Expression of Bacterial GshF in *Pichia pastoris* for Glutathione Production

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Conventionally, two consecutive enzymatic reactions catalyzed by γ-glutamylcysteine synthetase and glutathione synthetase are most commonly used for glutathione production. Here we demonstrate that bacterial bifunctional GshF can be used for glutathione production in a eukaryotic system without accumulation of the intermediate γ-glutamylcysteine.

Glutathione (γ-glutamylcysteinylglycine; GSH) is the predominant nonprotein thiol present in living organisms (16). It plays an important role in many physiological functions and has a number of diverse applications in the food, pharmaceutical, and cosmetic industries (12, 18). GSH is industrially produced by fermentation or enzymatic synthesis. By either process, GSH is synthesized through two consecutive enzymatic reactions catalyzed by γ-glutamylcysteine synthetase (γ-GCS, encoded by *gshA* in *Escherichia coli*, or *GSHI* in *Saccharomyces cerevisiae*) and GSH synthetase (GS, encoded by *gshB* in *E. coli* or *GSHII* in *S. cerevisiae*), which are widely present in eukaryotes and Gram-negative bacteria. These two enzymes, especially those derived from *E. coli* and *S. cerevisiae*, have been overexpressed in various model microorganisms to generate genetically engineered strains with higher GSH production capabilities. Previous studies have reported the overexpression of the *E. coli gshA* and *gshB* genes in *Lactococcus lactis* (13), *Clostridium acetobutylicum* (23), *S. cerevisiae* (17), and *E. coli* (8, 14). Overexpression of the *S. cerevisiae* *GSHI* and *GSHII* genes in *S. cerevisiae* (5, 20) and *Pichia pastoris* (6) has also been reported.

While researchers have focused mainly on improving GSH productivity, one fact that is often overlooked is accumulation of the intermediate metabolite, i.e., the dipeptide γ-glutamylcysteine (γ-GC), during these processes. For instance, when the *gshA* and *gshB* genes from *E. coli* were overexpressed in *L. lactis* (13), the intracellular molar ratio of γ-GC to GSH reached 9:1 in chemically defined medium and 1:1 in complex medium (M17 broth). Overexpression of the *E. coli gshA* and *gshB* genes in *C. acetobutylicum* also led to the intracellular accumulation of a significant amount of γ-GC (25% of the GSH produced; molar ratio) (23). When *GSHI* and *GSHII* from *S. cerevisiae* were overexpressed in *P. pastoris*, accumulation of γ-GC was also observed (this study; unpublished data). The accumulation of γ-GC might be attributed to the imbalanced activities of γ-GCS and GS. γ-GC accumulation is an undesirable feature in the production of GSH on an industrial scale, as it not only reduces the GSH yield but also increases the cost of GSH purification.

Recently, a novel bifunctional enzyme possessing both γ-GCS and GS activities (termed γ-GCS-GS or GshF, encoded by *gshF*) was identified and characterized in some Gram-positive bacteria, e.g., *Streptococcus agalactiae* (9, 19), *Listeria monocytogenes* (7), and *Pasteurella multocida* (19, 21). The emergence of this novel enzyme provides an alternative approach to solve the problem of γ-GC accumulation, because the colocalization of γ-GCS and γ-GS modules in GshF is expected to increase the effective concentration of γ-GC, thereby increasing the catalytic rate of the second enzymatic reaction. GshF also has other potential advantages over the conventional γ-GCS/GS systems when used for GSH production. First, the feedback inhibition of γ-GCS activity by GSH is considered a physiological mechanism to prevent the overaccumulation of GSH (1, 2). GshF from *S. agalactiae* and *Streptococcus thermophilus* was shown to be insensitive to an increased concentration of GSH (9, 11). Therefore, the use of GshF might be able to maintain the GSH biosynthesis rate even in the presence of a high concentration of GSH. Second, the manipulation and engineering of a single gene is supposed to be easier than the manipulation of two genes. To date, the value of GshF in the biotechnological production of GSH has not been fully exploited. Only recently, Li et al. (11) have successfully expressed GshF from *S. thermophilus* in *E. coli*. A recombinant *E. coli* strain with GshF activity was used for the enzymatic production of GSH using three precursor amino acids. However, due to the low ATP regeneration efficiency of *E. coli*, this process was coupled with an extraneous ATP regeneration system of *S. cerevisiae*, making further process optimization complex.

The objective of this study was to introduce bacterial GshF into eukaryotic cells so that the strong ATP-generating ability of the eukaryotic system can be used for one-step GSH biosynthesis. The methylotrophic yeast *P. pastoris* was chosen as the host because it had several attractive features beneficial for high-level GSH production, such as a low nutrient requirement and ease of growth to a high cell density (2a, 3, 4). The GshF proteins, from *L. monocytogenes*, *S. agalactiae*, and *Lactobacillus plantarum*, were each cloned and expressed in *P. pastoris* GS115. The GSH production capabilities and other characteristics of the resulting recombinant strains were then determined. The data demonstrated that bacterial bifunctional GshF can be used for GSH production in a eukaryotic system.

Cloning of *gshF* genes derived from bacteria. Lm-*gshF*, Sa-*gshF*, and Lp-*gshF*, derived from *L. monocytogenes*, *S. agalactiae*,

TABLE 1 PCR-related information for cloning of *gshF* genes

Gene name	Source	Primer pair used for amplification[a]	Gene size (bp)	Closest homologue(s) (% homology)
Lm-gshF	*L. monocytogenes*	LmgshF-F, 5′-AG<u>ATCGAT</u>ACCATGATAAAACTTGATATGAC-3′; LmgshF-R, 5′-CA<u>GCGGCCGC</u>TTAGTCAAATAAGAAATCTAA-3′	2,346	*L. monocytogenes* M7, L99, HCC23 (99)
Sa-gshF	*S. agalactiae*	SagshF-F, 5′-AG<u>ATCGAT</u>ACCATGATTATAGATCGACTGTTACAAAGAAGCCACTCTCATCTAC-3′; SagshF-R, 5′-CTA<u>GCGGCCGC</u>TTAGCCTAAGGAACTCTTTTTAGTTA-3′	2,276	*S. agalactiae* NEM316, 2603V/R, A909 (100)
Lp-gshF	*L. plantarum*	LpgshF-F, 5′-AG<u>ATCGAT</u>ACCATGGAATTAGATGCCGTTGGTA-3′; LpgshF-R, 5′-CA<u>GCGGCCGC</u>TTAATCTTCATTTTTAAACAATGC-3′	2,256	*L. plantarum* WCFS1 (100)

[a] ClaI and NotI sites are underlined.

and *L. plantarum*, respectively, were amplified by PCR from the respective genomic DNA. The sequences of the primers used and other PCR-related information are summarized in Table 1. The Lm-*gshF* gene was obtained from *L. monocytogenes* strain ATCC 19114-3 genomic DNA using LmgshF-F and LmgshF-R, creating ClaI and NotI sites (underlined in Table 1), respectively. Similarly, Sa-*gshF* and Lp-*gshF* were amplified using SagshF-F/SagshF-R and LpgshF-F/LpgshF-R, respectively. Three fragments of the expected size (around 2.3 kb) were thus obtained (Fig. 1A). The nucleotide sequence of Lm-*gshF* showed 99% identity with those of *L. monocytogenes* M7, L99, and HCC23. The nucleotide se-

quence of Sa-*gshF* had 100% identity with those of *S. agalactiae* NEM316, *S. agalactiae* 2603V/R and A909. The nucleotide sequence of Lp-*gshF* had 100% identity with that of *L. plantarum* WCFS1.

TABLE 2 Strains and plasmids used in this study

| Strain or plasmid	Relevant characteristics	Source or reference
Strains		
E. coli DH5α		TaKaRa
P. pastoris GS115	*his4*⁻ Mut⁺	Invitrogen
S. cerevisiae S288c		Our lab
L. monocytogenes		Our lab
S. agalactiae		Our lab
L. plantarum		Our lab
GS115/LmgshF	GS115 integrated with recombinant plasmid pGAPZH-LmgshF	This study
GS115/SagshF	GS115 integrated with recombinant plasmid pGAPZH-SagshF	This study
GS115/LpgshF	GS115 integrated with recombinant plasmid pGAPZH-LpgshF	This study
GS115/Sc288c	GS115 integrated with recombinant plasmid pGAPZH-ScGSH	This study
Plasmids		
pGAPZαA	Vector for constitutive secreted protein expression; Zeoʳ	Invitrogen
pPIC9k	Vector for inducible secreted protein expression; *HIS4*; Kanʳ Ampʳ	Invitrogen
pGAPZαAH	pGAPZαA-based expression vector carrying *HIS4* fragment from pPIC9k; Zeoʳ	This study
pGAPZH-Scgsh1	pGAPZαAH containing *gsh1* from *S. cerevisiae* S288c; Zeoʳ	This study
pGAPZH-Scgsh2	pGAPZαA containing *gsh2* from *S. cerevisiae* S288c; Zeoʳ	This study
pGAPZH-ScGSH	pGAPZαAH containing *gsh1 and gsh2* from *S. cerevisiae* S288c; Zeoʳ	This study
pGAPZH-LmgshF	pGAPZαAH containing *gshF* from *L. monocytogenes*; Zeoʳ	This study
pGAPZH-LpgshF	pGAPZαAH containing *gshF* from *L. plantarum*; Zeoʳ	This study
pGAPZH-SagshF	pGAPZαAH containing *gshF* from *S. agalactiae*; Zeoʳ	This study
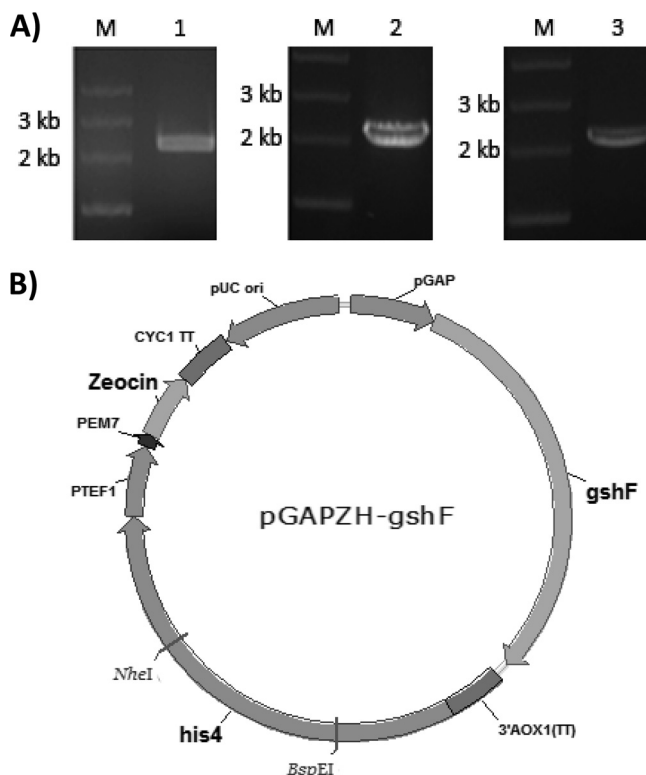
FIG 1 Cloning and expression of *gshF* genes from three different Gram-positive bacteria in *P. pastoris* GS115. (A) PCR amplification of the three *gshF* genes. Lanes M, molecular size markers; lane 1, *L. monocytogenes gshF*; lane 2, *S. agalactiae gshF*; lane 3, *L. plantarum gshF*. (B) Schematic representation of the construction of a recombinant expression vector containing the *gshF* genes derived from Gram-positive bacteria.
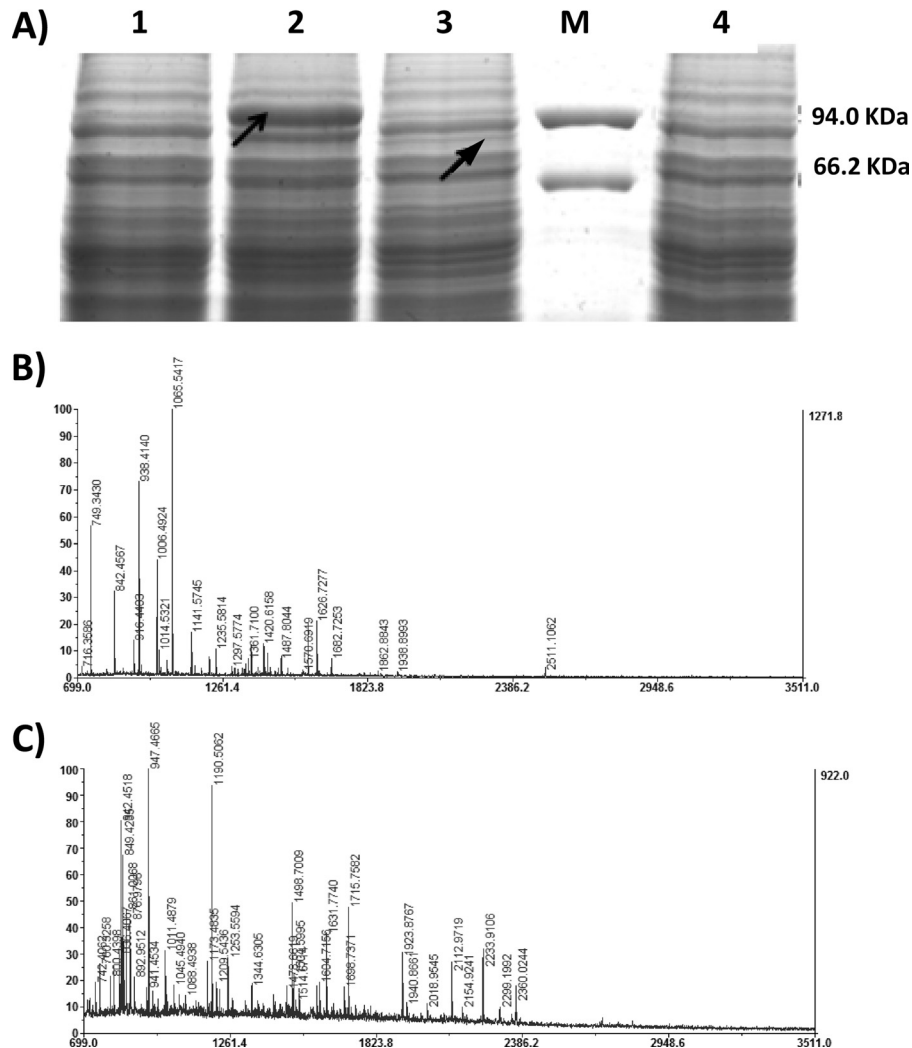
FIG 2 Expression of *gshF* genes of *L. monocytogenes*, *S. agalactiae*, and *L. plantarum* in *P. pastoris*. (A) GshF expression analysis by SDS-PAGE. Recombinant *P. pastoris* cells were harvested after culture for 42 h in YPD. Cell lysate samples were subjected to 10% SDS-PAGE and then stained with Coomassie blue. Lane 1, GS115(control); lane 2, GS115/lmgshF; lane 3, GS115/LpgshF; lane 4, GS115/SagshF; lane M, protein molecular size markers. The arrows indicate the positions of GshF bands. (B) Peptide mass fingerprint of overexpressed bands in cell lysate of GS115/lmgshF (indicated by the left arrow in panel A). A MASCOT score of >82 (the default MASCOT threshold for such searches) was accepted as significant (*P* < 0.05). (C) Peptide mass fingerprint of overexpressed bands in cell lysate of GS115/LpgshF (indicated by the right arrow in panel A). A MASCOT score of >82 was accepted as significant (*P* < 0.05).

Construction of recombinant strains. The nucleotide sequence of *HIS4* was amplified from plasmid pPIC9k by PCR using oligonucleotide primers his4-F (5′-CTAGATCTATGACATTTCCCTTGCTACCTGCA-3′ [BglII site underlined]) and his4-R (5′-CTGGATCCTTAAATAAGTCCCAGTTTCTC-3′ [BamHI site underlined]). All of the strains and plasmids used and generated in this study are summarized in Table 2. The 2,921-bp product obtained was inserted into the BamHI site of the vector pGAPZαA, yielding plasmid pGAPZαAH. The ClaI/NotI PCR fragments of Lm-*gshF*, Sa-*gshF*, and Lp-*gshF* were cloned into the pGAPZαAH expression vector to form pGAPZH-Lmgsh, pGAPZH-Sagsh, and pGAPZH-Lpgsh, respectively. A schematic representation of the recombinant expression vectors is shown in Fig. 1B. Three recombinant *P. pastoris* strains, GS115/LmgshF, GS115/SagshF, and GS115/LpgshF, were then generated by transforming linearized pGAPZH-Lmgsh, pGAPZH-Sagsh, and pGAPZH-Lpgsh, respectively, into *P. pastoris* GS115. The results of *gshF* gene expression are shown in Fig. 2A. The predicted molecular masses of the *L. monocytogenes*, *S. agalactiae*, and *L. plantarum* GshF proteins were 88.8, 86.3, and 83.0 kDa, respectively. A strongly overexpressed protein band (indicated by the left arrow) was observed at the expected position on SDS-PAGE of *L. monocytogenes* cell lysate, and a weak band (indicated by the right arrow) was obtained with *L. plantarum* cell lysate. The bands indicated by arrows in lanes 2 and 3 of Fig. 2A were excised and subjected to matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) analysis (15). Peptide mass fingerprinting results confirmed that the proteins in the two bands (from left to right) were GshF proteins from *L. monocytogenes* HCC23 and *L. plantarum* WCFS1, respectively (Fig. 2B and C). For *S. agalactiae* GshF, however, neither discernible protein band could be seen on SDS-PAGE and Coomassie blue staining, nor could its peptides be detected by MS analysis (data not shown). Therefore, although the three *gshF* genes were expressed by exactly the same strategy in *P.*
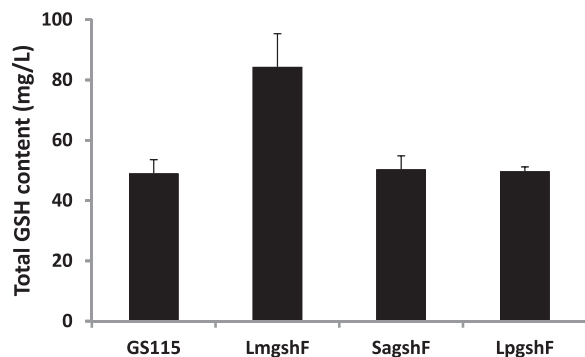
FIG 3 Determination of GSH contents of recombinant *P. pastoris* strains expressing bacterial *gshF* genes. Recombinant *P. pastoris* cells were harvested after culture for 2 days in YPD.

pastoris, their expression levels varied from around 7% of the total soluble protein (quantified by the Gel-Pro Analyzer program [Media Cybernetics]) for GshF from *L. monocytogenes* to not detectable for that from *S. agalactiae*. We hypothesized that the codon usage biases of the three *gshF* genes may account for their different expression levels in *P. pastoris*.

Synthesis of GSH using recombinant *P. pastoris* expressing GshF. To evaluate the GSH synthesis capabilities of GS115/LmgshF, GS115/SagshF, and GS115/LpgshF, three positive clones (confirmed by colony PCR) of each strain, together with control strain GS115, were cultured in YPD liquid medium (2% glucose, 1% yeast extract, 2% peptone) for 2 days, and the intracellular GSH content was extracted from the cells with 40% ethanol (22) and determined using the monobromobimane fluorescent labeling and high-performance liquid chromatography methods (23). Figure 3 shows that strain GS115/LmgshF had significantly improved GSH synthesis capability, compared with that of strain GS115. The intracellular GSH level was increased by almost 80%. No significant increase in intracellular GSH content was observed in strains GS115/SagshF and GS115/LpgshF. These results were not unexpected, because the GshF expression level of GS115/LmgshF was significantly higher than that of the other two strains.

Comparison of GSH synthesis capabilities of recombinant *P. pastoris* strains expressing *L. monocytogenes gshF* and *S. cerevisiae GSHI/GSHII*. In order to further evaluate the potential of bacterial GshF for GSH biosynthesis and the potential of reducing γ-GC accumulation, we compared the thiol-producing profile of strain GS115/LmgshF with that of another *P. pastoris* recombinant strain expressing the *S. cerevisiae GSHI* and *GSHII* genes (Fig. 4). The strain expressing the *GSHI* and *GSHII* genes of *S. cerevisiae* Sc288c was generated by exactly following the strategy of Fei et al. (6) and designated GS115/Sc288c in this work. Strains GS115, GS115/LmgshF, and GS115/Sc288c were cultured in YPD medium for 42 h, and their intracellular thiol concentration profiles were determined. No difference in the growth rates of these three strains was observed (data not shown). Figure 2A shows that the intracellular γ-GC content of GS115/Sc288c accounted for 11.2% of the GSH synthesized (molar ratio). This increased to 20.1% when three amino acid precursors (glycine, cysteine, and glutamic acid) were added (Fig. 2B). In contrast, as we hypothesized, no intracellular γ-GC was detected in GS115/LmgshF in the absence or presence of precursor amino acids. Although the catalytic efficiency of GshF seems slightly lower than that of GSHI/GSHII, as
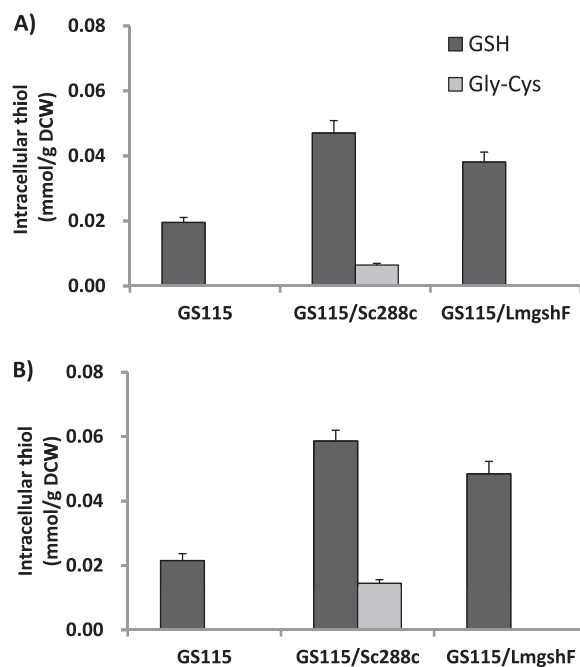

FIG 4 Evaluation of GSH synthesis capability of recombinant *P. pastoris* strains expressing *L. monocytogenes gshF* and *S. cerevisiae GSHI/GSHII* with and without feeding with three amino acid precursors. A, without amino acid precursors; B, with glycine, glutamate, and cysteine. Each amino acid at 5 mM was fed to the flask 1 h before the harvesting of cells.

the GSH synthesized by GS115/LmgshF is around 81 to 83% of that synthesized by GS115/Sc288c, this problem could be solved by increasing the GshF protein expression level in *P. pastoris* or improving the GshF turnover activity by directed evolution.

Conclusion. In order to test the possibility of using bacterial bifunctional GshF for GSH production in a eukaryotic system, three GshF proteins from the Gram-positive species *L. monocytogenes*, *S. agalactiae*, and *L. plantarum* were each cloned and expressed in the methylotrophic yeast *P. pastoris* GS115. Only *L. monocytogenes* GshF displayed significant protein expression and catalytic activity in *P. pastoris*. Compared with that of *S. cerevisiae* GSHI and GSHII, the expression of GshF in *P. pastoris* could significantly reduce the accumulation of the intermediate metabolite γ-GC.

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