RESEARCH ARTICLE
doi: 10.2306/scienceasia1513-1874.2025.088

ScienceAsia 51 (5): 2025: ID 2025088: **1–6**

Characterization of a novel bifunctional glutathione synthetase derived from *Enterococcus italicus*

Jing Li[a,*,†], Lei Yang[a,†], Hao Wang[b,†], Xueyang Li[a], Conghui Zhang[a], Kai Tian[a], Xue Yang[a], Jianyu Zheng[a], Yuese Liu[a], Meina Zhang[a], Guisheng Jia[a], Xiaofeng Zhang[a]

[a] Technical Center for Safety of Industrial Products, Tianjin Customs, Tianjin 300308 China
[b] Department of Critical Care Medicine, Central Hospital of Cangzhou City, Hebei 061000 China

*Corresponding author, e-mail: lj12345678901201@163.com
† These authors contributed equally to this work.

Received 23 Dec 2024, Accepted 26 Sep 2024
Available online 31 Oct 2025

ABSTRACT: Glutathione is a tripeptide containing a γ-amide bond and a sulfhydryl group, which is composed of glutamic acid, cysteine, and glycine. It possesses various important biological functions such as antioxidation. Its biosynthesis initially occurs through the action of glutamate cysteine ligase, which catalyzes the linkage of glutamic acid and cysteine to form γ-glutamylcysteine, and then glutathione synthetase catalyzes the addition of glycine to γ-glutamylcysteine to form γ-glutamylcysteine glycine. In this study, a strain with high glutathione production was isolated and identified as *Enterococcus italicus* through 16S rRNA gene sequencing. The 1600 bp bifunctional glutathione synthetase encoding gene *gshF* was obtained by PCR and heterogeneously expressed in *Escherichia coli* BL21(DE3) using a pET-22b(+) vector. After purification by Ni column, the molecular weight of bifunctional glutathione synthetase GshF was determined to be 60 kDa. Enzymatic analysis showed that the optimum reaction temperature of GshF was 37 °C and the optimum pH was 8.0. Moreover, 30 mmol/l Mg^{2+} significantly enhanced enzyme activity. These results provided a basis for understanding the mechanism of high glutathione production in *E. italicus* and offered a new enzyme source for glutathione biosynthesis.

KEYWORDS: glutathione, glutathione synthetase, *Enterococcus italicus*, protein expression, enzymatic property

INTRODUCTION

Glutathione (GSH), a tripeptide composed of glutamic acid, cysteine, and glycine, is a vital antioxidant in cells with diverse biological functions, including detoxification, immune modulation, and maintenance of cellular redox balance [1, 2]. The biosynthesis of GSH is orchestrated through a two-step enzymatic cascade. The first step involves the formation of a peptide bond between glutamic acid and cysteine, a reaction facilitated by γ-glutamylcysteine synthetase (GshA). This is succeeded by the formation of a peptide bond between γ-glutamylcysteine (γ-Glu-Cys) and glycine, which is mediated by glutathione synthetase (GshB) [3, 4]. Interestingly, certain Gram-positive bacteria have evolved a more streamlined biosynthetic pathway, employing a bifunctional glutathione synthetase GshF that catalyzes both of these reactions, thereby condensing the GSH synthesis process into a single catalytic event [5]. This unique feature streamlines the GSH production process and offers potential advantages in industrial-scale production.

Lactic acid bacteria (LAB) are a group of Gram-positive bacteria that ferment carbohydrates to produce lactic acid, playing a crucial role in food fermentation and gut health [6–9]. The bifunctional glutathione synthetase GshF has been identified in several LAB strains, such as *Lactobacillus plantarum*, *Lactobacillus casei*, *Streptococcus thermophilus* [10, 11]. These studies indicated that the bifunctional glutathione synthetase is typically insensitive to high concentrations of GSH. Thus, when using GshF for GSH biosynthesis, the GSH titer could be even higher by alleviating the feedback inhibition of GSH [10].

In this study, we screened and identified a high glutathione-producing strain of *E. italicus*. The bifunctional glutathione synthetase encoding gene *gshF* was obtained by PCR and heterogeneously expressed in *E. coli* BL21(DE3) using the pET-22b(+) vector. After purification, the enzymatic properties of GshF were studied, which laid the foundation for the industrial production of GSH in the future.

MATERIALS AND METHODS

Strains, medium, culture conditions, and reagents

E. italicus was cultured using MRS Broth (Haibo, Qingdao, China) under static condition of 37 °C. *E. coli* was cultured in LB Broth (Haibo) under shaking condition at 37 °C. *E. coli* the strain DH5α was used for plasmid construction, and the strain BL21(DE3) was used for protein expression. Bacteria genomic DNA extraction kit, gel electrophoresis recovery kit and plasmid extraction kit were purchased from Nanjing Vazyme Biotech Co., Ltd. (Vazyme, Nanjing, China). DNA polymerase, primers, dNTPs (dATP, dCTP, dTTP, and dGTP), ddH2O; nucleic acid dye, loading buffer, DNA Marker were purchased from Takara Biomedical Technology Co.,

Ltd. (Takara, Beijing, China). Other reagents such as glutamate, cysteine, glycine, γ-glutamylcysteine, and 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) were purchased from Sigma-Aldrich Company (Sigma-Aldrich, St. Louis, USA).

Gene cloning and recombinant plasmid construction

Genomic DNA from *E. italicus* was isolated utilizing the Bacteria Genomic DNA Extraction Kit (Vazyme), serving as the template for the amplification of the *gshF* gene. The amplification was conducted using a pair of primers with the following sequences: the forward primer 5′-TAAGAAGGAGATATACATATGGATTATCGCG CACTACT-3′ and the reverse primer 5′-TGGTGGTGGT GGTGCTCGAGTTGGCCTATCTCTTCGATTC-3′. The resultant 2,274 bp PCR product was cloned into the pET-22b(+) vector employing the One Step Cloning Kit (Vazyme), and the recombinant plasmid pET22b-*gshF* was obtained.

Protein expression and purification

The recombinant plasmid pET22b-*gshF* was transformed into the *E. coli* BL21(DE3) cells by the standard heat-shock method. A single colony was selected and cultivated at 200 rpm in LB broth containing 50 μg/ml of ampicillin at 37 °C for 12 h. Overnight culture was 50-times diluted in a 250 ml-glass flask with 100 ml of fresh LB broth. The culture was grown at 37 °C until the cell density reached an OD_{600} value of 0.6–0.8. Subsequently, 0.5 mM of isopropyl-β-D-thiogalactopyranoside (IPTG) was added to the culture, and the cultivation was continued at 16 °C for 12 h. Bacterial cells were harvested by centrifugation 6,000 *g* for 10 min, and resuspended in 20 mM sodium phosphate buffer (pH 7.0) and lysed by ultrasonic treatment on ice (power 400 W, pulse 5 s, pause 5 s, cycle 49). Cell debris was removed by centrifugation at 10,000 *g* for 10 min at 4 °C, and the filtered supernatant was subject to a 1 ml Ni-TED column (Sangon, Shanghai, China) to purify GshF. The expression and molecular weight of GshF in *E. coli* were estimated by SDS-PAGE and Native-PAGE.

Enzyme activity assay

In a 1 ml reaction system: 100 mmol/l Tris-HCl (pH 8.0), 40 mmol/l L-Glutamate, 20 mmol/l L-Cysteine, 40 mmol/l Glycine, 20 mmol/l $MgCl_2$, 20 mmol/l ATP, and 100 μl of enzyme solution. The reaction was conducted for 20 min, after which 0.5 ml of the above mixture was added to 2.5 ml of DTNB analytical solution, shaken well, and held in a water bath at 25 °C for 5 min. The absorbance at 412 nm was measured with water as a blank control.

Effect of temperature and pH on enzyme activity

The optimal temperature for GshF was investigated at temperatures ranging from 20 to 50 °C, with increments of 5 °C, in a 50 mmol/l phosphate buffer solution at pH 7.0. To assess thermostability, the residual activity of the enzyme was measured after incubation at 35 °C for 3 h. The optimum pH was determined by testing pH values of 2.0, 4.0, 6.0, 8.0, 10.0, and 12.0 using different buffer systems: citrate buffer, phosphate buffer, and Tris-HCl buffer, all at 50 mmol/l concentration and at the previously determined optimal temperature. To evaluate pH stability, the residual activity was measured after incubating the enzyme in buffers with pH values of 8.0 for 12 h at 4 °C.

Effect of metal ions on enzyme activity

To explore the effect of metal ions on GshF, different metal ions (Mg^{2+}, Co^{2+}, Ca^{2+}, Zn^{2+}, Mn^{2+}, and Fe^{2+}) were added to the purified enzyme solution at a final concentration of 10 mmol/l. Then, standard enzyme assays were performed to measure the effect of metal ions on the activity of GshF. In addition, the Mg^{2+} was added to the purified enzyme solution at a final concentration of 10, 20, 30, 40, 50, and 60 mmol/l to explore the effect on GshF.

GSH inhibition experiments

GSH was tested as a competitive inhibitor for both the glutamate cysteine ligase and glutathione synthetase activities of GshF using the ADP formation assays [12]. For glutamate cysteine ligase activity, substrate concentrations of glutamic acid and cysteine were 25 mM and 10 mM, respectively. For glutathione synthetase activity, substrate concentrations of γ-glutamylcysteine and glycine were 5 mM and 25 mM, respectively. GSH was added to the reaction mixture at concentrations ranging from 0 to 100 mM.

Statistical analysis

Each experiment was repeated at least three times. The results were shown as the mean ± standard deviation of the mean of 3 independent experiments. Statistical analysis of the data was carried out using one-way ANOVA (Tukey's test at $p < 0.05$) by employing statistical software (SPSS).

RESULTS

Gene cloning and vector construction

The *gshF* gene was successfully amplified from the genomic DNA of *E. italicus* using specific primers. The amplified product was confirmed to be within the expected size range of 2,000–3,000 bp by agarose gel electrophoresis, aligning with the theoretical length of 2,274 bp (Fig. 1). Despite the presence of additional bands, the specific band of interest within the range of 2,000–3,000 bp was recovered.

The *gshF* gene fragment was linked to the pET-22b(+) vector and subsequently transformed into *E. coli* DH5α giving rise to single colonies on LB plates containing ampicillin. Colony PCR of the single colony

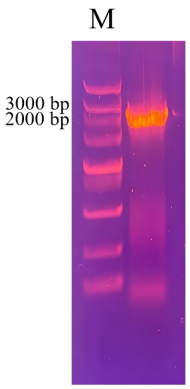

Fig. 1 PCR amplification of *gshF* gene from *E. italicus*.

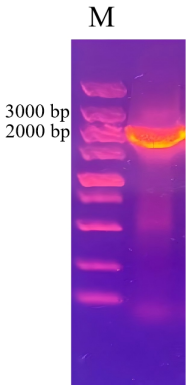

Fig. 2 PCR determination of recombinant plasmid pET22b-*gshF*.

confirmed the successful insertion of the *gshF* gene, and the clones produced the expected 2,274 bp fragment (Fig. 2), resulting in the recombinant plasmid pET22b-*gshF*.

Bioinformatics analysis of GshF

As shown in Fig. 3, through the homology comparison of glutathione synthetase sequences from different strains, it was found that the GshF of *E. italicus* has high similarity with glutathione synthetase from other

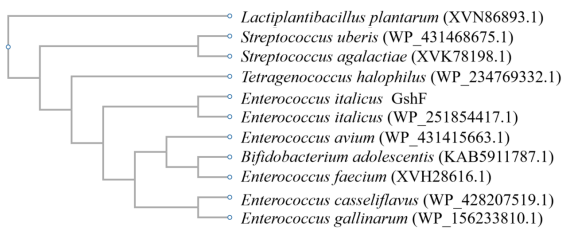

Fig. 3 The evolutionary tree of *E. italicus* GshF.

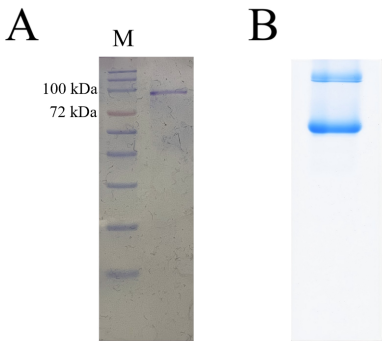

Fig. 4 SDS-PAGE (A) and Native-PAGE (B) analysis of the purified GshF by affinity chromatography.

enterococci strains. Specifically, the similarities with *Enterococcus gallinarum* (WP_156233810.1) and *Enterococcus casseliflavus* (WP_428207519.1) were 65.25% and 64.99%, respectively. Moreover, GshF showed relatively high similarity of 60.37% with *Bifidobacterium adolescentis* (KAB5911787.1) and *Tetragenococcus halophilus* (WP_234769332.1). Nevertheless, the similarity between GshF and *Lactiplantibacillus plantarum* (XVN86893.1) was the lowest, at only 25.17%. The alignment of the amino acid sequences of GshF from *E. italicus* and glutathione synthetases from other strains was provided in Fig. S1.

Expression and purification of GshF

The theoretical molecular weight (Mw) and isoelectric point (pI) were calculated using the Compute pI/Mw tool (http://web.expasy.org/compute_pi/). The complete *gshF* gene had an open reading frame of 2,274 bp, encoding a protein of 757 amino acids with a calculated molecular weight of 86.0 kDa and the theoretical pI of 4.97. To investigate the biochemical properties of GshF, the recombinant plasmid was transformed into *E. coli* BL21(DE3), and the resulting recombinant strain *E. coli* DE3/pET-gshF was induced with 0.5 mmol/l IPTG at 16 °C. The cells were harvested and disrupted by sonication in an ice-water bath. The cell lysate was found fully clear, and no inclusion bodies were formed, which suggested that the recombinant GshF was highly soluble. Subsequently, the recombinant GshF with a six-histidine tag was purified by Ni-NTA chromatography, and the purified recombinant protein were applied to SDS-PAGE to determine the expression level and molecular mass of GshF. SDS-PAGE analysis revealed a single band within the molecular weight range of 72–100 kDa (Fig. 4A), which was consistent with the calculated molecular mass (86.0 kDa). To determine whether GshF has a dimeric structure, a Native-PAGE experiment was conducted on the recombinant protein. Native-PAGE does not involve the addition of protein denaturants, allowing the protein to maintain its native structure during the separation process. As can be more clearly seen from Fig. 4B, the

purified GshF protein was separated into two distinct bands after electrophoresis, suggesting that GshF has a dimeric structure. Next, the purified GshF was used to study its biochemical properties.

Activity profiles and stability profiles

The optimal temperature for GshF activity was determined by assaying enzyme activity across a range of temperatures from 20 to 50 °C, revealing maximum activity at 35 °C (Fig. 5A). The stability of GshF at this optimal temperature demonstrated a gradual decline in activity over time, with the enzyme retaining approximately 60.3% of its initial activity after 3 h (Fig. 5B).

To investigate the optimal pH for GshF, enzymatic activity was measured at various pH levels while maintaining a constant temperature of 35 °C. The enzyme exhibited the highest activity at pH 8.0, with relative activities of only 52.7% and 43.2% observed at pH 2.0 and pH 12.0, respectively (Fig. 6A). Furthermore, GshF displayed good stability at pH 8.0, maintaining around 84.0% of its relative activity over a 12-h period under these conditions (Fig. 6B).

Influence of different metal ions and GSH

To know the effect of different metal ions on GshF activity, enzyme assays were conducted in the presence of various metal salts. The addition of Mg^{2+} in the reaction induced a significant improvement of the enzymatic activity, and the relative activity of GshF increased to 123.2%. However, the presence of 10 mmol/l Co^{2+} caused a strong inhibition of the enzymatic activity (Fig. 7A). Since Mg^{2+} has a positive effect on GshF, the influence of Mg^{2+} at concentrations of 10, 20, 30, 40, 50, and 60 mmol/l on GshF activity was further investigated. The results showed that the maximum enzyme activity was observed at 30 mmol/l of Mg^{2+} (Fig. 7B).

The ability of GSH to inhibit either the glutamate cysteine ligase or glutathione synthetase activity of GshF was investigated using GSH concentrations up to 100 mM. No significant inhibition was seen.

DISCUSSION

GSH is a tripeptide consisting of glutamic acid, cysteine, and glycine, and holds considerable value in diverse applications due to its distinctive properties. In the cellular environment, GSH essentially protects the cells from a wide range of free radicals, including reactive oxygen species, lipid hydroperoxides, xenobiotic toxicants, and heavy metals [13]. GSH is also a crucial determinant of redox signaling, vital for the detoxification of xenobiotics, and regulates cell proliferation, apoptosis, immune function, and fibrogenesis [14]. Kennedy et al [15] demonstrated the significance of glutathione in supporting immune function, showing that supplementation can enhance immune status, particularly in immunocompromised individuals. Due to its antioxidant nature, GSH is extensively utilized in the pharmaceutical, cosmetic, and food industries. Annually, more than 200 tons of pure crystalline GSH are produced worldwide [16]. The interest in glutathione and derived products is based on its reductive character and the resulting diverse possibilities of medical and industrial application.

The production methods of GSH encompass extraction method, chemical synthesis method, fermentation method, and enzyme method, among which the fermentation method and the enzyme method are the most prevalently used [17, 18]. The extraction method mainly refers to the utilization of extraction and precipitation and other approaches to extract GSH-rich tissues, but the content of GSH in the tissues is extremely low, resulting in a low extraction yield and purity. Harington and Mead described the chemical synthesis of GSH in 1935, which was commercialized in the 1950s [3, 19]. However, the obtained GSH is a mixture of L-form and D-form, which is not easily separable. Fermentation involves the use of microbial metabolic pathways to synthesize GSH, with commonly used strains being *Saccharomyces cerevisiae*, *Pichia pastoris*, and *E. coli*, etc., but the products are not easily separable and purified, and there are too many impurities [20–22]. The enzymatic synthesis of GSH is inherently highly specific and promises high yields as only the supply of the minimally required substances, namely, amino acids, ATP, and enzymes, is necessary, which simplifies purification as well [23, 24]. The glutathione synthetase enzyme series includes two-step enzymes GshA, GshB, and GshF. Currently, GshF is mainly selected because it does not have issues such as feedback inhibition and intermediate product accumulation, and is not sensitive to GSH [21]. Therefore, mining GshF from more microbial sources has high application value and significance.

In this study, the *gshF* gene of *E. italicus* was cloned and expressed in *E. coli*, and its enzymatic properties were investigated. The determination of the optimal temperature and pH for GshF activity offered valuable insights into the enzyme's performance under various circumstances. The maximum activity at 35 °C and pH 8.0, along with its stability profile, indicated that GshF was well-adapted to mesophilic and mildly alkaline environments. These conditions are particularly relevant for industrial processes where temperature and pH control are crucial. The significant enhancement of GshF activity in the presence of Mg^{2+} and the inhibition caused by Co^{2+} highlight the significance of metal ions in modulating enzyme activity. The optimal concentration of Mg^{2+} at 30 mmol/l for maximum enzyme activity provided an understanding of the enzyme's metal ion requirements for maximal activity. These findings are essential for optimizing reaction conditions in industrial applications and for

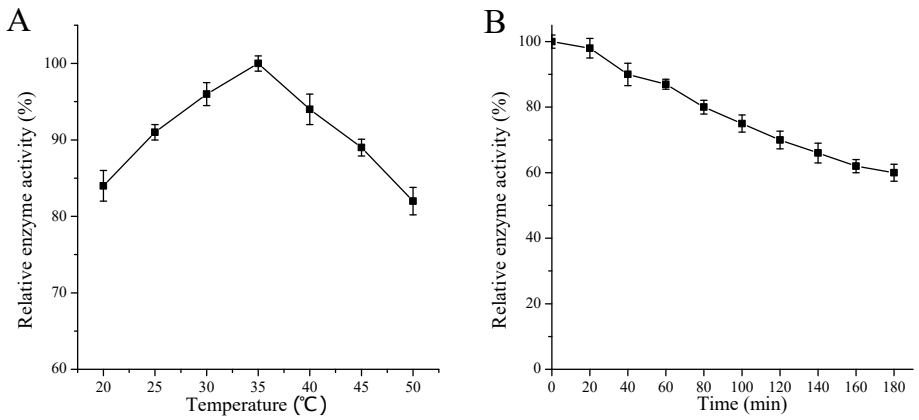

Fig. 5 Effect of temperature from 20 to 50 °C on the GshF activity (A), and the residual activity of GshF incubated at 35 °C for 3 h (B).

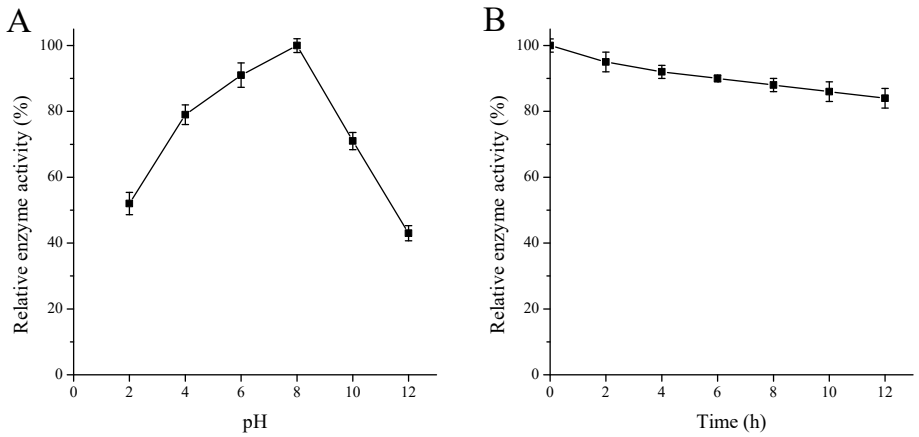

Fig. 6 Effect of pH from 2.0 to 12.0 on the GshF activity (A), and the residual activity of GshF incubated at pH 8.0 for 12 h (B).

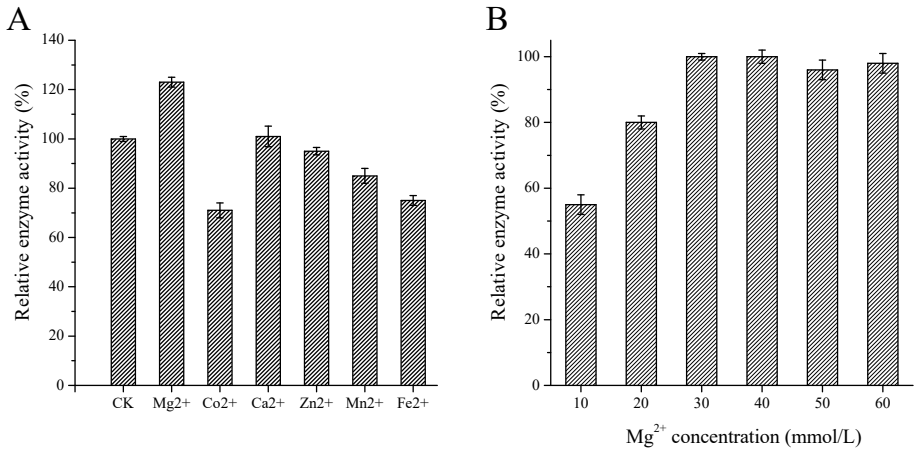

Fig. 7 Effect of different metal ions on the GshF activity (A), and the influence of Mg^{2+} concentration ranging from 10 to 60 mmol/l on the GshF activity (B). CK represents the enzyme activity determined without the addition of any metal ions.

comprehending the mechanism of GshF.

In conclusion, the biochemical characterization of GshF from *E. italicus* provided a foundation for understanding its role in glutathione synthesis and its potential applications in biotechnology. The enzyme's temperature and pH optima, stability, and metal ion sensitivity offer valuable insights for future studies aimed at enhancing GshF activity and exploring its applications in the production of glutathione, a molecule with significant health and industrial benefits.

Appendix A. Supplementary data

Supplementary data associated with this article can be found at https://dx.doi.org/10.2306/scienceasia1513-1874.2025.088.

Acknowledgements: This study was financially supported by Research on the Integration and Application Demonstration of Key Technologies for Quality Assurance of Drug Identification (Grant No. 2021YFF0602502).

REFERENCES

1. Wu G, Fang Y, Yang S, Lupton J, Turner N (2004) Glutathione metabolism and its implications for health. *J Nutr* **134**, 489–492.
2. Simpalipan P, Pattaradilokrat S, Sawaswong V, Uthayopase K, Phutthawong K, Harnyuttanakorn P (2025) Genetic analysis of glutathione synthetase of *Plasmodium falciparum*, a potential candidate for antimalarial drug development. *ScienceAsia* **51**, ID 2025035.
3. Li Y, Wei G, Chen J (2004) Glutathione: a review on biotechnological production. *Appl Microbiol Biotechnol* **66**, 233–242.
4. Pophaly S, Singh R, Pophaly S, Kaushik J, Tomar S (2012) Current status and emerging role of glutathione in food grade lactic acid bacteria. *Microb Cell Fact* **11**, 114.
5. Gopal S, Borovok I, Ofer A, Yanku M, Cohen G, Goebel W, Kreft J, Aharonowitz Y (2005) A multidomain fusion protein in *Listeria monocytogenes* catalyzes the two primary activities for glutathione biosynthesis. *J Bacteriol* **187**, 3839–3847.
6. Choovet N, Jirakanjanasit T, Subphon P, Thepsombat S, Kingkaew E, Tanaka N, Shiwa Y, Tanasupawat S, et al (2014) Antimicrobial activity, genome analysis, and gene cluster encoding the plantaricin production of *Lactiplantibacillus plantarum* ZBK1-5. *ScienceAsia* **50**, ID 2024046.
7. De Filippis F, Pasolli E, Ercolini D (2020) The food-gut axis: lactic acid bacteria and their link to food, the gut microbiome and human health. *FEMS Microbiol Rev* **44**, 454–489.
8. Sitdhipol J, Niwasabutra K, Chaiyawan N, Teerawet S, Thaveethaptaikul P, Phuengjayaem S, Taweechotipatr M, Tanasupawat S, et al (2022) Probiotic characterization and *in vitro* functional properties of lactic acid bacteria isolated in Thailand. *ScienceAsia* **49**, 29–37.
9. Tsai C, Lin L, Chou L (2022) The effects of lactic acid bacteria-fermented lemon juice on blood pressure regulation and allergic responses in rodents. *ScienceAsia* **48**, 181–187.
10. Li W, Li Z, Yang J, Ye Q (2011) Production of glutathione using a bifunctional enzyme encoded by *gshF* from *Streptococcus thermophilus* expressed in *Escherichia coli*. *J Biotechnol* **154**, 261–268.
11. Xiong Z, Kong L, Wang G, Xia Y, Zhang H, Yin B, Ai L (2018) Functional analysis and heterologous expression of bifunctional glutathione synthetase from *Lactobacillus*. *J Dairy Sci* **101**, 6937–6945.
12. Janowiak B, Griffith O (2005) Glutathione synthesis in *Streptococcus agalactiae*. One protein accounts for gamma-glutamylcysteine synthetase and glutathione synthetase activities. *J Biol Chem* **280**, 11829–11839.
13. Narayanankutty A, Job J, Narayanankutty V (2019) Glutathione, an antioxidant tripeptide: dual roles in carcinogenesis and chemoprevention. *Curr Protein Pept Sci* **20**, 907–917.
14. Lu S (2013) Glutathione synthesis. *Biochim Biophys Acta* **1830**, 3143–3153.
15. Kennedy L, Sandhu J, Harper M, Cuperlovic-Culf M (2020) Role of glutathione in cancer: from mechanisms to therapies. *Biomolecules* **10**, 1429.
16. Orumets K, Kevvai K, Nisamedtinov I, Tamm T, Paalme T (2012) YAP1 over-expression in *Saccharomyces cerevisiae* enhances glutathione accumulation at its biosynthesis and substrate availability levels. *Biotechnol J* **7**, 566–568.
17. Fei L, Wang Y, Chen S (2009) Improved glutathione production by gene expression in *Pichia pastoris*. *Bioprocess Biosyst Eng* **32**, 729–735.
18. Wang C, Zhang J, Wu H, Li Z, Ye Q (2015) Heterologous *gshF* gene expression in various vector systems in *Escherichia coli* for enhanced glutathione production. *J Biotechnol* **214**, 63–68.
19. Schmacht M, Lorenz E, Senz M (2017) Microbial production of glutathione. *World J Microbiol Biotechnol* **33**, 106.
20. Patzschke A, Steiger M, Holz C, Lang C, Mattanovich D, Sauer M (2015) Enhanced glutathione production by evolutionary engineering of *Saccharomyces cerevisiae* strains. *Biotechnol J* **10**, 1719–1726.
21. Zhang J, Quan C, Wang C, Wu H, Li Z, Ye Q (2016) Systematic manipulation of glutathione metabolism in *Escherichia coli* for improved glutathione production. *Microb Cell Fact* **15**, 38.
22. Gao Y, Liu N, Zhu Y, Yu S, Liu Q, Shi X, Xu J, Xu G, et al (2022) Improving glutathione production by engineered *Pichia pastoris*: strain construction and optimal precursor feeding. *Appl Microbiol Biotechnol* **106**, 1905–1917.
23. Li W, Li Z, Ye Q (2010) Enzymatic synthesis of glutathione using yeast cells in two-stage reaction. *Bioprocess Biosyst Eng* **33**, 675–682.
24. Chen J, Xie L, Cai J, Yang C, Duan X (2013) Enzymatic synthesis of glutathione using engineered *Saccharomyces cerevisiae*. *Biotechnol Lett* **35**, 1259–1264.

Appendix A. Supplementary data

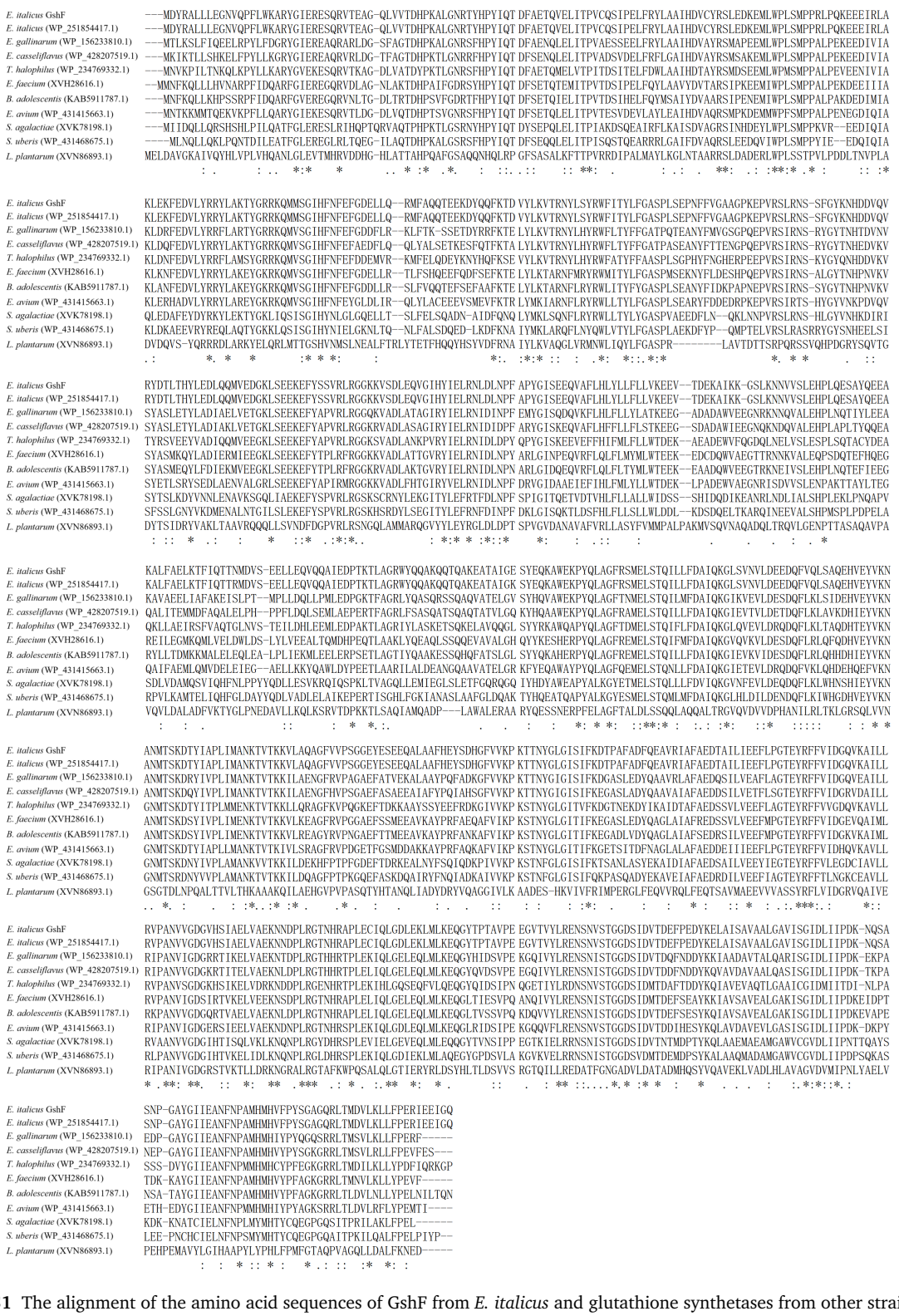

Fig. S1 The alignment of the amino acid sequences of GshF from *E. italicus* and glutathione synthetases from other strains.