Functional analysis and heterologous expression of bifunctional glutathione synthetase from *Lactobacillus*

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ABSTRACT

Bifunctional glutathione synthetase (GshF) has recently been reported to simultaneously catalyze the 2-step ATP-dependent biosynthesis of reduced glutathione (GSH). In this work, 19 putative *gshF* were mined from the complete sequenced genome of 20 representative *Lactobacillus* species. To functionally analyze these putative GshF, GshF from *Lactobacillus plantarum* and *Lactobacillus casei* were selected and successfully expressed in *Escherichia coli*. Compared with the control without expressing GshF, GSH titers were enhanced significantly in *E. coli* with overexpression of GshF, demonstrating that putative GshF from *Lactobacillus* have functional activities on GSH biosynthesis. Moreover, with the expression of GshF from *L. plantarum* in *E. coli* as a paradigm, GSH yield (286.5 μ M) was strongly improved by 177.9% with optimized induced conditions and precursor concentration compared with the control under unoptimized conditions. Transcriptional analysis showed that key genes of endogenous GSH metabolism and precursor biosynthesis were remarkably suppressed by GshF expression, indicating that the increase of GSH titer was attributed to heterologous expression of GshF. Overall, our results suggested that *gshF* is enriched in *Lactobacillus* and that heterologous expression of GshF is an efficient strategy for improving GSH biosynthesis.

Key words: bifunctional glutathione synthetase, glutathione biosynthesis, heterologous expression, *Lactobacillus*

INTRODUCTION

The genus *Lactobacillus*, as a member of versatile probiotic lactic acid bacteria (LAB), is widely present

in dairy products and other fermented foods, naturally inhabits the human environment, and has potential health-associated properties (Settachaimongkon et al., 2016; Xiong et al., 2017). Many species of *Lactobacillus* (e.g., *Lactobacillus plantarum* and *Lactobacillus casei*) have been commercialized in the probiotic market and widely applied in the food industry, such as milk fermentation and cheese production. With the advent of omics technologies, *Lactobacillus* have emerged as a group of model organisms for studies and research on LAB. For example, the whole-genome sequencing of *L. plantarum* WCFS1 isolated from human saliva has provided insight into potential probiotic properties such as adhesion and immunomodulation capacity (Kleerebezem et al., 2003; Siezen et al., 2012).

Reduced glutathione (GSH; γ -glutamyl-L-cysteinylglycine), a bioactive tripeptide consisting of L-Glu, L-Cys, and Gly, is one of the most abundant nonprotein thiol compounds in living organisms (Wang et al., 2016). Due to its physiological functions, including resistance to oxidative stresses, regulation of cellular redox homeostasis, and enhancement of host immunity (Yang et al., 2016), GSH is widely used in food, pharmaceutical, and cosmetic industries (Li et al., 2004). Typically, GSH is the ATP-dependent synthesis via 2 consecutively biosynthetic steps using soluble enzymes in the cytosol (Figure 1), γ -glutamylcysteine synthetase (also known as glutamate-cysteine ligase, EC 6.3.2.2; γ -GCS) and GSH synthetase (EC 6.3.2.3, GS; Li et al., 2004). First, L-Glu and L-Cys are catalyzed by γ -GCS to form the dipeptide γ -L-glutamyl-L-cysteine, which subsequently connects with Gly by GS to form GSH (Yang et al., 2016). The first reaction catalyzed by γ -GCS is the rate-limiting step of GSH biosynthesis because of the feedback inhibition of GSH at both the transcriptional and posttranslational levels (Xiong et al., 2015). Moreover, GSH peroxidase (EC 1.11.1.9; GPx) and GSH reductase (EC 1.8.1.7; GR) are the key enzymes involved in GSH metabolism. The former catalyzes the conversion of reduced GSH into

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Figure 1. The simplified pathway of reduced glutathione (GSH) synthesis. γ -GCS = γ -glutamylcysteine synthetase; GS = GSH synthetase; GshF = bifunctional GSH synthetase; GPx = GSH peroxidase; GR = GSH reductase; GSSG = GSH disulfide. Minus sign (–) denotes feedback inhibition. Color version available online.

its oxidized form glutathione disulfide, and the latter enzyme regenerates GSH from glutathione disulfide for maintaining thiol-redox homeostasis in cells (Pophaly et al., 2017).

Reduced glutathione is widely distributed in eukaryotic microorganisms and gram-negative bacteria (Pophaly et al., 2012) but rarely in gram-positive bacteria except some low-GC-content bacteria (i.e., *Streptococcus*, *Lactococcus*, *Lactobacillus*, *Enterococcus*, *Clostridium*, and *Listeria*). Interestingly, some gram-positive bacteria (e.g., *Streptococcus thermophilus*, *Streptococcus agalactiae*, and *Enterococcus faecalis*) have been reported to synthesize GSH, but no direct evidence shows the presence of either the activities of γ -GCS and GS or the genes *GGCS* and *GS* in the genome sequences of those strains (Li et al., 2005). Until recently, bifunctional GSH synthetase (**GshF**) was reported to simultaneously catalyze the 2-step ATP-dependent biosynthesis of GSH in those strains because GSH biosynthesis generally requires the consecutive reaction by γ -GCS and GS (Vergauwen et al., 2006; Ge et al., 2012). Bifunctional GSH synthetase contains an N-terminal γ -GCS-like domain followed by a typical ATP-grasp GS-like domain, but this GS-like domain

has no sequence homology with any known GS (Stout et al., 2012). Based on sequence alignment, more than 20 microbial species, most of which are gram-positive bacteria such as *L. plantarum* and *S. thermophilus*, have been found to contain GshF, of which most have not been studied by cloning and expression (Li et al., 2011). Bifunctional GSH synthetase is normally insensitive to high concentrations of GSH. Hence, using GshF for GSH biosynthesis, GSH titer could be even higher by alleviating the feedback inhibition of GSH (Li et al., 2011). In this work, the distribution of *gshF* in *Lactobacillus* was examined based on the complete sequenced genome of *Lactobacillus*. Biosynthesis of GSH was significantly improved in *E. coli* with heterologous overexpression of GshF from *Lactobacillus*. To our knowledge, it is still unclear how heterologous GshF affects exogenous metabolism in the host. Our study investigates the effect of heterologous GshF on exogenous GSH metabolism and GSH precursor biosynthesis in *E. coli* at the transcriptional level by real-time quantitative reverse transcription PCR (**RT-qPCR**) analysis.

MATERIALS AND METHODS

Plasmids, Strains, Regents, and Culture Conditions

All plasmids, strains, and primers in this study are listed in Tables 1 and 2. *Escherichia coli* Top10 was used as the host for plasmid construction. *Escherichia coli* BL21(DE3) was used for gene expression. Restriction enzymes and T4 DNA ligase were purchased from Takara Biomedical Technology Co. Ltd. (Beijing, China). The PrimeSTAR HS DNA polymerase (Takara) was used for DNA amplification. Kits for DNA fragment purification and plasmid extraction were provided by Axygen Biosciences (Corning Inc., Jiangsu, China). All primers and other reagents were purchased by Shanghai Sangon Biotech Co. Ltd. (Shanghai, China). Luria-Bertani medium was used for plasmid construction and gene expression. According to the resistance marker of plasmids, kanamycin (50 mg/L) was added in each culture. *Lactobacillus plantarum* WCFS1 and *L. casei*

Table 1. Strains and plasmids used in this study

Strain/plasmid	Relevant characteristics	Source/reference
<i>Escherichia coli</i> Top10	F [–] mcrA Δ(mrr-hsdRMS-mcrBC) ϕ80lacZΔM15 ΔlacX74 nupG recA1 araD139 Δ(ara-leu)7697 galE15 galK16 rpsL(Str ^R) endA1 λ [–]	Invitrogen (Carlsbad, CA)
<i>E. coli</i> BL21(DE3)	F [–] ompT gal dcm lon hsdSB(rB [–] mB [–]) λ(DE3 [lacI lacUV5-T7p07 ind1 sam7 nin5])	Invitrogen
<i>Lactobacillus plantarum</i> WCFS1	Bifunctional GSH1 synthetase gene (<i>gshF</i>); 2,256 bp	Our laboratory
<i>Lactobacillus casei</i> WCFS1	Bifunctional GSH synthetase gene (<i>gshF</i>); 1,965 bp	Xiong et al. (2017)
pET24a	Protein expression vector of <i>E. coli</i> , kanamycin resistance	Invitrogen
pZX12	pET24a derived, carrying a <i>gshF</i> gene from <i>L. plantarum</i> at <i>NdeI</i> and <i>XhoI</i> sites, kanamycin resistance	This work

¹GSH = reduced glutathione.

LC2W were cultured in de Man, Rogosa and Sharpe medium under anaerobic conditions at 37°C (Xiong et al., 2017).

Mining and Phylogenetic Analyses of *GshF* Based on the Genomes of *Lactobacillus*

The complete genome sequences of 20 representative *Lactobacillus* species, including *L. acidophilus* NCFM (NC_006814), *L. amylovorus* GRL 1112 (NC_014724), *L. brevis* ATCC 367 (NC_008497), *L. buchneri* NRRL B-30929 (NC_015428), *L. casei* LC2W (NC_017473), *L. crispatus* ST1 (NC_014106), *L. delbrueckii* bulgaricus ATCC 11842 (NC_008054), *L. fermentum* CECT 5716 (NC_017465), *L. gasserii* ATCC 33323 (NC_008530), *L. helveticus* CNRZ32 (NC_021744), *L. johnsonii* DPC 6026 (NC_017477), *L. kefirifaciens* ZW3 (NC_015602), *L. paracasei* 8700:2 (NC_022112), *L. plantarum* WCFS1 (NC_004567), *L. reuteri* DSM 20016 (NC_009513), *L. rhamnosus* ATCC 8530 (NC_017491), *L. ruminis* ATCC 27782 (NC_015975), *L. sakei* 23K (NC_007576), *L. salivarius* CECT 5713 (NC_017481), and *L. sanfranciscensis* TMW 1.1304 (NC_015978), were downloaded from NCBI (<https://www.ncbi.nlm.nih.gov>) and used to construct a local database using Bioedit software (<http://www.mbio.ncsu.edu/bioedit/bioedit.html>). Mining of *GshF* was performed by using Blast (BLOSUM62 scoring matrix) searches of a local database of *Lactobacillus* to identify sequences homologous to the reported functional *GshF* from *L. plantarum* and *S. thermophilus* (Li et al., 2011;

Ge et al., 2012) with a minimum 25% identity and an e-value of 1^{-15} or lower. The respective sequences were extracted from the local database using Bioedit software. Amino acid sequences of *GshF* were aligned and used to construct a phylogenetic tree using a neighbor-joining algorithm with bootstrap replication of 1,000 in Mega6 software (Tamura et al., 2013).

Plasmid Construction

All molecular manipulations including restriction enzyme digestions, gene cloning, plasmid transformation, and protein expression were performed as described by Sambrook and Russell (2001). The *gshF* genes from *L. plantarum* and *L. casei* (designated as *gshF-Lp* and *gshF-Lc*, respectively) were cloned by PCR amplification using primers *gshF-Lp-F/gshF-Lp-R* and *gshF-Lc-F/gshF-Lc-R*, respectively. The purified PCR products of *gshF* were excised by *NdeI* and *XhoI* and inserted into plasmid pET24a to create pZX12 and pZX08, respectively.

Fermentation of Engineered *E. coli* for *GshF* Expression and GSH Production

Plasmids pZX12, pZX08, and pET24a (the control) were transformed into *E. coli* BL21(DE3). The clones were grown in Luria-Bertani medium at 37°C overnight and preserved at -80°C using 30% glycerol in 1:1 ratio with the cultures as frozen stock cultures. The frozen seeds were inoculated by a 1:100 dilution in a 250-mL

Table 2. Primers used in this study

Primer ¹	Sequence ² (5' → 3')	Restriction enzyme
<i>gshF-Lp-F</i>	GGAATTCCATATGATGGAATTAGATGCCGTTGGTAAGGAATTG	<i>NdeI</i>
<i>gshF-Lp-R</i>	CCGCTGAGATCTTCATTTTAAACAATGCATCCAACAA	<i>XhoI</i>
<i>gshF-Lc-F</i>	GGAATTCCATATGATGTTGAATCATTATTGGCAACT	<i>NdeI</i>
<i>gshF-Lc-R</i>	CCGCTGAGGCTAGCTTATTTTCGCTAAACAGATACGTG	<i>XhoI</i>
<i>GGCS</i> -realtime-F	CTGACTTTCTCTGCCCCGATC	
<i>GGCS</i> -realtime-R	TCCGCTACAAAATCCTCTTCG	
<i>GS</i> -realtime-F	CCCATCGCAAACATCAACATC	
<i>GS</i> -realtime-R	GATCGCCCATCTCCATATAGTG	
<i>GPx</i> -realtime-F	AGAAGTTCGCCGGTAATGTG	
<i>GPx</i> -realtime-R	TCTCCAACGTGCTCATATTGCG	
<i>GR</i> -realtime-F	ACTATGATTACATCGCCATCGG	
<i>GR</i> -realtime-R	CACAGCCAACATTTACGCAG	
<i>gltB</i> -realtime-F	TTTATCTGGATCTTGCGGACC	
<i>gltB</i> -realtime-R	GTACGGTGTAGTGGAGAAGC	
<i>cysK</i> -realtime-F	ACCATGAGTATTGAACGCCG	
<i>cysK</i> -realtime-R	AGGTATTTCTCTGGATTGCTGG	
<i>glyA</i> -realtime-F	CAAGCCAAAGAACAAGCC	
<i>glyA</i> -realtime-R	CATATCAACGAACAGGTAAGCAC	
<i>ltaE</i> -realtime-F	GACCCTACCGTTAATGCTCTG	
<i>ltaE</i> -realtime-R	ATATACTCTTCGCCACGTTTCG	
<i>kbl</i> -realtime-F	TTGATGCTAACGGTGGACTG	
<i>kbl</i> -realtime-R	TGGCATAGCGATAGCGTTTAG	

¹F = forward; R = reverse.

²Underlining denotes site of restriction enzyme.

Erlenmeyer flask containing 30 mL of production medium. Isopropyl β -D-1-thiogalactopyranoside (IPTG) was initially added to 0.1 mM and the cultures were incubated at 37°C and 250 rpm for 6 h. The expression and molecular weights of GshF in *E. coli* were estimated by SDS-PAGE. The IPTG and addition time of IPTG as well as the precursors Gly, L-Glu, and L-Cys were tested to determine the optimal concentrations of each component for GSH production.

RNA Preparation and RT-qPCR Analysis

The *E. coli* cells from fermentation medium were collected and total RNA was isolated with an RNA isolation kit (Axygen Biosciences). Genome DNA was removed by digestion with DNase I (Takara) and verified by PCR analysis with the RNA as the template. The quantification and integrity of RNA were determined by both Nanodrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA) and agarose gel electrophoresis. Data were verified in 3 independent experiments. The cDNA was prepared by PrimeScriptRT reagent kit (Takara) and used as template for RT-qPCR, which was performed with a new Lightcycle 96 real-time PCR system (Roche Diagnostics, Risch-Rotkreuz, Switzerland) using an SYBR Premix Ex TaqII kit (Takara). The primers of RT-qPCR are listed in Table 2. The data of each gene were analyzed and normalized using $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001) with the *clpB* gene encoding ClpB chaperone as an internal control (Wang et al., 2015).

Analytical Methods

Cell growth was measured by optical density (OD) at 600 nm with a UV-visible spectrophotometer (UV-1600PC, Mapada Ltd., Shanghai, China). The cells of fermentation broths were harvested by centrifugation at $13,201 \times g$ for 1 min. The cell pellets were resuspended and sonicated for 20 min. The cell lysate was centrifuged at 12,000 rpm for 10 min and analyzed by SDS-PAGE. The gel was stained with Coomassie Brilliant Blue staining (R250) and was used to determine protein expression by a low-molecular-weight protein marker (Takara) using an image analysis system (Bio-Rad, Hercules, CA).

Ten milliliters of the culture broth was centrifuged at 12,000 rpm for 1 min, and the supernatant was decanted for GSH assay. The GSH was extracted from *E. coli* cells by 40% ethanol and detected with the alloxan method (Xiong et al., 2015). Reduced glutathione reacted with alloxan (1 g/L) in a solution of glycine (0.1 M) and NaH_2PO_4 - NaH_2PO_4 buffer solution (0.24 M, pH 7.6) for 20 min and was determined by OD₃₀₅

with UV spectrophotometry. Specific GSH yield (%) was calculated by GSH concentration/OD₆₀₀. All of the assays and experiments were performed in triplicate for each *E. coli* strain with the same transformant.

RESULTS AND DISCUSSION

Distribution of GshF in Lactobacillus Genomes

The distribution of putative *gshF* was examined by genomic analysis of the complete genomes of 20 representative *Lactobacillus* species. In silico analysis revealed the presence of putative *gshF* in 11 *Lactobacillus* species, including *L. brevis*, *L. buchneri*, *L. casei*, *L. fermentum*, *L. paracasei*, *L. plantarum*, *L. reuteri*, *L. rhamnosus*, *L. ruminis*, *L. sakei*, and *L. salivarius* (Figure 2A). The domain analysis of 19 putative GshF using the NCBI conserved domain search tool confirmed that all putative GshF possessed the domain of the PRK02471 superfamily (accession no. cl25875) responsible for bifunctional γ -GCS/GS, suggesting that they have the potential to synthesize GSH using the precursor AA. The presence of putative *gshF* in genomes indicated the ability of GSH biosynthesis in *Lactobacillus*, which directly demonstrated why many *Lactobacillus* species could produce GSH (Pophaly et al., 2012). In addition, *L. buchneri*, *L. fermentum*, *L. plantarum*, and *L. reuteri* have multiple gene numbers of *gshF* (designated *gshF-n*, $n \geq 2$) distributed in the genomes, suggesting that they may be highly resistant to extreme environments due to the stronger ability of GSH biosynthesis.

Phylogenetic tree analysis showed that GshF in *Lactobacillus* species could be divided into 3 distinct types that had low sequence identities and longer genetic distances but were highly conserved in evolution (Figure 2B). For evolution distances, GshF in *Lactobacillus* were located relatively close to γ -GCS of *E. coli*, not GS of *E. coli*, implying that evolutionary GS may appear earlier than γ -GCS and GshF (Copley and Dhillon, 2002). The GshF-n in the same *Lactobacillus* species showed low identities with each other and had longer distances to each other. For example, 4 GshF (from GshF-1 to GshF-4) were discovered from the genome of *L. fermentum*; their sequence identities were less than 50% with each other using NCBI Blastp search (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>).

Heterologous Expression of GshF from Lactobacillus in E. coli

The majority of GshF in *Lactobacillus* (12/19 GshF) were assigned to type I based on a phylogenetic tree (Figure 2B). Thus, type I GshF from *L. plantarum* and

L. casei (GshF-Lp and GshF-Lc) were selected as the model GshF for functional analysis. The genes *gshF-Lp* and *gshF-Lc* were cloned from *L. plantarum* WCFS1 and *L. casei* LC2W by PCR and inserted into the expression vector pET24a to create pZX12 and pZX08 (Figure 3). The plasmids were sequenced by Sangon Biotech (Shanghai, China), showing that *gshF-Lp* and *gshF-Lc* with high identities of 100% using NCBI Blastx analysis were successfully inserted into pET24a. When pZX12 and pZX08 were transformed into the ex-

pression host *E. coli* BL21(DE3), SDS-PAGE was used to analyze the protein expression. Using Vector NTI Advance software (Invitrogen, Carlsbad, CA), GshF-Lp and GshF-Lc had theoretical molecular weights of 83.1 and 71.3 kDa as well as isoelectric points of 6.65 and 5.85, respectively. Compared with the control *E. coli* harboring empty vector pET24a, *E. coli* containing pZX12 or pZX08 had a desired band at 70 to 85 kDa, suggesting that *E. coli* could significantly express heterologous GshF proteins from *Lactobacillus* (Figure 4A).

Figure 2. Presence and phylogenetic analysis of bifunctional glutathione synthetase (GshF) in representative *Lactobacillus* genomes. (A) Gene number of *gshF* in 20 selected *Lactobacillus* species. (B) Phylogenetic analysis of GshF in *Lactobacillus*. The protein sequences were used to construct a phylogenetic tree using a neighbor-joining algorithm with bootstrap replication of 1,000 with γ-glutamylcysteine synthetase (γ-GCS) and reduced glutathione synthetase (GS) of *Escherichia coli* BL21(DE3) as the control. Color version available online.

Figure 3. Schematic representation of the recombinant expression vectors containing the *gshF* genes from *Lactobacillus plantarum* WCFS1 and *Lactobacillus casei* LC2W. Color version available online.

The feedback inhibition of γ -GCS activity by GSH at both the transcriptional and posttranslational levels is considered to be a biochemical mechanism that prevents the overaccumulation of GSH in living cells (Ge et al., 2012). To overcome this problem in GSH production, a strategy with the use of GshF that is insensitive to GSH feedback inhibition was developed to improve the rate of GSH biosynthesis even in the presence of a high concentration of GSH (Wang et al., 2011, 2016; Ge et al., 2012; Yang et al., 2016). Hence, to demonstrate that GshF-Lp and GshF-Lc were functionally expressed in *E. coli*, GSH biosynthesis was examined in this work.

If GSH concentration was significantly improved in cells with expressing GshF, it indicated functional expression and strong activities of GshF. For intracellular GSH titer, *E. coli* harboring pZX12 and pZX08 had 103.1 and 96.7 μ M GSH, respectively, in comparison with *E. coli*/pET24a (8.3 μ M GSH, the control). Hence, GshF definitely enhanced GSH biosynthesis in *E. coli*, suggesting that GshF from *Lactobacillus* had functional activities for GSH biosynthesis (Figure 4B).

Effects of Culture Condition on GSH Biosynthesis in *E. coli* Expressing GshF from *Lactobacillus*

Vast literature has reported that culture condition (i.e., induction condition) could strongly affect protein expression and activity and eventually affect the titer of target product (Fucinos et al., 2014). In this study, *E. coli*/pZX12 containing *gshF*-Lp had higher GSH titer than *E. coli*/pZX08 containing *gshF*-Lc. Therefore, *E. coli*/pZX12 was used for the next experiments to examine whether GSH biosynthesis could further improve by affecting GshF expression with the change of culture condition. For the expression of recombinant proteins using the pET-series vectors with T7 promoter, 1 mM IPTG is recommended for full induction; however, proper induction was generally obtained at low IPTG concentrations (e.g., 0.01 mM; Fucinos et al., 2014). Thus, the effects of induction conditions such as IPTG concentration and induction time were investigated in this study. The cells were initially induced with a range of IPTG concentrations between 0.1 and 1 mM. There was a marked increase in the GSH titer when IPTG

Figure 4. Expression of bifunctional glutathione synthetase (GshF) and reduced glutathione (GSH) titer in *Escherichia coli* BL21(DE3). (A) Expression of GshF by SDS-PAGE. Lane M = low-molecular-weight marker (Takara Biomedical Technology Co. Ltd., Beijing, China); lane 1 = control *E. coli* BL21(DE3)/pET24a; lane 2 = *E. coli* BL21(DE3)/pZX08; lane 3 = *E. coli* BL21(DE3)/pZX12. Arrows indicate the expression of GshF. (B) The GSH titers in *E. coli* BL21(DE3)/pZX12 and *E. coli* BL21(DE3)/pZX08 when isopropyl β -D-1-thiogalactopyranoside was initially added to 0.1 mM and the cultures were incubated at 37°C and 250 rpm for 6 h. Error bars indicate SD of the mean of triplicate. Color version available online.

Figure 5. The effects of (A) isopropyl β -D-1-thiogalactopyranoside (IPTG) concentration and (B) induction time on reduced glutathione (GSH) production in *Escherichia coli* BL21(DE3)/pZX12. Error bars indicate SD of the mean of triplicate. Color version available online.

concentration was increased from 0.1 to 0.8 mM (Figure 5A). The highest yield of GSH (220 mM) was reached with 0.8 mM IPTG. Various induction time between 2 and 6 h was also investigated for GSH production. It was apparent that 5 h of induction time gave the highest GSH titer (Figure 5B).

The addition of GSH precursors Gly, L-Glu, and L-Cys can significantly stimulate GSH biosynthesis (Xiong et al., 2015). Yield of GSH could also gradually improve with the increasing concentration of precursors (Fei et al., 2009; Wang et al., 2012). For example, using *Pichia pastoris* harboring γ -GCS and GS of GSH biosynthetic pathway from *S. cerevisiae*, GSH titer was obviously improved from 2.03 g/L to 4.15 g/L when the precursor concentration was increased from 5 to 15 mmol/L (Fei et al., 2009). Various concentrations of Gly, L-Glu, and L-Cys ranging from 0 to 20 mM were initially added into the medium for GSH biosynthesis in this work (Figure 6). When precursor concentration was increased from 0 to 5 mM, cell density was almost not changed, but GSH titer and specific GSH yield were significantly increased. Cell density and GSH titer were gradually decreased with the increase of precursor concentration from 5 to 20 mM; cell growth was inhibited at 20 mM precursor (Figure 6A). The highest GSH titer and specific GSH yield were 286.5 μ M and 173.3 μ M/OD₆₀₀ (Figure 6B and 6C), which were improved by 27.9 and 31.9%, respectively, in comparison with the control (without addition of precursors).

Transcriptional Analysis of GSH Metabolism and Precursor Biosynthesis in *E. coli* Expressing GshF from *Lactobacillus*

To our knowledge, there is no report on the systemic effect of heterologous expression of GshF in the host. Hence, to assess how GshF expression affects endogenous metabolism in *E. coli*, transcriptional change

Figure 6. The effect of reduced glutathione (GSH) precursors on (A) cell growth, (B) GSH titer, and (C) specific GSH yield in *Escherichia coli* BL21(DE3)/pZX12. OD = optical density. Error bars indicate SD of the mean of triplicate. Color version available online.

Figure 7. The transcriptional change in the key genes of (A) reduced glutathione (GSH) metabolism and (B) GSH precursor (Gly, L-Glu, and L-Cys) biosynthesis in *Escherichia coli* BL21(DE3)/pZX12 in comparison with the control strain BL21(DE3)/pET24a. Error bars indicate SD of the mean of triplicate. Color version available online.

of the key genes of endogenous GSH metabolism and GSH precursors Gly, L-Glu, and L-Cys biosynthesis was investigated by RT-qPCR. Compared with the control *E. coli*/pET24a (without expressing GshF), all genes of endogenous GSH metabolism (*GGCS*, *GS*, *GPx*, and *GR*) were clearly suppressed more than 5-fold at the transcriptional level in *E. coli*/pZX12 (Figure 7A), suggesting that GshF expression resulted in the decrease of endogenous GSH biosynthesis. It also may be partly due to the feedback inhibition of high GSH concentration. Moreover, it further showed that high GSH titer in *E. coli*/pZX12 should be attributed to heterologous expression of GshF. Interestingly, the key genes of GSH precursor biosynthesis—*gltB* (responsible for glutamate synthase, large subunit), *cysK* (responsible for cysteine synthase A), *glyA* (responsible for serine hydroxymethyltransferase), *ltaE* (responsible for L-allo-threonine aldolase), and *kbl* (responsible for glycine C-acetyltransferase)—were also strongly inhibited more than 4-fold in *E. coli*/pZX12 (Figure 7B). Notable was the >130-fold suppression of *cysK*, the key gene for cysteine biosynthesis, at 6 h. Our result indicated that GshF expression reduces or even inhibits the biosynthesis of precursor AA. It seemed contradictory to high GSH titer in *E. coli*/pZX12, but it was reasonable by the effect of exogenous addition of abundant precursors. To our knowledge, this is the first report about the effect of heterologous expression of GshF on endogenous metabolism in the host.

Based on the literature (Li et al., 2011; Hara et al., 2012; Zhang et al., 2016b), construction of the high-yield strains is important to GSH biosynthesis. Except engineered target genes of GSH and precursor metabolism [i.e., *GGCS*, *GS*, *GPx*, *GR*, *ggt* (encoding glutathione hydrolase), *pepT* (encoding peptidase T), *sdaA* (encoding L-Ser deaminase I), *MET14* (encoding

adenylylsulfate kinase), *MET16* (encoding 3'-phospho-adenylylsulfate reductase), and *CYS4* (encoding cystathionine β -synthase) in the host (Suzuki et al., 2011; Ge et al., 2012; Hara et al., 2012; Zhang et al., 2016b)], our study showed that heterologous expression of GshF could be an efficient strategy for constructing microbial cell factories with high GSH yield. In recent years, GSH have been reported to protect LAB against various environmental stresses (Lee et al., 2010; Zhang et al., 2010, 2012, 2016a). Therefore, overexpression of *gshF* could provide a useful method for the increased viability and productivity of LAB during industrial processes and gastrointestinal transit.

CONCLUSIONS

Nineteen putative *gshF* genes were mined from representative *Lactobacillus* genomes in this study, indicating that *gshF* is enriched in *Lactobacillus*. To characterize these putative GshF, GshF-Lp and GshF-Lc were selected and overexpressed in *E. coli* BL21(DE3). Our result showed the functional activities of putative GshF from *Lactobacillus*, which could remarkably improve GSH biosynthesis. Compared with the control, GSH titer (286.5 μ M) was significantly increased by 177.9% through optimizing induced condition and precursor concentration. Based on RT-qPCR analysis, the key genes of GSH metabolism and precursor biosynthesis were strongly inhibited by heterologous GshF expression, suggesting that the increase of GSH titer was attributed to GshF expression.

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