

Marker-free chromosomal integration of the manganese superoxide dismutase gene (*sodA*) from *Streptococcus thermophilus* into *Lactobacillus gasseri*

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Abstract

A strategy for functional gene replacement in the chromosome of *Lactobacillus gasseri* is described. The phospho- β -galactosidase II gene (*lacII*) was functionally replaced by the manganese superoxide dismutase (MnSOD) gene (*sodA*) from *Streptococcus thermophilus*, by adapting the insertional inactivation method described for lactobacilli [Russell, W.M. and Klaenhammer, T.R. 2001 Efficient system for directed integration into the *Lactobacillus acidophilus* and *Lactobacillus gasseri* chromosomes via homologous recombination. Appl. Environ. Microbiol. 67, 4361–4364]. *L. gasseri* carrying the heterologous *sodA* gene grew on lactose as efficiently as the wild-type parent. An active MnSOD was expressed in the transgenic strain, and the enzyme migrated on PAGE-SOD activity gels to the same position as that of MnSOD from *S. thermophilus*. The expression of MnSOD from a single copy of *sodA* integrated in the chromosome of *L. gasseri* provided enhanced tolerance to hydrogen peroxide, and extended the viability of carbon/energy starved cultures stored at 25 °C. This is the first report showing the successful utilization of the pORI plasmid system to generate marker-free gene integration in *L. gasseri* strains.

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1. Introduction

Lactobacillus spp. are Gram-positive, non-pathogenic and microaerophilic organisms that belong to a heterogeneous group of organisms known as lactic acid bacteria (LAB). While a number of tools have been designed to generate chromosomal and plasmid modifications in

other members of the LAB group like *Lactococcus lactis* [1], less attention has been given to the heterogeneous and genetically recalcitrant lactobacilli. Recently, a vector was developed to integrate a gene into the *Lactobacillus gasseri* chromosome, via a single cross-over event [2]. In the same way, the lactococcal two plasmid system [3] was adapted for gene disruption in *L. gasseri* and *L. acidophilus* [4] and proven useful in the insertional inactivation of acid-stress related genes in *L. acidophilus* [5]. In these studies, gene disruption was achieved by a single cross-over mechanism, and the integrated plasmids

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carried antibiotic markers. However, the generation of “food-grade” organisms free of antibiotic markers is essential for the development of safe and stable new products [6].

Growth of lactobacilli in aerobic environments results in oxidative stress due to the generation of the partially reduced and highly reactive oxygen intermediates. In general, *Lactobacillus* spp. do not require molecular oxygen for growth, and lack a functional electron transport chain. However, most *Lactobacillus* spp. contain enzymes capable of using molecular oxygen such as NADH oxidase, NADH peroxidase, or pyruvate oxidase [7–9] and generate partially reduced reactive oxygen intermediates. Most lactobacilli do not possess superoxide dismutase (SOD) or catalase (KAT), which are important in the disposal of toxic oxygen byproducts formed under aerobic conditions [10]. Furthermore, superoxide reductase (SOR) which is found in most oxygen-sensitive microbes [11], has not been reported in lactobacilli. Recently, we demonstrated that the expression of a plasmid containing a heterologous Mn-superoxide dismutase gene (*sodA*) in intestinal lactobacilli conferred a significant protection against the toxicity of hydrogen peroxide [12].

In the present study, we successfully adapted the two-plasmid pORI method to functionally replace a *L. gasseri* gene through two sequential cross-over events to

create a transgenic strain free of antibiotic markers. The superoxide dismutase gene (*sodA*) and its flanking sequences, from *Streptococcus thermophilus* A054 [21,12,28], were integrated into the chromosomal phospho- β -galactosidase II (*lacII*) gene of *L. gasseri* NCK334. Stress resistance assays were carried out and demonstrated that the new constructs were more resistant to hydrogen peroxide and showed greater viability during carbon/energy starvation at 25 °C.

2. Materials and methods

2.1. Bacterial strains and growth conditions

The bacterial strains and plasmids used in this study are shown in Table 1. *Escherichia coli* strains were grown in LB medium at 37 °C. *Lactobacillus* strains were grown in MRS [13], MRS supplemented with 1 mM MnSO₄, or APT broth [14] (Difco) at 37 °C. *S. thermophilus* AO54 [15] was grown at 42 °C in M17 medium [16] supplemented with 0.5% (w/v) glucose (M17G). Solid media for plating were prepared by adding 1.5% (w/v) agar to the appropriate liquid media. Antibiotic resistant *E. coli* or *Lactobacillus* strains were selected on BHI agar (Difco) or MRS agar containing the appropriate antibiotics, respectively. When required

Table 1
Bacterial strains & plasmids used in this study

Strains	Relevant characteristics	Source or reference
<i>Escherichia coli</i>		
MC1061	λ^{-} F ⁻ Δ (<i>araA-leu</i>)7697 <i>araD139</i> Δ (<i>codB-lac3</i>) = Δ <i>lac74 galK16 galE15 mcrA0 relA1 rpsL150 spoT1 mcrB9999 hsdR2</i> (Str ^r)	Stratagene [34]
NCKO	As MC1061, but (<i>sodA-lacZ</i>)49 Lac ⁺ ; (<i>sodB-Kan</i>) 1- Δ_2 (Cm ^r) (Km ^r)	[12]
EC1000	RepA ⁺ MC1000, Km ^r , carrying a single copy of the pWV01 <i>repA</i> gene in <i>glgB</i> ; host for pORI28-based plasmids	[27]
<i>Streptococcus thermophilus</i> AO54	Wild-type industrial strain	[15]
<i>Lactobacillus gasseri</i>		
NCK334	Type strain. Human isolate ATCC33323	Klaenhammer
NCK334/pTRK669	NCK334 transformed with pTRK669	This study
NC1500	NCK334 transformed with pTRK563	[12]
NC1501	NCK334 transformed with pSodA	[12]
NC1502	NCK 334 integrant. pJB34-S is integrated at the <i>lacII</i> gene	This study
NC1503	NCK 334 integrant. 2nd cross-over derivative leaving only <i>sodA</i> integrated at the <i>lacII</i> gene	This study
Plasmids		
pSodA	As pTRK563, but with 1.2 kb <i>sodA</i> from <i>S. thermophilus</i>	[12]
pTRK669	Ori (pWV01), Cm ^r , RepA ⁺	[4]
pTRK563	Em ^r ; Δ <i>cat</i> derivative of pGK12 with <i>lacZ</i> from pBluescript II KS(+)	[35]
pORI28	Em ^r , ori (pWV01), replicates only with <i>repA</i> provided <i>in trans</i>	[27]
pJB34	3074 bp; pORI28 with 1408 bp internal <i>L. gasseri lacII</i> internal fragment	This study
pJB34-B	3001 bp; pJB34 cut with <i>Bam</i> HI & <i>Bgl</i> II to remove the <i>Eco</i> RI site	This study
pJB34-E	2921 bp; pJB34-B Δ 80-bp at the <i>lacII</i> internal fragment. An <i>Eco</i> RI site was introduced by PCR	This study
pJB34-S	4121 bp; pJB34-E containing 1.2 kb <i>sodA</i> from <i>S. thermophilus</i>	This study

Amp^r, ampicillin resistance; Cm^r, chloramphenicol resistance; Em^r, erythromycin resistance; Km^r, Kanamycin resistance. NC & NCK culture collection at North Carolina State University, Raleigh, NC. ATCC, American type culture collection.

erythromycin (Em, 150 or 2 $\mu\text{g ml}^{-1}$) or chloramphenicol (Cm, 20 or 4 $\mu\text{g ml}^{-1}$) were used for *E. coli* and *Lactobacillus*, respectively.

2.2. Chemicals and enzymes

Cytochrome c^{3+} , xanthine, xanthine oxidase, riboflavin, nitro-blue tetrazolium, lysozyme, proteinase K, phenol, chloroform, and all antibiotics used were purchased from Sigma (St. Louis, MO). All other chemicals and bacteriological media were purchased from Fisher Scientific (Pittsburgh, PA). Restriction enzymes, DNA ligase, and Taq polymerase were purchased from Promega (Madison, WI), New England BioLabs (Beverly, MA), Qiagen (Valencia, CA), or Roche (Indianapolis, IN). PCR primers were purchased from Integrated DNA Technologies (Coralville, IA).

2.3. DNA isolation and manipulation

Escherichia coli strains were transformed using calcium chloride [17]. *L. gasseri* was transformed by electroporation using the protocol described by Walker et al. [18] using a Bio-Rad Gene Pulser (BioRad, Richmond, CA). *E. coli* plasmid DNA and *Lactobacillus* total DNA were isolated using the Qiagen Mini Spin isolation kit (Qiagen, Valencia, CA), and the DNA easy Tissue Kit (Qiagen), respectively. PCRs were performed in a Perkin Elmer Biosystems GeneAmp 2400 PCR System (Boston, MA) or in an iCycler iQ Real-Time PCR Detection System (Bio-Rad), using standard procedures [19]. DNA-probes were labeled and luminescence detected with the digoxigenin DNA-labeling and detection kit (Roche) according to the manufacturer's protocol.

2.4. Oligonucleotides for PCR amplification

The primers utilized in this study are listed in Table 2. Oligonucleotides containing the appropriate restriction sites were designed to amplify internal fragments of the phospho- β -galactosidase II gene (*lacII* [Genebank Accession No. AB011419]) [20]. Primers ggalF5 (*XbaI*) and ggalR5 (*EcoRI*) were used to amplify the region between 53 and 726 bp. Primers ggalF3 (*EcoRI*) and ggalR3 (*BamHI*) were used to amplify the fragment between 806 and 1461 bp. Primers ggalEF5-EmF and ggalER3-EmR were designed to confirm pJB34-S integration by PCR amplification of junction fragments. Probes for Southern hybridization analysis were generated using primers ggalEF5-ggalER3 (to amplify a 1.4 kb fragment of *lacII* from *L. gasseri* NCK334) and STSODF–STSODR (to amplify a 1.2 kb DNA fragment containing the *sodA* from *S. thermophilus* [Genebank Accession No. AF538722]).

2.5. Preparation of cell-free extracts

Cultures of *S. thermophilus* and *L. gasseri* were grown aerobically with shaking at 37 °C and 200 r.p.m. overnight (16 h). The overnight cultures were used to inoculate fresh media to an initial OD₆₀₀ of 0.05, and cells were allowed to grow to mid-exponential growth phase (OD₆₀₀ of 0.2–0.4) before they were harvested by centrifugation at 5000g for 30 min at 4 °C. The cells were washed twice in equal volumes of 0.05 M phosphate buffer containing 10⁻⁴ M EDTA (pH 7.8) (phosphate/EDTA buffer), pelleted by centrifugation (5000g), and resuspended in the same buffer at 1/40 the original volume. Dialyzed cell-free extracts were prepared as described [21].

Table 2
Primers utilized in this study

Primer	Sequence	Reference
ggalF5	5'-GAT CTC TAG ATT GAA GGT GCG TAT GA-3'	This study
ggalR5	5'-GAT CGA ATT CTG CAT GAG CAA TCT TA-3'	This study
ggalF3	5'-GAT CGA ATT CTG GCT AAC CGT CAT A-3'	This study
ggalR3	5'-GAT CGG ATC CTC CAT CAG ATT CAA TA-3'	This study
ggalEF5	5'-CTA CTG CCG CAA ATC AAA-3'	This study
ggalER3	5'-CTT TTC CAT CAG ATT C-3'	This study
EmF	5'-AAT CGT GGA ATA CGG GTT TGC-3'	Klaenhammer Lab
EmR	5'-GCC TTT TCC TGA GCC GAT TTC-3'	Klaenhammer Lab
RTLact16SF ^a	5'-GTA GGG AAT CTT CCA CAA TG-3'	[12]
RTLact16SR ^a	5'-TAG TTA GCC GTG ACT TTC TG-3'	[12]
KOF ^a	5'-GGA ATT CCC TTC CTT ACG CTT ACG ATG TTT GG-3'	[21]
RTSodAR ^a	5'-TGC AAC TTA CGT GGC GAA TG-3'	[12]
STSODF	5'-GAG AGG ACA GAT TCA AGA TG-3'	[21]
STSODR	5'-GTT TTG GCG GCT CC-3'	[21]
startSODF	5'-GGA CCT TTC ATA TGG CTA TTA TCC-3'	[12]
stopSODR	5'-TCA AGA CTG AGG ATC CTT CTA GAC-3'	[12]

^a Primers utilized for qRT-PCR experiments.

2.6. Biochemical assays

Dialyzed cell-free extracts were assayed for protein concentration by the Bradford method [22], using bovine serum albumin as standard. The proteins were separated by electrophoresis on a 10% (w/v) non-denaturing polyacrylamide gel and SOD activity was detected by the nitro-blue tetrazolium method [23]. Specific activity of SOD in the cell-free extracts was assayed using the cytochrome c^{+3} method [24].

2.7. Preparation of antibodies against *S. thermophilus* MnSOD and Western-blot hybridization

Antiserum containing polyclonal antibodies against the purified SodA protein (Anti-SodA) was prepared as described [12]. For the detection of SodA in cell-free extracts, samples were dot blotted onto a Hybond-ECL nitrocellulose membranes (Amersham Bioscience, Piscataway, NJ), or were subjected to electrophoresis on 4–15% (w/v) gradient SDS-polyacrylamide minigels before electro-transferring to nitrocellulose membranes. Membranes were blocked, treated with the primary (Anti-SodA) and Secondary (goat anti rabbit IgG)/Horseradish peroxidases, and the signal was detected as previously described [12].

2.8. Quantitative real-time PCR

One-step quantitative real-time PCR (qRT-PCR) was used for the detection and quantification of 16S rRNA and *sodA* mRNA in *S. thermophilus* and in all strains used. Specific primers meeting qRT-PCR criteria were utilized (Integrated DNA Technologies; Table 2). Total RNA from log phase cultures (OD_{600} 0.2–0.4) were extracted with the RNeasy Mini Kit (Qiagen) and assessed for quality by electrophoresis on 1.2% (w/v) agarose gel. DNase treatment and SYBR Green I RT-PCR were carried out as suggested by the manufacturer (Qiagen). RT-PCR amplification mixtures (20 μ l) contained 50 ng template of total mRNA, 2 \times SYBR Green I Master Mix buffer (10 μ l) (Qiagen), and 200 nM forward and reverse primers. Reactions were run on an iCycler iQ Real-time PCR Detection System (Bio-Rad). The cycling conditions comprised 30 min of reverse-transcriptase reaction at 50 °C, 15 min polymerase activation at 95 °C, 40 cycles at 95 °C for 15 s, and 50 °C for 30 s, followed by 31 cycles to obtain the melting curve. Each assay included (in triplicate): a standard curve of four serial dilution points of *sodA* DNA (ranging from 50 ng to 50 fg equivalent to 10^{10} – 10^4 molecules), a no-template control, and a template control without reverse transcriptase added. Experiments were repeated twice and all PCR efficiencies were above 95%. Sequence Detection iCycler iQ optical system software (version 3.0 a) (Bio-Rad) were utilized for analysis. The median

coefficient of variation (based on calculated quantities) of duplicated samples was lower than 6%. Results were expressed and presented as the *sodA/16S* mRNA ratio, and the absolute copy number of mRNA of each molecule as previously described [17].

2.9. Growth kinetics

The maximum specific growth rate (μ_{max} , h^{-1}) of each strain in APT broth containing lactose as carbon source was determined as follows: Each culture was grown aerobically overnight (16 h) in APT lactose broth to the exponential growth phase (OD_{600} 0.2–0.4). Cells from log-phase were harvested via centrifugation, washed in sterile media, and resuspended in APT media. Standardized inocula were utilized to inoculate broth containing lactose [0.5% (w/v)] to an OD_{600} of ca. 0.1 (total volume = 200 μ l APT per well) and incubated at 37 °C with continuous shaking in microtiter plates. Growth was automatically monitored by measuring the changes in OD_{600} as a function of time using a Bioscreen-C microbiological analyzer (Labsystems, Frankfurt, Germany). For each culture, the maximum specific growth rate (μ_{max} , h^{-1}) was calculated from the slope of fitting to a linear regression of the exponential growth phase data with correlation coefficients, r^2 , of 0.99. Each point represents the mean of five independent cultures.

2.10. Survival of *L. gasseri* in the presence of hydrogen peroxide

The survival of each strain in APT media, without sugar, and containing hydrogen peroxide at different concentrations, was determined as follows: 16 h cultures of *L. gasseri* grown aerobically at 37 °C were used to inoculate 50 ml of APT broth to an initial OD_{600} of 0.1. Cultures were grown at 37 °C aerobically to exponential growth phase (OD_{600} 0.2–0.4), centrifuged, washed in sterile media and resuspended in the initial volume in APT media without sugar (to avoid growth of the organism during exposure to H_2O_2) containing various concentrations of hydrogen peroxide and incubated at 37 °C and 200 r.p.m. for 90 min. Following this incubation, the cells were serially diluted, spread onto fresh MRS plates, and incubated aerobically for 48 h at 37 °C before colonies were enumerated. Each point represents the mean of three independent exposures to H_2O_2 .

3. Results

3.1. Construction of a site-specific integration plasmid for the insertional inactivation of *lacII* of *L. gasseri* NCK334

Our objective was to select a gene target that allows us to generate a genetically enhanced strain without

modifying other important characteristic. Since the draft annotation of *L. gasseri* NCK334 was not available at the time this project was initiated, we designed specific PCR primers (Table 2) based on the sequence for the *lacII* gene from *L. gasseri* JCM1031, a human intestine isolate that produces two distinctive and functional phospho- β -galactosidase enzymes [25,26]. Later, DNA sequence comparison analysis between JCM1031 [20] and NCK334 phospho- β -galactosidase genes (http://www.jgi.doe.gov/JGI_microbial/html/index.html) showed a 94% and a 95% identity between *lacI* and *lacII*, respectively. The designed primers allowed us to amplify the expected PCR fragments from *L. gasseri* NCK334.

The construction of the integrative pORI28-based [3] plasmid pJB34-S, developed to generate a site-specific directional (i.e., orientation-dependent) insertion, is represented in Fig. 1. A 1.4-kb internal fragment of the *lacII* gene from *L. gasseri* NCK334 was cloned into pORI28 generating plasmid pJB34 (3.1 kb). *Bam*HI/*Bgl*II restriction and subsequent self-ligation eliminated the *Eco*RI site in the polylinker region and created a new *Xho*II site. Thus, an 80-bp internal fragment of the cloned *lacII* fragment was removed by inverse PCR (using *ggalR5* and *ggalF3* primers). The resulting 2.9-kb fragment was treated with *Eco*RI and re-ligated generating pJB34-E. The entire *sodA* gene and flanking regions, which include the original upstream promoter region and stop codon [21,12], were then cloned via an *Eco*RI site into pJB34-E, generating a plasmid pJB34-S, which contained *sodA* flanked by two *lacII* fragments of 655 and 673 bp (Fig. 1). The orientation of *sodA* transcription was obtained by PCR amplification using the four combinations of the primers *ggalF5*, *ggalR3*, startSODF, and stopSODR listed in Table 2. Only the primer couples *ggalF5*-stopSODR and *ggalR3*-startSODF amplified the expected fragment sizes.

3.2. Functional chromosomal replacement of *L. gasseri* NCK334 *lacII*

The two-plasmid lactococcal integration strategy [27] adapted for use in *Lactobacillus* by Russell and Klaenhammer [4] was utilized to establish the non-replicative vector pJB34-S in *L. gasseri* NCK334. RepA function was provided by the helper plasmid pTRK669, which is stable at 37 °C, but not at 43 °C. Plasmid pJB34-S was introduced by electroporation into *L. gasseri* containing pTRK669. Transformants carrying both plasmids were transferred five times (overnight transfers) and allowed to grow at 43 °C in MRS broth containing a low concentration of erythromycin (2 μ g/ml⁻¹) to avoid the insertion of multiple copies of the vector. The occurrence of single cross-over events was verified by PCR amplification of the junction fragments from chromosomal DNA of Em^r-Cm^s colonies, using *lacII* specific external primers and specific internal primers

for the Em gene in the vector (Table 2). Only the strains containing pJB34-S inserted in the desired orientation produced the expected PCR fragments (data not shown). Three randomly picked Em^r-Cm^s colonies were selected for subsequent steps. The selected integrants were grown for ca. 50 generations (generation time = 51 min.) in non-selective medium to allow the second cross-over to occur. After 50 generations, cells were plated on MRS agar and screened for Em^s, Cm^s, and for MnSOD⁺. Em^s-Cm^s/MnSOD⁺ colonies were obtained at a frequency of 10⁻³.

PCR and Southern hybridization assays using internal fragments of *lacII* (Fig. 2(a) and (b)) or *sodA* (data not shown) genes as probes confirmed the occurrence of the desired gene replacement. The *lacII* probe hybridized to a *Hind*III fragment of \approx 4.0 kb in *L. gasseri* NCK334. In the integrants, this band was replaced by two junction fragments due to the presence of a *Hind*III site in the plasmid sequence. Fragments of 6.0 and 2.1 kb were visualized in NC1502 (single cross-over) and of 3.2 and 2.1 kb in NC1503 (double cross-over) strains. The results were similar for the different clones tested indicating the presence of a pure population with a single copy of the plasmid integrated (data not shown).

No differences were observed in the DNA sequence of the integrated copy of *sodA*. Moreover, native PAGE-SOD activity gels (data not shown) and SDS-PAGE analyzed by Western-blotting (Fig. 3) revealed an active and functional MnSOD present only in cell-free extracts of the integrant strains. The MnSOD protein expressed in *L. gasseri* strains NC1501, NC1502, and NC1503 migrated to the same position as that from *S. thermophilus* AO54, indicating that the active protein was being expressed (i.e., not a fusion protein). The amounts of *sodA* transcript, SodA protein, and the specific activity of the MnSOD correlated with the activity expressed from the single copy of *sodA* integrated into the genome (400 mU mg⁻¹ of protein) in the integrants grown on MRS (Fig. 3). Furthermore, growing the different strains in MRS media supplemented with MnSO₄ (1 mM) doubled the specific activity of MnSOD (i.e., 100% increase) in the cell-free extracts (data not shown). No differences in the expression levels of MnSOD were observed between the single cross-over integrants (NC1502) and the double cross-over derivative (NC1503), where only *sodA* was integrated into *lacII*.

3.3. Effect of the insertional inactivation of *lacII* on the growth of *L. gasseri* on lactose

To verify that the ability of *L. gasseri* to grow on lactose was intact, strains NC1502 and NC1503 carrying the *sodA* gene were grown for 48 h in modified APT broth supplemented with 0.5% (w/v) of lactose as sole carbon source. The growth rate and the final pH of the integrants were similar to that of the wild-type strain

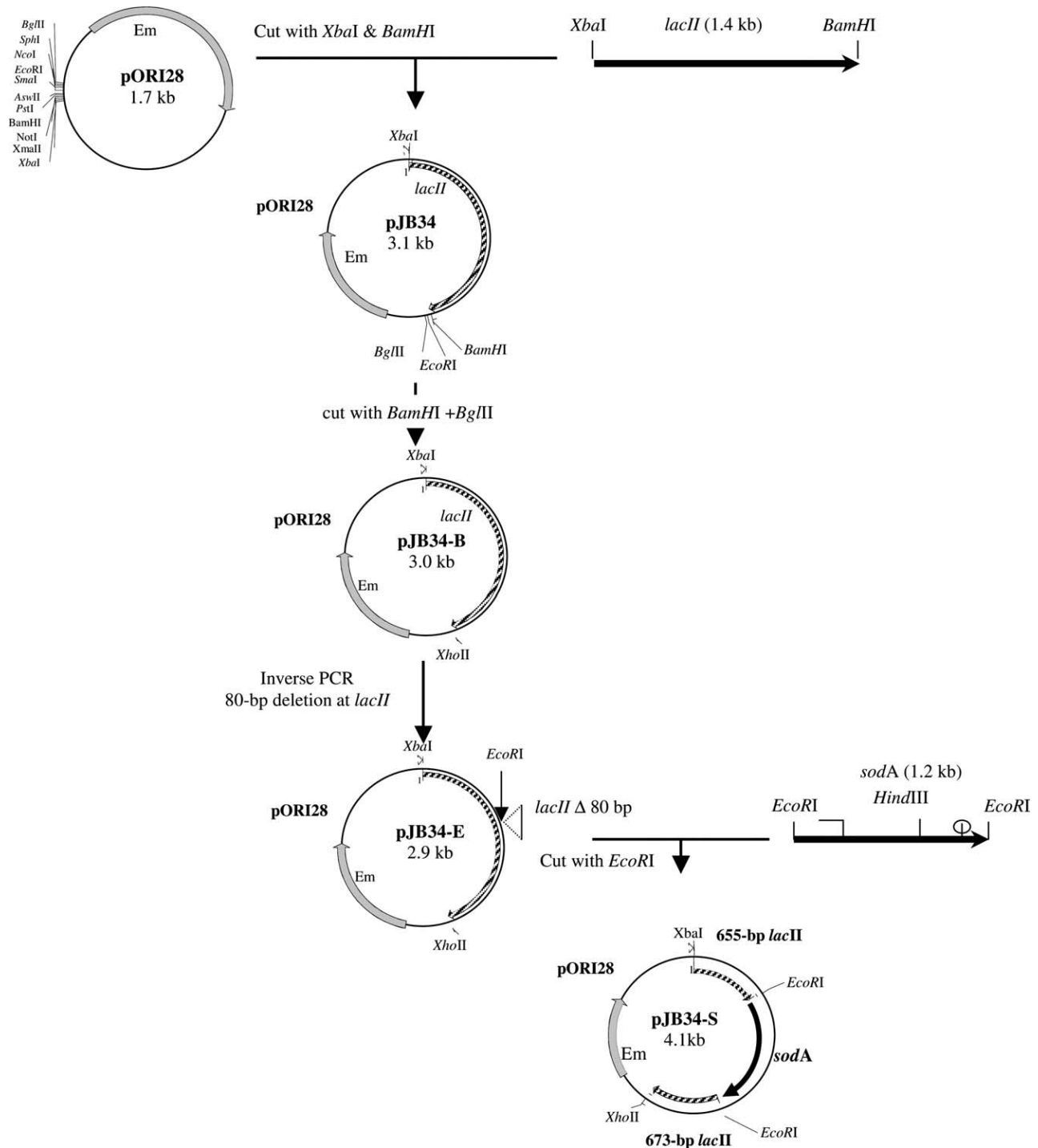


Fig. 1. Construction of pJB34-S; an integrative plasmid containing the *sodA* gene from *S. thermophilus* AO54 & the two flanking sequences of *lacII* from *L. gasseri* NCK334. Symbols localize promoter (←) and Rho independent terminator (ϕ) regions in *sodA*.

L. gasseri NCK334 (Table 3). These results show that the disruption of the *lacII* did not affect lactose utilization by *L. gasseri* NCK334-derivative strains, suggesting that lactose was hydrolyzed by the second phospho- β -galactosidase (*lacI*) [http://www.jgi.doe.gov/JGI_microbial/html/index.html], which is highly homologous to the *lacI* gene from *L. gasseri* JCM1031 [26].

3.4. Protection against hydrogen peroxide

We have previously shown [21,12,28] that paraquat and other known redox cycling compounds cannot be used as superoxide generators (i.e., oxidative stress agents) in the Gram-positive organisms used in this study, since those compounds cannot be transported into

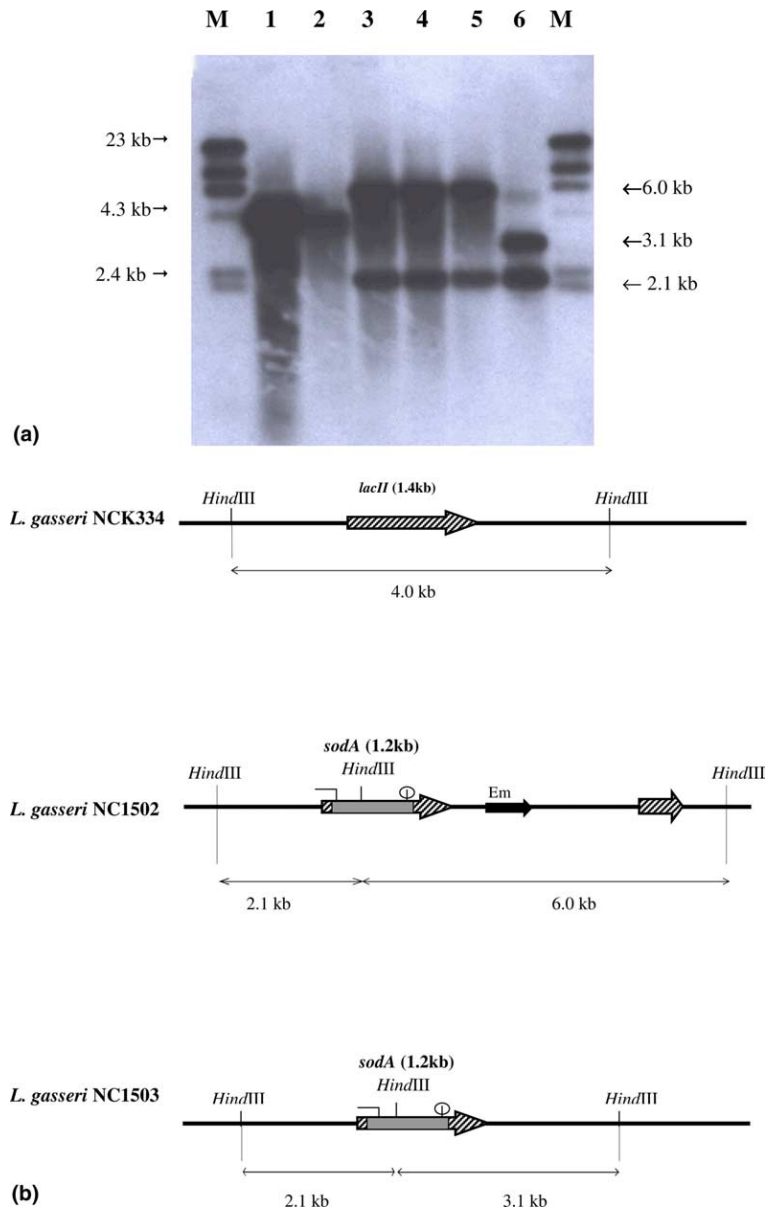


Fig. 2. (a) Southern hybridization analysis of chromosomal DNA from *L. gasseri* strains. DNA was digested with *Hind*III (lane 1, plasmid pJB34-S; lane 2, NCK334; lanes 3–5, NC1502; lane 6, NC1503) and hybridized with a 1.43-kb internal fragment of *lacII*. (b) Schematic representation (i.e., not drawn to scale) of the relevant regions and *Hind*III fragments sizes of *L. gasseri* strains NCK334 (wild-type); NC1502 (single cross-over) and NC1503 (double cross-over). Symbols localize promoter (–) and Rho independent terminator (ϕ) regions.

the cytoplasm. However, it has been demonstrated that MnSOD protects against hydrogen peroxide stress [12,29]. Therefore, we examined the effects of different concentrations of H₂O₂ on the survival of the different *L. gasseri* strains expressing *sodA* and the parental strain (Fig. 4). Significant differences were observed in the survival of the wild-type *L. gasseri* (NCK334) and the derivative strains NC1501, NC1502, and NC1503 expressing the *sodA* gene. Thus, a four-log reduction in viability was achieved after 90 min of exposure of NCK 334, NC 1501, NC 1502, and NC 1503 to 23.8, 44.6, 41.6, and 42.7 mM of hydrogen peroxide, respectively (Fig. 4).

3.5. Protection against carbon starvation

To evaluate the effect of MnSOD on cell survival under carbon/energy starvation conditions, cells of *L. gasseri* NCK334, NC1501, NC1502, and NC1503 were held at 25 and 4 °C over a 41-day period in APT broth maintained under static/aerobic conditions (Fig. 5). The data showed that cells of the different *L. gasseri* strains expressing MnSOD survived for longer periods of starvation at 25 °C than the wild-type strain (Fig. 5(a)). Thus, at day 41, the cultures with a single copy of *sodA* integrated in the chromosome (NC 1502 & 1503)

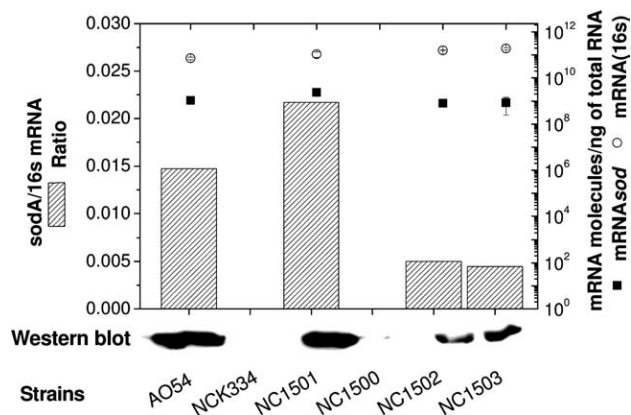


Fig. 3. MnSOD-protein concentration (Western-blot) in cell-free extracts and transcription level of *sodA* (qRT-PCR) using RNA extracted from *S. thermophilus* AO54 and the different *Lactobacillus* strains harboring either pSodA or a single copy of *sodA* integrated into *L. gasseri* chromosome. Each lane was loaded with 10 μ g of total protein that corresponded 300, 15.7, 2.25 and 2.64 mU of MnSOD, respectively. SD \pm value for duplicated experiments was lower than 6%.

Table 3

Maximum specific growth rates and final pH determined for the *L. gasseri* derivatives

Strain	μ_{max} (h ⁻¹)	Final pH
NCK334	0.33 \pm 0.05	4.15 \pm 0.15
NC1502	0.30 \pm 0.02	4.20 \pm 0.10
NC1503	0.31 \pm 0.02	4.10 \pm 0.15

Strains were grown aerobically at 37 °C in APT broth containing 0.5% (w/v) lactose.

No significant growth in the base APT medium without carbohydrate was observed. Results are based on three independent experiments, \pm SD.

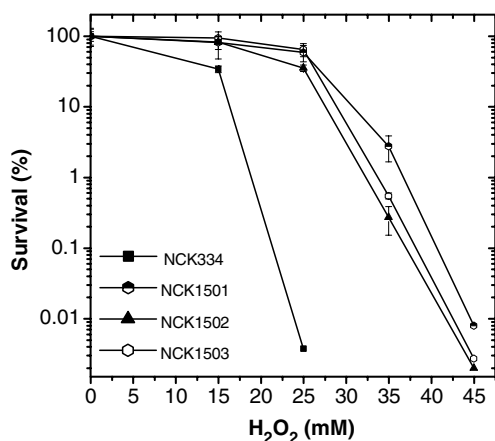


Fig. 4. Effects of hydrogen peroxide on the viability of wild-type *L. gasseri* and the different *sodA* bearing strains. Cells from exponential phase of growth were exposed to increasing concentrations of hydrogen peroxide for 90 min. The entire procedure was carried out in APT broth without sugar and under aerobic conditions.

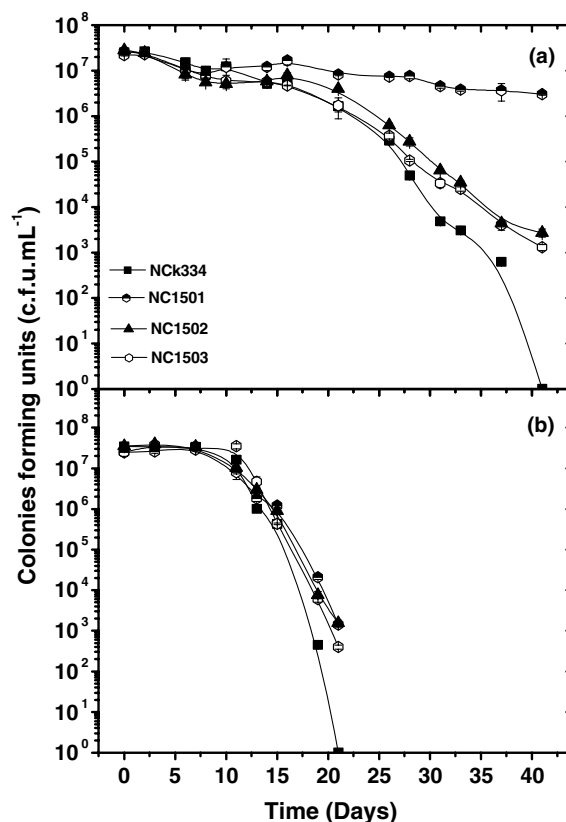


Fig. 5. Effects of carbon starvation on the survival of the different strains of *L. gasseri*. Cells from the exponential growth phase were placed at 25 °C (a) or 4 °C (b). The entire procedure was carried out in APT broth without sugar under aerobic conditions.

showed 3-logs (1000-fold) better survival than the wild-type strain (NCK334), while the culture with a multi-copy plasmid containing *sodA* showed >6-logs of better survival than the wild-type strain (Fig. 5(a)). Indeed, the degree of survival of the different strains at 25 °C positively correlates with the specific activity of MnSOD expressed in the cells. On the other hand, cells starved at 4 °C died at much faster rate than those starved at 25 °C regardless of the level of active MnSOD (Fig. 5(b)).

4. Discussion

Previous studies have demonstrated the presence of two phospho- β -galactosidase genes (*lacI* and *lacII*) and that their respective gene products were active in *L. gasseri* JCM1031 [20,25,26]. Also, the accessibility of *L. gasseri* NCK334 genome sequence (http://www.jgi.doe.gov/JGI_microbial/html/index.html) enabled us to confirm the high homology between the pair of phospho- β -galactosidase sequences from *L. gasseri* NCK334 and JCM1031. Since the objective of this study was to generate genetically enhanced strains of *L. gasseri*

NCK334 without disrupting any of their technologically important genes, we targeted the second phospho- β -galactosidase gene (*lacII*) for the insertion of *sodA*. In the present study, the strategy developed for site-specific insertion of DNA sequences into *Lactobacillus* spp. [4], was successfully adapted to integrate a marker-free single copy of *sodA* gene from *S. thermophilus* AO54 [21] in the genomic phospho- β -galactosidase *lacII* of *L. gasseri*. The non-replicating vector.

pJB34-S containing the *sodA* gene flanked by *lacII* regions of ≈ 700 bp each was established in *L. gasseri* NCK334 with the RepA function provided by pTRK669 [4]. We followed the strategy described by Russell and Klaenhammer [4] to prompt single cross-over events. However, we kept the concentrations of the antibiotic very low to obtain strains containing a single copy of pJB34-S inserted into the chromosome. Subsequently, we removed the antibiotic pressure to allow for the second cross-over event to take place. Southern hybridization analysis demonstrated that only the DNA containing the desired gene was integrated into the chromosome of the host strain. Additionally, DNA sequence analysis showed that *sodA* was intact and suffered no mutations during the genetic manipulation. Also, native and SDS-PAGE gels were analyzed for SOD-activity and Western-blotting, respectively, and revealed the presence of a functional MnSOD that migrated to the same position as that of *S. thermophilus* AO54. MnSOD activity differences in NC 1501 vs. NC 1502 & 1503 is due to the obvious differences in gene copy number and the subsequent transcription and translation levels (Fig. 3). On the other hand, the differences in the enzyme activity seen in *S. thermophilus* AO54 as compared to NC 1502 & NC 1503 (Fig. 3), where MnSOD is expressed from a single copy of *sodA*, may be due to many factors that are not clear at the present time. However, one of the most likely explanations is the differences in the rate of manganese transport in these organisms, where Mn^{2+} is required to activate the apo-protein to an active MnSOD. This conclusion is supported by the 100% increase in the specific activity of MnSOD observed upon supplementing the growth medium with 1 mM manganese. Studies to improve Mn^{2+} transport in *L. gasseri* and other probiotic lactic acid bacteria are greatly needed.

As intended, the disruption of *lacII* did not affect the ability of the new constructs to utilize lactose, as no variations in the specific growth rates or the final pHs were observed between the *L. gasseri* parent strain and the new constructs when grown in APT-containing lactose as the sole source of energy. Also, there was no difference in MnSOD specific activity in *L. gasseri* cells growing either on lactose or glucose (data not shown). Thus, a stable gene replacement was indeed achieved in *L. gasseri* by targeting the redundant phospho- β -galactosidase gene (*lacII*).

Previous studies determined that MnSOD protects against H_2O_2 by inhibiting the superoxide-dependent reduction of ferric iron to ferrous iron and thus prevent the generation of hydroxyl radical (HO^\bullet) from hydrogen peroxide by the Fenton reaction [12]. To verify whether a single copy of *sodA* integrated in the chromosome of *L. gasseri* could provide sufficient protection against H_2O_2 , we tested the survival of the different constructs and the parent strain during 90 min exposure to H_2O_2 (Fig. 4). The data showed that the presence of active MnSOD significantly enhanced the organism's tolerance to H_2O_2 challenge, and the protection provided by a single copy of *sodA* (NC 1502 & 1503) was optimal and not significantly different from that seen in the strain with a multicopy plasmid (NC 1501) (Fig. 4). To understand these results, one needs to know that the toxicity of H_2O_2 could result from its reaction with reduced iron (Fe^{2+}) to generate hydroxyl radicals (HO^\bullet), or by its ability to directly oxidize the cellular components especially at very high concentrations of H_2O_2 . Furthermore, the reduction of iron can be mediated by O_2^- or by other reducing agents in the cell. Thus, MnSOD can only protect against superoxide-mediated iron reduction and prevent the generation of HO^\bullet (6); however, SOD cannot protect against the direct killing by H_2O_2 or against the HO^\bullet generated by the reaction between H_2O_2 and Fe^{2+} reduced by other reducing agents. The fact that protection against H_2O_2 killing was not significantly different among the *L. gasseri* strains with different concentrations of MnSOD (i.e., NC 1501, NC 1502, or NC 1503) indicates that the rate of O_2^- generated in these cells is relatively low and is efficiently removed by the level of MnSOD expressed from single copy of *sodA*.

The expression of MnSOD also provided the cells with significant (i.e., 10^3 to $>10^6$ -fold) better protection during carbon/energy starvation at 25 °C (Fig. 5(a)). However, this protective effect was less pronounced at 4 °C, and the cells died at a faster rate than at 25 °C (Fig. 5(b)). At the present time, we cannot explain the reason for this greater rate of death during starvation for carbon/energy source at 4 °C (Fig. 5(b)). However, the reduced rate of protection by MnSOD against starvation at 4 °C may be due to the higher solubility of oxygen and the reduced activity of the enzyme at the low temperature.

In summary, the results reported here demonstrated the successful integration of *sodA* from *S. thermophilus* AO54 into the chromosome of *L. gasseri*, and that the expression of MnSOD provided significant protection against hydrogen peroxide and carbon/energy starvation at 25 °C. However, it is possible to further improve resistance to carbon starvation in *L. gasseri* harboring a single copy of the heterologous *sodA* gene (strains NC 1502 and 1503) by improving its transcription, translation and/or post-translation modification to produce higher

activity of this antioxidant enzyme. SODs have also been shown to provide protection against heat-shock [30], freeze-thaw [31], and acid pH [32]; however, the exact mechanism(s) is not presently understood but is most likely due to preventing a free radical-mediated stress. Experiments are underway to examine the added benefits that these genetically improved MnSOD-enriched cells could provide in the treatment of gastrointestinal disorders. Strains with such desirable characteristics could also be used as vehicles for the delivery of pharmaceutical and/or nutraceutical molecules to the gastrointestinal tract [33].

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References

- [1] Cotter, P.D., Hill, C. and Ross, R.P. (2003) A food-grade approach for functional analysis and modification of native plasmids in *Lactococcus lactis*. *Appl. Environ. Microbiol.* 69, 702–706.
- [2] Neu, T. and Henrich, B. (2003) New thermosensitive delivery vector and its use to enable nisin-controlled gene expression in *Lactobacillus gasseri*. *Appl. Environ. Microbiol.* 69, 1377–1382.
- [3] Leenhouts, K., Buist, G., Bolhuis, A., ten Berge, A., Kiel, J., Mierau, I., Dabrowska, M., Venema, G. and Kok, J. (1996) A general system for generating unlabelled gene replacements in bacterial chromosomes. *Mol. Gen. Genet.* 253, 217–224.
- [4] Russell, W.M. and Klaenhammer, T.R. (2001) Efficient system for directed integration into the *Lactobacillus acidophilus* and *Lactobacillus gasseri* chromosomes via homologous recombination. *Appl. Environ. Microbiol.* 67, 4361–4364.
- [5] Azcárate-Peril, M.A., Altermann, E., Hoover-Fitzula, R.L., Cano, R.J. and Klaenhammer, T.R. (2004) Identification and inactivation of genetic loci involved with acid tolerance in *Lactobacillus acidophilus*. *Appl. Environ. Microbiol.* 70, 5315–5322.
- [6] Bruno-Bárcena, J.M., Azcárate-Peril, M.A. and Sineriz, F. (2001) Genetically modified organisms (GMO) in food products: potential risks and the reaction of the consumer In: *Food Microbiology Protocols* (Spencer, J.F.T. and Ragout de Spencer, A.L., Eds.), pp. 481–484. Humana Press, Totowa-New Jersey.
- [7] Condon, S. (1987) Responses of lactic acid bacteria to oxygen. *FEMS Microbiol. Rev.* 46, 269–280.
- [8] Sedewitz, B., Schleifer, K.H. and Götz, F. (1984) Physiological role of pyruvate oxidase in the aerobic metabolism of *Lactobacillus plantarum*. *J. Bacteriol.* 160, 462–465.
- [9] Sedewitz, B., Schleifer, K.H. and Götz, F. (1984) Purification and biochemical characterization of pyruvate oxidase from *Lactobacillus plantarum*. *J. Bacteriol.* 160, 273–278.
- [10] Hassan, H.M. (1989) Microbial superoxide dismutases. *Adv. Genetic* 26, 65–97.
- [11] Jenney, F.E., Verhagen, M.F.J.M., Cui, X.Y. and Adams, M.W.W. (1999) Anaerobic microbes: oxygen detoxification without superoxide dismutase. *Science* 286, 306–309.
- [12] Bruno-Bárcena, J.M., Andrus, J.M., Libby, S.L., Klaenhammer, T.R. and Hassan, H.M. (2004) Expression of a heterologous manganese superoxide dismutase gene in intestinal lactobacilli provides protection against the toxicity of hydrogen peroxide. *Appl. Environ. Microbiol.* 70, 4702–4710.
- [13] de Man, J.C., Rogosa, M. and Sharpe, M.E. (1960) A medium for the cultivation of lactobacilli. *J. Appl. Bacteriol.* 23, 130–135.
- [14] Evans, J.B. and Niven, C.R. (1951) Nutrition of the heterofermentative lactobacilli that cause greening of cured meat products. *J. Bacteriol.* 62, 599–603.
- [15] Mercenier, A., Robert, C., Romero, D.A., Slos, P. and Lemoine, Y. (1987) Transformation of *Streptococcus thermophilus* spheroplasts In: *Streptococcal Genetics* (Ferretti, J.J. and Curtiss, R. III, Eds.), pp. 234–237. American Society for Microbiology, Washington, DC.
- [16] Terzaghi, B.E. and Sandine, W.E. (1975) Improved medium for lactic streptococci and their bacteriophages. *Appl. Microbiol.* 29, 807–813.
- [17] Sambrook, J. and Russell, D.W. (2001) *Molecular Cloning: a Laboratory Manual*. Cold Spring Harbor Laboratory, NY.
- [18] Walker, D.C., Aoyama, K. and Klaenhammer, T.R. (1996) Electrotransformation of *Lactobacillus acidophilus* group A1. *FEMS Microbiol. Lett.* 138, 233–237.
- [19] Innis, M.A., Gelfand, D.H., Sninsky, J.J. and White, T.J. (1990) *PCR Protocols: a Guide to Methods and Applications*. Academic Press, San Diego.
- [20] Saito, T., Suzuki, M., Konno, K., Kitazawa, H., Kawai, Y., Itoh, T. and Kamio, Y. (1998) Molecular cloning and sequencing of two phospho-beta-galactosidase I and II genes of *Lactobacillus gasseri* JCM1031 isolated from human intestine. *Biosci. Biotechnol. Biochem.* 62, 2318–2327.
- [21] Andrus, J.M., Bowen, S.W., Klaenhammer, T.R. and Hassan, H.M. (2003) Molecular characterization and functional analysis of the manganese-containing superoxide dismutase gene (*sodA*) from *Streptococcus thermophilus* AO54. *Arch. Biochem. Biophys.* 420, 103–113.
- [22] Bradford, M.M. (1976) Rapid and sensitive method for quantitation of microgram quantities of protein utilizing principle of protein-dye binding. *Anal. Biochem.* 72, 248–254.
- [23] Beauchamp, C. and Fridovich, I. (1971) Superoxide dismutase: improved assays and an assay applicable to acrylamide gels. *Anal. Biochem.* 44, 276–287.
- [24] McCord, J.M. and Fridovich, I. (1969) Superoxide dismutase an enzymatic function for erythrocyte hemocuprein. *J. Biol. Chem.* 244, 6049–6055.
- [25] Suzuki, M., Saito, T. and Itoh, T. (1996) Coexistence of two kinds of 6-phospho-beta-galactosidase in the cytosol of *Lactobacillus gasseri* JCM1031 purification and characterization of 6-phospho-beta-galactosidase II. *Biosci. Biotechnol. Biochem.* 60, 708–710.
- [26] Suzuki, M., Saito, T. and Itoh, T. (1996) Purification and characterization of 6-phospho-beta-galactosidase from *Lactobacillus gasseri* JCM 1031. *Biosci. Biotechnol. Biochem.* 60, 139–141.
- [27] Law, J., Buist, G., Haandrikman, A., Kok, J., Venema, G. and Leenhouts, K. (1995) A system to generate chromosomal mutations in *Lactococcus lactis* which allows fast analysis of targeted genes. *J. Bacteriol.* 177, 7011–7018.
- [28] Chang, S.K. and Hassan, H.M. (1997) Characterization of superoxide dismutase in *Streptococcus thermophilus*. *Appl. Environ. Microbiol.* 63, 3732–3735.
- [29] Carlouz, A. and Touati, D. (1986) Isolation of superoxide dismutase mutants in *Escherichia coli*: is superoxide dismutase necessary for aerobic life. *EMBO J.* 5, 623–630.
- [30] Benov, L.T. and Fridovich, I. (1995) Superoxide dismutase protects against aerobic heat shock in *Escherichia coli*. *J. Bacteriol.* 117, 3344–3346.

- [31] Park, J.I., Grant, C.M., Davies, M.J. and Dawes, I.W. (1998) The cytoplasmic Cu,Zn superoxide dismutase of *Saccharomyces cerevisiae* is required for resistance to freeze-thaw stress – generation of free radicals during freezing and thawing. *J. Biol. Chem.* 273, 22921–22928.
- [32] Sanders, J.W., Leenhouts, K.J., Haandrikman, A.J., Venema, G. and Kook, J. (1995) Stress response in *Lactococcus lactis*: cloning, expression analysis and mutation of the lactococcal superoxide dismutase gene. *J. Bacteriol.* 177, 5254–5260.
- [33] Kaur, I.P., Chopra, K. and Saini, A. (2002) Probiotics: potential pharmaceutical applications. *Eur. J. Pharm. Sci.* 15, 1–9.
- [34] Casadaban, M.J. and Cohen, S.N. (1980) Analysis of gene control signals by DNA fusion and cloning in *Escherichia coli*. *J. Mol. Biol.* 138, 179–207.
- [35] Russell, W.M. and Klaenhammer, T.R. (2001) Identification and cloning of *gusA*, encoding a new beta-glucuronidase from *Lactobacillus gasseri* ADH. *Appl. Environ. Microbiol.* 67, 1253–1261.