Characterization of GshAB of Tetragenococcus halophilus: a two-domain glutathione synthetase

Characterization of GshAB of *Tetragenococcus halophilus*, a two domain glutathione synthetase

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Abstract

The γ-glutamyl tripeptide glutathione (γ-Glu-Cys-Gly) is a low molecular thiol that acts as antioxidant in response to oxidative stress in eukaryotes and prokaryotes. γ-Glutamyl dipeptides including γ-Glu-Cys, γ-Glu-Glu, γ-Glu-Gly also have kokumi activity. Glutathione is synthesized by first ligating Glu with Cys by γ-glutamylcysteine ligase (Gcl/GshA), then the resulting dipeptide γ-glutamylcysteine is ligated with Gly by glutathione synthetase (Gs/GshB). GshAB/GshF enzymes that contain both Gcl and Gs domains are capable of catalysing both reactions. The current study aimed to characterize GshAB from *Tetragenococcus halophilus* after heterologous expression in *Escherichia coli*. The optimal conditions for GshAB from *T. halophilus* were pH 8.0 and 25 °C. The substrate specificity of the Gcl reaction of GshAB was also determined. GshAB has a high affinity to Cys. γ-Glu-Cys was the only dipeptide generated when Glu, Cys, Gly and other amino acids were present in the reaction system. This specificity differentiates GshAB from *T. halophilyus* from Gcl of heterofermentative lactobacilli and GshAB of *Streptococcus agalactiae*, which also use amino acids other than Cys as glutamyl-acceptor. Quantification of *gshAB* in cDNA libraries from *T. halophilus* revealed that *gshAB* was overexpressed in response to oxidative stress but not in response to acid, osmotic, or cold stress. In conclusion, GshAB in *T. halophilus* served as part of the oxidative stress response but this study did not provide any evidence for a contribution to the resistance to other stressors.

Keywords: γ-Glutamylcysteine ligase, γ-Glutamyl dipeptides, kokumi, Glutathione

synthesis, *Tetragenococcus halophilus*, osmotolerance, oxidative stress, *Lactobacillus*.

Key points:

- Glutathione synthesis in *Tetragenococcus halophilus* is carried out by the two-domain enzyme GshAB.
- GshAB is inhibited by glutathione and is highly specific for Cys as acceptor.
- *T. halophilus* synthesizes glutathione in response to oxidative stress.

Introduction

The γ-glutamyl tripeptide glutathione (γ-Glu-Cys-Gly) is a low molecular thiol that acts as antioxidant in response to oxidative stress in eukaryotes and prokaryotes. A protective effects of glutathione against oxidative damage has also been demonstrated in lactic acid bacteria (Kim et al. 2008; Yoon and Byun 2004). Glutathione supplementation also enhanced tolerance of lactic acid bacteria against acid, cold and osmotic stresses (Lee et al. 2010; Pophaly et al. 2012; Zhang et al. 2010a; Zhang et al. 2007; Zhang et al. 2010b).

In addition to the role of glutathione as part of the oxidative stress response of LAB, γ-glutamyl peptides including γ-Glu-Cys, γ-Glu-Glu, γ-Glu-Gly, γ-Glu-Gln, γ-Glu-Met and γ-Glu-Leu impart kokumi activity. These dipeptides or tripeptides activate the human calcium-sensing receptor (CasR) and enhance and extend the perception of primary tastes including salty, umami and sweet tastes. Kokumi activity has been described as increasing thickness, continuity, complexity and mouthfulness (Kuroda et al. 2013; Roudot-Algaron et al. 1994; Toelstede et al. 2009; Ueda et al. 1997; Yan et al. 2018; Zhao et al. 2016). γ-Glutamyl peptides in cheese were generated from enzymes in raw milk (Hillmann et al. 2016), but microorganism also play an essential role in the generation of kokumi peptides in food fermentations such as soy sauce and sourdough (Minami et al. 2003; Zhao and Gänzle 2016).

The γ-glutamyl tripeptide glutathione is composed of glutamate, cysteine and glycine. The non-ribosomal synthesis of glutathione is a two-step enzyme reaction; each step

consumes one molecule of ATP. γ-Glutamylcysteine ligase or γ-glutamylcysteine synthetase (γ-Gcl, EC 6.3.2.2) ligates cysteine to glutamate, and glutathione synthetase (GS, EC 6.3.2.3) catalyzes the ligation of glycine to γ-glutamylcysteine, resulting in the tripeptide glutathione (Meister and Anderson 1983). The ability to synthesize glutathione was initially reported for eukaryotes, Gram-negative bacteria and Gram-positive bacteria including lactic acid bacteria (Fahey et al. 1978; Kim et al. 2008; Pophaly et al. 2012). *Limosilactobacillus reuteri* (previously *Lactobacillus reuteri*) (Zheng et al. 2020) produces several γ-glutamyl dipeptides during sourdough fermentation, the gene responsible for γ-glutamyl-cysteine ligation was termed as *gcl* (Yan et al. 2018). Species in several genera of heterofermentative lactobacilli, particularly of the genera *Lentilactobacillus* and *Limosilactobacillus*, encode for up to three Gcls per genome (Xie and Gänzle 2021; Yan et al. 2018). Biochemical characterization of three Gcls from *Lm. reuteri* revealed that all three enzymes use cysteine as preferred substrate but differ in their substrate specificity towards other amino acids (Xie and Gänzle 2021) and thus differentially contribute to formation of γ-glutamyl peptides in food fermentations. However, most lactobacilli lack the gene encoding glutathione synthetase (*gshB*), cannot synthesize glutathione (Pophaly et al. 2012) and import glutathione from the medium (Pophaly et al. 2017).

In some gram-positive bacteria, a single gene encoding a bifunctional protein, *gshAB*/*gshF*, composed of the Gcl domain at the N-terminus and GS domain at the C-terminus, mediates the biosynthesis of γ-glutamyl dipeptides and glutathione. The gene

encoding the multidomain bifunctional protein GshAB/GshF was first identified in *Streptococcus agalactiae* (Janowiak and Griffith 2005) and in *Listeria monocytogenes* (Gopal et al. 2005) but is also present in *Streptococcus thermophilus* and *Enterococcus* spp.. (Pophaly et al. 2017; Vergauwen et al. 2006; Wang et al. 2015). To date, most of the strains predicted to encode *gshAB*/*gshF* are pathogens (Gopal et al. 2005).

Tetragenococcus halophilus is a halophilic lactic acid bacterium growing in a broad range of salinity conditions including saturated salt solutions. It is used in food fermentation processes that are characterized by high salt concentrations (Gänzle 2022; Kobayashi et al. 2003; Roling and Van Verseveld 1996; Thongsanit et al. 2002; Villar et al. 1985). Compatible solutes such as glycine betaine and some amino acids mediate resistance of *T. halophilus* to high salinity (Lin et al. 2017; Robert et al. 2000). Genomes of *T. halophilus* encode for the bifunctional protein GshAB (e.g. WP_253214445), which has, however, not been characterized in *T. halophilus*. γ-Glutamyl peptides contribute to kokumi flavor in soy sauce (Frerot and Chen 2013; Kuroda et al. 2013), however the details in how these compounds are produced during fermentation of soy sauce of fish sauces remains to be investigated. The current study aims to characterize the GshAB enzyme from *T. halophilus,* to characterize the enzyme with respect to optimal conditions and substrate specificity, and to assess a potential role of the enzyme in stress resistance of *T. halophilus*.

Materials and Methods

Strains and Growth Conditions

T. halophilus CICC10469 was grown in the modified Man, Rogosa, and Sharpe medium (mMRS) with the following ingredients per liter: 5 g beef extract, 5 g yeast extract, 10 g peptone, 10 g malt extract, 10 g maltose, 5 g fructose, 5 g glucose, 2.6 g KH_2PO_4, 4 g K_2HPO_4, 3 g NH_4CL, 0.5 g cysteine HCl, 1 g Tween 80, 0.1 mg $MgSO_4 \bullet 7H_2O$, and 0.05 g $MnSO_4 \bullet H_2O$ at 30 °C with an optimum NaCl concentration of 1 M. *Escherichia coli* DH5α and BL21 which served as hosts for plasmids in the cloning procedures, were grown at 37 °C in Luria broth (LB) mediums; and media were supplemented with kanamycin (50 μg/ml), or isopropyl-β-D-1-thiogalactopyranoside (IPTG) for maintenance of plasmid or for protein overexpression.

DNA manipulation

Genomic DNA was isolated with the Blood & Tissue Kit (Promega, Madison, WI, U.S.A.). The gene encoding for GshAB was amplified from the chromosomal DNA of *T. halophilus* using primers listed in Table S1; primers were synthesized by Integrated DNA Technologies (San Diego, CA, U.S.A.). PCR amlicons were cloned into the *Nhe*I *Bam*HI restriction sites of the expression plasmid pET28a, which introduced a N-terminal His6 tag to the recombinant protein. After verification by DNA sequencing, plasmids were transferred into *E. coli* strain BL21(DE3) for protein overexpression. The nucleotide sequence of the *gshAB* insert cloned into pET28a was deposited at Genbank with the accession number OQ632271.

Overexpression and purification of recombinant His6-tagged GshAB

Recombinant strains were grown at 37 °C until the OD_{600} reached 0.5. Protein

overexpression was induced with the addition of 0.2 mM IPTG and growth at 20 °C overnight. After overexpression, cells were harvested by centrifugation at 8000×*g* for 10 min; cells were resuspended in PBS buffer and lysed by bead-beating. The resulting crude cell extract was purified using HisPur™ Ni-NTA Spin Columns (Thermo Fischer Scientific; Waltham, MA, USA) according to the manufacturer's instructions. The recombinant protein was eluted with imidazole and dialyzed with 50 mM Tris-HCl buffer (pH 8.0). Glycerol was added to a final concentration of 20% to preserve proteins. Protein concentrations were determined with Bradford reagents and purities were assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Determination of the optimum pH and temperature of γ-glutamylcysteine synthetase activity of GshAB

The GshAB activity at different pH and temperature conditions were assayed using a fluorescence-based method according to White et al. (White et al. 2003) with some modifications. Briefly, 50 µL aliquots of Gcl reaction buffer (400 mM Tris, 40 mM ATP, 20 mM L-glutamic acid, 20 mM EDTA, 40 mM $MgCl_2$) were pipetted into 96-well plates, 8 wells per samples, 4 of which were independent replicates for γ-glutamyl-cysteine synthesis reaction, and 4 for the negative control. The 96-wells plate was kept on ice, and 50 µL of purified GshAB was added to each well to a final concentration of 0.16 g/L. For control reactions, 50 µL 500 mM 5-sulfosalicylic acid (SSA) was added prior to GshAB addition, and mixed by pipetting for several times to denature the enzyme. After a 5 min pre-incubation, 50 µL of 10 mM L-cysteine was added to all

wells to a final concentration of 3.33 mM. After a 45 min incubation, the Gcl reaction was stopped by SSA addition. The plates were then centrifuged at 3500 rpm for 10 min to precipitate protein, and 20 μL aliquots of supernatant from each well were transferred to a 96-wells plate designed for fluorescence detection. For the determination of optimum pH conditions for Gcl activity of GshAB, all components were dissolved in either Citrate-Phosphate Buffer, Tris-HCl buffer or Carbonate-Bicarbonate Buffer to adjust their pH values.

The γ-glutamylcysteine generation was determined by measuring the fluorescence intensity after derivatization with naphthalene dicarboxaldehyde (NDA). After protein precipitation, 20 μL supernatant of each well was transferred to a 96-wells plate for further fluorescence detection, followed by addition of 180 μL NDA derivatization solution (50mM Tris, pH 10, 0.5N NaOH, and 10mM NDA in Me2SO, v/v/v 1.4/0.2/0.2). Standard curves were constructed for each experiment and 3 independent replicates were conducted for each experiment. The plate was then covered to be protected from light and incubated in room temperature for 30 min, and then NDA-γ-Glu-Cys fluorescence intensity was measured (472 ex/528 em) using a SynergyTM Mx Monochromator-Based Multi-Mode Microplate Reader (BioTek Instruments, Winooski, VT, USA).

Determination of effect of glutathione on the Gcl activity of GshAB

Glutathione was tested as a competitive inhibitor for the Gcl activity of *T. halophilus* GshAB. The γ-glutamylcysteine synthesis reactions were conducted as described above

under the optimum temperature and pH conditions, and with the addition of glutathione to a final concentration ranging from 0 to 6 μM. The generation of γ-glutamylcysteine was measured by the above-described fluorescence-based method. 3 replicates were conducted for each sample.

Effect of the cysteine concentration on γ-Gcl activity of *T. halophilus* GshAB

The Gcl reaction was conducted with a final concentration of cysteine ranging from 0 to 10 mM to determine whether higher concentration of cysteine inhibit the Gcl activity of GshAB. γ-Glutamylcysteine generation with cysteine concentration ranging from 0 to 1 mM was also measured in order to determine the lowest concentration of cysteine to be used in the further competition experiments of other amino acid substrate. 3 replicates were conducted for each sample.

Substrate specificity of the Gcl domain of GshAB determined by phosphate assay

To test if GshAB can ligate other amino acids with glutamate besides cysteine and generate glutamyl dipeptides, purified GshAB was incubated with glutamate and one of 18 other amino acids, and the phosphate generated during the reaction was measured using the Phosphate Assay Kit (Abcam, Cambridge, United Kingdom). Briefly, 50 μL Gcl reaction buffer was mixed with purified GshAB, then the enzyme reaction in the negative controls was blocked by SSA addition. Then, one amino acid each dissolved in 50 mM Tris-HCl buffer was added to each well. The following amino acids were tested: glycine (Gly), L-serine (Ser), L-threonine (Thr), L-asparagine (Asn), L-glutamine (Gln), L-aspartic acid (Asp), L-glutamic acid (Glu), L-cysteine (Cys), L-

lysine (Lys), L-arginine (Arg), L-histidine (His), L-alanine (Ala), L-valine (Val), L-leucine (Leu), L-isoleucine (Ile), L-proline (Pro), L-phenylalanine (Phe), L-methionine (Met), L-tryptophan (Trp). For each amino acid, 3 replicates were included. After incubation at the optimum temperatures for 45 min, glutamyl dipeptide synthesis was stopped by adding SSA to reaction wells and held on ice for 20 min. The plates were then centrifuged at 3500 rpm for 10 min to precipitate protein, and 20-μL aliquots of supernatant from each well were transferred to another 96-wells plate and mixed with 180 μL ddH_2O. The amount of phosphate generated was assayed to determine whether the amino acid acts as substrate of GshAB during glutamyl dipeptides synthesis.

Inhibition effect of other amino acids on γ-glutamyl-cysteine synthesis

To test whether other amino acids can compete with cysteine to bind GshAB, competition experiments were conducted by mixing glutamate, cysteine and one of the other amino acids with the enzyme, and measured γ-glutamyl-cysteine synthesis. The lowest cysteine concentration needed for Gcl reaction was determined as described above. In brief, Gcl reaction was conducted with 3 replicates by mixing Gcl reaction buffer (containing 20 mM glutamate) with GshAB, 5mM other substrate amino acids and 1 mM L-cysteine, SSA was added to control reactions in advance to block the enzymatic reaction. The plates were then incubated in 25°C for 45 min and the reaction was stopped by adding SSA, the concentration of γ-glutamyl-cysteine generated was measured by the fluorescence-based method described above.

Quantitation of γ-glutamyl dipeptides generated by GshAB using HPLC-MS/MS

HPLC-MS/MS was used to determine the γ-glutamyl dipeptides generated by GshAB during fermentation process. GshAB was mixed with amino acid mixtures containing Ala, Gly, Ser, Leu, Ile, Phe, Met, Cys, Pro, Gln and Asp, each with a final concentration of 3 mM, and with Gcl reaction buffer. The final concentration of Glu was 30 mM. Final protein concentration of GshAB was about 0.16 g/L. All reactions were carried out for 3 h at 25 °C, then terminated by 150 μl 500 mM SSA. For the controls, GshAB was added only after the addition of SSA. All samples were conducted with three biological replicates.

To avoid the formation of disulfide bonds, γ-Glu-Cys was derivatized by mixing 180 μl of samples with 20 μl of 2 M NaOH to achieve a final pH of about 8.5, and followed by addition of 20 μl of 100 mM dithiothreitol (DTT, freshly made) and incubation at 50 °C for 30 min. Subsequently, 20 μl of 200 mM iodoacetamide (IAM) was added to the solution, and the resulting mixture was kept in dark for 20 min at room temperature to alkylate cysteine; th6en, 20 μl of 500 mM SSA was added to acidify the solution to about pH 3.0. The alkylated samples (190 μl) were concentrated by 20 μl under vacuum, then resuspended by using 90 μl of 45% acetonitrile (ACN) with 0.1% formic acid (FA, v/v) and 0.5 μg/ml internal dipeptide tyrosine-phenylalanine (YF) for HPLC-MS/MS analysis. The γ-Glu-Cys standard was also alkylated as described above.

Standards of γ-Glu-Ala, γ-Glu-Gly, γ-Glu-Ser, γ-Glu-Met, alkylated γ-Glu-Cys, γ-Glu-Pro, γ-Glu-Gln and γ-Glu-Asp were obtained from United Biosystems (Herndon, VA, USA) and were diluted using 45% ACN with 0.1% FA and 0.5 mg/L YF to a range

from 0.5 to 100 mg/L, and separated using a Wasters Acquity UPLC unit equipped with a InfinityLab Poroshell 120 HILIC-Z, P column (2.7 μm, 50 × 2.1 mm, Phenomenex, Torrance, CA, U.S.A.) and detected using a Xevo TQ MS system (Waters Corporation, Milford, Massachusetts, U.S.A) with multiple reaction monitoring (MRM). Mobile phase A consisted of 0.1 % formic acid in Milli-Q water, while mobile phase B consisted of 0.1 % formic acid in 90% acetonitrile. Samples were eluted as the following procedure: 0−0.50 min, 0.6 ml/min, 100% B; 0.51−11 min, 0.5 ml/min, 100−70% B; 11.01−12.00 min, 0.6 ml/min, 70−100% B; column re-equilibration with 100% B for 6 min at a flow of 0.6 ml/min. Data acquisition was interfaced to the Masslynx v4.1 software (Waters Corporation, Milford, Massachusetts, U.S.A).

For quantification of γ-Glu-Ile, γ-Glu-Leu and γ-Glu-Phe, 200 μl of dipeptides samples were concentrated by SpeedVac vacuum concentrators to 20 μl, then resuspended by using 100 μl of H_2O with 0.1% FA and 0.05 mg/L YF. The standards of these three dipeptides were diluted using H_2O with 0.1% FA and 0.05 mg/L YF and ranged from 0.001 to 1 mg/L. γ-Glu-Ile, γ-Glu-Leu and γ-Glu-Phe were then separated using a Wasters Acquity UPLC unit equipped with a Luna Omega polar C18 column (1.6 μm, 50 × 2.1 mm, Phenomenex, Torrance, CA, U.S.A.) and detected using a Xevo TQ MS system (Waters Corporation, Milford, Massachusetts, U.S.A) with multiple reaction monitoring (MRM). Mobile phase A consisted of 0.1 % formic acid in Milli-Q water, and mobile phase B consisted of 0.1 % formic acid in acetonitrile. Samples were eluted at a flow rate of 0.3 ml/min with the following gradients: 0−0.50 min, 99% A; 0.51-

6.00 min, 99−88% A; 6.01−8.00 min, 88-80% A; column re-equilibration with 99% A for 5 min at a flow rate of 0.5 ml/min. Data acquisition was interfaced to the Masslynx v4.1 software (Waters Corporation, Milford, Massachusetts, U.S.A).

Stress shock, RNA isolation, and reverse transcriptase quantitative PCR (RT-qPCR)

T. halophilus cells were grown in MRS medium supplemented with 1 M of NaCl until exponential phase of growth and harvested by centrifugation. The cell pellets were then resuspended for various stress conditions. Cell suspended in MRS with 1 M NaCl were cultured at different temperature ranging from 4 °C to 37 °C for 4 h; for oxidative stress, cultures were rotated at 160 rpm to create aerobic condition; for salt stress experiments, the cells were incubated in MRS mediums with a final NaCl concentration of 0%, 5.85% (1 M), 20% and 25%, respectively; cells were also incubated in MRS with different pH value ranging from 4-10. At least three biological replicates were conducted for each condition.

After exposure to stress, total RNA of each sample was extracted with RNAiso™ Plus (TaKaRa Bio Inc., Otsu, Shiga, Japan) and treated with RNase-free DNase I (Fermentas, Thermo Scientific, Waltham, USA) for 45 min at 37 °C to remove residual DNA according to the manufacturer's instructions. Total RNA was converted to cDNA with random primers using the PrimeScript RT reagent kit (Takara, Dalian, China).

Quantitative real-time PCR was performed using the 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) with the primers listed in Table S1. The

following procedures were applied: 95 °C for 30 s, followed by 40 cycles at 94 °C for 5 s and at 60 °C for 34 s. The 16S rRNA gene was used to as reference gene and cells grown in a NaCl concentration of 1M at 30 °C and a pH of 7 without shaking were used as reference conditions. Results were calculated using the $\Delta\Delta C_T$ method (Pfaffl 2001).

Amino acid sequences alignment and phylogenetic analysis

Amino acid sequences of previously reported GshAB/GshF were obtained from the Universal Protein Resource (UniProt) database (https://www.uniprot.org/). Multiple sequence alignments of amino acid sequences were performed with CLUSTAL W (Thompson et al. 1994) program using MEGA 6.0 (Tamura et al. 2013). Phylogenetic analysis was conducted by Neighbor-Joining method (Saitou and Nei 1987) with the bootstrap replications of 1000, evolutionary distances were computed according to the Poisson correction method.

Results

Purification of *T. halophilus* GshAB and determination of optimal pH and temperature for enzyme reaction

Recombinant GshAB of *T. halophilus* was overexpressed and purified by affinity chromatography (Fig S1). The overexpressed GshAB was soluble with a molecular weight between 70 kDa and 100 kDa, which conforms to the predicted molecular weight. Gcl enzyme activity of the purified GshAB was highest at 25 °C (Fig. 1A). Generally, the enzyme activity was higher at low-temperatures than at higher temperatures, and the enzyme activity drops significantly above 45 °C (Fig 1A).

Enzyme activity of GshAB was also tested in buffer systems with pH values ranging from 4.4 to 10 (Fig. 1B). The enzyme activity was normalized relative to the activity at pH 8. Gcl activity of GshAB was highest at pH 8. Optimal conditions for the Gcl reaction of GshAB were thus at pH 8 and 25 °C; these conditions were applied for further experiments.

Inhibition effects of cysteine and glutathione on GCS activity of GshAB

The effects of cysteine and glutathione on Gcl activity were determined (Fig. 2). Synthesis of γ-glutamyl-cysteine by the Gcl domain of GshAB increased slightly when the concentration of Cys increased from 1 mM to 6.6 mM. At higher Cys concentrations, Gcl activity decreased. Addition of glutathione inhibited the GCS activity of GshAB (Fig. 2). In conclusion, the Gcl activity of GshAB was inhibited by glutathione and concentrations of Cys exceeding 6 mM.

Synthesis of other γ-glutamyl dipeptides by GshAB

To identify whether GshAB ligates glutamate with amino acids other than cysteine, the purified protein was initially incubated with glutamate and other amino acids. The enzyme reaction was monitored by quantification of phosphate released by Gcl-mediated ATP hydrolysis (Fig. 3). GshAB hydrolysed ATP when mixed with glutamate and one of the other amino acids tested, including Gly, Ser, Thr, Asn, Asp, Lys, Arg, His, Ala, Val, Leu, Ile, Pro, Phe, Met and Trp; phosphate release was lower but still detectable in presence of Gln and Glu. Results thus indicated that in addition to cysteine, GshAB has ATPase activity when other amino acids are present.

Competition experiment evaluating the effects of the presence of other amino acids on Gcl activity

A competition experiment was conducted by mixing the enzyme with Glu, Cys (1 mM, as determined in Fig. S2) and one of the other amino acids, followed by quantification of γ-glutamylcysteine generated by GshAB. The GshAB reaction with Glu and Cys served as control. The fold changes of γ-glutamyl cysteine generated in presence of one other amino acid was used to evaluate the effect of the amino acid on Gcl activity of GshAB (Fig 4). Addition of Ser decreased the γ-glutamyl cysteine concentration while addition of Lys, Arg and Val increased levels of γ-glutamyl cysteine. These results indicated that beside Ser, Cys has a higher affinity to bind GshAB and is ligated to Glu, producing γ-glutamyl cysteine.

Quantification of γ-glutamyl dipeptides generated by GshAB

To determine the synthesis of γ-glutamyl dipeptides other than γ-glutamylcysteine, LC-MS/MS was used to quantify γ-glutamyl dipeptides generated by GshAB (Fig. 5). In the enzyme reaction, Ala, Gly, Ser, Leu, Ile, Phe, Met, Cys, Pro, Gln and Asp were present in equal concentrations; Glu was added to a 10-fold higher concentration. LC-MS/MS detected only γ-glutamyl-cysteine, other glutamyl dipeptides were not detected by LC-MS/MS. The result indicate that GshAB ligate Glu to Cys in priority when mixed with multiple amino acids and generates γ-glutamylcysteine as the main or sole product.

Gene expression of GshAB under different stress conditions

Products of GshAB have been reported to protect LAB cells against various stress conditions, thus the expression of *gshAB* in *T. halophilus* GshAB under various environmental conditions was assessed by quantification of mRNA by RT-qPCR. Experiments were performed at temperatures, oxygen levels, NaCl concentrations, and pH levels (Fig. 6). Different osmotic and pH conditions did not alter the expression level of GshAB significantly. Expression of *gshAB* was highest at around 30°C. Transcription levels of *gshAB* was also higher at aerobic conditions when compared to anaerobic conditions.

Discussion

Glutathione has been considered as the most important thiol compound and protects LAB from multiple stress conditions (Zhang et al. 2010b). The synthesis of glutathione is a two-step ATP-dependent enzyme reaction. The genes responsible for the two steps were suggested to have evolved independently, *gcl* (*gshA*) may have spread from cyanobacteria through horizontal gene transfer while the glutathione synthetase gene (*gshB*) may have evolved from ATP-grasp superfamily protein (Copley and Dhillon 2002; Galperin and Koonin 1997). In *Lactobacillales* and related organisms, glutathione is synthesized through the two-domain protein GshAB/GshF, which was characterized in *Listeria monocytogenes* and *Streptococcus agalactiae* but not in food-fermenting lactic acid bacteria (Gopal et al. 2005; Janowiak and Griffith 2005). The N-terminus of GshAB/GshF shares homology with GshA from cyanobacteria, while the C-terminus ATP-binding domain shows higher identity to D-Ala - D-Ala ligase than

glutathione synthetases (Gopal et al. 2005; Janowiak and Griffith 2005). Analyses of genome sequences indicated that GshAB/GshF genes in streptococci, *Listeria*, and enterococci were disseminated by horizontal gene transfer (Gopal et al. 2005; Janowiak and Griffith 2005). Over-expression of GshA and GshB from *E. coli* in *Lactococcus lactis* demonstrated that the protein is functional but the wild type strain accumulates glutathione by transport rather than *de novo* synthesis (Li et al. 2005). Likewise, GshAB/GshF from *S. agalactiae* and *Streptococcus thermophilus* have been used in producing glutathione (Li et al. 2011; Xu et al. 2019). Remarkably, genomes of heterofermentative lactobacilli frequently encode for Gcl which synthesizes γ-glutamyl dipeptides but the GshB domain that is required for synthesis of glutathione is generally lacking (Xie and Gänzle 2021; Yan et al. 2018).

T. halophilus is a key organism in the fermentation process of foods with high-salt content such as soy sauce or fish sauce. These products are produced for use as condiments with a high salty and umami taste intensity, which is enhanced by the presence of kokumi active compounds (Mouritsen et al. 2017; Zhao et al. 2016). γ-Glutamyl dipeptides in soy sauce contribute to the kokumi activity of the product (Kuroda et al. 2013; Zhao et al. 2016). The current study characterized the bifunctional enzyme GshAB responsible for glutathione synthesis by *T. halophilus*. In particular, we aimed to determine whether the two-domain GshAB is also capable of accumulating γ-glutamyl peptides, as previously shown for three Gcls of *Limosilactobacillus reuteri*, and thus contributes to formation of peptides with kokumi activity in food

fermentations peptides.

The activity of GshAB from *T. halophilus* was inhibited by addition of glutathione. GshAB/GshF from *E. faecalis* and *L. monocytogenes* were also subject to feedback inhibition by glutathione (Gopal et al. 2005; Janowiak et al. 2006; Vergauwen et al. 2006) but GshAB/GshF of *S. thermophilus* and *S. agalactiae* were insensitive to glutathione (Janowiak and Griffith 2005; Li et al. 2011). Structural analysis suggested that glutathione abolished Gcl activity by competitive inhibition of substrate binding to the Gcl domain (Griffith and Mulcahy 1999). Sequence analysis of *T. halophilus* GshAB sequence indicated high conservation of many key residues identified in *E. coli* Gcl such as the Mg^{2+} and substrate binding residue H150 (Hibi et al. 2004), except of a Val for Ile substitution at residue 141 of *T. halophilus* GshAB (Fig. 7A). The amino acid sequence E494-R508 of *S. agalactiae* GshAB was reported to mediate resistance to glutathione inhibition and has a higher sequence identity to the corresponding sequence in *S. thermophilus* (53.3%) than in *T. halophilus* (46.7%). A study with truncated GshAB indicated that this short protein sequence may induce conformational changes that impede glutathione binding (Janowiak et al. 2006) and glutathione inhibition.

GshAB of *S. agalactiae* ligated amino acids other than cysteine to glutamate with a γ-glutamyl bond to generate several different γ-glutamyl dipeptides and γ-Glu-X_{aa}-Gly (Kino et al. 2007). In the phylogenetic tree shown in Fig. 7B, the Gcl domains from *T. halophilus* clusters with the Gcl domains of *E. faecalis* and *E. faecium*, indicating a

closer evolution relationship, but the substrate specificity of the enterococcal GshAB has not been described. LC-MS/MS analysis detected only γ-glutamylcysteine when GshAB activity was assayed in presence of multiple amino acids. Taken together, the available data suggest that glutathione synthesis by GshAB is an optional part of the lifestyle of lactic acid bacteria. The available data on the substrate specificity of the Gcl/GshA domain, and on enzyme inhibition by glutathione suggest that synthesis of glutathione in response to oxidative stress is the main function of the enzyme in most homofermentative lactic acid bacteria including *T. halohilus*. GshAB in *S. agalacticae* and *S. thermophilus* are not inhibited by glutathione, and produce γ-glutamyl di- and tripeptides other than γ-glutamyl cysteine and glutathione, respectively, and may serve additional metabolic or ecological functions. Likewise, heterofermentative lactobacilli do not synthesize glutathione as only the Gcl domain is present. These Gcl enzymes also produce γ-glutamyl dipeptides other than γ-glutamyl cysteine and also have an unknown metabolic or ecological function. The regulation of gene expression of *gshAB* in *T. halophilus*, the substrate specificity, and the inhibition by cysteine and glutathione strongly indicate that GshAB activity in this organism serves to produce glutathione but does not synthesize other γ-glutamyl di- or tripeptides.

The role of glutathione in mitigating intracellular oxidative stress in many organisms as the predominant non-protein thiol compound is well described (Kino et al. 2007). In the present study, RT-qPCR documented that the expression of *gshAB* in *T. halophilus* was up-regulated under aerobic condition to generate glutathione to protect the cell

from reactive oxygen species (Copley and Dhillon 2002). In *Fl. sanfranciscensis*, intracellular glutathione also protected against cold stress including refrigerated storage and freeze-thaw cycles (Zhang et al. 2010a; Zhang et al. 2012). The quantification of *gshAB* expression by *T. halophilus* at lower temperatures, however, did not provide indication of a comparable role in this organism. Likewise, glutathione was reported to protect *Lactococcus lactis* and *Leuconostoc mesenteroides* against acid stress (Kim et al. 2012; Zhang et al. 2007), but quantification of gene expression did not support such a role in *T. halophilus.* Moreover, the activity of GshAB of *T. halophilus* was very low at acidic conditions. Although previous studies have found positive roles of glutathione addition in LAB resistance to osmotic stress (Zhang et al. 2010b), results from current study indicated GshAB was not regulated under different salinity condition and may not play a role in the salinity adaptation process of *T. halophilus*.

In conclusion, the current study characterized the bifunctional protein GshAB from *T. halophilus*, which contributes to the generation of glutathione in in response to oxidative stress. The optimal conditions for enzyme reaction were evaluated and its substrate specificity of the Gcl activity was identified, measuring the ATP consumption verified enzyme reaction when mixing GshAB with Glu and other amino acids, but competition experiments also found that the affinity to Cys is higher. *T. halophilus* GshAB is closely related to GshAB from enterococci and the identification of GshAB from the food-grade LAB *T. halophilus* will contribute to future applications.

Author Contribution Statement

JL and MG conceived and designed research. JL and JX conducted experiments. JL, LL and MG analyzed data. JL and MG wrote the manuscript. All authors read and approved the manuscript

Data Availability Statement.

All data generated or analyzed during this study are included in this published article or were deposited in public databases.

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Statements and Declarations

Ethical approval

The article does not contain any studies with human participants or animals performed by any of the authors

Conflicts of interest

The authors declare no conflicts of interest.

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Figure captions

Figure 1. (A) The γ-glutamyl cysteine generated by GshAB at temperatures ranging from from 4°C to 55°C. (B) The γ-glutamyl cysteine generated by GshAB at different pH-values. Results were normalized to that of pH 8.

Figure 2. Effects of different glutathione or cysteine concentrations on the GCS activity of GshAB. White symbols represent the γ-glu cys generated with glutathione addition, while black symbols represent that of cysteine addition.

Figure 3. Characterization of the substrate specificity of GshAB by assaying the phosphate generated by ATP consumption during enzyme reaction.

Figure 4. Amino acids were added to evaluate their effects on γ-glu-cys synthesis activity of GshAB, results were normalized to the control (only Cys was mixed with the enzyme and Glu) and data obtained from 3 replicates were subjected to one-way analysis of variance (one-way ANOVA), comparisons were made between the mean of each condition and that of the control; significant difference is shown by asterisks.

Figure 5. Concentration of γ-glutamyl dipeptides generated by activity of *T. halophilus* GshAB in presence of multiple amino acids.

Figure 6. Fold change of *gshAB* expression under different temperatures (A), oxygen level (B), NaCl concentration (C) and pH level (D). Cell grown in a NaCl concentration of 1M at 30 °C and a pH of 7 without shaking was used as reference. Gene expression data under different conditions obtained from 3 biological replicates were subjected to one-way analysis of variance (one-way ANOVA) or t test, significant differences ($P <$

0.05) were indicated if the bars do not share a common superscript lowercase letters or indicated with asterisks.

Figure 7. (A) Sequence alignment of *T. halophilus* GshAB with GshAB/GshF from *S. agalactiae* (protein UniProt number: Q8DXM9), *S. thermophilus* (D4N891), *L. monocytogenes* (Q8Y3R3), *P. multocida* (Q9CM00), *E. faecalis* (Q82ZG8) and GCS/GshA from *E. coli*; key residues identified in *E. coli* GCS were indicated in red boxes and the peptide essential for resistance of glutathione inhibition in *S. agalactiae* was showed in blue box (B) Phylogenetic tree showing the evolutionary relationship of the GCS domain in the N-terminal of previously reported GshAB and the GCS of *E. coli*, the percentages of replicate trees (1000 replicates) higher than 80 are shown next to the branches.

Figure 1

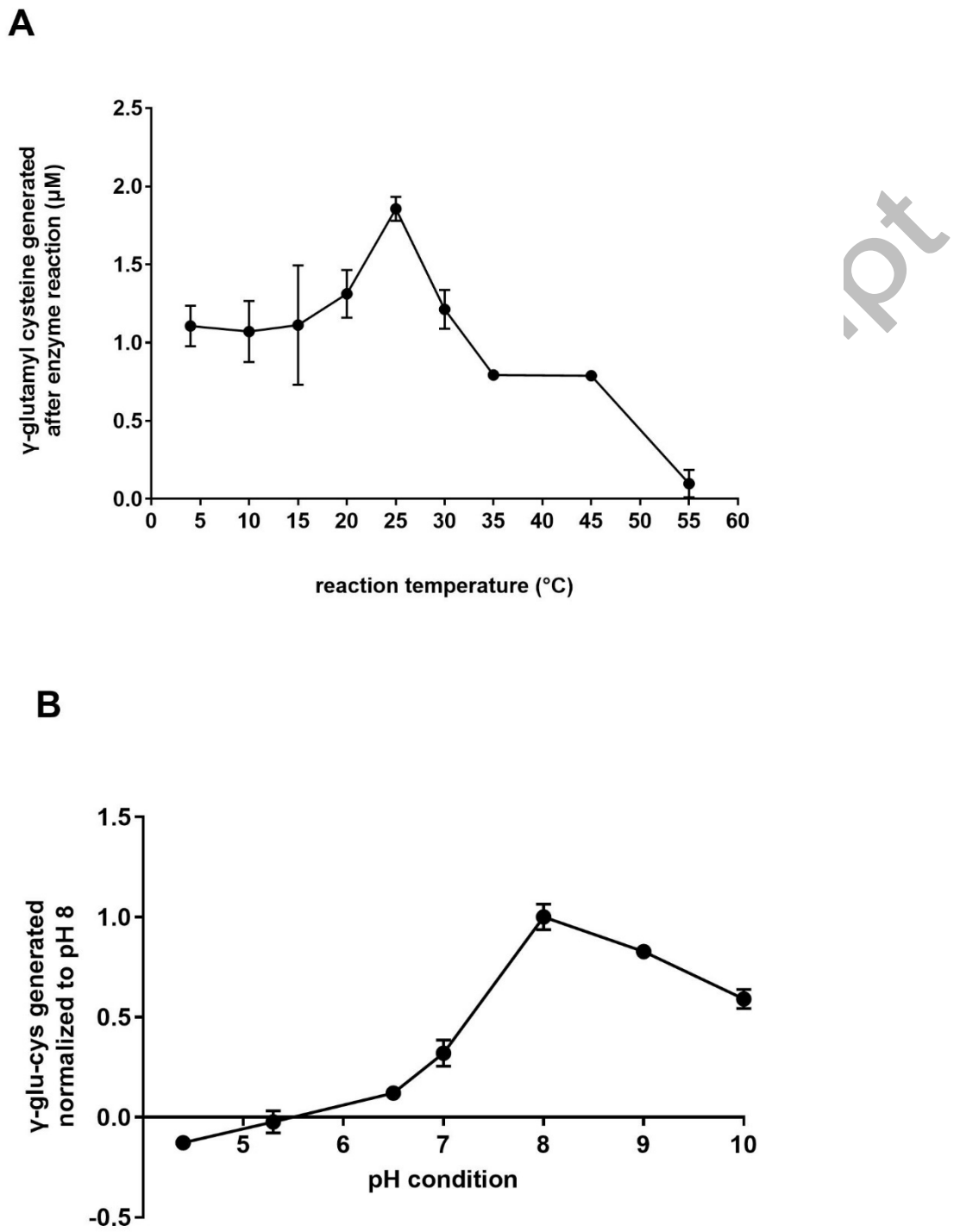

A
γ-glutamyl cysteine generated after enzyme reaction (μM)
2.5
2.0
1.5
1.0
0.5
0.0
0 5 10 15 20 25 30 35 40 45 50 55 60
reaction temperature (°C)
B
γ-glu-cys generated normalized to pH 8
1.5
1.0
0.5
0.0
-0.5
5 6 7 8 9 10
pH condition

Figure 2

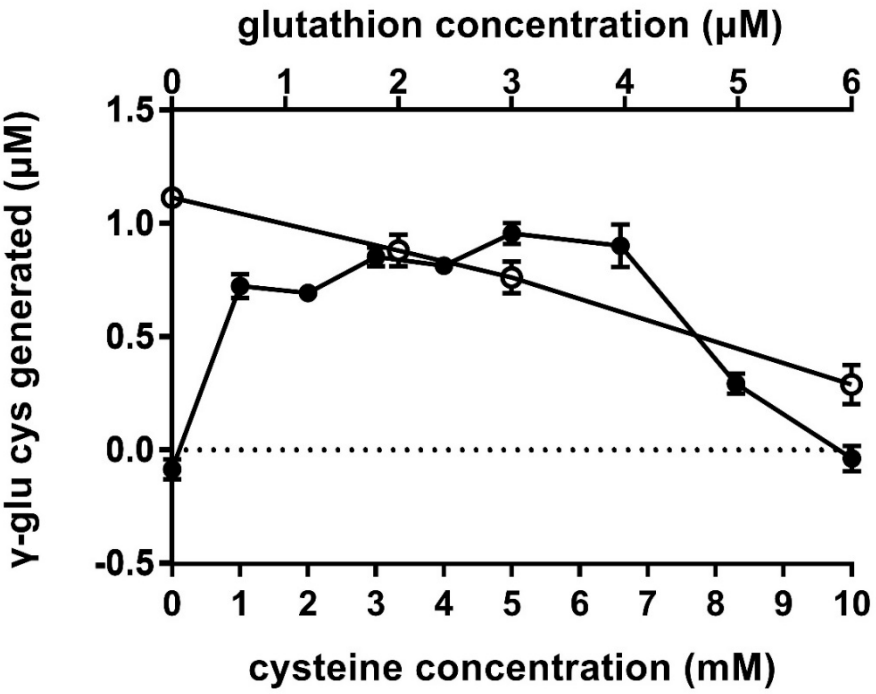


Figure 3

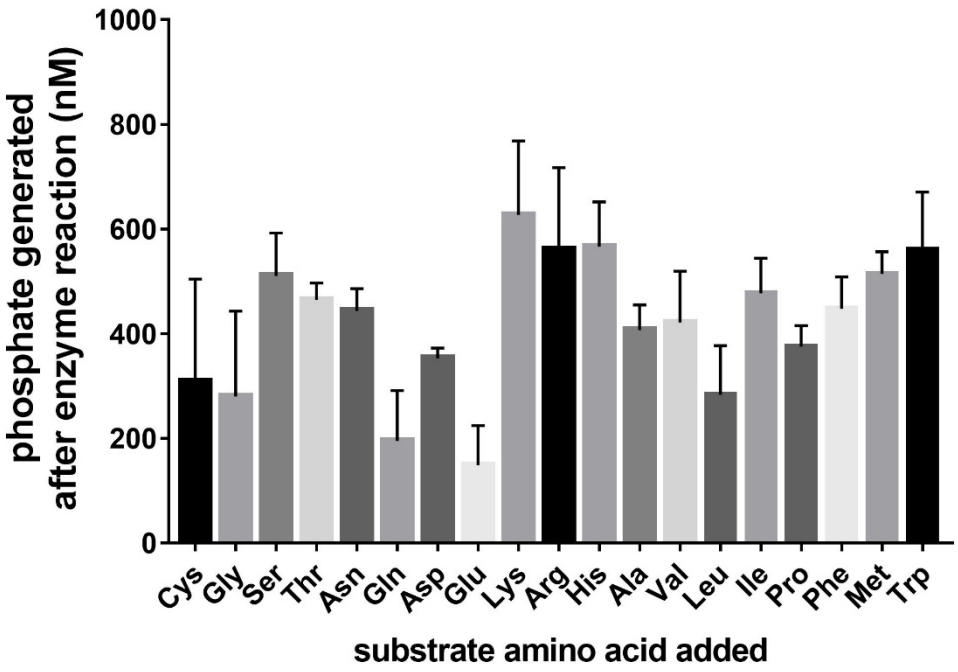


Figure 4

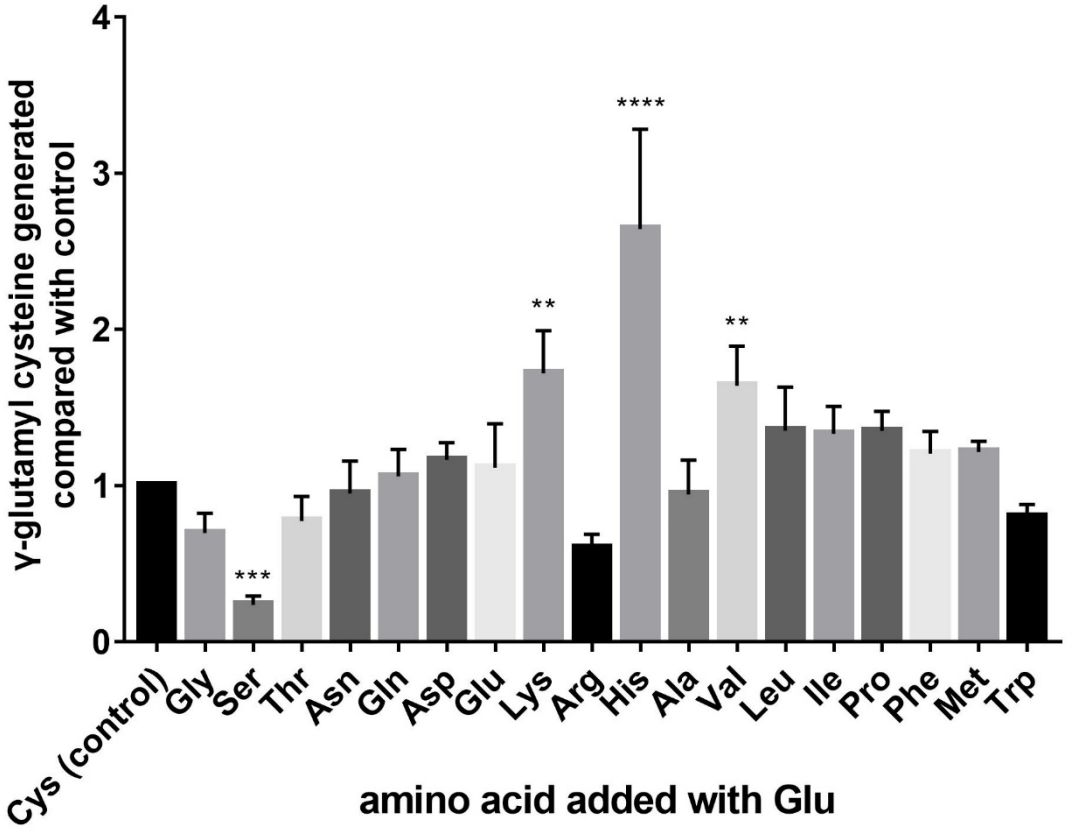


Figure 5

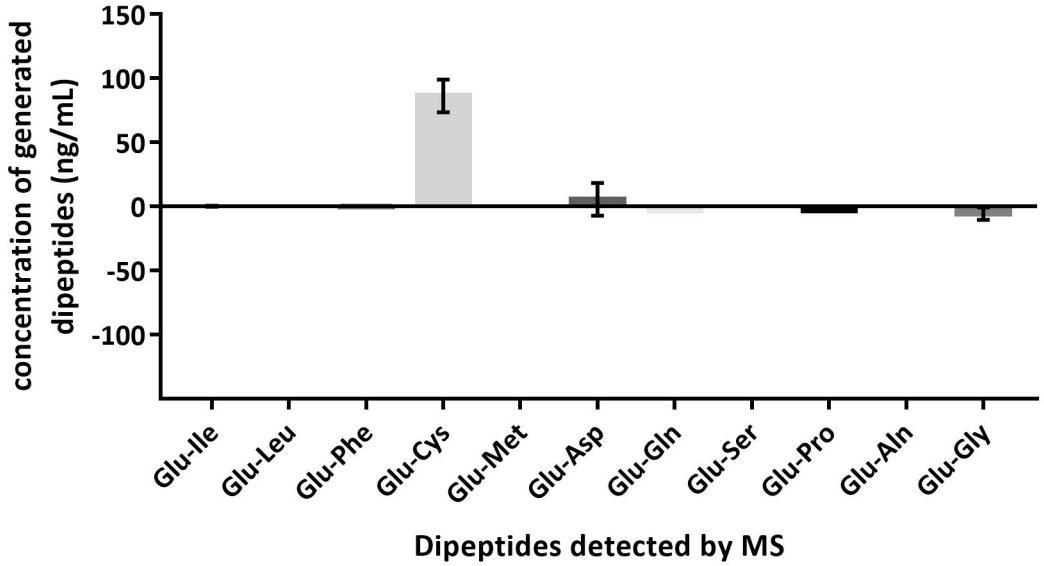


Figure 6

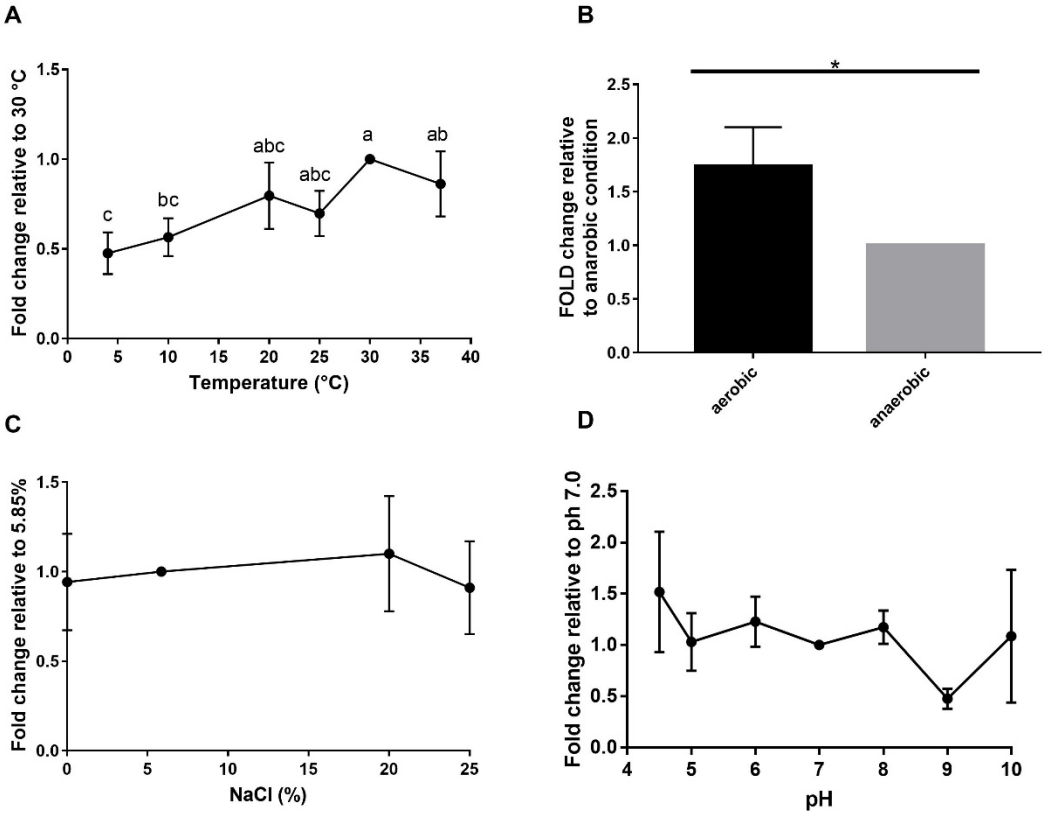
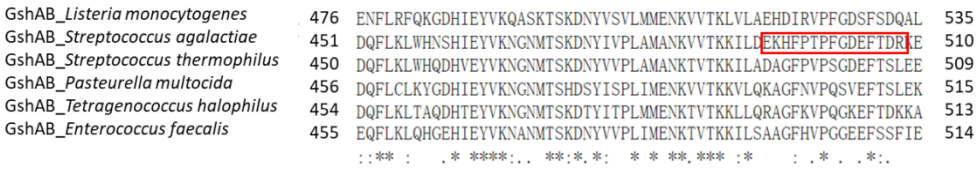


Figure 7

Panel A



Panel B: