J Ind Microbiol Biotechnol (2013) 40:489–494
DOI 10.1007/s10295-013-1239-9

Genomic analysis reveals the biotechnological ability of *Enterococcus italicus* to produce glutathione

Francesca Borgo · Aristodemo Carpen · Chiara Ferrario · Stefania Iametti · Maria Grazia Fortina

Received: 23 December 2012 / Accepted: 1 February 2013 / Published online: 5 March 2013

Abstract Through the analysis of the recently available genome shotgun sequence of *Enterococcus italicus* DSM 15952^T type strain (Accession PRJNA61487, ID 61487), we found the presence of a gene encoding a bifunctional enzyme, termed γ-GCS-GS or GshF, involved in glutathione production and not influenced by feedback inhibition. The *gshF* gene exhibited high nucleotide and amino acid sequence similarity to other reported sequences from the *Enterococcus* genus and was constitutively expressed both in osmotic shock or in common cultural conditions. Several experimental studies concerning the culture medium, physiological stress, cell extract obtainment, and scaling-up showed that in selected conditions *E. italicus* was able to accumulate up to 250 μM of intracellular glutathione, which represented the main thiol group present into the cells. This is the first report regarding the production of glutathione by *E. italicus*, a species that could be used as a safe adjunct culture for glutathione-enriched dairy foods.

Keywords *Enterococcus italicus* · GshF · qRT-PCR · Intracellular glutathione · Fermentation optimization

Abbreviations

GSH	Reduced glutathione
GSSG	Oxidized glutathione
γ-GCS-GS or GshF	Glutamate-cysteine ligase/gamma-glutamylcysteine synthetase
TTG	Total thiol groups
TIG	Total intracellular glutathione

Introduction

Glutathione, a biological active tripeptide consisting of L-glutamate, L-cysteine, and glycine, is an abundant and ubiquitous low molecular mass thiol widely distributed in living organisms, including human, yeast, and bacterial cells [21, 26]. Its biological significance is mainly related to the free sulfhydryl moiety of the cysteine residue, which confers unique redox and nucleophilic properties [23]. Glutathione assumes a pivotal role in bioreductive reactions, transport, in sulfur metabolism [22], in protection against UV [28] and heavy metals [24], and against many exogenous organic substances [14]. During oxidative stress cells are exposed to elevated levels of reactive oxygen species (ROS) [5]. The adaptive response to ROS induces the expression of a number of genes including glutathione reductase and glutaredoxin 1 (*grxA*). This suggests a possible role of glutathione in protective action towards oxidative stress [20].

Owing to its bioactive properties, the commercial demand for GSH is expanding in the pharmaceutical and food industry [17]. Glutathione can be produced by enzymatic methods in the presence of ATP and its precursor amino acids (L-glutamic acid, L-cysteine, glycine) [17].

Electronic supplementary material The online version of this article (doi:10.1007/s10295-013-1239-9) contains supplementary material, which is available to authorized users.

F. Borgo (✉) · C. Ferrario · M. G. Fortina
Department of Food, Environmental and Nutritional Sciences (DeFENS), Division of Food Microbiology and Bioprocesses, Università degli Studi di Milano, 2 Celoria, 20134 Milan, Italy
e-mail: francesca.borgo@unimi.it

A. Carpen · S. Iametti
Department of Food, Environmental and Nutritional Sciences (DeFENS), Division of Chemical and Biomolecular Sciences, Università degli Studi di Milano, 2 Celoria, 20134 Milan, Italy

Alternatively, the molecule can be obtained by direct fermentative methods using sugar as a starting material and *Saccharomyces cerevisiae* and *Candida utilis* as producer species on an industrial scale, by supplementing cultures with precursor amino acids, salts, and carbon and nitrogen sources [32].

The biosynthesis of glutathione in cells usually involves two sequential ATP-dependent reactions catalyzed by γ-glutamylcysteine (γ-GC) synthetase (γ-GCS, EC 6.3.2.2) and GSH synthetase (GS, EC 6.3.2.3). In most cells, these two enzymes are encoded by separate genes, *gshA* and *gshA* (*gsh1* and *gsh2* in eukaryotes), respectively. The activity of γ-GCS is subjected to feedback inhibition by the final product to avoid overaccumulation of glutathione, and formation of γ-GC is the rate-limiting step in the biosynthesis pathway.

In contrast to the extensive studies in eukaryotic cells, relatively little is known about glutathione in prokaryotes [18]. A survey of protein databases like PFAM and NCBI reveals that genes with high similarity to *gshA*, but not to *gshB*, are found in Gram-positive bacteria, suggesting that these bacteria lack the capability to synthesize glutathione, although they are able to import the molecule from the surroundings [18]. However, there is evidence that some lactic acid bacteria are able to synthesize glutathione in relatively high concentration, as do *Enterococcus faecalis* and *Streptococcus thermophilus* [19, 27].

Through the analysis of the recently available genome shotgun sequence of *Enterococcus italicus* DSM 15952^T type strain (Accession PRJNA61487, ID 61487) [3], we found in this enterococcal species a gene coding for an enzyme possessing both γ-GCS and GS activities. This bifunctional enzyme, termed γ-GCS-GS or GshF, identified in a few of Gram-positive bacteria, is reported to remove the feedback inhibition, thereby enhancing glutathione biosynthesis [31].

The species *E. italicus*, proposed a few years ago [11], was found in several cheeses coming from different geographic areas. In previous studies [2, 12] we showed that this novel enterococcal species is characterized by a low potential virulence profile (low antibiotic resistance rates and absence of virulence factors) in comparison with other enterococcal clinical strains [6]. For these reasons *E. italicus* could represent a new safe adjunct culture for the dairy industry.

The aim of this study was to provide evidence that *E. italicus* species is characterized by the presence of a *gshF* gene encoding the bifunctional enzyme γ-GCS-GS, induced upon oxidative stress. Moreover, we studied how the glutathione-producing capacity of *E. italicus* may depend on parameters of cultivation during batch fermentation. This is the first report on *E. italicus* as a promising producer of glutathione in a simple medium without addition of precursors. The results reported here make this species especially attractive for tailoring of cheeses with enhanced flavor and for development of bio-preservation strategies.

Materials and methods

Bacterial strains

Enterococcus italicus type strain (TP1.5^T-DSM 15952^T German Collection of Microorganism and Cell Cultures, Braunschweig, Germany) was studied in comparison with *Lactococcus lactis* (IL1403-NIZO Food Research, the Netherlands) used as negative control for the production of glutathione [18]. For *gshF* detection studies a collection of 18 *E. italicus* strains, previously isolated from different dairy sources [3], were used. The strains, stored frozen (−80 °C) in M17 (Difco, Detroit, MI, USA) supplemented with 15 % (w/v) glycerol, were grown at 37 °C for 24 h in M17.

gshF detection

Total DNA was extracted as described by Borgo et al. [3]. The *gshF* primers were designed on the basis of the *E. italicus* DSM 15952^T whole genome shotgun sequence (Accession PRJNA61487, ID 61487). Before use, the sequence of the forward primer was checked with the BLAST tool (Basic Local Alignment Search Tool) at the National Center for Biotechnology Information (NCBI) to ensure that no matches with other genes were present [1]. The primers were synthesized by PRIMM (Milan, Italy) and the sequences were 5′-CGGAGCAGCTGGACCGAAAG-3′ and 5′-CGGCTTCACGACAAAGCCGT-3′ (forward and reverse, respectively). Each 25 µl reaction mixture contained 100 ng of bacterial DNA, 2.5 µl of 10X reaction buffer (Dream *Taq* buffer, MBI-Fermentas, Vilnius, Lithuania), 200 mM of a deoxynucleoside triphosphate mixture, 2.5 mM $MgCl_2$, 0.5 µM of each primer, and 0.5 U of *Taq* polymerase (Dream *Taq* DNA-polymerase, MBI-Fermentas). The amplification was performed in a Gene Amp PCR System 2400 (Perkin-Elmer, Norwalk, CT, USA) and conducted through 30 cycles of denaturation at 94 °C for 1 min and annealing at 58 °C for 1 min, with extension at 72 °C for 2 min. The final cycle was followed by an additional 7-min elongation period at 72 °C. PCR products were separated on 1.5 % agarose gel, stained with ethidium bromide in 1X Tris acetate–EDTA buffer (40 mM Tris acetate, 1 mM EDTA, pH 8.0), and photographed under UV light.

Quantitative RT-PCR

RNA was extracted with a NucleoSpin RNA II extraction kit (Macherey–Nagel, GmbH&Co. KG, Düren, Germany) according to the manufacturer's instructions. The mRNA concentration and purity were optically determined using a UV–Vis spectrophotometer (SmartSpec^{TM} Plus, Bio-Rad, Milan, Italy), which measured sample absorbencies at wavelengths of 260 and 280 nm. Based upon the final concentrations, 100 ng of mRNA was rewritten into cDNA using a RevertAid First Strand cDNA synthesis kit (MBI-Fermentas) through the following thermal cycle: 5 min at 25 °C, 30 min at 42 °C, and 5 min at 85 °C. The mRNA expression levels of *gshF* were analyzed with SYBR green technology in real-time quantitative PCR (qPCR) using SsoFast EvaGreen Supermix (Bio-Rad, Milan, Italy) on a Bio-Rad CFX96 system according to the manufacturer's instructions. In order to quantitatively measure the expression of the *E. italicus gshF*, amplification of the 16SrDNA gene as the endogenous control gene was performed simultaneously, and the relative expression level was determined according to the method reported by Pfaffi [25]. The primers used were *gshF*rRNA forward, 5′-GCCATC GAAGATCCTACCAA-3′, and *gsh*RvRNA reverse, 5′-GACCGACAATCCCTTTTGAA-3′ and 5′-GAAGTCGT AACAAGG-3′ AND 5′-GTGTCTCAGTCCAGTGTGG-3′ for 16SrDNA. The specificity of the primers was tested with melting curves during amplification and by 1 % agarose gels. Quantitative PCR was carried out according to the following cycle: initial hold at 96 °C for 30 s, followed by 40 cycles at 96 °C for 2 s and 60 °C for 5 s. The amount of template cDNA used for each sample was 1.25 ng.

Batch cultivation in flasks and fermentor

A frozen culture of bacterial cells was inoculated into 10 ml of primary M17 culture medium in a 50-ml Erlenmeyer flask and incubated for 16 h in stationary conditions at 37 °C. Primary seed culture was used to inoculate (1 %) 100 ml of secondary modified culture media (see below) in a 500-ml flask and grown for 16 h at 37 °C. For the evaluation of intracellular total thiol group (TTG) production, two different M17-modified media were checked: M17 without yeast extract (M17-m1) and M17-m2 (without yeast extract and triptone). To investigate the import ability of the strains, the media were supplemented with 50 mg l^{-1} of GSH (Sigma-Aldrich, Inc., St Louis, Mo, USA). In a further step, the effects of NaCl concentration (3–10 %, w/v), of different incubation temperatures (15, 25, 37, and 42 °C), and of anaerobic and aerobic conditions were also studied.

Batch fermentation for glutathione intracellular content was carried out in a 6-l bioreactor (Prélude, Biolafitte & Moritz, Pierre Guerin Technologies, Mauze, France). The initial culture conditions were as follows: working volume 3 l, 1 % inoculum, temperature 37 °C, pH 6.5 (maintained by automatic addition of NaOH or HCl 1 M), shaking rate 40*g*. At the end of the fermentation period, the culture was harvested by centrifugation (Beckman model J2-21 Centrifuge, Beckman Coulter Inc., USA) at 6,000*g* for 20 min and the pellet was stored at −20 °C until further use. Each growth experiment was carried out in duplicate.

Preparation of cell-free extracts and protein analysis

The cell pellet was washed twice with 100 mM phosphate buffer (pH 7) and suspended in 1 ml of the same buffer. Glass beads (∅ 0.1 mm, Sigma; 0.5 g ml^{-1}) were added to the cell suspension, which was then sonicated in an ultrasonic disintegrator (SoniPrep 150, Fisons®, Sussex, England) operating with 45-s pulses at a 30-s interval for a total of 6 cycles. Cell debris was removed by centrifugation at 13,000*g* at 4 °C for 15 min to give a cell-free extract (CFE). Protein concentration in the CFE was determined by the method of Bradford [4] with bovine serum albumin (Sigma) as a standard.

Total thiol groups (TTG) and total intracellular glutathione (TIG) determination

The CFEs were deproteinized by addition of sulfosalicyclic acid (5 % w/v, final concentration). Precipitated proteins were removed by centrifugation (11,000*g*, 10 min) and the clear supernatants were used for total thiols (TTG) and total intracellular glutathione (TIG) determination. TTG were determined spectrophotometrically according to the method of Ellman [7], and using a molar extinction coefficient of 13,600 at 412 nm. Total intracellular glutathione (GSH plus GSSG) was determined through the recycling assay of Tietze [30], in which GSSG is reduced enzymatically to GSH, which is then allowed to react with an excess of Ellman's reagent. The intracellular GSH content was also evaluated by fluorescent labeling with monobromobimane (mBBr) essentially as described by Fahey and Newton [8]. Briefly, water (340 µl) and the supernatant from the sulfosalycylic acid treatment (120 µl) were pipetted into a 1.5-ml plastic tube. *N*-Ethylmorpholine (100 µl, 1 M) was added to the wall of the tube and mBBr (20 µl, 0.1 M) was immediately added with mixing. The reaction was allowed to proceed for 20 min in the dark. After addition of glacial acetic acid (20 µl), samples were stored at −20 °C in the dark until being analyzed. The mixture was centrifuged at 18,000*g* for 5 min before injection into a µBONDRACK column (4.6 × 250 mm)

packed with 5-μm reversed-phase material (Polymer Laboratories, Cartersville, Georgia, USA). A Waters 600E high-performance liquid chromatograph equipped with a Dilutor 401 injector (Gilson) and a Waters 470 fluorometer-detector (excitation wavelength, 394 nm; emission wavelength, 480 nm) was used. Elution solvent A contained 14.2 % methanol and aqueous 0.25 % acetic acid adjusted to pH 3.9 with sodium hydroxide (50 %), and solvent B contained 90 % methanol in the same acetate-based buffer. The following elution profile was used: isocratic (100 % A) for 18 min, followed by 100 % B for 8 min, at a flow of 0.8 ml min^{-1}. All experiments were repeated twice and averaged for analyses.

Results and discussion

Detection of *gshF* gene in *Enterococcus italicus* and expression studies

Since the genome sequence of *E. italicus* TP1.5^T (DSM 15952^T) revealed the presence of the *gshF* gene encoding a bifunctional enzyme, termed γ-GCS-GS, a primer set based on this sequence was used to amplify a 970-bp internal fragment and to verify the presence of the gene in various strains. Eighteen collection strains, coming from different dairy sources, tested positive for the *gshF*-specific amplification, suggesting that this gene could be considered a common genetic trait of the species.

A comparison between the nucleotide sequence of *E. italicus gshF* and other reported nucleotide sequences in related and unrelated species showed high similarity (75 %) with *gshF* genes of other *Enterococcus* species. A lower level of similarity was found with the *gshF* genes of *Streptococcus* spp. (64 %), *Listeria* spp. (50 %), and *Clostridium* spp. (50 %) [15, 29]. *E. faecalis* and *E. faecium* are reportedly able to produce glutathione [27], but their presence as adjunct cultures is not desirable. *E. italicus* could thus be considered an interesting alternative for the production of high antioxidant-content foods.

Figure S1 shows the primary structure of the *E. italicus* 757 amino acid GshF protein (Accession PRJNA61487, ID 61487) in comparison with sequences in the same organisms discussed above. BLAST analysis of the amino acid sequence in GshF showed that its N-terminal portion (residues 14–474) exhibits moderate sequence identity to domains identified in the glutamylcysteine ligase domain (PF04262) of bacterial glutamylcysteine ligase (EC 6.3.2.2). The C-terminal portion of GshF contains an ATP-grasp domain (residues 486–591). This is one of several distinct ATP-binding folds, and it is found in enzymes that catalyze the formation of amide bonds through the ATP-dependent ligation of a carboxylate-containing molecule to an amine or thiol group [13]. This fold is found in many different enzyme families, including various peptide synthetases, biotin carboxylase, synapsin, succinyl-CoA synthetase, pyruvate phosphate dikinase, and glutathione synthetase [9].

To confirm that the *gshF* gene from *E. italicus* encodes functional enzymes, we employed qRT-PCR to quantify transcript levels of the *gshF* gene in cells grown under different growth conditions. Expression levels were monitored both in standard cultural conditions (M17 medium, 37 °C, 16 h incubation) or in a M17 medium containing additional 3 % NaCl. In the latter case, a 10-fold increase in the expression level of *gshF* was observed. This could suggest that *gshF* encodes a constitutive enzyme, whose expression is positively modulated in the presence of an osmotic stress. As reported by other authors [19, 27] the increase of intracellular glutathione during osmotic shock could be due to the role played by glutathione in maintaining cell viability, to its involvement in the regulation of K^+ export channels, and to its role in increasing inducible antioxidant enzymes.

Effects of different media and growth conditions on total thiol group production (TTG)

Several media and growth conditions were screened to assess the possibility of modulating the ability of *E. italicus* to produce and accumulate TIG. Yeast extract provides 95 % of total glutathione in the M17 medium, the remainder coming from peptone [16]. In modified M17 media lacking one or both of the glutathione sources, *E. italicus* growth was lowered by 30 %, but the levels of total thiol production were still much higher than those in *L. lactis* (Table 1), thus demonstrating the ability of *E. italicus* to perform de novo synthesis of glutathione. An increase in cell biomass could lead to increased GSH production, but an increased cell density often corresponds to a decrease in cellular GSH content. The experiments reported in Table 1 also indicate that *E. italicus* is able to import glutathione from its environment, as reported for other Gram-positive bacteria.

Another approach that can be exploited to improve GSH accumulation within producing cells is the addition of precursor amino acids to the medium [17]. However, this approach increases cost. NaCl-induced osmotic stress protocols—originally developed for some yeasts [10]—were investigated as an alternative and inexpensive method of GSH overproduction in bacteria. The application of osmotic stress to growing *E. italicus* cells led to a 116 % increase in TTG (from 26 to 71 μM in a minimal medium (M17-m1) containing 3 % NaCl, see Table 1).

The same medium was therefore used to evaluate the effect of different temperatures on cell growth and TTG

Table 1 Effects of different media on dry cell weight (DCW) and total thiol group (TTG) production in *E. italicus* TP1.5^T and *L. lactis* IL1403

Medium	DCW ± SD (g l^{-1})		TTG ± SD (μM)	
	E. italicus	*L. lactis*	*E. italicus*	*L. lactis*
M17[a]	0.7 ± 0.035	0.7 ± 0.035	80 ± 4.00	75 ± 3.75
M17-m1[b]	0.5 ± 0.025	0.5 ± 0.025	26 ± 1.30	<18 ± 0.90
M17-m2[c]	0.4 ± 0.020	0.36 ± 0.018	23 ± 1.15	<18 ± 0.90
M17-m1 ST[d]	1.0 ± 0.050	0.9 ± 0.045	160 ± 8.00	140 ± 7.00
M17-m2 ST	0.9 ± 0.045	0.86 ± 0.043	152 ± 7.60	145 ± 7.25
M17-m1 3 % NaCl	0.4 ± 0.020	0.2 ± 0.010	71 ± 3.55	ND

Significant differences ($p < 0.05$) among means were determined with a one-way analysis of variance

SD standard deviation, *ND* not detected

[a] Control medium contained (g l^{-1}) 5.0 triptone, 5.0 soy peptone, 5.0 meat extract, 2.5 yeast extract, 0.5 ascorbic acid, 0.25 magnesium sulfate, 19.0 disodium glycerophosphate, and 10 glucose (pH 6.5)

[b] M17-m1 yeast extract free

[c] M17-m2 yeast extract and triptone free

[d] ST = 50 mg l^{-1} of GSH added to the tested media

production. In *E. italicus* TP1.5^T, the highest TTG content was observed at 37 °C, whereas the best biomass yield was observed at 42 °C.

Thus, conditions maximizing cell growth did not exactly match those maximizing TTG production. Similar results were obtained by Wei et al. [32] studying the effect of temperature on growth and GSH production by *Candida utilis*.

The influence of oxygen was also studied, by growing *E. italicus* TP1.5^T under anaerobic, static aerobic, and agitated aerobic conditions in M17-m1 containing 3 % NaCl. Cellular yield increased slightly during anaerobic growth, but intracellular TTG concentration did not vary between cells grown anaerobically or statically under aerobic conditions (data not shown). The lowest extraction yield was obtained when *E. italicus* TP1.5^T was grown aerobically with shaking, likely as a consequence of a change in the morphological arrangement of cells, which in these conditions afforded long cell chains and cell aggregates (Fig. S2).

Batch fermentation

GSH production *E. italicus* TP1.5^T was scaled up by carrying out batch fermentation in a 6-l bioreactor. The experiments were carried out either in standard complete M17 medium or under osmotic stress (M17 + 3 % NaCl).

Table 2 Total thiol groups (TTG), total intracellular glutathione (TIG), and GSH content in *Enterococcus italicus* grown in a 6-l bioreactor

Average ± SD	M17	M17 + 3 % NaCl
DCW (g l^{-1})	2.1 ± 0.11	1.4 ± 0.07
% Extraction yield	15.0 ± 0.75	12.3 ± 0.61
TTG (μM)	150.0 ± 7.50	247.5 ± 12.37
TIG (μM)	112.0 ± 5.60	250.0 ± 12.50
TIG/DCW (μmol g^{-1})	53.3 ± 2.66	178.5 ± 8.92
Cytosolic protein (g l^{-1})	21.1 ± 1.05	14.5 ± 0.72
TIG/cytosolic protein (μmol g^{-1})	5.3 ± 0.26	17.2 ± 0.86
GSH (μM)	26.9 ± 1.34	45.0 ± 2.25

Significant differences ($p < 0.05$) among means were determined with a one-way analysis of variance

SD standard deviation

As shown in Table 2, *E. italicus* produced more TIG under osmotic stress and this seemed to turn on glutathione biosynthesis despite reduced growth. Under osmotic stress the maximal TIG concentration achieved was 250 μM, as determined by a simple enzyme assay able to evaluate the total TIG (GSH + GSSG). These species accounted for most or all of the reactive thiol groups present in the cells. Under osmotic stress, the intracellular concentration of the reduced form of glutathione (GSH) almost doubled (as determined by the fluorometric monobromobimane assay) with respect to controls, confirming the relevance of this form of the compound for cell survival under osmotic stress.

Conclusion

The results obtained in this study showed the ability of *E. italicus* species to perform de novo synthesis (and import) of glutathione. This synthetic activity relates to the presence of a functional *gshF* gene encoding a bifunctional enzyme not influenced by feedback inhibition. With the development of a simple fermentation process employing a cheap medium (without the need for specific precursors), the concentration of intracellular glutathione (oxidized and reduced) reached a concentration of 250 μM.

These preliminary results are promising in terms of providing an alternative way to produce glutathione. More relevantly, they could provide a straightforward approach to glutathione-enriched food. This is of relevance for dairy products in particular, because in this case *E. italicus* can be used as a safe adjunct culture.

Acknowledgments This work was supported by the "Post genomica batterica per la qualità e sicurezza degli alimenti" project from the Lombardy region (Italy). The authors thank F. Bonomi for helpful comments about the manuscript.

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