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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 μM) 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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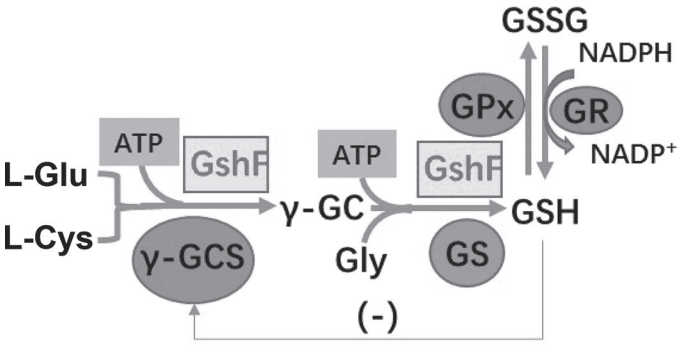

Figure 1. The simplified pathway of reduced glutathione (GSH) synthesis. γ-GCS = γ-glutamylcysteine synthetase; GS = GSH synthetase; GshF = bifunctional GSH synthetase; GPx = GSH peroxidase; GR = GSH reductase; GSSG = GSH disulfide. Minus sign (−) denotes feedback inhibition. Color version available 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
Escherichia coli 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)
E. coli BL21(DE3)	F^- ompT gal dcm lon hsdSB(rB^- mB^-) λ(DE3 [lacI lacUV5-T7p07 ind1 sam7 nin5])	Invitrogen
Lactobacillus plantarum WCFS1	Bifunctional GSH1 synthetase gene (*gshF*); 2,256 bp	Our laboratory
Lactobacillus casei WCFS1	Bifunctional GSH synthetase gene (*gshF*); 1,965 bp	Xiong et al. (2017)
pET24a	Protein expression vector of *E. coli*, kanamycin resistance	Invitrogen
pZX12	pET24a derived, carrying a *gshF* gene from *L. plantarum* at *Nde*I and *Xho*I sites, kanamycin resistance	This work

[1]GSH = reduced glutathione.