion of protocatechuate to β-carboxy-*cis*, *cis*-muconate by a ring-cleavage reaction (Shen et al. 2012).

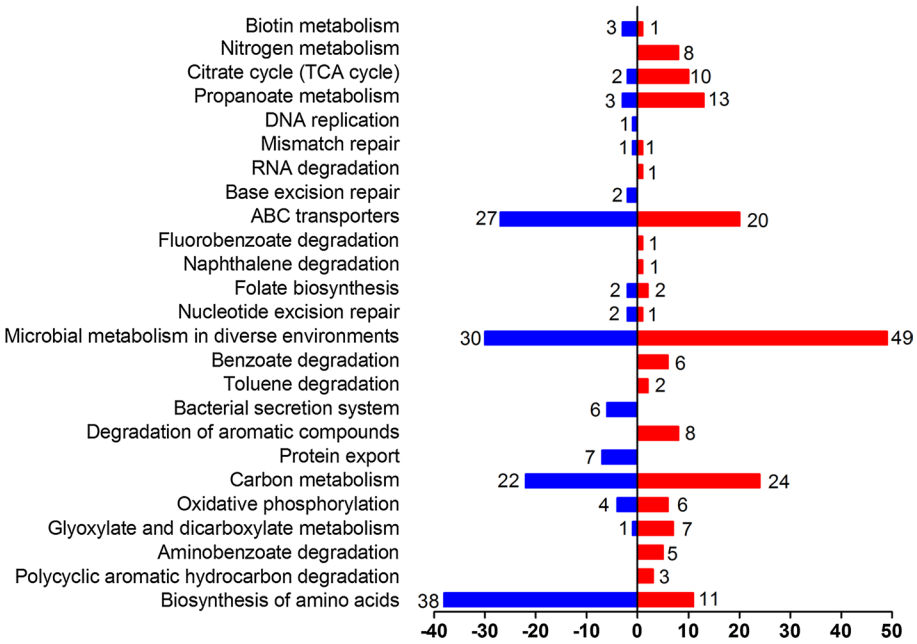

Fig. 3 Differentially expressed genes (ferulic acid vs. glucose). The *red* and *blue bars* represent up- and down-regulated genes, respectively, and the numeric labels represent the number of genes with that function pathway

Table 1 Differentially expressed genes of ferulic acid degradation

Locus tag	Gene name	Gene description	Fold change
ncgl0278	*phdT*	Major facilitator superfamily permease	2.24
ncgl0279	*phdA*	Acyl-coA synthetase	4.71
ncgl0280	*phdR*	Transcription regulator protein	2.42
ncgl0281	*phdB*	Dehydrogenase	7.92
ncgl0282	*phdC*	Metal-dependent hydrolase of the TIM-barrel fold	6.56
ncgl0283	*phdD*	Acyl-coA dehydrogenase	2.79
ncgl0284	*phdE*	Acyl dehydratase	4.41
ncgl2300	*vanA*	Ferredoxin subunits of nitrite reductase and ring-hydroxylating dioxygenase	44.68
ncgl2301	*vanB*	Flavodoxin reductase 1	3.57
ncgl2302	*vanK*	Major facilitator superfamily permease	5.17
ncgl2314	*pcaG*	Protocatechuate 3,4-dioxygenase subunit alpha	4.48
ncgl2315	*pcaH*	Protocatechuate 3,4-dioxygenase subunit beta	10.88

Differentially expressed genes related to the stress response

Stress responses are very important to microbes, and osmolarity, temperature and nutrient availability are highly variable environment factors to them (Estruch 2000). Therefore, under environmental stresses, such as heat, cold, heavy metal or oxidative agent exposure, transcription factors are activated to regulate production of functional proteins to prevent further damage.

The extracytoplasmic function (ECF) σ factors contain many alternative σ factors, and in numerous organisms, the ECF σ factor genes have been identified (Helmann 2002). In the *C. glutamicum* genome, several putative ECF σ factor genes have been detected; some are related to the stress response. In our microarray data, the alternative σ factor genes *sigB* and *sigE* were up-regulated (Table 2).

sigB encodes the nonessential σ factor SigB, which plays a role in the stress response (Larisch et al. 2007). However, the survival rate showed no significant difference between wild type (WT) and Δ*sigB* mutant under the stress of ferulic acid (data not shown).

The ECF σ factor *sigE*, an alternative sigma factor in *C. glutamicum*, is involved in the response to cell surface stressors (Park et al. 2008). The strain of Δ*sigE* was more sensitive to stressors of cell surface, nitric oxide (NO), and acidic pH in *Corynebacterium pseudotuberculosis* (Pacheco et al. 2012). *sigE* expression was increased by ferulic acid in *C. glutamicum* (Table 2). Moreover, the *sigE* mutant was more sensitive to ferulic acid stress (15 mM)

Table 2 Differentially expressed genes on stress response

Locus tag	Gene name	Gene description	Fold change
ncgl1844	*sigB*	RNA polymerase sigma factor *sigB*	4.06
ncgl1075	*sigE*	RNA polymerase sigma factor *sigE*	4.21
ncgl0251	*katA*	Catalase	11.13
ncgl0455	*cgl0472*	Oxidoreductase	2.01
ncgl2955	*cgl3060*	Oxidoreductase	2.16

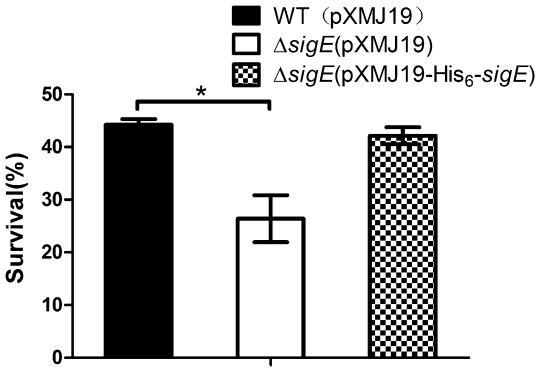

Fig. 4 Mutant lacking SigE was highly sensitive to ferulic acid stress. Survival of the *C. glutamicum* WT(pXMJ19), Δ*sigE*(pXMJ19) and Δ*sigE*(pXMJ19-His_6-*sigE*) strains was assessed after exposure to ferulic acid (15 mM) for 30 min. Mean values with standard deviations (*error bars*) from at least three replicates are shown. $^*P \leq 0.05$

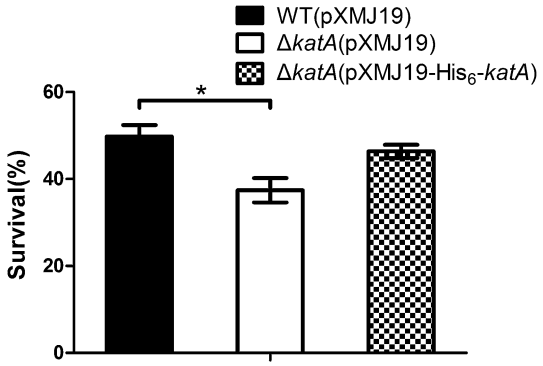

Fig. 5 Mutant lacking KatA was highly sensitive to ferulic acid stress. Survival of the *C. glutamicum* WT(pXMJ19), Δ*katA*(pXMJ19) and Δ*katA*(pXMJ19-His_6-*katA*) strains was assessed after challenge with ferulic acid (15 mM) for 30 min. Mean values with standard deviations (*error bars*) from at least three replicates are shown. $^*P \leq 0.05$

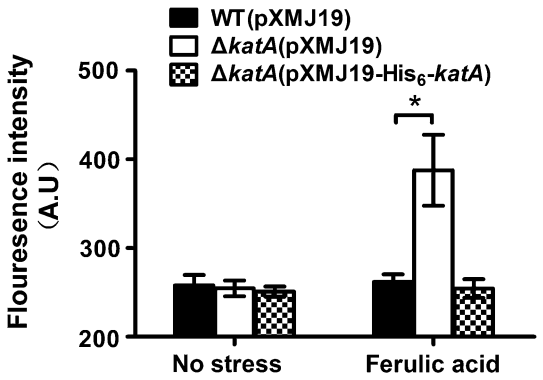

Fig. 6 Mutant lacking KatA exhibited increased ROS production under ferulic acid stress. A quantitative assay of intracellular ROS under ferulic acid stress was performed. Mean values with standard deviations (*error bars*) from at least three replicates are shown. $^*P \leq 0.05$. The ROS levels in the indicated *C. glutamicum* strains are measured by a DCFH-DA fluorescence assay after exposure to oxidative stress-inducing reagents. *AU* arbitrary units

than was the WT strain, while the complemented strain had a survival rate similar to that of the WT (Fig. 4).

Intracellular reactive oxygen species (ROS), which are generated by environmental factors, can cause oxidative stress. Microbes must evolve mechanisms to protect them against oxidative stress. These mechanisms involve enzymes such as superoxide dismutase and catalase, small proteins such as thioredoxin and glutaredoxin and other molecules such as glutathione. Catalase is responsible for clearing H_2O_2, the breakdown of which yields H_2O and O_2. The catalase gene (*katA*) was up-regulated by ferulic acid stress. The mutant was more sensitive to ferulic acid stress (15 mM) than was the WT, and the complemented strain had a survival rate similar to that of the WT (Fig. 5). To evaluate the function of KatA in ROS reduction in the presence of ferulic acid stress, ROS levels were examined using DCFH-DA, a membrane-permeable dye that diffuses passively into cells. As shown in Fig. 6, the *katA* mutant had significantly higher ROS levels than those of the WT after ferulic acid stress treatment. However, ROS levels in the *katA* mutant were restored completely by complementation to levels in the WT (Fig. 6). *ncgl0455* and *ncgl2955*, which encode two oxidoreductases, were up-regulated by ferulic acid stress.

Differentially expressed genes related to protein protection/degradation and DNA repair (the SOS response)

Microorganisms evolve some proteins to reduce damaged DNA/proteins or protect functional proteins to maintain normal metabolism (Jiang et al. 1999). Protein disulfide isomerase (PDI) is useful for correct folding and formation of disulfide bond to functional proteins (Jiang et al. 1999). *ncgl0877*, which encodes a thiol–disulfide isomerase in *C. glutamicum,* was up-regulated, which could protect proteins

from further damage by ferulic acid stress. Microbes produce some ATP-dependent enzymes to degrade harmful or damaged proteins for preventing accumulation of these proteins and making precursors for protein synthesis of a new round (Engels et al. 2005). The holoenzyme of Clp contains two separate and functionally distinct subunits (Engels et al. 2004). The proteolytic subunits of ClpP can hydrolyze substrates, but their active sites are buried within the proteolytic core, formed by fourteen ClpP subunits (Engels et al. 2004). Therefore, associate with the core, the members of the Clp/Hsp100 superfamily: ClpS, ClpC or ClpX (hexamers of ATPase subunits) (Schirmer et al. 1996), which are needed for substrates recognition, unfolding and ultimately translocation (Hlavácek and Váchová 2002). In *C. glutamicum* (Engels et al. 2005) and *Streptomyces coelicolor* (Bellier and Mazodier 2004), the transcriptional activator ClgR induces expression of *clpCP* genes. Our transcriptomic analysis showed that *clpP*, *clpX*, *clpS*, *clpC* and *clgR* were up-regulated by ferulic acid stress (Table 3). Therefore, the up-regulation of *clp* genes suggests increased degradation of damaged proteins under ferulic acid stress.

In microorganisms, some environmental agents that may damage DNA can induce several genes responsible for DNA repair (Davis et al. 2002). This process is termed the SOS response. LexA is an important regulator of the SOS response, and it was up-regulated by ferulic acid stress. Moreover, *recO* and *dnaE2*, which encode DNA damage repair proteins, were up-regulated by ferulic acid stress.

Differentially expressed genes related to replication, transcription and translation

Ferulic acid as a growth inhibitor could make a stress situation for *C. glutamicum* that causes the slower metabolism of the cells. The microarray analysis showed that the gene encoding DNA polymerase III subunit beta (*ncgl0002*), which is related to DNA replication, was down-regulated. *ncgl0540*, which encodes DNA-directed RNA polymerase subunit alpha, was down-regulated. The transcription elongation factor *greA* (*ncgl0946*), the encoded protein of which induces the nucleolytic activity of bacterial RNA polymerase (RNAP) (Stepanova et al. 2007), was down-regulated. In prokaryotes and archaea, the N-utilizing substance A protein (NusA) is an essential transcription factor (Li et al. 2013). NusA plays important roles in transcriptional anti-termination, termination, pausing and elongation (Li et al. 2013). The NusA-RNA complex structures in *Mycobacterium tuberculosis* show that like the function of many cold-shock proteins, as an RNA chaperone function, it can bind to nascently forming RNA (Beuth et al. 2005). Our transcriptomic data showed that the *C. glutamicum* NusA (*ncgl1912*) was down-regulated by ferulic acid stress. Two genes related to translation were differentially expressed. The translation initiation inhibitor *ncgl0277* was up-regulated, and the translation initiation factor IF-1 gene (*ncgl0536*) was down-regulated by ferulic acid stress (Table 4).

Table 3 Differentially expressed genes related to protein protection/degradation and DNA repair (the SOS response)

Locus tag	Gene name	Gene description	Fold change
ncgl0877	*cgl0914*	Thiol–disulfide isomerase	6.24
ncgl1887	*clgR*	Transcriptional regulator	2.35
ncgl2328	*clpP*	ATP-dependent Clp protease proteolytic subunit	3.30
ncgl2304	*clpX*	ATP-dependent protease ATP-binding subunit ClpX	3.10
ncgl2585	*clpC*	ATP-dependent protease ATP-binding subunit ClpC	4.18
ncgl2429	*clpS*	ATP-dependent Clp protease adaptor protein ClpS	2.14
ncgl1855	*lexA*	LexA repressor	2.87
ncgl0611	*dnaE2*	Error-prone DNA polymerase	2.52
ncgl2204	*recO*	DNA repair protein RecO	2.81

Table 4 Differentially expressed genes related to replication, transcription and translation

Locus tag	Gene name	Gene description	Fold change
ncgl0002	*cgl0003*	DNA polymerase III subunit beta	0.44
ncgl0540	*cgl0564*	DNA-directed RNA polymerase subunit alpha	0.29
ncgl0946	*greA*	Transcription elongation factor GreA	0.36
ncgl1912	*nusA*	Transcription elongation factor NusA	0.40
ncgl0277	*cgl0282*	Translation initiation inhibitor	2.40
ncgl0536	*infA*	Translation initiation factor IF-1	0.14

Therefore, replication, transcription and translation were down-regulated. This represents a mechanism of *C. glutamicum* to reduce the effect of ferulic acid stress and the damage to DNA, RNA and proteins under unfavorable conditions.

Differentially expressed genes related to the cell envelope

The cell envelope of *C. glutamicum* consists of plasma membrane, thick arabinogalactan–peptidoglycan polymer, mycomembrane and outer lipid layer (Bayan et al. 2003). This can enhance tolerance to some stress conditions including ferulic acid.

The transcriptional profile of *Lactobacillus brevis* revealed that ferulic acid induces the expression of some membrane proteins, possibly to reduce ferulic acid-induced effects on ion leakage and fluidity of membrane (Winkler and Kao 2011). Therefore, the effect of ferulic acid on the *C. glutamicum* cell envelope was determined. The microarray data suggested that some genes related to the cell envelope were differentially expressed. *ncgl0995*, which encodes a glycosyltransferase (likely involved in cell wall biogenesis), was up-regulated. This suggests strengthening of the cell wall to resist the effect of ferulic acid. Moreover, numerous membrane proteins were differentially expressed (Table 5), which may influence the membrane fluidity.

Table 5 Differentially expressed genes on cell envelope

Locus tag	Gene name	Gene description	Fold change
ncgl0995	*cgl1039*	Glycosyltransferase, probably involved in cell wall biogenesis	2.01
ncgl2802	*cgl2902*	Cell envelope-related transcriptional regulator	0.33
ncgl0626	*cgl0654*	Carbon starvation protein, membrane protein	4.76
ncgl0192	*cgl0195*	Membrane protein	3.80
ncgl2498	*cgl2587*	Membrane protein	3.42
cg1910	–	Membrane protein	2.55
cg0231	–	Membrane protein	0.19
ncgl0680	*cgl0710*	Membrane protein	0.15
ncgl0824	*cgl0858*	Metalloendopeptidase-like membrane protein	0.14
cg2147	–	BioY family membrane protein	0.12
ncgl1476	*cgl1534*	Membrane protein	0.20

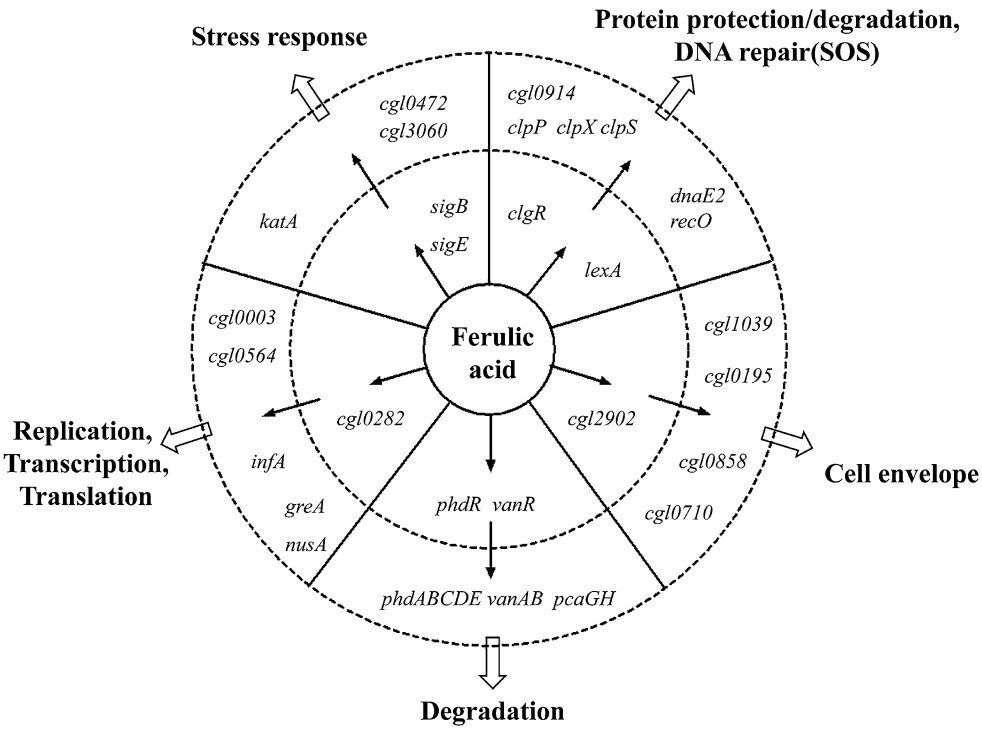

Fig. 7 Response of *Corynebacterium glutamicum* to ferulic acid. Schematic diagram of the gene regulatory networks and significant regulatory elements involved in the response of *C. glutamicum* to ferulic acid stress

Conclusions

The mechanisms of tolerance to an inhibitor generated by lignocellulose pretreatment of *C. glutamicum* were as follows: First, *C. glutamicum* was able to degrade ferulic acid. Second, the *C. glutamicum* cell envelope has a greater protective effect than do those of other bacteria. Third, growth of *C. glutamicum* cells was slower (genes related to replication, transcription and translation were down-regulated) than that under control conditions, which could reduce the damage to DNA, RNA and proteins. Fourth, cells activated various mechanisms to cope with the effect of ferulic acid (induction of the SOS response, production of Clp family proteins to degrade damaged proteins and protein modifications) (Fig. 7). The *sigE* and *katA* mutants were more sensitive to ferulic acid stress. Therefore, *C. glutamicum* can degrade ferulic acid to reduce the damage caused. Moreover, this microorganism possesses defense and damage repair mechanisms.

In previous studies, ferulic acid was reported to reduce biofilm formation in *E. coli* (Kot et al. 2015), up-regulate the expression of heat shock proteins, efflux systems and the *groESL* operon in *C. beijerinckii* (Lee et al. 2015), induce the expression of currently uncharacterized membrane proteins (possibly affecting ion leakage and membrane fluidity) and trigger generalized stress responses in *L. brevis* (Winkler and Kao 2011). In this study, several stress response genes and genes encoding membrane proteins were differentially expressed under ferulic acid stress in *C. glutamicum*.

As a soil bacterium, *C. glutamicum* may be exposed to aromatic compounds, which are important soil pollutants and difficult to biodegrade. *C. glutamicum* may adapt to the presence of aromatic compounds, including ferulic acid, and acquire gene clusters that allow it to degrade this compound. The results provide insight into the mechanisms of *C. glutamicum* adaptation and tolerance to ferulic acid, an important lignocellulose-derived inhibitor. This provides a theoretical foundation for the engineering of industrial strains tolerant to ferulic acid in the future.

Acknowledgments This work was supported by the National Natural Science Foundation of China (Nos. 31270078, 31370150 and 31500087), Key Science and Technology R&D Program of Shaanxi Province, China (2014K02-12-01) and the Natural Science Foundation of Shandong Province, China (ZR2015CM012).

References

Abdelkafi S, Sayadi S, Ben Ali Gam Z, Casalot L, Labat M (2006) Bioconversion of ferulic acid to vanillic acid by *Halomonas elongata* isolated from table-olive fermentation. FEMS Microbiol Lett 262:115–120

Almeida JRM, Modig T, Petersson A, Hähn-Hägerdal B, Lidén G, Gorwa-Grauslund MF (2007) Increased tolerance and conversion of inhibitors in lignocellulosic hydrolysates by *Saccharomyces cerevisiae*. J Chem Technol Biotechnol 82:340–349

Bayan N, Houssin C, Chami M, Leblon G (2003) Mycomembrane and S-layer: two important structures of *Corynebacterium glutamicum* cell envelope with promising biotechnology applications. J Biotechnol 104:55–67

Bellier A, Mazodier P (2004) ClgR, a novel regulator of *clp* and *lon* expression in *Streptomyces*. J Bacteriol 186:3238–3248

Beuth B, Pennell S, Arnvig KB, Martin SR, Taylor IA (2005) Structure of a *Mycobacterium tuberculosis* NusA-RNA complex. EMBO J 24:3576–3587

Bonnina E, Brunel M, Gouy Y, Lesage-Meessen L, Asther M, Thibault J (2001) *Aspergillus niger* I-1472 and *Pycnoporus cinnabarinus* MUCL39533, selected for the biotransformation of ferulic acid to vanillin, are also able to produce cell wall polysaccharide-degrading enzymes and feruloyl esterases. Enzyme Microb Technol 28:70–80

Chen X, Kohl TA, Rückert C, Rodionov DA, Li LH, Ding JY, Kalinowski J, Liu SJ (2012) Phenylacetic acid catabolism and its transcriptional regulation in *Corynebacterium glutamicum*. Appl Environ Microbiol 78:5796–5804

Civolani C, Barghini P, Roncetti AR, Ruzzi M, Schiesser A (2000) Bioconversion of ferulic acid into vanillic acid by means of a vanillate-negative mutant of *Pseudomonas fluorescens* strain BF13. Appl Environ Microbiol 66:2311–2317

Davis EO, Dullaghan EM, Rand L (2002) Definition of the mycobacterial SOS box and use to identify LexA-regulated genes in *Mycobacterium tuberculosis*. J Bacteriol 184:3287–3295

Ding W, Si MR, Zhang WP, Zhang YL, Chen C, Zhang L, Lu ZQ, Chen SL, Shen XH (2015) Functional and biochemical characterization of a vanillin dehydrogenase in *Corynebacterium glutamicum*. Sci Rep 5:8044

Du L, Ma L, Qi F, Zheng X, Jiang C, Li A, Wan X, Liu SJ, Li S (2016) Characterization of a unique pathway for 4-Cresol catabolism initiated by phosphorylation in *Corynebacterium glutamicum*. J Biol Chem 291:6583–6594

Engels S, Schweitzer JE, Ludwig C, Bott M, Schaffer S (2004) clpC and clpP1P2 gene expression in *Corynebacterium glutamicum* is controlled by a regulatory network involving the transcriptional regulators ClgR and HspR as well as the ECF sigma factor σ^H. Mol Microbiol 52:285–302

Engels S, Ludwig C, Schweitzer JE, Mack C, Bott M, Schaffer S (2005) The transcriptional activator ClgR controls transcription of genes involved in proteolysis and DNA repair in *Corynebacterium glutamicum*. Mol Microbiol 57:576–591

Estruch F (2000) Stress-controlled transcription factors, stress-induced genes and stress tolerance in budding yeast. FEMS Microbiol Rev 24:469–486

Helmann JD (2002) The extracytoplasmic function (ECF) sigma factors. Adv Microb Physiol 46:47–110

Hlavácek O, Váchová L (2002) ATP-dependent proteinases in bacteria. Folia Microbiol (Praha) 47:203–212

Huang Y, Zhao KX, Shen XH, Jiang CY, Liu SJ (2008) Genetic and biochemical characterization of a 4-hydroxybenzoate hydroxylase from *Corynebacterium glutamicum*. Appl Microbiol Biotechnol 78:75–83

Jiang XM, Fitzgerald M, Grant CM, Hogg PJ (1999) Redox control of exofacial protein thiols/disulfides by protein disulfide isomerase. J Biol Chem 274:2416–2423

Jönsson LJ, Alriksson B, Nilvebrant NO (2013) Bioconversion of lignocellulose: inhibitors and detoxification. Biotechnol Biofuels 6:16

Kallscheuer N, Vogt M, Kappelmann J, Krumbach K, Noack S, Bott M, Marienhagen J (2016) Identification of the phd gene cluster responsible for phenylpropanoid utilization in *Corynebacterium glutamicum*. Appl Microbiol Biotechnol 100:1871–1881

Kot B, Wicha J, Piechota M, Wolska K, Gruzewska A (2015) Antibiofilm activity of trans-cinnamaldehyde, *p*-coumaric, and ferulic acids on uropathogenic *Escherichia coli*. Turk J Med Sci 45:919–924

Larisch C, Nakunst D, Hüser AT, Tauch A, Kalinowski J (2007) The alternative sigma factor SigB of *Corynebacterium glutamicum* modulates global gene expression during transition from exponential growth to stationary phase. BMC Genom 8:4

Lee S, Lee JH, Mitchell RJ (2015) Analysis of *Clostridium beijerinckii* NCIMB 8052's transcriptional response to ferulic acid and its application to enhance the strain tolerance. Biotechnol Biofuels 8:68

Li K, Jiang T, Yu B, Wang L, Gao C, Ma C, Xu P, Ma Y (2013) *Escherichia coli* transcription termination factor NusA: heat-induced oligomerization and chaperone activity. Sci Rep 3:2347

Li T, Chen X, Chaudhry MT, Zhang B, Jiang CY, Liu SJ (2014) Genetic characterization of 4-cresol catabolism in *Corynebacterium glutamicum*. J Biotechnol 192(Pt B):355–365

Liu YB, Long MX, Yin YJ, Si MR, Zhang L, Lu ZQ, Wang Y, Shen XH (2013) Physiological roles of mycothiol in detoxification and tolerance to multiple poisonous chemicals in *Corynebacterium glutamicum*. Arch Microbiol 195:419–429

Liu Y, Chen C, Chaudhry MT, Si M, Zhang L, Wang Y, Shen X (2014) Enhancing *Corynebacterium glutamicum* robustness by overexpressing a gene, *mshA*, for mycothiol glycosyltransferase. Biotechnol Lett 36:1453–1459

Merkens H, Beckers G, Wirtz A, Burkovski A (2005) Vanillate metabolism in *Corynebacterium glutamicum*. Curr Microbiol 51:59–65

Mills TY, Sandoval NR, Gill RT (2009) Cellulosic hydrolysate toxicity and tolerance mechanisms in *Escherichia coli*. Biotechnol Biofuels 2:26

Pacheco LG, Castro TL, Carvalho RD, Moraes PM, Dorella FA, Carvalho NB, Slade SE, Scrivens JH, Feelisch M, Meyer R, Miyoshi A, Oliveira SC, Dowson CG, Azevedo V (2012) A role for sigma factor σ^E in *Corynebacterium pseudotuberculosis* resistance to nitric oxide/peroxide stress. Front Microbiol 3:126

Parawira W, Tekere M (2011) Biotechnological strategies to overcome inhibitors in lignocellulose hydrolysates for ethanol production: review. Crit Rev Biotechnol 31:20–31

Park SD, Youn JW, Kim YJ, Lee SM, Kim Y, Lee HS (2008) *Corynebacterium glutamicum* σ^E is involved in responses to cell surface stresses and its activity is controlled by the anti-σ factor CseE. Microbiology 154:915–923

Plaggenborg R, Overhage J, Loos A, Archer JA, Lessard P, Sinskey AJ, Steinbüchel A, Priefert H (2006) Potential of *Rhodococcus* strains for biotechnological vanillin production from ferulic acid and eugenol. Appl Microbiol Biotechnol 72:745–755

Schirmer EC, Glover JR, Singer MA, Lindquist S (1996) HSP100/Clp proteins: a common mechanism explains diverse functions. Trends Biochem Sci 21:289–296

Schurig-Briccio LA, Farías RN, Rodríguez-Montelongo L, Rintoul MR, Rapisarda VA (2009) Protection against oxidative stress in *Escherichia coli* stationary phase. Arch Biochem Biophys 483:106–110

Shen X, Jiang C, Huang Y, Liu Z, Liu S (2005) Functional identification of novel genes involved in the glutathione-independent gentisate pathway in *Corynebacterium glutamicum*. Appl Environ Microbiol 71:3442–3452

Shen X, Zhou N, Liu S (2012) Degradation and assimilation of aromatic compounds by *Corynebacterium glutamicum*: another potential for applications for this bacterium? Appl Microbiol Biotechnol 95:77–89

Si MR, Long MX, Chaudhry MT, Xu Y, Zhang P, Zhang L, Shen X (2014) Functional characterization of *Corynebacterium glutamicum* mycothiol S-conjugate amidase. PLoS ONE 9:e115075

Si M, Xu Y, Wang T, Long M, Ding W, Chen C, Guan X, Liu Y, Wang Y, Shen X, Liu SJ (2015) Functional characterization of a mycothiol peroxidase in *Corynebacterium glutamicum* that uses both mycoredoxin and thioredoxin reducing systems in the response to oxidative stress. Biochem J 469:45–57

Stepanova E, Lee J, Ozerova M, Semenova E, Datsenko K, Wanner BL, Severinov K, Borukhov S (2007) Analysis of promoter targets for *Escherichia coli* transcription elongation factor GreA in vivo and in vitro. J Bacteriol 189:8772–8785

Tsujiyama S, Ueno M (2008) Formation of 4-vinyl guaiacol as an intermediate in bioconversion of ferulic acid by *Schizophyllum commune*. Biosci Biotechnol Biochem 72:212–215

Wang T, Si M, Song Y, Zhu W, Gao F, Wang Y, Zhang L, Zhang W, Wei G, Luo ZQ, Shen X (2015) Type VI secretion system transports Zn^{2+} to combat multiple stresses and host immunity. PLoS Pathog 11:e1005020

Winkler J, Kao KC (2011) Transcriptional analysis of *Lactobacillus brevis* to N-butanol and ferulic acid stress responses. PLoS ONE 6:e21438