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ScienceDirect
Metabolic Engineering 8 (2006) 662–671
METABOLIC ENGINEERING
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Introducing glutathione biosynthetic capability into *Lactococcus lactis* subsp. *cremoris* NZ9000 improves the oxidative-stress resistance of the host

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Received 29 May 2006; received in revised form 28 June 2006; accepted 25 July 2006
Available online 4 August 2006

Abstract

This study describes how a metabolic engineering approach can be used to improve bacterial stress resistance. Some *Lactococcus lactis* strains are capable of taking up glutathione, and the imported glutathione protects this organism against H_2O_2-induced oxidative stress. *L. lactis* subsp. *cremoris* NZ9000, a model organism of this species that is widely used in the study of metabolic engineering, can neither synthesize nor take up glutathione. The study described here aimed to improve the oxidative-stress resistance of strain NZ9000 by introducing a glutathione biosynthetic capability. We show that the glutathione produced by strain NZ9000 conferred stronger resistance on the host following exposure to H_2O_2 (150 mM) and a superoxide generator, menadione (30 μM). To explore whether glutathione can complement the existing oxidative-stress defense systems, we constructed a superoxide dismutase deficient mutant of strain NZ9000, designated as NZ4504, which is more sensitive to oxidative stress, and introduced the glutathione biosynthetic capability into this strain. Glutathione produced by strain NZ4504(pNZ3203) significantly shortens the lag phase of the host when grown aerobically, especially in the presence of menadione. In addition, cells of NZ4504(pNZ3203) capable of producing glutathione restored the resistance of the host to H_2O_2-induced oxidative stress, back to the wild-type level. We conclude that the resistance of *L. lactis* subsp. *cremoris* NZ9000 to oxidative stress can be increased in engineered cells with glutathione producing capability.

Keywords: Glutathione; Lactococcus lactis; Oxidative-stress resistance; Metabolic engineering

1. Introduction

Lactococcus lactis is widely used in the manufacture of cheese and other fermented dairy products. The limited biosynthetic capacity, relatively simple carbon metabolism, and genetic accessibility have made *L. lactis* an ideal platform for metabolic engineering. It has become one of the most commonly used Gram-positive gene expression hosts (Mierau and Kleerebezem, 2005). As a facultative anaerobe, *L. lactis* can, under certain circumstances, tolerate and utilize oxygen (Miyoshi et al., 2003). Reactive oxygen species (ROS), such as H_2O_2, O_2^- (superoxide anion radical), and $OH^\bullet$ (hydroxyl radical), are generated through the stepwise reduction of O_2 to H_2O. ROS will attack many cellular components, like lipids, proteins, DNA and RNA, causing loss of their functions and cell death (Miller and Britigan, 1997). In *L. lactis*, a manganese containing superoxide dismutase (Mn-SOD) and NADH

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doi:10.1016/j.ymben.2006.07.004

oxidase/NADH peroxidase, which eliminates O_2^- and H_2O_2, play an important role in oxidative-stress resistance (van de Guchte et al., 2002; van Niel et al., 2002; Miyoshi et al., 2003). However, due to lack of other efficient oxidative-stress defense mechanisms, such as catalase, H_2O_2 becomes the primary cause of cell component destruction when *L. lactis* is grown aerobically (Condon, 1987). Previous studies have focused on identifying oxidative-stress defense systems in *L. lactis* and understanding their mechanisms. Attempts have also been made to isolate and/or construct both non-recombinant and recombinant *L. lactis* strains resistant to oxidative stress (Miyoshi et al., 2003; Rochat et al., 2005a). A recent successful example was the introduction of a haem catalase, KatE, from *Bacillus subtilis* into *L. lactis* subsp. *cremoris* NZ9000, which resulted in a significantly improved oxidative-stress resistance when challenged with 4 mM H_2O_2 (Rochat et al., 2005b).

The tripeptide glutathione (γ-GluCysGly, reduced form GSH) is the predominant low-molecular-weight thiol in living organisms. GSH plays important physiological roles in many cellular processes including protection against the deleterious effects of ROS (Meister and Anderson, 1983). In our previous study, we presented evidence showing that GSH taken up by *L. lactis* subsp. *cremoris* SK11 protects the host against oxidative stress generated by H_2O_2 (Li et al., 2003). *L. lactis* subsp. *cremoris* NZ9000 is the host strain for the nisin controlled gene expression (NICE) system (Kuipers et al., 1998), which is widely used in the metabolic engineering study of *L. lactis* (Hugenholtz et al., 2002). Although belonging to the same subspecies, strain NZ9000 cannot synthesize GSH (neither can strain SK11), nor take up glutathione from the environment (while strain SK11 can take up GSH) (Li et al., 2003).

Following up the discovery of the physiological role of GSH in *L. lactis* SK11 and other *L. lactis* strains that can take up GSH, we were interested to investigate if we could overproduce GSH in *L. lactis* strains that cannot take up GSH. The engineered strain can subsequently be used as a model system to explore the effect of in situ production of GSH on properties of fermented food like cheeses. More importantly, we wonder if the introduced GSH producing capability in the engineered strains can result in an improved oxidative-stress response, as this can increase the robustness of starters used in dairy industries. To this end, we have successfully introduced a GSH biosynthetic capability into strain NZ9000(pNZ3203) (Li et al., 2005). This was achieved by using the NICE system and the genes *gshA* and *gshB* from *Escherichia coli* encoding the enzymes γ-glutamylcysteine synthetase and glutathione synthetase (Li et al., 2005). This metabolic engineering approach allowed strain NZ9000, a lactic acid bacterium that is unable to synthesize or take up GSH on its own, to produce GSH. The aim of the present study was to investigate if GSH produced by strain NZ9000 and its derivatives can improve the oxidative-stress resistance of the engineered host. We demonstrated that the GSH produced in strain NZ9000(pNZ3203) or strain NZ4504(pNZ3203), a superoxide dismutase (SOD) mutant of strain NZ9000, conferred stronger resistance on the host following exposure to oxidative stresses generated by H_2O_2 and menadione. To our knowledge, this is the first report showing the oxidative-stress resistance of *L. lactis* can be improved by using metabolic engineering approach. This demonstrates that introducing a specific metabolic pathway can result in a targeted improvement of stress resistance, one of the most important industrial microbial functionalities. Our insights into the protective role of GSH in *L. lactis* NZ9000 will also contribute to our understanding on the role of the GSH-dependant redox system in Gram-positive bacteria, and provide useful information for further engineering aero-tolerant bacterial strains.

2. Materials and methods

2.1. Chemicals

Reduced glutathione (GSH), NADPH, glutathione reductase (GR), 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), *N*-ethylmaleimide (NEM) were purchased from Sigma (St. Louis, USA). All inorganic compounds were of reagent grade or higher quality.

Table 1
Strains and plasmids used in this study

Strain or plasmids	Relevant characteristics[a]	Source or reference
Strains		
E. coli MC1061		Casabadan and Cohen (1980)
L. lactis NZ9000	MG1363 *pepN::nisRK*	Kuipers et al. (1998)
L. lactis NZ4504	Tet^r, *sodA::tetM*, SodA-negative derivative of NZ9000	This study
Plasmids		
pCI182	Tet^r, 8.0-kb pBR322 derivative carrying the Tn*919 tetM* gene	Hill et al. (1988)
pUC18	Amp^r	Sambrook et al. (1989)
pNZ4504	Tet^{r}Amp^r	This work
pNZ8148	Cm^r, inducible expression vector, carrying the *nisA* promoter	Mierau and Kleerebezem (2005)
pNZ3203	Cm^r, pNZ8148 derivative containing a functional *E. coli gshA* and *gshB* gene	Li et al. (2005)

[a]Tet^r, tetracycline resistant; Cm^r, chloramphenicol resistant; Amp^r, ampicillin resistant.

2.2. Bacterial strains, plasmids and culture conditions

The strains and plasmids used in this study are listed in Table 1. Inocula of *L. lactis* NZ9000 and its derived strains were transferred from −70 °C stock cultures to M17 broth (Oxoid) supplemented with 5 g/L glucose (refer to as GM17 broth), and incubated at 30 °C for 16 h as preculture. The precultures were used to inoculate fresh GM17 broth. Strains were grown statically or aerobically (shaken at 200 rpm of small-volume cultures in large-volume culture flasks (ratio > 5)) at 30 °C. When appropriate, chloramphenicol (10 μg/ml) was added to GM17 broth as a selection marker for strain NZ9000 harboring plasmid pNZ8148 and pNZ3203, tetracycline (3 μg/ml) and chloramphenicol (10 μg/ml) for strain NZ4504 harboring plasmid pNZ8148 and pNZ3203. Unless otherwise specified, nisin (2 ng/ml) and cysteine (5 mM) were added to the culture of *L. lactis* at 2 h of incubation.

2.3. DNA manipulations

Restriction endonucleases, Mung Bean Nuclease, the Klenow fragment of DNA polymerase and T4 DNA ligase were purchased from Roche (Mannheim, Germany), Amersham Pharmacia Biotech (Roosendaal, The Netherlands) or Gibco BRL Life Technologies (Breda, The Netherlands) and were used according to manufacturers' protocols. PCR amplifications were performed with 10 pmol of each primer (Proligo, Paris, France) and 10–100 ng of template DNA with amplification cycles according to the manufacturer's protocol (Roche, Mannheim, Germany) with a DNA thermocycler (Perkin-Elmer, Shelton, CT). *E. coli* MC1061 (Casabadan and Cohen, 1980) was used as a cloning host, and plasmid DNA was isolated as described before (Birnboim and Doly, 1979) and then subjected to anion-exchange chromatography on Jetstar columns (Genomed, Oberhausen, Germany). Transformations of plasmid DNA to *L. lactis* was performed as previously described (de Vos et al., 1989). DNA sequences of cloned PCR products were confirmed by DNA sequence analysis (BaseClear, Leiden, The Netherlands).

2.4. Construction of plasmids and strains

To construct the *sodA* disrupted *L. lactis* strain, an integration vector for single cross over was constructed. To this end pCI182 (Hill et al., 1988) was digested with HincII. The 4.2 kb *tetM* containing fragment was ligated into PstI digested pUC18 after removal of the 3′-recessive PstI ends by Mung Bean Nuclease. The resulting 6.9 kb vector in which the *tetM* gene was ligated divergently with respect to the *amp* gene was digested with EcoRI and HincII. An 850 bp *sodA* gene containing PCR fragment was amplified using primers SodAF (5′-AATACCATGGCATTTACATTACCTG-3′) and SodAR1 (5′-CCTCGAAGCTTATTTTATTTTGCCTTAGCG-3′) and chromosomal DNA of *L. lactis* NZ9000 as a template. The PCR product was digested with NdeI (endogenous restriction site) and the cohesive end was filled using Klenow. After digestion with EcoRI (endogenous restriction site) the 334 bp internal fragment of *sodA* was ligated in the digested vector, yielding the 7.2 kb integration vector pNZ4504. The junctions of ligated fragments were confirmed by sequencing. After transformation of this plasmid to *L. lactis* NZ9000 tetracycline resistant colonies were checked by PCR using primers SodAF, SodAR1 and TetMF (5′- TGAGGTCATTCTTAGTGGAG -3′) establishing correct integration at the *sodA* locus. A tetracycline resistant *sodA::tetM* single colony isolate was designated as *L. lactis* NZ4504. Its genetic integrity was confirmed by Southern analysis using the NdeI-EcoRI internal *sodA* fragment as a probe (data not shown).

Plasmids pNZ3203 (Li et al., 2005) and pNZ8148 (Mierau and Kleerebezem, 2005) (see Table 1) were introduced into *L. lactis* strain NZ4504 by electroporation transformation as previously described (de Vos et al., 1989), yielding strain NZ4504(pNZ3203) and NZ4504 (pNZ8148).

2.5. Preparation of cell free extracts and glutathione assay

Strain NZ9000 and its derivatives were grown aerobically for 16 h. The bacteria were (Tietze, 1969) 4 °C. The cell pellets were washed twice with 10 ml ice-cold buffer B (0.2 M potassium phosphate, 2 mM EDTA; pH 7.0) and resuspended in 10 ml of the same buffer. Cells were disrupted by sonication (VCX 400 sonicator, 20 kHz, Sonics and Materials, Newton, Mass.) on ice for 10 min with 3 s cooling interval per 1 s. Cell debris was removed by centrifugation (10,000 × *g* for 10 min at 4 °C), resulting in a cell free extract (CFE). The CFE was used for glutathione quantification assay and SDS-PAGE analysis. Total GSH (GSH + GSSG) was assayed by the enzymatic recycling procedure modified based on the literature (Tietze 1969). Seven hundred microliters of 0.3 mM NADPH, 100 μl of 6 mM DTNB, and enough of a GSH sample or water to give a final volume of 1.0 ml were mixed in a cuvette with a l-cm light path and equilibrated at 25 °C. To the warmed solution 10 μl of a 50-U/ml glutathione reductase solution was added, and the change in absorbance at 412 nm was monitored continuously. The GSH concentration of the aliquot assayed was determined by comparing the observed reaction rate to a standard curve generated with known amounts of GSH. The working solutions of NADPH, DTNB and GR were prepared with phosphate buffer A (125 mM potassium phosphate, 6.3 mM EDTA; pH 7.5).

2.6. Sensitivity to oxidants

For determining the sensitivities for H_2O_2 and menadione, *L. lactis* NZ9000 and its derivative cultures or *L. lactis* SK11 were grown aerobically in GM17 at 30 °C, centrifuged (10,000 × *g* for 10 min at 4 °C), washed in

buffer B, and resuspended in 400 μl of buffer B to achieve cell suspensions with comparable cell densities ($(8\pm1)\times10^8$ cfu/ml). Four hundred microliters of H_2O_2 or menadione solution of different concentrations (detailed in the individual figure legends) were added to 400 μl of cell suspension in buffer B, and immediately mixed, to achieve a desired final oxidant concentration. After treatment for a certain time (detailed in the individual Figure legends), cells were pelleted by centrifugation ($20{,}000\times g$ for 1 min), washed again in buffer B to remove the residual H_2O_2 or menadione. Cells were resuspended in 400 μl of buffer B, and spread on GM17 plates at serial dilutions to measure survival after incubation at 30 °C for 48 h. Results were expressed as the mean ± standard deviation of three independent experiments.

2.7. Statistical analysis

The Student's *t*-test was used to investigate statistical differences. Samples with *P*-values <0.05 were considered to be statistically different.

3. Results

3.1. Effects of cysteine on the aerobic growth of L. lactis NZ9000(pNZ3203)

The average specific growth rate of strain NZ9000(pNZ3203) after nisin induction ($0.46\,h^{-1}$) was significantly lower than that of strain NZ9000(pNZ8148) ($0.73\,h^{-1}$) under aerobic conditions (Fig. 1). We found that addition of 5 or 10 mM cysteine restored the growth rate of NZ9000(pNZ3203) to the level of strain NZ9000 (pNZ8148) (Fig. 1). The growth rate of strain NZ9000 (pNZ8148) was not affected by addition of cysteine (data not shown). The comparable aerobic growth rates of strain NZ9000(pNZ3203) and NZ9000(pNZ8148) enabled us to assess the difference between the oxidative-stress resistance of these strains.

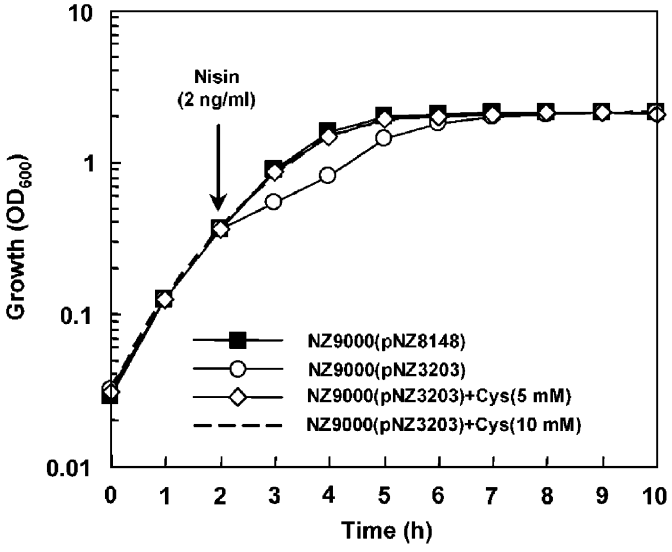

Fig. 1. Growth curves of *L. lactis* NZ9000(pNZ3203) and NZ9000(pNZ8148) under aerobic conditions. Precultures of *L. lactis* were inoculated to achieve an initial cell number of $(5.0\pm0.6)\times10^7$ cfu/ml. Nisin was added to all cultures at 2 h of cultivation to induce gene expression, whereas cysteine was added to the cultures of NZ9000(pNZ3203). Growth was monitored by measuring the optical density at 600 nm. Results were expressed as the mean of three independent experiments and the standard deviations were lower than 5% of the values.

3.2. Glutathione protects strain NZ9000 against H_2O_2-induced oxidative stress

The protective role of GSH in *L. lactis* ssp. *cremoris* SK11 was observed at a low-dose stress (5 mM H_2O_2, 5 min) (Li et al., 2003). In the present study, strain NZ9000(pNZ3203) harvested from aerobic cultures did not exhibit improved oxidative-stress resistance to treatment with 15 mM H_2O_2, as compared to that of the control strain NZ9000(pNZ8148). We therefore compared the response of strain NZ9000 and strain SK11 to H_2O_2-induced oxidative stress. Strain NZ9000 showed an 11.5-fold higher resistance to treatment with 50 mM H_2O_2 for 15 min compared to the resistance of strain SK11. This indicates that strain NZ9000 is more resistant to oxidative stress than strain SK11. We therefore hypothesized that the protective role of GSH in strain NZ9000 may not be observed unless the cells are exposed to extreme oxidative-stress conditions. No statistically significant difference was observed when strains NZ9000(pNZ3203) and NZ9000 (pNZ8148) were treated with 50 mM H_2O_2 for 15 min (Fig. 2A). However, a 2.6-fold increased resistance to H_2O_2 treatment for strain NZ9000(pNZ3203) compared to strain NZ9000(pNZ8148) was observed when the concentration of H_2O_2 increased to 150 mM (Fig. 2A). This suggests that the GSH produced by strain NZ9000(pNZ3203) protects the host, which can neither synthesize nor take up GSH on its own, against oxidative stress under high-dose H_2O_2 treatment.

To investigate the effect of different growth phase on the oxidative-stress resistance of strain NZ9000(pNZ3203), cultures of strain NZ9000(pNZ3203) and strain NZ9000 (pNZ8148) were withdrawn from early exponential phase (3 h), late exponential phase (5 h) and stationary phase (7 h) of aerobic cultivation, where nisin induction was initiated at 2 h. Cells were exposed to 150 mM H_2O_2 for 15 min. As shown in Fig. 2B, no statistically significant difference was observed between the survival of strain NZ9000(pNZ3203) and strain NZ9000(pNZ8148) harvested from early exponential phase. However, 2.6- and 2.9-fold increased resistances were observed between the survival of strain NZ9000(pNZ3203) and NZ9000(pNZ8148) harvested from late exponential phase and stationary phase. Interestingly, the increased H_2O_2 resistance of strain NZ9000(pNZ3203) correlated positively with the intracellular GSH content (Table 2), suggesting that the increased production of GSH

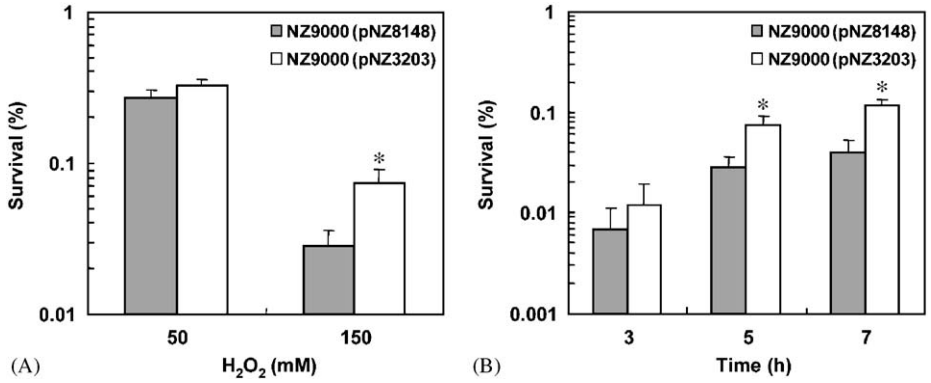

Fig. 2. Sensitivity of *L. lactis* NZ9000 cells with or without glutathione to H_2O_2 treatment. Cells were grown under standard conditions as detailed in Materials and methods. Panel A: Effect of different H_2O_2 concentrations. Cells of both strains were withdrawn after 5 h incubation, resuspended in buffer B, and treated with 50 mM or 150 mM H_2O_2 for 15 min as described in Materials and Methods. Panel B: Effect of different growth phase. Cells of both strains were withdrawn after 3, 5, and 7 h of incubation, resuspended in buffer B, and treated with 150 mM H_2O_2 for 15 min. Refer to Fig. 1 for the growth curve of strain NZ9000(pNZ3203) and NZ9000(pNZ8148) in the presence of 5 mM cysteine. Statistically significant differences ($P<0.05$) were determined by Student's *t*-test and are indicated with an asterisk.

Table 2
The GSH contents of strain NZ9000(pNZ3203) at different growth phases[a]

Cultivation time (h)	Intracellular GSH content (nmol/mg of protein)
0	0.0±0.02
3	23.5±0.1
5	59.2±0.1
7	95.6±0.2

[a]2 ng/ml nisin and 5 mM cysteine were added to the culture at 2 h of cultivation.

by strain NZ9000(pNZ3203) is physiologically beneficial for the host cells.

3.3. Glutathione protects strain NZ9000 against menadione-induced oxidative stress

Menadione (2-methyl-1,4-naphthoquinone) has been used in numerous oxidative-stress studies because it can generate superoxide anion (O_2^-) through a redox-cycling mechanism (Hassan and Fridovich, 1979; Keyer and Imlay, 1996; Fridovich, 1998; Gort and Imlay, 1998). Since the protective role of GSH in strain NZ9000(pNZ3203) was observed at a very high dose of H_2O_2 (150 mM, 15 min), we expected to see a similar, or even stronger, protective role of GSH in strain NZ9000(pNZ3203) when exposed to menadione, a more potent oxidative-stress generating compound. To achieve this, we first developed a simple assay to quickly assess the sensitivity of lactococcal cells to menadione. This was done by spotting serially diluted stationary phase lactococcal cultures onto GM17 plates containing different combinations of nisin, cysteine and menadione (Fig. 3). Sensitivity to menadione can be qualitatively assessed by simply comparing the colony forming units at different dilution levels.

After 24 h of incubation, colonies were observed at the 10^{-6} dilution for strain NZ9000(pNZ3203) and strain NZ9000(pNZ8148) in the presence of nisin and cysteine, although that of the former is slightly less (Fig. 3, row A). This indicates that the initial cell numbers in the cultures of both strains were comparable. Fig. 3, row B shows that strain NZ9000(pNZ3203) without nisin induction had a one log higher survival rate in the presence of 0.4 mM menadione, suggesting the expression of the genes *gshA* and *gshB* driven by leakage of the *nisA* promoter. Strain NZ9000(pNZ3203) with nisin induction had a two log higher survival rate in the presence of menadione compared to NZ9000(pNZ8148), which suggests that GSH plays a role in protecting the cells against chronic oxidative-stress induced by menadione (Fig. 3, row C). Cysteine uptake by lactococcal cells might be enhanced when 5 mM cysteine was added to the media. Strain NZ9000(pNZ8148) in the presence of 5 mM cysteine had a one log higher survival rate in the presence of menadione than that in the absence of cysteine (Fig. 3, row D compared to row C). This clearly shows that cysteine taken up by strain NZ9000(pNZ8148) also plays a protective role against menadione. However, cysteine taken up by strain NZ9000(pNZ3203) did not significantly increase the resistance of strain NZ9000(pNZ3203) to menadione-induced oxidative stress (Fig. 3, row D compared to row C). Our previous study showed that the intracellular cysteine concentration of strain NZ9000(pNZ3203) was 2.5-fold higher than that of strain NZ9000(pNZ8148) when both organisms were grown in chemically defined medium supplemented with 5 mM additional cysteine under nisin induction conditions (Li et al., 2005). Apparently, the high intracellular concentration of cysteine in strain NZ9000(pNZ3203) did not significantly contribute to menadione resistance in the presence of glutathione (Fig. 3, row D compared to row C); or the protective effect of cysteine was masked by that of glutathione in

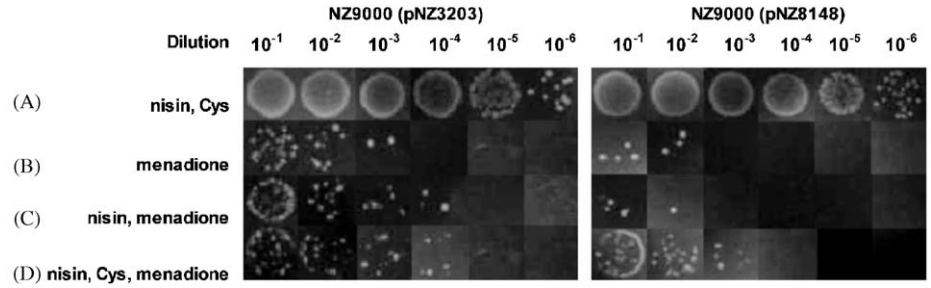

Fig. 3. Qualitative assay of sensitivity of lactococcal cells to menadione. Strain NZ9000(pNZ3203) and strain NZ9000(pNZ8148) were grown in GM17 broth aerobically at 30 °C for 16 h. 10 μl of serially diluted culture was spotted onto GM17 agar supplemented with different additives. (A): 2 ng/ml nisin + 5 mM cysteine; (B): 0.4 mM menadione; (C): 2 ng/ml nisin + 0.4 mM menadione; (D): 2 ng/ml nisin + 5 mM Cysteine + 0.4 mM menadione. Plates were incubated at 30 °C for 24 h before photography.

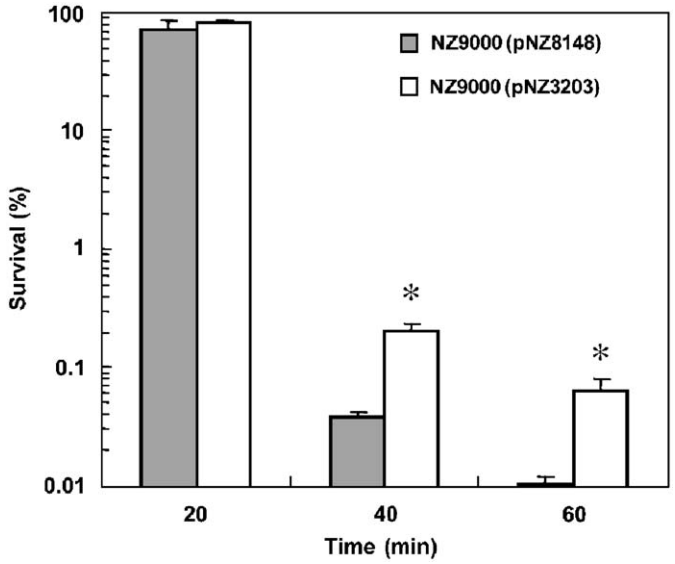

Fig. 4. Sensitivity of *L. lactis* NZ9000 cells with or without glutathione to menadione treatment. Cells were grown under standard conditions as detailed in Materials and methods. Cells were withdrawn after 5 h of incubation, resuspended in buffer B, and then treated with 20 mM menadione for 20, 40 and 60 min. Statistically significant differences ($P<0.05$) were determined by Student's *t*-test and are indicated with an asterisk.

strain NZ9000(pNZ3203). Collectively, even taking the enhanced cysteine uptake into account, Fig. 3 suggests, at least qualitatively, that GSH produced by strain NZ9000 protects the host cells against menadioneinduced oxidative stress.

To compare the sensitivity of strain NZ9000(pNZ3203) and strain NZ9000(pNZ8148) menadione quantitatively, a time-course of survival of lactococcal cells to menadione treatment was generated (Fig. 4). No significant difference was observed between the survival of the two strains when treated with 20 mM menadione for 20 min. However, the protective role of GSH in strain NZ9000(pNZ3203) was clearly seen when increasing menadione treatment time. Strain NZ9000(pNZ3203) treated with 20 mM menadione for 40 and 60 min showed 5.2- and 6.2-fold higher resistance compared to that of strain NZ9000(pNZ8148). Taken together, these results indicate that the GSH produced in strain NZ9000(pNZ3203) increases the resistance of the host cells to the oxidative stress induced by H_2O_2 and O_2^-.

3.4. Glutathione improves oxygen tolerance and oxidative-stress resistance of SOD deficient mutant *L. lactis* NZ4504

The protective role of glutathione in strain NZ9000 was observed under extreme stress conditions when the existing resistant mechanisms were not sufficient to cope with the oxidative-stress. We thus anticipated that glutathione may exhibit a more significant protective effect on the oxidative-stress resistance in NZ9000 derivative cells which have increased sensitivity to oxidant. To test this hypothesis, we constructed a SodA deficient mutant of *L. lactis* NZ9000, designated as NZ4504.

As compared to the wild-type strain NZ9000(pNZ8148), the lag phase of strain NZ4504(pNZ8148) was significantly longer, while the average specific growth rate of strain NZ4504(pNZ8148) in the logarithmic growth phase was 30% lower under aerobic conditions (Fig. 5). However, the lag phases and the growth rates of both strains were comparable in static cultures (data not shown). This suggests that SodA plays an important role in the response of *L. lactis* NZ9000 to oxygenation, which is consistent with previous observations in SodA deficient mutants of other facultative anaerobic Gram positive cocci such as *Streptococcus mutans*, *S. pneumoniae*, and *S. agalactiae* (Nakayama, 1992; Yesilkaya et al., 2000; Poyart et al., 2001).

To assess the physiological role of GSH in a SodA deficient mutant, plasmid pNZ3203 was transformed into strain NZ4504. The two enzymes involved in GSH biosynthesis, γ-glutamylcysteine synthase and glutathione synthase, were expressed after nisin induction, as previously observed in strain NZ9000 (Li et al., 2005). The GSH concentration of NZ4504(pNZ3203) cells after 4-h nisin induction was determined to be 81.7 ± 0.2 nmol/mg protein. This means GSH biosynthesis capability was successfully introduced into strain NZ4504.

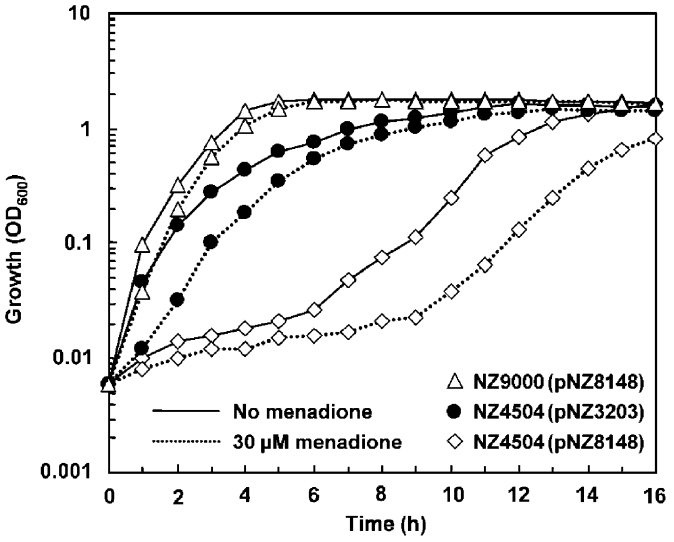

Fig. 5. Growth curves of *L. lactis* NZ9000(pNZ8148), NZ4504(pNZ8148) and NZ4504(pNZ3203) under aerobic conditions with 30 μM menadione and without menadione. Precultures of *L. lactis* were inoculated into GM17 broth to achieve an initial cell number of $(5.5 \pm 0.4) \times 10^7$ cfu/ml. Cultivations were performed on a shaker at 200 rpm for 16 h. Results were expressed as the mean of two independent experiments.

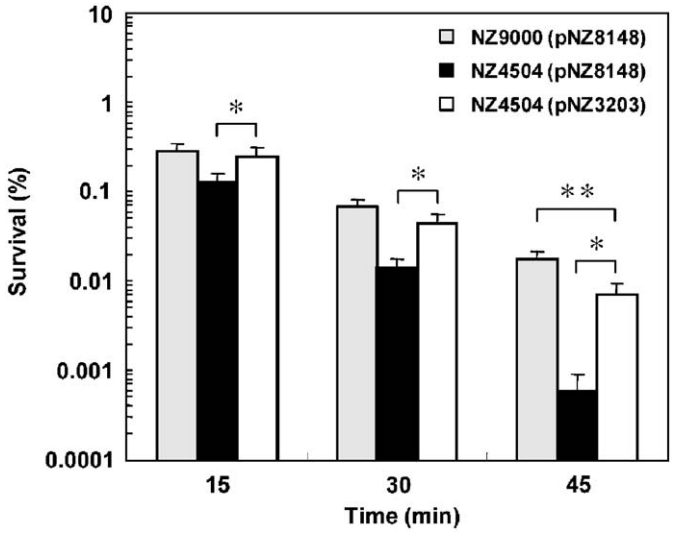

Fig. 6. Sensitivity of *L. lactis* NZ9000(pNZ8148), NZ4504(pNZ3203), and NZ4504(pNZ8148) to H_2O_2 treatment. Precultures of strain NZ4504(pNZ8148) were inoculated into GM17 broth 8 h before inoculation of strain NZ9000(pNZ8148) and NZ4504(pNZ3203). 2 ng/ml nisin and 5 mM cysteine was added to all three cultures after incubating strain NZ9000(pNZ8148) and NZ4504(pNZ3203) for 2 h. Cells were withdrawn after nisin induction for 3 h, resuspended in buffer B to achieve initial cell numbers of $(8.0 \pm 0.5) \times 10^8$ cfu/ml, then treated with 50 mM H_2O_2 for 15, 30 and 45 min. Statistically significant differences ($P < 0.05$) between the survival rates of strain NZ4504(pNZ8148) and NZ4504(pNZ3203) or between the survival rates of strain NZ9000(pNZ8148) and NZ4504(pNZ3203) are indicated a single asterisk or a double asterisk, respectively.

The lag phase of strain NZ4504(pNZ3203) was markedly shorter than that of strain NZ4504(pNZ8148) (Fig. 5), suggesting the GSH produced in strain NZ4504(pNZ3203) partially restored the impaired oxygenic growth of strain NZ4504. Although the average specific growth rate of strain NZ4504(pNZ8148) was 30% lower than that of strain NZ9000(pNZ8148), strain NZ4504(pNZ8148) was able to achieve a comparable final cell density as compared to that of strain NZ9000(pNZ8148). This suggests that *L. lactis* NZ4504(pNZ8148) has an inducible antioxidant defense system that allows the cells to adapt to an oxygenic environment (Sanders et al., 1995). Nevertheless, strain NZ4504(pNZ8148) was more sensitive to menadione (Fig. 5). A concentration of 30 μM menadione, which had a little negative effect on the growth of strain NZ9000(pNZ8148) and strain NZ4504(pNZ3203), further prolonged the lag phase and decreased the growth rate of strain NZ4504(pNZ8148).

To investigate whether GSH in strain NZ4504 (pNZ3203) increases the resistance of the host to oxidative stress, cells of strains NZ9000(pNZ8148), NZ4504 (pNZ3203), and NZ4504(pNZ8148) harvested in the late-exponential growth phase were exposed to 50 mM H_2O_2 (Fig. 6). Strain NZ4504(pNZ8148) was more sensitive to H_2O_2 than strain NZ9000(pNZ8148). This is consistent with the fact shown in Fig. 5 that strain NZ4504(pNZ8148) had a prolonged lag phase and grew slowly in aerobic cultures compared to strain NZ9000(pNZ8148). Notably, no statistically significant differences were observed between the survival rates of strain NZ9000(pNZ8148) and NZ4504(pNZ3203) when cells were exposed to 50 mM H_2O_2 treatment up to 30 min. This suggests that the introduction of a GSH biosynthetic ability into strain NZ4504 restored the oxidative-stress resistance of the SodA deficient mutant to the wild-type level. Increasing the treatment time to 45 min resulted in a statistically significant difference (2.6-fold) between the survival rate of strain NZ9000(pNZ8148) and NZ4504(pNZ3203). However, the survival rate of strain NZ4504(pNZ3203) was still 10.8-fold higher than that of strain NZ4504 (pNZ8148) (Fig. 6). This suggests a protective role for glutathione in strain NZ4504(pNZ3203).

4. Discussion

GSH is rarely found in Gram-positive bacteria (Fahey et al., 1978). In fact, Gram-positive bacteria were considered not to be able to synthesize GSH until two recent publications showed that *Streptococcus agalactiae* (Vergauwen et al., 2005) and *Listeria monocytogenes* (Gopal et al., 2005) can synthesize GSH. A survey of protein databases like PFAM and NCBI reveals that genes with high similarity to *gshA* are found in Gram-positive bacteria, including *L. lactis* IL1403 (Bolotin et al., 2001), *L. lactis* SK11 (http://genome.ornl.gov/microbial/lcre/). However, genes with similarity to *gshB* have so far not been observed in Gram-positive bacteria (Li et al., 2005). *S. agalactiae* and *L. monocytogenes* do not have *gshB* homologues; the reason that these two microorganisms can synthesize GSH is because they have a novel multidomain

protein, GshF, which consists of a γ-glutamylcysteine synthetase domain fused to an ATP-grasp domain, catalyzes the formation of GSH from its constituent amino acids (Gopal et al., 2005; Vergauwen et al., 2005). Still, little is known about the physiological role of glutathione in Gram-positive bacteria. In this study, we present evidence that GSH produced by engineered *L. lactis* NZ9000 improves resistance to the oxidative stress generated by H_2O_2 and menadione. We further show that GSH produced by engineered *L. lactis* NZ4504(pNZ3203), which is deficient in SOD activity, partially restored the oxygen tolerance and oxidative-stress resistance of the host. These results demonstrate that, although strain NZ9000 is not able to synthesize or take up GSH on its own, the oxidative-stress resistance of the host could be improved by introducing GSH biosynthetic capability.

L. lactis strains may generally lack the ability to synthesize GSH, but some strains of subspecies *cremoris* and *lactis* are able to take up GSH from the environment (Li et al., 2003). Interestingly, our previous survey showed the increased resistance to H_2O_2-induced oxidative stress is dependent on the pre-accumulation of GSH in 16 strains of *L. lactis* tested (Li et al., 2003). This suggests that the behavior of taking up GSH by some *L. lactis* strains is of physiological significance. The role of the GSH-dependent reduction system in *E. coli* and *Saccharomyces cerevisiae* has been well summarized (Carmel-Harel and Storz, 2000). This system basically comprises GSH, glutathione reductase (GR), and glutathione peroxidase (GPx). The activities of GR have been detected in many strains of *L. lactis*, including *L. lactis* SK11, NZ9000 and IL1403 (Li et al., 2003). Activities of glutathione peroxidase were also detected in *L. lactis* SK11 incubated statically and aerobically (Li et al., 2003), and in strain NZ9000 when incubated aerobically (Li et al., unpublished data). The amino acid sequence of GR shares 85% identity between *L. lactis* ssp. *lactis* IL1403 (Bolotin et al., 2001) and *L. lactis* ssp. *cremoris* SK11 (http://genome.ornl.gov/microbial/lcre/), while the amino acid sequence of GPx shares 93% identity between *L. lactis* ssp. *lactis* IL1403 (Bolotin et al., 2001) and *L. lactis* ssp. *cremoris* SK11 (http://genome.ornl.gov/microbial/lcre/). Therefore, the presence of GR and GPx appears to be a quite general feature of *L. lactis*.

Most *L. lactis* subsp. *cremoris* strains can take up GSH, with *L. lactis* ssp. *cremoris* NZ9000 being an exception (Li et al., 2003). Apparently, the GSH-specific transporter system is missing in strain NZ9000. This could be an evolutionary event, which forced strain NZ9000 to develop an alternative antioxidant system. Once GSH is introduced into the engineered strain NZ9000, a functional GSH–GR–GPx system may be present, which increases the resistance of the host cells to extreme oxidative stress. The GPx activity of strain NZ9000 grown statically was below the detection threshold (data not shown). This might explain why the protective role of GSH in strain NZ9000(pNZ3203) was not observed using cells grown statically. An alternative system involving GSH that plays a role in stress resistance is the glyoxalase system (Pocsi et al., 2004). However, the genome of *L. lactis* does not encode a glyoxalase system (Bolotin et al., 2001), which precludes this possibility.

The SOD enzymes catalyze the dismutation of the superoxide radical and are of vital importance for the oxygen tolerance of organisms (Fridovich, 1998). In prokaryotes three types of SOD are recognized and can be distinguished depending on the metal cofactor contained in the corresponding enzymes (Cu-Zn, Fe or Mn) (Sadosky et al., 1994). *L. lactis* appears to have a manganese-containing SOD (Mn-SOD) (Zitzelsberger et al., 1984), which might be because Mn-SOD tolerates higher concentrations of H_2O_2 compared to Cu–Zn-SOD and Fe-SOD (Fridovich, 1975). When *L. lactis* is exposed to O_2^-, the SOD is responsible for reducing the O_2^- to H_2O_2 by the dismutation reaction. If the rate of scavenging O_2^- by SOD is lower than the rate of O_2^- generation induced by menadione, NZ9000 cells may encounter dual oxidative stress (O_2^- and H_2O_2). An in vitro study showed GSH could directly react with O_2^- (Winterbourn and Metodiewa, 1994), resulting in the formation of GSSG. Therefore, upon menadione treatment, GSH protects the host cells from O_2^- by two possible mechanisms, the non-enzymatic reaction with O_2^- and the GPx-catalyzed enzymatic reaction with H_2O_2. This explains why the protective role of GSH in strain NZ9000 is more significant when cells were exposed to menadione than exposed to H_2O_2.

A SodA deficient mutant of *L. lactis* MG1363 has been constructed by Sanders and coworkers (Sanders et al., 1995) using the pVE6007/pORI19 system (Law et al., 1995). They found the Sod-negative mutant *L. lactis* MGSOD7 had an impeded growth in aerobic cultures. The reason that we constructed a SodA deficient mutant in strain NZ9000 is simply because strain NZ9000 is the host of the NICE system, which allows glutathione to be produced following introduction of pNZ3203. The GSH produced by the SodA deficient mutant NZ4504 significantly shortened the growth lag phase of the host. However, upon entering the logarithmic phase, the average specific growth rate of strain NZ4504(pNZ3203) was approximately 20% lower than that of strain NZ4504 (pNZ8148) (Fig. 5). O_2^- is generated during aerobic growth. As discussed above, GSH could directly react with O_2^-, leading to the formation of GSSG. Intracellular GSSG accumulation can occur if the GSSG was not completely reduced by GR. This may result in retarded cell growth as GSSG can inhibit protein synthesis initiation (Ernst et al., 1978). Notably, addition of 30 μM menadione to the media had less effect on the lag phase and growth rate of strain NZ4504(pNZ3203) as compared to the negative effects on strain NZ4504(pNZ8148) (Fig. 5). Furthermore, the resistance of strain NZ4504(pNZ8148) to H_2O_2-induced oxidative stress could be restored to the wild type level by introducing GSH producing capability (Fig. 6). Taken together, our data suggest that the GSH-dependent

reduction system plays an important physiological role in strain NZ9000 when the existing oxidative-stress defense system is impaired.

L. lactis is an excellent prokaryotic host for heterologous protein production (Kunji et al., 2003). However, oxygen challenges are often encountered when *L. lactis* cultures are exposed to industrial environments. Improving the oxygen tolerance and oxidative-stress resistance is therefore of industrial importance. Although previous studies on metabolic engineering of *L. lactis* were mainly focused on production of nutraceuticals (Hugenholtz et al., 2002), we demonstrate that introducing a complete metabolic pathway into *L. lactis* via metabolic engineering can result in targeted improvement of microbial functionalities, e.g. stress resistance. By simply introducing the glutathione biosynthetic capability into *L. lactis*, our investigation provides a novel approach to improve the oxidative-stress resistance of *L. lactis*.

Acknowledgements

This work was supported by the National Natural Sciences Foundation of China (contract No. 30300009). Sequence data for *L. lactis* ssp. *cremoris* SK11 were produced by the US Department of Energy Joint Genome Institute http://www.jgi.doe.gov/. We thank Kieran A. Ryan for critically reading the manuscript.

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