Appl Microbiol Biotechnol (2005) 67: 83–90
DOI 10.1007/s00253-004-1762-8

Yin Li · Jeroen Hugenholtz · Wilbert Sybesma · Tjakko Abee · Douwe Molenaar

Using *Lactococcus lactis* for glutathione overproduction

Received: 19 May 2004 / Accepted: 31 August 2004 / Published online: 13 October 2004

Abstract Glutathione and γ-glutamylcysteine were produced in *Lactococcus lactis* using a controlled expression system and the genes *gshA* and *gshB* from *Escherichia coli* encoding the enzymes γ-glutamylcysteine synthetase and glutathione synthetase. High levels of γ-glutamylcysteine were found in strains growing on chemically defined medium and expressing either *gshA* alone or both *gshA* and *gshB*. As anticipated, glutathione was found in a strain expressing *gshA* and *gshB*. The level of glutathione production could be increased by addition of the precursor amino acid cysteine to the medium. The addition of cysteine led to an increased activity of glutathione synthetase, which is remarkable because the amino acid is not a substrate of this enzyme. The final intracellular glutathione concentration attained was 358 nmol mg^{-1} protein, which is the highest concentration reported for a bacterium, demonstrating the suitability of engineered *L. lactis* for fine-chemical production and as a model for studies of the impact of glutathione on flavour formation and other properties of food.

Introduction

Glutathione (γ-glutamyl-L-cysteinylglycine, reduced form GSH) is the major non-protein thiol compound present in living organisms and is involved, predominantly, in buffering redox status of the cytoplasm and protecting cells against oxidative damage (Carmel-Harel and Storz 2000). It is synthesised in two ATP-dependent steps (Meister and Anderson 1983). The dipeptide γ-glutamylcysteine (γ-GC) is first synthesised from L-glutamic acid and L-cysteine by γ-glutamylcysteine synthetase (also known as glutamate-cysteine ligase, EC 6.3.2.2, γ-GCS). In the second step, catalysed by glutathione synthetase (EC 6.3.2.3, GS), glycine is added to the C-terminal end of γ-GC to form GSH. In microorganisms, GSH is found primarily in eukaryotes and in Gram-negative bacteria, but rarely in Gram-positive bacteria (Fahey et al. 1978). Of the latter, GSH is found in some low-GC content Gram-positive bacteria (*Streptococcus*, *Lactococcus*, *Lactobacillus*, *Enterococcus*, *Clostridium* and *Listeria*) (Fahey et al. 1978; Fernández and Steele 1993; Newton et al. 1996). Other Gram-positive bacteria (i.e. *Streptococcus thermophilus*, *Streptococcus agalactiae* and *Enterococcus faecalis*) were reported to synthesise GSH (Newton et al. 1996). However, there is, to the best of our knowledge, no direct evidence showing the presence of the activities of γ-GCS and GS in those strains. In addition, analysis of the genome databases reveals that the genes *gshA* and *gshB*, encoding γ-GCS and GS, respectively, are present neither in the genome sequences of *S. agalactiae* and *E. faecalis* (KEGG genes database 2004) nor in that of *S. thermophilus* (Genome sequencing project of *S. thermophilus*2004), which casts doubts on the conclusions drawn by Newton et al. (1996).

A survey of protein databases like PFAM and NCBI reveals that genes with high similarity to *gshA* are found in Gram-positive bacteria. However, genes with similarity to *gshB* have until now not been observed in Gram-positives (see also Copley and Dhillon 2002). The assignment of a function to the corresponding γ-GCS-like hypothetical proteins is uncertain, particularly because these organisms are not known to synthesise GSH (Newton et al. 1993, 1996). These observations do suggest that Gram-positive bacteria generally lack the capability to synthesise glutathione, although some strains are able to import glutathione from the environment (Sherrill and Fahey 1998).

Y. Li · J. Hugenholtz · W. Sybesma · D. Molenaar (✉)
Wageningen Centre for Food Sciences, NIZO food research,
P.O. Box 20, 6710 Ede, The Netherlands
e-mail: douwe.molenaar@nizo.nl
Tel.: +31-318-659674
Fax: +31-318-650400

Y. Li
The Key Laboratory of Industrial Biotechnology, Ministry of Education, Southern Yangtze University,
Huihe Road 170,
Wuxi, 214036, People's Republic of China

T. Abee
Laboratory of Food Microbiology, Wageningen University,
6703 Wageningen, The Netherlands

In a previous study (Li et al. 2003), we provided evidence that dairy strains of *Lactococcus lactis* cannot synthesise glutathione, but are able to take it up from the growth medium. Using *L. lactis* ssp. *cremoris* SK11 as a model strain, we found that glutathione taken up by strain SK11 increases its resistance to oxidative stress. As a consequence of these studies we were interested in producing glutathione in *L. lactis*. This could putatively render the cells more resistant to oxidative stress, but could also have beneficial results on fermentation products made using this organism. For example, it has been shown that flavour development in cheese curds is enhanced when glutathione is added (Singh and Kristoffersen 1971). In the present study, using the nisin-controlled expression system (NICE) for *L. lactis* (de Ruyter et al. 1996; Kleerebezem et al. 2000), a very high intracellular content of GSH, 358 nmol mg^{-1} protein, was attained by simultaneously expressing *gshA* and *gshB* genes from *Escherichia coli*. To our knowledge, this is the first example of an engineered Gram-positive bacterium producing glutathione. Furthermore, the glutathione content found in recombinant *L. lactis* is the highest reported in bacteria. This strain also offers the opportunity to study the effect of in situ glutathione production on the properties of fermented foods.

Materials and methods

Chemicals

GSH, γ-GC, ATP, and all amino acids were purchased from Sigma (St. Louis, Mo.). Monobromobimane (mBBr) was purchased from Calbiochem (San Diego, Calif.). All inorganic compounds were of reagent grade or high quality.

Bacterial strains, plasmids, and media

The strains and plasmids used in this study are listed in Table 1. *Escherichia coli* TG1, which was used as a chromosome DNA template for amplifying genes *gshA* and *gshB*, was grown with aeration in tryptone yeast (TY) extract broth at 37°C for 16 h. Strains of *L. lactis* were grown without aeration at 30°C in a chemically defined medium (CDM) as described previously (Li et al. 2003) or in M17 broth (Merck, Darmstadt, Germany), supplemented with 5 g l^{-1} glucose. When appropriate, the medium contained chloramphenicol (10 μg ml^{-1}) as a selection marker. To analyse the effect of gene over-expression, the NICE system was used (de Ruyter et al. 1996; Kleerebezem et al. 2000). For glutathione production and enzyme activity analysis, *L. lactis* cells were grown until an optical density (OD) at 600 nm of approximately 0.4 was achieved. Subsequently, the culture was split into two cultures. Nisin (2 ng ml^{-1}) was added to one of the two cultures, and both cultures were grown for an additional 4–8 h to reach the stationary phase.

DNA manipulations

Isolation of chromosome DNA of *E. coli* and standard recombinant DNA techniques were performed as described by Sambrook et al. (1989). Isolation and transformation of *L. lactis* DNA were performed as previously described (de Vos et al. 1989).

Construction of strains and plasmids

The *E. coli gshA* gene was amplified by PCR using *Pwo* DNA polymerase and chromosomal DNA of *E. coli* TG1 as template DNA with the primers 5′-GAGGCCATGG-CAATCCCGGACGTATCACAGGCGC-3′ and 5′-TTCTTCTAGATCAGGCGTGTTTTTCCAGCCACAC-3′. The 1,574-bp PCR product generated was cloned into pNZ8148 using the *Nco*I and *Xba*I sites that were introduced by the primers used, yielding the *gshA* overexpression plasmid pNZ3201.

The *E. coli gshB* gene was amplified by PCR using *Pwo* DNA polymerase and chromosomal DNA of *E. coli* TG1 as template DNA with the primers 5′-TTGGTCTAGAG-GAGAAGAATAATGATCAAGCTCG-3′ and 5′-AAG-GAAGCTTTTACTGCTGCTGTAAACGTGCTTC-3′. The 982-bp PCR product generated, containing the Shine-Dalgarno sequence upstream of the *gshB* gene of *E. coli*, was cloned into pNZ8148 and pNZ3201, respectively, using the *Xba*I and *Hind*III sites that were introduced by the primers used, yielding the *gshB* overexpression

Table 1 Strains and plasmids used in this study. *Cm*^{r} Chloramphenicol resistant

Strain or plasmid	Relevant characteristics	Source or reference
Strains		
Lactococcus lactis NZ9000	MG1363 *pepN::nisRK*	Kuipers et al. 1998
Escherichia coli TG1	Derivative of *E. coli* K-12	
Plasmids		
pNZ3201	Cm^r, pNZ8148 derivative containing a functional *E. coli gshA* gene	This work
pNZ3202	Cm^r, pNZ8148 derivative containing a functional *E. coli gshB* gene	This work
pNZ3203	Cm^r, pNZ8148 derivative containing a functional *E. coli gshA* and *gshB* gene	This work
pNZ8148	Cm^r, inducible expression vector, carrying the *nisA* promoter	

plasmid pNZ3202, and the *gshA* and *gshB* overexpression plasmid pNZ3203.

Plasmids pNZ3201, pNZ3202 or pNZ3203 were introduced into *L. lactis* strain NZ9000 by electroporation. Subsequently, thiol producing capacity was evaluated by HPLC.

Preparation of cell-free extracts and protein analysis

Pre-chilled, overnight grown culture (50 ml) was harvested by centrifugation (10,000 *g*, 10 min, 4°C). Harvested cells were washed twice with ice-cold saline (0.85% NaCl, w/v) and re-suspended in 1 ml 200 mM phosphate buffer (pH 7.0) containing 2 mM EDTA. From this cell suspension, 1 ml was added to a vial with 1 g glass beads and broken using a mini bead beater (FastPrep FP120, Savant, Farmingdale, N.Y.) at 4°C for 30 s, following which the cell debris was removed by centrifugation (10,000 *g*, 10 min, 4°C), resulting in cell-free extract (CFE). Protein concentrations were determined using a BCA protein assay kit using bovine serum albumin as the standard (Pierce, Rockford, Ill.). For protein analysis lactococcal CFE was mixed with an equal amount of 2-fold concentrated Laemmli buffer and, after heating at 95°C for 10 min, 10 μl of each sample was analysed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

Thiol assays

mBBr fluorescent labelling and HPLC methods were used to determine the intracellular thiol (cysteine, γ-GC and glutathione) contents according to methods in the literature (Fahey and Newton 1987). A 90 μl aliquot of a cell suspension was added to 200 μl 50 mM HEPPSO (pH 7.5) containing 50% aqueous acetonitrile, 1 mM dithiothreitol, and 5 mM DTPA, following which 5 μl mBBr (50 mM in acetonitrile) was added. The mixture was heated for 30 min at 60°C, and was subsequently acidified by adding 5 μl 1.2 M methanesulfonic acid. The mixture was centrifuged at 20,000 *g* for 5 min prior to injection to a PLRP-S 300 Å column (4.6 mm ×250 mm) packed with 5 μm reversed-phase material (Polymer Laboratories, Church Stretton, UK), using a Waters 600E HPLC (Waters, Milford, Mass.) equipped with a Dilutor 401 injector (Gilson, Villiers-le-Bel, France) and an FL2000 fluorometer-detector (Spectra Physics, Darmstadt, Germany) (excitation at 390 nm, emission measured at 480 nm). The elution solvent A is 5% (v/v) aqueous acetonitrile, 0.25% (v/v) glacial acetic acid, pH 3.5, and solvent B is 90% acetonitrile and 0.25% glacial acetic acid in water. The following elution profile was used. Isocratic conditions (100% solvent A) were maintained for 2 min, stepping directly to 35% solvent B in 35 min and to 100% solvent B in further 3 min and maintained for 5 min, followed by an immediate return to initial conditions for re-equilibration (15 min) before loading the next sample. Flow rate was maintained at 1 ml min^{-1} throughout.

Enzyme assays

The activity of γ-GCS was measured by the method of Jackson (1969) with a slight modification. The reaction mixture contained 30 mmol L-glutamate, 30 mmol L-cysteine, 30 mmol ATP, 20 mmol $MgSO_4 \cdot 7H_2O$, 200 mmol KCl, 200 mmol diethanolamine-HCl buffer (pH 9.15), and CFE in a final volume of 500 μl. Incubation was carried out at 37°C for 15 min. The reactions were terminated by adding 825 μl ice-cold 3.2% sulphosalicylic acid. After removing the protein precipitates by centrifugation, 90 μl sample (or control) supernatant was used to measure the γ-GC concentrations using the mBBr fluorescent labelling HPLC method described above.

The activity of GS was measured according to the method of Gushima et al. (1983a). The reaction was carried out in 200 μl of a mixture containing 5.0 mM γ-GC, 10 mM glycine, 10 mM ATP, 10 mM $MgCl_2$, 100 mM Tris-HCl buffer (pH 7.5), and CFE at 37°C for 15 min. The reactions were terminated by adding 500 μl ice-cold 3.2% sulphosalicylic acid. After removing the protein precipitates by centrifugation, the thiol concentrations were measured in 90 μl sample (or control) by the mBBr fluorescent labelling HPLC method.

In the above two assays, the composition of the control was the same as the reaction mixture except that sulphosalicylic acid was added prior to addition of CFE. One unit of specific enzyme activity was defined as the amount of enzyme that catalysed the synthesis of 1 nmol γ-GC or GSH per minute per milligram of protein.

H_2O_2 treatment

L. lactis NZ9000 cells harbouring plasmids pNZ8148, pNZ3201, pNZ3202, and pNZ3203 were grown anaerobically in CDM at 30°C until an OD_{600} of approximately 0.4 was achieved, to which 2 ng ml^{-1} nisin was added to induce gene expression. The induction phase lasted until cell growth reached stationary phase (OD 2.5, usually after 4 h induction). Samples (10 ml) of the cultures were harvested, washed twice in cold saline, and re-suspended in a volume of saline to achieve a final OD_{600} of 2.5. Of this cell suspension, 1 ml was treated with 10 mM H_2O_2 for 30 min, pelleted by centrifugation at 10,000 *g* for 1 min, then washed again with saline to remove the residual H_2O_2. Cells were re-suspended in the same volume of saline, and either spread on M17 plates at different dilutions to measure survival, or inoculated into CDM with an inoculum size of 1% (v/v) for growth experiments. Colony counting results were the mean of three plates incubated at 30°C for 48 h with standard deviation shown as error bars.

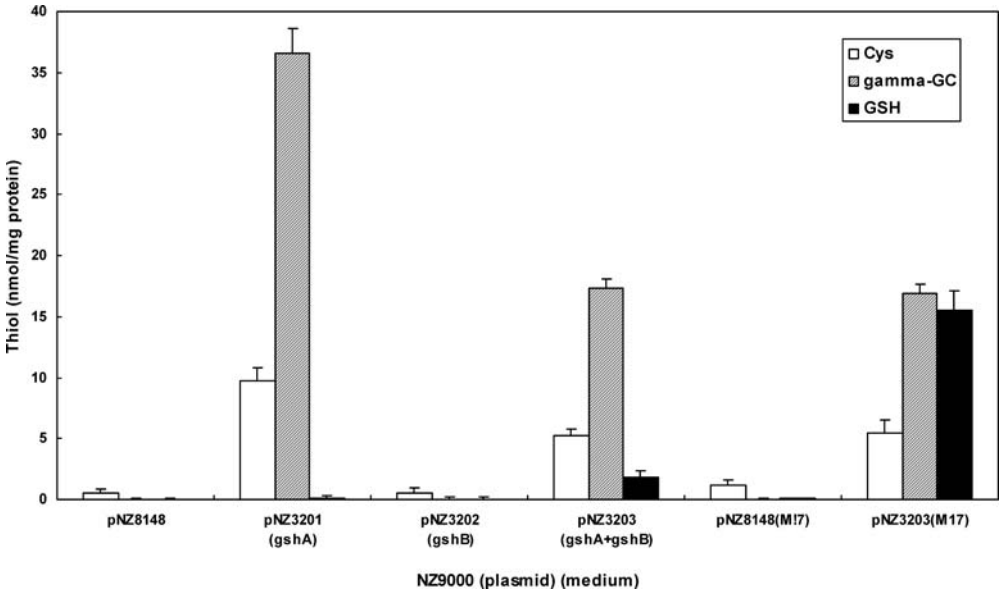

Fig. 1 Intracellular thiol content of *Lactococcus lactis* NZ9000 harbouring different plasmids grown in chemically defined medium (CDM) (not shown) or M17 broth (illustrated here). Cells were harvested after 4 h induction with 2 ng ml^{-1} nisin. *Cys* Cysteine, *gamma-GC* γ-glutamylcysteine, *GSH* glutathione

Growth experiments

A 10 µl aliquot of the H_2O_2-treated and saline-washed cell suspension was inoculated into 1 ml fresh CDM. Subsequently, 200 µl culture was transferred into a well of a microtitre plate (Corning, Corning, N.Y.). Growth was monitored at 600 nm in a kinetic microtitre plate reader (SPECTRAmax PLUS 384; Molecular Devices, Sunnyvale, Calif.). Each growth experiment was carried out in triplicate. The effect of the duration of the lag phase was estimated by measuring the time needed to reach five times the initial optical density. Results represent the mean of these time intervals estimated from three independent growth curves.

Results

Production of glutathione in CDM

To introduce glutathione producing capability in *L. lactis*, the NICE system was used. As glutathione is not present in most Gram-positive bacteria (Fahey et al. 1978), and the combined *gshA* and *gshB* genes could not be found in the currently available genome sequences of Gram-positive bacteria, *gshA* and *gshB* genes were amplified from *E. coli*, cloned and introduced into *L. lactis* NZ9000. Figure 1 shows that, after nisin induction, a high amount of γ-GC was produced in strain NZ9000 (pNZ3201), while a small amount of glutathione was produced in strain NZ9000 (pNZ3203). There was no detectable glutathione in cell extracts of strain NZ9000 (pNZ3201) and strain NZ9000 (pNZ3202), indicating that *L. lactis* NZ9000 lacks γ-GCS and GS activities.

To evaluate the expression level of γ-GCS and GS, *L. lactis* NZ9000 harbouring the empty vector pNZ8148 (as a control), pNZ3201, pNZ3202 and pNZ3203 were grown under nisin inducing conditions, and extracts of the cultures were prepared and analysed by SDS-PAGE (Fig. 2). Growth of strain NZ9000 (pNZ3201) and NZ9000 (pNZ3203) in the presence of nisin resulted in the appearance of an additional protein band with an apparent molecular mass of approximately 58 kDa, which is the expected size of γ-GCS. In addition, growth of strain NZ9000 (pNZ3202) and NZ9000 (pNZ3203) in the presence of nisin resulted in the appearance of an additional protein band with an apparent molecular mass of approximately 36 kDa, which is the expected size of GS. The high and functional expression of *gshA* in strain NZ9000 (pNZ3201) resulted in a high level of γ-GC production (Fig. 1), and the expression of *gshA* and *gshB* in strain NZ9000 (pNZ3203) resulted in the production of GSH. Remarkably, the intracellular level of γ-GC in strain NZ9000 (pNZ3203) was only half that of strain NZ9000 (pNZ3201), and the level of GSH in strain NZ9000 (pNZ3203) was very low (Fig. 1). This might be due to the fact that the expression levels of *gshA* and *gshB* in strain NZ9000 (pNZ3203) were lower than those in strains with

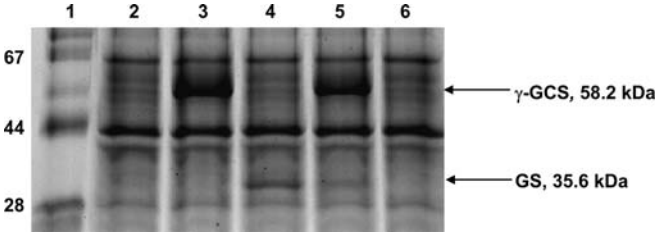

Fig. 2 Coomassie blue-stained gel after sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of cell-free extract (CFE) of *L. lactis* NZ9000 grown in CDM in the presence (*lanes 2–5*) or absence (*lane 6*) of nisin. Lanes: *1* Protein standards (molecular masses in kDa indicated on the left), *2–6L. lactis* NZ9000 harbouring pNZ8148 (*2*), pNZ3201 (*3*), pNZ3202 (*4*), pNZ3203 (*5*, *6*). Additional bands resulting from nisin induction and representing γ-glutamylcysteine synthetase (*γ-GCS*) protein (*lanes 3, 5*) and glutathione synthetase (*GS*) protein (*lanes 4, 5*) are indicated by *arrows*

Table 2 Effect of addition of amino acids mixture on production of thiol by *L. lactis* NZ9000 (pNZ3203). Cells were grown in M17 broth and harvested after 6 h induction with 2 ng ml^{-1} nisin. *γ-GC* γ-glutamylcysteine, *GSH* glutathione

Concentration (mM)[a]	Intracellular thiol content (nmol mg^{-1} protein)			
	Cysteine	γ-GC	GSH	GSH/γ-GC
0	9±2	46±7	47±5	1
0.5	33±4	34±5	71±9	2
5	95±4	15±4	294±25	20
10	121±15	3±1	358±17	120

[a]A concentrated, freshly prepared mixture containing glutamate, cysteine, and glycine (200 mM each) was filter-sterilised and added to the culture together with 2 ng ml^{-1} nisin when an OD_{600} of 0.4 was achieved

the individually expressed genes (Fig. 2). In addition, the expression level of *gshB* in strain NZ9000 (pNZ3202) was significantly lower than that of *gshA* in strain NZ9000 (pNZ3201), suggesting that the presence of *gshA* upstream of the *gshB* gene decreased the efficiency of transcription.

Increased production of glutathione by *L. lactis* ssp. *cremoris* NZ9000 (pNZ3203)

The low intracellular level of glutathione in strain NZ9000 (pNZ3203) grown in CDM might be due to the poor nutrient supply in CDM. Therefore, instead of CDM, M17 broth was used to assess the overproduction level of GSH in strain NZ9000 (pNZ3203), since strain NZ9000 does not take up GSH present in M17 broth. *L. lactis* NZ9000 is a derivative of *L. lactis* ssp. *cremoris* MG1363, which was demonstrated to lack the capability of taking up GSH from the environment in a previous study (Li et al. 2003). Growth in M17 broth with nisin induction resulted in a nearly 8-fold increased GSH level compared to that of the culture grown in CDM (Fig. 1), suggesting that nutrient composition can greatly affect the production of glutathione.

Remarkably, the intracellular molar ratio of GSH to γ-GC of the culture grown in CDM (1:9) decreased to nearly 1:1 when cells were grown in M17 broth (Fig. 1). The latter ratio was still unusually low, since γ-GC, as an intermediate, does not accumulate to such a high level in *E. coli* (Carmel-Harel and Storz 2000). As a first approach to increase the production of GSH on CDM, the three precursor amino acids of glutathione, namely L-glutamate (Glu), L-cysteine (Cys), and glycine (Gly) were added to the medium. It has been reported that addition of precursor amino acids enhanced the production of GSH by *E. coli* (Li et al. 1998). In *L. lactis* NZ9000 (pNZ3203), upon addition of 5 mM and 10 mM of the three precursor amino acids, the levels of GSH appeared to be increased 6-fold and 8-fold, respectively, relative to the level without added amino acids (Table 2). More importantly, the ratio of GSH to γ-GC increased along with the increase of the concentration of mixed amino acids. A very high ratio of 120:1 was achieved upon addition of 10 mM mixed amino acids (Table 2), suggesting that addition of precursor amino acids significantly stimulated the conversion of γ-GC to GSH.

To study which amino acid was responsible for the increased production of GSH, different combinations of Glu, Cys, and Gly (5 mM of each) were added to nisin-

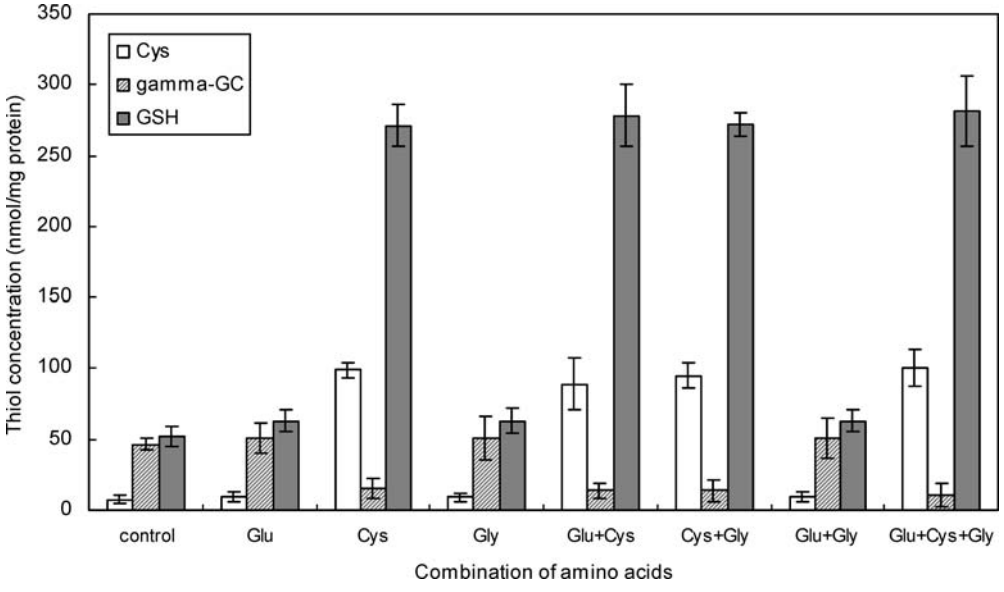

Fig. 3 Effect of different combinations of glutamate, cysteine, and glycine (5 mM of each) on the production of thiol by *L. lactis* NZ9000 (pNZ3203)

Table 3 Activities of γ-GCS and GS in *L. lactis* ssp. *cremoris* NZ9000 (pNZ3203) grown in M17 broth supplemented with different concentrations of cysteine. Cells were grown in M17 broth and harvested after 6 h induction with 2 ng ml^{-1} nisin.

Cysteine concentration (mM)	Enzyme activity (nmol mg^{-1} protein min^{-1})	
	γ-GCS	GS
0	122±12	2.3±0.3
0.5	125±18	3.7±0.7
5	117±15	9.6±1.9
10	114±8	12±2
E. coli TG1[a]	11±1	3.0±0.5

[a]*E. coli* TG1 was freshly inoculated into TY broth and cultivated until stationary phase was achieved

induced cultures. Figure 3 clearly demonstrates that the increased production of GSH upon addition of mixed amino acids can be fully attributed to the addition of cysteine. Although glycine might be expected to be a putative stimulator of the conversion of γ-GC to GSH, this was shown not to be the case (Fig. 3). The stimulating effect of cysteine on the conversion of γ-GC to GSH is remarkable, since cysteine itself is not involved in the conversion process of γ-GC to GSH. However, analysis of γ-GCS and GS activities in strain NZ9000 (pNZ3203) revealed the interesting phenomenon that GS activity increased by roughly 4-fold and 5-fold when 5 mM and 10 mM cysteine, respectively, were added to the culture, while the activity of γ-GCS remained at a high level (Table 3). The increased activity of GS upon cysteine addition may account for the increased conversion of γ-GC to GSH, but the mechanism behind this effect remains unknown.

Expression of γ-GCS enhances cysteine uptake

The expression of γ-GCS in strain NZ9000 (pNZ3201) and the expression of γ-GCS and GS in strain NZ9000 (pNZ3203) resulted in intracellular levels of cysteine increased 20-fold and 10-fold, respectively, compared to the control (Fig. 1). In addition, the effect of adding mixed amino acids on the production of GSH by strain NZ9000 (pNZ3203) also indicated that the intracellular level of cysteine increased (up to 13-fold) upon addition of 10 mM mixed amino acids (Table 2). Therefore, it would be interesting to know whether the increased intracellular level of cysteine was caused by increased biosynthesis or increased uptake capability. To elucidate this phenomenon, strain NZ9000 (pNZ3203) and strain NZ9000 (pNZ8148) were grown in CDM free of nisin and no growth difference was found (Fig. 4). When nisin was added to the medium at the beginning of cultivation, the growth of strain NZ9000 (pNZ8148) was almost unaltered, while the growth rate of strain NZ9000 (pNZ3203) was severely decreased (Fig. 4), possibly due to the protein burden. However, the addition of 0.5 mM and 5 mM cysteine

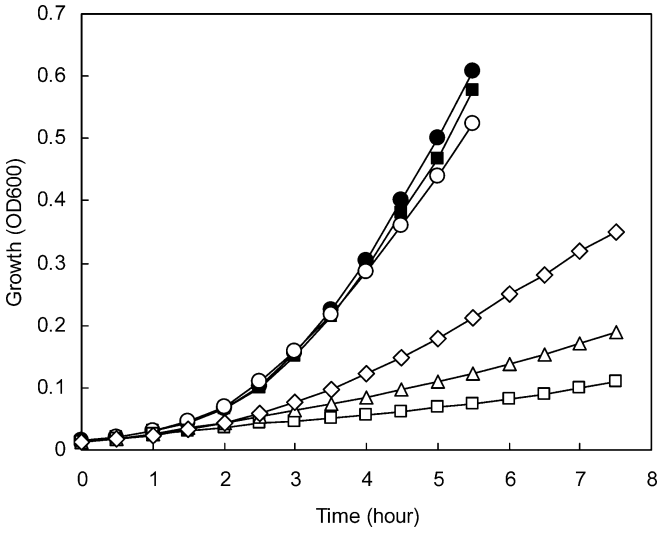

Fig. 4 Growth of *L. lactis* ssp. *cremoris* NZ9000 (pNZ8148) (*circles*) and NZ9000 (pNZ3203) (*squares*) in CDM in the absence (*closed symbols*) and presence (*open symbols*) of 2 ng ml^{-1} nisin. *Triangles* and *rhombuses* represent the growth of strain NZ9000 (pNZ3203) in the presence of 0.5 mM and 5 mM cysteine, respectively. Nisin was added to the medium at the beginning of cultivation and growth was monitored in a kinetic microtitre plate reader. The pre-culture was cultivated in CDM without nisin

partly restored the growth of strain NZ9000 (pNZ3203) in the presence of nisin, indicating that the cells took up more cysteine from the medium to satisfy physiological demand. The effect also demonstrates that the capacity for cysteine biosynthesis limits growth in strain NZ9000 (pNZ3203). Furthermore, when grown in medium supplemented with 5 mM additional cysteine under nisin induction, the intracellular cysteine level of strain NZ9000 (pNZ3203) (±100 nmol mg^{-1} protein) was 2.5-fold higher than that of strain NZ9000 (pNZ8148) (±40 nmol mg^{-1} protein). This result indicated that the expression of *gshA* and *gshB* significantly enhanced cysteine uptake.

Glutathione does not protect strain NZ9000 (pNZ3203) against oxidative stress

To investigate whether the GSH produced in strain NZ9000 (pNZ3203) leads to an increased resistance to oxidative stress as shown in *L. lactis* ssp. *cremoris* SK11 (Li et al. 2003), cells of NZ9000 harbouring plasmid pNZ8148, pNZ3201, pNZ3202, and pNZ3203 were grown in CDM and induced by 2 ng ml^{-1} nisin. Cells induced for 4 h and 20 h were washed and exposed to 10 mM H_2O_2 for 15 min followed by measurement of the survival rate of colony forming units. Unexpectedly, strain NZ9000 (pNZ3203) did not exhibit increased resistance to treatment with H_2O_2 (data not shown). On the contrary, the resistance to H_2O_2 treatment of strain NZ9000 (pNZ3203) was slightly decreased compared to that of strain NZ9000 (pNZ8148). To investigate whether the resistance of strain NZ9000 (pNZ3203) to H_2O_2 treatment is growth phase

dependent, cells grown in difference phases after nisin induction were withdrawn and exposed to H_2O_2 treatment, upon which the growth of subcultures was monitored. The duration of the lag phase of strain NZ9000 (pNZ3203) in subcultures was longer than that of strain NZ9000 (pNZ8148), independent of the growth phase (data not shown). This confirms that the GSH produced in strain NZ9000 (pNZ3203) does not lead to a higher level of resistance to oxidative stress.

Discussion

L. lactis has become a routine prokaryotic host for metabolic engineering, and a variety of strategies have been proven to be successful (Kleerebezem and Hugenholtz 2003). The biosynthesis of GSH is a relatively simple pathway but is not present in *L. lactis*. The genes encoding γ-GCS and GS cannot be found in the currently available genome sequence of *L. lactis* ssp. *lactis* IL1403 and experimental evidence for the lack of GSH biosynthetic capacity was provided in a previous study (Li et al. 2003). However, some strains of *L. lactis* do have the ability to take up glutathione from the environment. Glutathione taken up by *L. lactis* ssp. *cremoris* SK11 increases resistance to H_2O_2 stress (Li et al. 2003).

In this study, GSH biosynthetic capability was introduced into *L. lactis* ssp. *cremoris* NZ9000 by over-expressing the *gshA* and *gshB* genes from *E. coli* using the NICE system. Cloning of *gshA* and *gshB* genes from *E. coli* is not new but the attempt to express these two genes in Gram-positive bacteria has never been made. Nearly 20 years ago the *gshA* and *gshB* genes were separately cloned from *E. coli* B (Murata et al. 1983; Murata and Kimura 1982) and sequenced (Gushima et al. 1984; Watanabe et al. 1986a). Although the introduction of the hybrid plasmid pGS500 carrying both *gshA* and *gshB* genes into *E. coli* RC912 resulted in a simultaneous increase in the activities of γ-GCS (10-fold) and GS (14.5-fold), the intracellular GSH concentration in *E. coli* RC912 (pGS500) cells increased only 1.3-fold compared to the wild type (Gushima et al. 1983b). The reason for this disappointing result was ascribed to the fact that the activity of γ-GCS in *E. coli* is feedback inhibited by GSH. The GSH concentration required for 50% inhibition of γ-GCS, 2.5 mM (Watanabe et al. 1986b), is comparable to the intracellular level measured in stationary phase cells of *E. coli* B (approximately 3 μmol ml^{-1} packed cells) (Gushima et al. 1983b; Murata et al. 1981). A similar phenomenon was observed in recombinant *Saccharomyces cerevisiae* over-expressing *gshA* and *gshB* genes from *E. coli* B. There, the intracellular glutathione content increased only 2-fold although the expression of γ-GCS and GS increased 1,039-fold and 33-fold, respectively (Ohtake et al. 1988, 1989).

Upon addition of 10 mM cysteine, the activities of γ-GCS and GS in *L. lactis* NZ9000 (pNZ3203) were 10-fold and 4-fold, respectively, of those measured in *E. coli* TG1 (Table 3), indicating that both genes were over-expressed in *L. lactis*. However, compared with the enzyme activities in *E. coli* RC912 (pGS500) cells calculated from literature (Gushima et al. 1983b), the activities of γ-GCS and GS in *L. lactis* NZ9000 (pNZ3203) were not exceptionally high: the activity of γ-GCS was 2.7-fold that in *E. coli* RC912 (pGS500) while the activity of GS was only one-fifth that in *E. coli*. In spite of this, the intracellular GSH concentration in *L. lactis* NZ9000 (pNZ3203), 358 nmol mg^{-1} protein (approximately 140 mM, assuming an intracellular volume of 2–3 μl per milligram of cell protein, Table 2), was roughly 20-fold of that of *E. coli* RC912 (pGS500) (Gushima et al. 1983b) and 3-fold of that of *S. cerevisiae* YNN27 (pGSX120E) (Ohtake et al. 1989). This unexpectedly high concentration of GSH in *L. lactis* NZ9000 (pNZ3203) indicates (1) that there is no γ-glutamyltranspeptidase activity present in *L. lactis* (in *E. coli* and *S. cerevisiae* this enzyme degrades GSH); (2) the feedback inhibition of GSH on γ-GCS seems not to be effective in *L. lactis* NZ9000 (pNZ3203), since the GSH concentration of 140 mM is obviously higher than the concentration required for feedback inhibition. The latter phenomenon is interesting, since it has been proposed that feedback inhibition of γ-GCS by GSH is the rate-limiting step in glutathione biosynthesis (Anderson 1998; Huang et al. 1988). There are three possible interpretations: (1) the glutathione produced by *L. lactis* is an oxidised form (GSSG) or bound to protein. This possibility was excluded because the presence of dithiothreitol (a strong thiol reducing agent) in the pre-treatment buffer for thiol analysis did not affect the GSH concentration measured by HPLC (data not shown); (2) the structure of γ-GCS was modified by the host, which is an unlikely possibility; (3) the unexpectedly high intracellular concentration of cysteine, caused by increased cysteine uptake, releases (or desensitises) γ-GCS from feedback inhibition by GSH.

Cysteine availability is normally the key rate-limiting factor in glutathione biosynthesis, and the maintenance of adequate intracellular GSH levels is dependent upon the extracellular availability and transport of cysteine into cells (Anderson 1998). In this study, we found that expression of *gshA* enhanced the activity of the cysteine transport system of *L. lactis*, although the exact mechanism is unknown. More strikingly, the activity of GS in *L. lactis* was improved by increasing the extracellular cysteine availability. SDS-PAGE did not show significant differences in GS levels under different cysteine concentrations (data not shown).

The GSH produced by *L. lactis* NZ9000 (pNZ3203) does not increase resistance to H_2O_2 treatment. Since strain NZ9000 neither produces nor imports glutathione, it possibly lacks the enzymatic machinery to use glutathione for the reduction of oxidative stress. This might explain why NZ9000 does not benefit from glutathione production. Alternatively, a strain like SK11 that is known to benefit from glutathione could be used as a host to produce glutathione.

The strain or its method of construction described here can be used to investigate the effects of in situ glutathione production by lactic acid bacteria on properties of

fermented foods like cheeses, where beneficial effects of the addition of glutathione have already been shown (Singh and Kristoffersen 1971).

Acknowledgements The authors want to thank Mr. Jan van Riel, for his assistance in HPLC analysis. This project was partly supported by National Science Foundation of China (Contract No. 30300009).

References

Anderson ME (1998) Glutathione: an overview of biosynthesis and modulation. Chem Biol Interact 111–112:1–14

Carmel-Harel O, Storz G (2000) Roles of the glutathione- and thioredoxin-dependent reduction systems in the *Escherichia coli* and *Saccharomyces cerevisiae* responses to oxidative stress. Annu Rev Microbiol 54:439–461

Copley SD, Dhillon JK (2002) Lateral gene transfer and parallel evolution in the history of glutathione biosynthesis genes. Genome Biol 3:research0025.1–research0025.16

Fahey RC, Newton GL (1987) Determination of low-molecular-weight thiols using monobromobimane fluorescent labeling and high-performance liquid chromatography. Methods Enzymol 143:85–96

Fahey RC, Brown WC, Adams WB, Worsham MB (1978) Occurrence of glutathione in bacteria. J Bacteriol 133:1126–1129

Fernández L, Steele JL (1993) Glutathione content of lactic acid bacteria. J Dairy Sci 76:1233

Genome sequencing project of *Streptococcus thermophilus* (2004) http://www.biol.ucl.ac.be/gene/genome

Gushima H, Miya T, Murata K, Kimura A (1983a) Purification and characterization of glutathione synthetase from *Escherichia coli* B. J Appl Biochem 5:210–218

Gushima H, Miya T, Murata K, Kimura A (1983b) Construction of glutathione-producing strains of *Escherichia coli* B by recombinant DNA techniques. J Appl Biochem 5:43–52

Gushima H, Yasuda S, Soeda E, Yokota M, Kondo M, Kimura A (1984) Complete nucleotide sequence of the *E. coli* glutathione synthetase gsh-II. Nucleic Acids Res 12:9299–9307

Huang CS, Moore WR, Meister A (1988) On the active site thiol of gamma-glutamylcysteine synthetase: relationships to catalysis, inhibition, and regulation. Proc Natl Acad Sci USA 85:2464–2468

Jackson CR (1969) Studies in the enzymology of glutathione metabolism in human erythrocytes. Biochem J 111:309–313

KEGG genes database (2004) http://www.genome.ad.jp

Kleerebezem M, Hugenholtz J (2003) Metabolic pathway engineering in lactic acid bacteria. Curr Opin Biotechnol 14:232–237

Kleerebezem M, Hols P, Hugenholtz J (2000) Lactic acid bacteria as a cell factory: rerouting of carbon metabolism in *Lactococcus lactis* by metabolic engineering. Enzyme Microb Technol 26:840–848

Kuipers OP, de Ruyter PG, Kleerebezem M, de Vos WM (1998) Quorum sensing-controlled gene expression in lactic acid bacteria. J Biotechnol 64:15–21

Li Y, Chen J, Mao YY, Lun SY, Koo YM (1998) Effect of additives and fed-batch culture strategies on the production of glutathione by recombinant *Escherichia coli*. Process Biochem 33:709–714

Li Y, Hugenholtz J, Abee T, Molenaar D (2003) Glutathione protects *Lactococcus lactis* against oxidative stress. Appl Environ Microbiol 69:5739–5745

Meister A, Anderson ME (1983) Glutathione. Annu Rev Biochem 52:711–760

Murata K, Kimura A (1982) Cloning of a gene responsible for the biosynthesis of glutathione in *Escherichia coli* B. Appl Environ Microbiol 44:1444–1448

Murata K, Kato J, Chibata I (1981) Enzymatic preparation of glutathione. Ferment Ind 39:900–908

Murata K, Miya T, Gushima H, Kimura A (1983) Cloning and amplification of a gene for glutathione synthetase in *Escherichia coli* B. Agric Biol Chem 47:1381–1383

Newton GL, Fahey RC, Cohen G, Aharonowitz Y (1993) Low-molecular-weight thiols in streptomycetes and their potential role as antioxidants. J Bacteriol 175:2734–2742

Newton GL, Arnold K, Price MS, Sherrill C, delCardayre SB, Aharonowitz Y, Cohen G, Davies J, Fahey RC, Davis C (1996) Distribution of thiols in microorganisms: mycothiol is a major thiol in most actinomycetes. J Bacteriol 178:1990–1995

Ohtake Y, Watanabe K, Tezuka H, Ogata T, Yabuuchi S, Murata K, Kimura A (1988) The expression of γ-glutamylcysteine synthetase gene of *Escherichia coli* in *Saccharomyces cerevisiae*. Agric Biol Chem 52:2753–2762

Ohtake Y, Watanabe K, Tezuka H, Ogata T, Yabuuchi S, Murata K, Kimura A (1989) Expression of glutathione synthetase gene of *Escherichia coli* B in *Saccharomyces cerevisiae*. J Ferment Bioeng 68:390–399

Ruyter PG de, Kuipers OP, de Vos WM (1996) Controlled gene expression systems for *Lactococcus lactis* with the food-grade inducer nisin. Appl Environ Microbiol 62:3662–3667

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual, 2nd edn. Cold Spring Harbor Laboratory Press, New York

Sherrill C, Fahey RC (1998) Import and metabolism of glutathione by *Streptococcus mutans*. J Bacteriol 180:1454–1459

Singh VK, Kristoffersen T (1971) Accelerated ripening of Swiss cheese curd. J Dairy Sci 55:744–749

Vos WM de, Vos P, de Haard H, Boerrigter I (1989) Cloning and expression of the *Lactococcus lactis* subsp. *cremoris* SK11 gene encoding an extracellular serine proteinase. Gene 85:169–176

Watanabe K, Yamano Y, Murata K, Kimura A (1986a) The nucleotide sequence of the gene for gamma-glutamylcysteine synthetase of *Escherichia coli*. Nucleic Acids Res 14:4393–4400

Watanabe K, Murata K, Kimura A (1986b) Purification and characterization of γ-glutamylcysteine synthetase of *Escherichia coli* B. Agric Biol Chem 50:1925–1930