High-level resistance to oxidative stress in *Lactococcus lactis* conferred by *Bacillus subtilis* catalase KatE

T. Rochat,1†§ A. Miyoshi,2‡ § J. J. Gratadoux,1† P. Duwat,1|| S. Sourice,1‡ V. Azevedo2 and P. Langella1†

1Unité de Recherches Laitières et de Génétique Appliquée, Institut National de la Recherche Agronomique, Domaine de Vilvert, 78352 Jouy-en-Josas cedex, France
2Institute of Biological Sciences, Federal University of Minas Gerais (UFMG-ICB), Belo Horizonte, MG, Brazil

*Lactococcus lactis*, a lactic acid bacterium widely used for food fermentations, is often exposed to damaging stress conditions. In particular, oxidative stress leads to DNA, protein and membrane damages that can be lethal. As *L. lactis* has no catalase, the impact of production of the *Bacillus subtilis* haem catalase KatE on its oxidative stress resistance was tested. This cytoplasmic catalase was engineered for extracellular expression in *L. lactis* with an optimization strategy based on fusion to the nisin-inducible promoter and a lactococcal signal peptide (SPUsp45). The production of KatE by *L. lactis* conferred an 800-fold increase in survival after 1 h exposure to 4 mM hydrogen peroxide, and a 160-fold greater survival in long-term (3 days) survival of aerated cultures in a cydA mutant, which is unable to respire. The presence of KatE protected DNA from oxidative damage and limited its degradation after long-term aeration in a cydArecA mutant, defective in DNA repair. *L. lactis* is thus able to produce active catalase that can provide efficient antioxidant activity.

INTRODUCTION

Oxidative stress can be defined as an excess of reactive oxygen species (ROS) that have strong oxidizing potential for cells (Farr & Kogoma, 1991; Fridovich, 1998). ROS cause damage to macromolecular constituents such as DNA, RNA, proteins and lipids (Berlett & Stadtman, 1997). Toxicity occurs when the degree of oxidative stress exceeds the capacity of cell defence systems (Farr & Kogoma, 1991). ROS originate from partial reduction of molecular oxygen (O2) to superoxide (O2•−), hydrogen peroxide (H2O2) and hydroxyl radical (OH•) (Storz & Imlay, 1999). The biological sources of ROS are numerous, e.g. they can be generated in aerobiosis by flavoproteins (Condon, 1987), and by macrophages during inflammatory reactions (Ross, 1991). Thus, oxidative stress plays an important role in pathologies of the gastrointestinal tract of humans such as inflammatory bowel diseases (Kruidenier & Verspaget, 2002; Kruidenier et al., 2003d) and in the radio-induced tissue injury that may occur during radiotherapy (Sun et al., 1998).

Bacterial species are more or less sensitive to oxygen according to the enzymic equipment they possess to prevent or repair ROS damage. Some lactic acid bacteria, such as *Lactococcus lactis*, widely used in the production of fermented food products, produce a superoxide dismutase, which degrades O2•− to generate H2O2 (Sanders et al., 1995). However, they lack catalases, antioxidant metalloenzymes that catalyse the reaction in which toxic H2O2 is reduced to two H2O molecules and O2. In contrast, catalase activity has been reported in several *Lactobacillus* species during the last decade (Igarashi et al., 1996; Knauf et al., 1992). In the absence of catalase, H2O2 produced by the cell or present in the environment accumulates, and may lead, especially in presence of iron, to the production of the more toxic OH• (Fridovich, 1998; Imlay, 2003).

Bacterial catalases are widespread in aerobes (facultative or not) such as *Escherichia coli* and *Bacillus subtilis*. Two classes of catalases have been distinguished, according to their active-site composition: one is haem-dependent, and the other, also named pseudocatalase, is manganese-dependent.
for the reduction of H$_2$O$_2$. Catalases of two lactobacilli have been successfully transferred and phenotypically expressed in heterologous hosts deficient in catalase activity (Abriouel et al., 2004; Knauf et al., 1992; Noonpakdee et al., 2004). Strains of lactic acid bacteria expressing high levels of catalases could be useful in both traditional food applications and new therapeutic uses. A probiotic antioxidant strain able to eliminate ROS in the digestive tract of animals and humans could have applications for treatment of inflammatory diseases or post-cancer drug treatments.

In this work, we tested the effect of the production of the B. subtilis haem-catalase KatE (Engelmann et al., 1995) on the oxidative stress resistance of L. lactis.

**METHODS**

**Bacterial strains and growth conditions.** The bacterial strains and plasmids used in this work are listed in Table 1. L. lactis strains were grown in M17 medium supplemented with 0-5% glucose (GM17) or Brain Heart Infusion at 30 °C without agitation. E. coli strains were grown in aerated Luria–Bertani medium at 37 °C. When required, antibiotics were used at the following concentrations: for L. lactis, erythromycin (Em; 5 µg ml$^{-1}$), chloramphenicol (Cm; 10 µg ml$^{-1}$), tetracycline (Tc; 2 µg ml$^{-1}$), streptomycin (Sm; 1.5 µg ml$^{-1}$), rifampicin (Rif; 100 µg ml$^{-1}$); and for E. coli, ampicillin (100 µg ml$^{-1}$) or chloramphenicol (12.5 µg ml$^{-1}$). For enumeration, appropriate dilutions of samples (prepared in cold peptone water; 1 g l$^{-1}$) were plated on GM17 and incubated at 30 °C for 48 h. Aerated cultures (45 ml) were performed in 250 ml Erlenmeyer flasks; non-aerated cultures (10 ml) were grown in closed 25 ml tubes. Aeration was performed by stirring the cultures in circular agitators (Infors) at 240 r.p.m. Expression of the katE gene placed under the control of the nisin-inducible promoter P$_{nisA}$ (de Ruyter et al., 1996a) was induced as follows. An overnight culture was used to inoculate fresh medium at a dilution of 1/50. At OD$_{600}$ around 0-5, 1 ng ml$^{-1}$ of nisin (Sigma) was added to the culture, which was further incubated for 1 h. For the activity of KatE, 10 µM haemin (Sigma) was added to the medium.

**DNA manipulations.** General molecular biology techniques and isolation of chromosomal DNA from L. lactis and plasmid DNA from E. coli were performed as described by Sambrook et al. (1989). DNA restriction and modification enzymes were used as recommended by the suppliers. When required, DNA fragments were isolated from agarose gels by using the Concert Rapid Gel Extraction System (Gibco-BRL). PCR amplifications, using Taq DNA polymerase (Invitrogen), were performed with a Perkin-Elmer DNA Thermal Cycler. Unless otherwise indicated, DNA fragments were isolated by gel electrophoresis and DNA manipulations. General molecular biology techniques and isolation of chromosomal DNA from L. lactis and plasmid DNA from E. coli were performed as described by Sambrook et al. (1989). DNA restriction and modification enzymes were used as recommended by the suppliers. When required, DNA fragments were isolated from agarose gels using the Concert Rapid Gel Extraction System (Gibco-BRL). PCR amplifications, using Taq DNA polymerase (Invitrogen), were performed with a Perkin-Elmer DNA Thermal Cycler. Unless otherwise indicated, DNA fragments were isolated by gel electrophoresis and from agarose gels using the Concert Rapid Gel Extraction System (Gibco-BRL). PCR amplifications, using Taq DNA polymerase (Invitrogen), were performed with a Perkin-Elmer DNA Thermal Cycler. Unless otherwise indicated, DNA fragments were isolated by gel electrophoresis and DNA manipulations.

**Table 1. Bacterial strains and plasmids**

<table>
<thead>
<tr>
<th>Strain/plasmid</th>
<th>Characteristics</th>
<th>Source/reference</th>
</tr>
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<tbody>
<tr>
<td>E. coli TOP10</td>
<td>F$^{-}$ mcrA Δ(mrr–hsdRMS–mcrBC) Δ80lacZΔM15 ΔlacX74 recA1</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>E. coli TG1</td>
<td>supE hsd Δ5 thi Δ(lac–proAB) F’ (traD36 proAB–lacZ ΔM15)</td>
<td>Gibson (1984)</td>
</tr>
<tr>
<td>L. lactis MG1363</td>
<td>Wild-type strain</td>
<td>Gasson (1983)</td>
</tr>
<tr>
<td>L. lactis NZ9000</td>
<td>MG1363 carrying nisRK genes on the chromosome</td>
<td>Kuipers et al. (1998)</td>
</tr>
<tr>
<td>L. lactis MG1363 cylA</td>
<td>cylA mutant (Em’)$^2$</td>
<td>Duwat et al. (2001)</td>
</tr>
<tr>
<td>L. lactis MG1363 recA</td>
<td>recA mutant (Tc’)$^2$</td>
<td>Duwat et al. (1995)</td>
</tr>
<tr>
<td>L. lactis NZ9000 cylA</td>
<td>MG1363::cylA/ΔcylA $^5$ carrying nisRK genes on the chromosome; Em’</td>
<td>This work</td>
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<tr>
<td>L. lactis NZ9000 cylA</td>
<td></td>
<td>This work</td>
</tr>
<tr>
<td>pVE3655</td>
<td>pWV01/Cm’; carrying the nisin-inducible promoter P$_{nisA}$</td>
<td>Le Loir et al. (2001)</td>
</tr>
<tr>
<td>pSEC::Nuc</td>
<td>pWV01/Cm’; expression vector containing a fusion between the signal peptide of Usp45 (Sp$<em>{Usp}$) and Nuc, expressed under the control of P$</em>{nisA}$</td>
<td>Bermudez-Humaran et al. (2003)</td>
</tr>
<tr>
<td>pLL253</td>
<td>pAM βI/Em’</td>
<td>Simon &amp; Chopin (1988)</td>
</tr>
<tr>
<td>pUC1318: P$_{23}$</td>
<td>Ap’, carrying the constitutive promoter P$_{23}$</td>
<td>Y. Le Loir$^*$</td>
</tr>
<tr>
<td>pILLN12::katE</td>
<td>pILLN12 (Renault et al., 1996) carrying the B. subtilis katE gene under the control of P$_{23}$ constitutive promoter; Tet$^R$</td>
<td>This work</td>
</tr>
<tr>
<td>pBS::P$_{23}$::katE</td>
<td>Ap’, pBS:Ks+ carrying the B. subtilis katE gene under the control of P$_{23}$ constitutive promoter</td>
<td>This work</td>
</tr>
<tr>
<td>pCRII-TOPO</td>
<td>Ap’ Km’</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>pTPS::katE</td>
<td>pCRII-TOPO carrying PCR fragment with the B. subtilis katE gene</td>
<td>This work</td>
</tr>
<tr>
<td>pSEC::KatE</td>
<td>pWV01/Cm’; expression vector where the Nuc coding sequence is replaced by the B. subtilis katE gene; under the control of P$_{nisA}$</td>
<td>This work</td>
</tr>
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$^*$Laboratoire de Microbiologie, École Nationale Supérieure d’Agronomie, Institut National de la Recherche Agronomique, UMR1055, 65 rue de Saint Brieuc, CS84215 35042 Rennes cedex, France.
DNA sequencing was carried out on double-stranded plasmid DNA by the dideoxy chain-termination method with the MegaBACE Sequencing System (Amersham Biosciences).

**Cloning of *B. subtilis* katE.** The katE gene was amplified by PCR from the chromosome of *B. subtilis* 168 using the primers 5′-AA-TGTTGCTTACAGATTGTCTTCTTTAAA and 3′-TTTTCCTCGAG- TTGTAAGCTTAGCTGAG, designed using the sequenced genome of *B. subtilis* (Kunst et al., 1997). The restriction sites used for subsequent cloning are underlined: Xbal and Xhol for the 5′ and 3′ primers, respectively. The PCR product was then inserted into pBS:KS+ (Stratagene; hereafter called pBS) cut by Xhol and Xbal in *E. coli* TG1, resulting in pBS:katE. The promoter P23 fragment (van der Vossen et al., 1987) was obtained after digestion of plasmid pUC1318: P23 (kindly provided by Y. Le Loir) by EcoRI and Xhol. It was then cloned in pBS:katE cut by EcoRI and Xhol, resulting in pBS:P23:katE. The expression cassette P23:katE obtained after double digestion of pBS:P23:katE by EcoRI and Xhol, was inserted into ECO/R uhol-cut high-copy-number plpILN12, resulting in pILN12:P23:katE obtained in *L. lactis*. The plasmid pSEC:Nuc (Table 1; Bermudez-Humaran et al., 2003) was used in this work: (i) to clone the katE gene under transcriptional control of the lactococcal nisin-inducible promoter PnisL (de Ruyter et al., 1996a) and (ii) to secrete the KatE catalase into the extracellular medium. This plasmid contains the ribosome-binding site (RBSUsp45) and the sequence encoding the signal peptide (SP Usp45) of the *B. subtilis* katE gene (Van Asseldonk et al., 1990) and the mature part of the staphylococcal nuclease (Nuc; Le Loir et al., 1998). An NsiI site was introduced at the 3′ end of RBSUsp45 to allow replacement of the nuc coding sequence by a DNA fragment encoding the *B. subtilis* katE. Thus, the 2172 bp katE gene was PCR-amplified from the plasmid pBS:P23:katE (Table 1). Primers, containing one artificial restriction site at each end, were designed according to the genomic DNA sequence of katE (GenBank accession no. D83026). Two primers were used: (i) SCKatE5′ for the coding strand 5′-GGATG CATCAAGTGTAGCAGCAAAAC-3′, where the NsiI site is underlined and CA (in bold) was added to adapt the reading frame of pILNps:katE; and (ii) SCKatE3′ for the complementary strand 5′-GGTCGCAGCTGTCAAATCTGCTTACCC-3′, where the Xhol site is underlined. The resulting amplified product was then cloned into the pCRII-TOPO vector (Table 1), generating the intermediate construction pTPS: katE (Table 1) in *E. coli* TOP10 (Table 1). This construction was then digested with NsiI and Xhol, allowing purification of fragments containing the katE gene. This fragment was cloned into NsiI/Xhol-cut pSEC:Nuc vector, resulting in plasmid pSEC:KatE (Table 1), established in *E. coli* TG1 (Table 1). The sequence of the insert was checked.

**Construction of *L. lactis* strains to analyse the impact of KatE production.** To analyse the effects of katE expression on bacterial physiology, two strains were constructed: *L. lactis* MG1363 containing pILN12:KatE, hereafter called MG(pILN12:KatE), and *L. lactis* NZ9000 (carrying the nisRK genes necessary for PnisL induction; Table 1; Kuipers et al., 1998) containing pSEC: KatE, hereafter called NZ(pSEC: KatE). The production and the activity of KatE and its potential impact on the survival rate of these KatE+ strains after H2O2 exposure were successively analysed. Finally, we evaluated the influence of katE expression (using the construction pSEC: KatE) on long-term survival in aerated cultures. As the wild-type strain of *L. lactis* is not appropriate for these experiments, we constructed a derivative of *L. lactis* NZ9000 unable to respire (inactivated in the cytochrome oxidase gene cydA; NZ9000 cydA, or NZ cydA for short) by conjugation as previously described (Langella et al., 1993). The donor strain was the erythromycin-resistant (*Em*E) MG1363 cydA (Duwat et al., 2001) and the recipient was a spontaneously streptomycin- and rifampicin-resistant (*Sm*R *Rif*R) mutant of NZ9000 (Bermudez-Humaran et al., 2002). Transconjugants were first isolated on GM17 containing Em, Sm and Rif. Then, one transconjugant that contained nisRK genes was selected; pSEC: Nuc was introduced into this transconjugant and a TBD-agar test was performed in the presence of 1 ng nisin ml⁻¹ to check nuc induction by detection of Nuc activity as previously described (Le Loir et al., 1994). OD600 and pH measurements of aerated cultures, supplemented with haemin, of the selected transconjugant showed that it was still unable to respire, like the parental cydA strain (OD600 <2.5 and pH <6). As the negative control we used the plasmid pVE655 (Le Loir et al., 2001), which was obtained after Xhol digestion of pNZ28010 (de Ruyter et al., 1996b) to delete the gas gene and maintain only PnisL. The two plasmids pVE655 and pSEC: KatE were introduced into this transconjugant, giving strains hereafter called NZ cydA(pVE655) and NZ cydA(pSEC: KatE), respectively. In this context, where addition of haemin has no influence on O2 utilization (Duwat et al., 2001), the impact of KatE on *L. lactis* survival could be analysed in aerated stress conditions. Finally, the strain NZ9000 cydA/recA (NZ cydA/recA for short), unable to respire (with aeration and in the presence of haemin) and unable to repair DNA damage (because of recA inactivation) was constructed by the same procedure: a mating was performed between MG1363 recA (Duwat et al., 1995) and NZ cydA. The two plasmids pVE655 and pSEC: KatE were introduced into a selected transconjugant, giving strains hereafter called respectively NZ cydA/rec(ApVE655) and NZ cydA/rec(ApSEC: KatE), and the influence of KatE on DNA integrity in cultures exposed to long-term aeration was analysed. Noninduced cultures and *L. lactis* NZ strains harbouring the control plasmid pVE655 (Table 1) were used as negative controls.

**Detection of catalase activity in *L. lactis* strains.** Two millilitres of exponentially growing cultures of each *L. lactis* strains, induced or not for 1 hour by addition of 1 ng nisin ml⁻¹, were harvested and resuspended in 30 μl TES buffer (50 mM Tris/HCl pH 8.0, 1 mM EDTA, 25 % Sucrose). Samples (20 μl) of TES-resuspended cells were mixed with 10 μl H2O2 (8 M). To detect catalase activity in the culture medium, H2O2 was directly mixed with 20 μl harvested supernatant. The presence of catalase activity leads to bubble formation resulting from the transformation of H2O2 to H2O and gaseous O2. Quantitative assay of catalase activity was performed on cell suspensions by the method of Sinha (1972). Briefly, exponentially growing cells (induced for NZ derivative strains) were centrifuged, resuspended in phosphate buffer (0.1 M, pH 7) at 10⁸ c.f.u. ml⁻¹, and mixed with 0.8 mmol H2O2 in phosphate buffer. H2O2 concentration was determined each minute by mixing, at a ratio of 1:3, an aliquot with a solution of dichromate in acetic acid (1/3 dipotassium chromate 50 g l⁻¹; 2/3 glacial acetic acid). The samples were then boiled and centrifuged to remove cells; the absorbance was measured at 570 nm. Catalase activity was expressed as μmol H2O2 degraded per min per 10⁸ c.f.u. The results presented correspond to the mean of three different assays.

**Preparation of cellular and supernatant protein fractions of *L. lactis* for SDS-PAGE.** For fractionation between cell and supernatant fractions, 2 ml samples of nisin-induced *L. lactis* cultures were centrifuged for 5 min at 6000 g at 4 °C. Protein extracts were then prepared as previously described (Le Loir et al., 1998).

**Amino-terminal sequencing.** To determine the nature of the catalase form detected in the cell fraction of NZ(pSEC: KatE), the corresponding band was cut from the SDS-PAGE gel and submitted to N-terminal microsequencing (performed by I. C. Huet at UBSP, INSRA Jouy-en-Josas) on a gas-phase sequencer (model 477A/HPLC 120A; Perkin Elmer).

**Survival after H2O2 exposure.** Cultures in stationary phase were diluted 1/50 in GM17, supplemented with haemin, grown to OD600 0-5 and divided into two samples, nisin-induced or not, for the construction containing PnisL. To measure the survival of the
Production of an active catalase in *L. lactis*

The katE gene encoding the *B. subtilis* haem catalase KatE was introduced into *L. lactis* with the aim of improving its antioxidant properties. The katE ORF was first placed under the control of the constitutive promoter P23 for its cytoplasmic production, resulting in the plasmid pILN12 : KatE, and introduced into *L. lactis* MG1363. We previously reported that fusion of exported proteins to a signal peptide led in numerous cases to higher production levels than those of the same protein devoid of signal peptide and expressed in the cytoplasm (for a review, see Nouaille et al., 2003). We therefore expected to achieve a higher protein yield, possibly combined with efficient secretion of the enzyme produced, by this procedure. Thus, we fused katE in-frame downstream of the signal peptide of the major secreted lactococcal protein Usp45 (SP_Usp45). The fusion was expressed under the transcriptional control of the inducible promoter P_nisA. Catalase activity of both MG(pILN12 : KatE) and NZ(pSEC : KatE) strains was evaluated. When H₂O₂ was added to cell culture samples, bubbles due to O₂ formation appeared only with samples of MG(pILN12 : KatE) and of induced NZ(pSEC : KatE) cultures (Fig. 1a). No activity was detected in supernatant samples of this induced culture. Comparative assays of the catalase activity resulting from the constitutive or inducible production of KatE [expressed by MG(pILN12 : KatE) and NZ(pSEC : KatE), respectively] were performed. The level of activity in MG(pILN12 : KatE) cells was not significantly different from that observed with the control strain (Fig. 1b). In contrast, a threefold higher activity [≈ 3 μmol H₂O₂ min⁻¹ (10⁹ c.f.u.)⁻¹] was measured for NZ(pSEC : KatE).

We then determined the cellular distribution of KatE in induced NZ(pSEC : KatE) cultures. Cellular and supernatant protein fractions of NZ(pVE3655) and NZ(pSEC : KatE)

![Fig. 1.](image1.png)  
**Fig. 1.** *L. lactis* produces an active catalase KatE. (a) Exponential-phase cultures from *L. lactis* MG(pIL253) and MG(pILN12 : KatE), or from nisin-induced or noninduced NZ(pVE3655) and NZ(pSEC : KatE), were concentrated then mixed with H₂O₂ solution. Catalase activity was detected by bubble formation due to O₂ liberation. (b) Comparative assay of catalase activity in cell suspensions was performed using a colorimetric method (Sinha, 1972) and expressed as μmol H₂O₂ decomposed per min per 10⁹ c.f.u.

![Fig. 2.](image2.png)  
**Fig. 2.** The majority of KatE produced in *L. lactis* is present as a nonprocessed intracellular precursor. Cellular (C) and supernatant (S) protein fractions of nisin-induced exponential-phase cultures of *L. lactis* NZ(pVE3655) and NZ(pSEC : KatE) were separated by SDS-PAGE and stained with Coomassie blue. M, molecular mass marker.
were run on SDS-PAGE and stained with Coomassie blue. In the cellular fraction, one ~80 kDa migrating band (for an expected band at ~79 kDa) was detected and present only in the NZ(pSEC:KatE) extract, suggesting that it could correspond to KatE (Fig. 2). No corresponding band was detected in the supernatant fractions of either strain. N-terminal microsequencing of this extracted band revealed the first nine amino acid residues of SPUsp45 suggesting that the major protein produced by NZ(pSEC:KatE) is the intracellular precursor SPUsp:KatE. The absence of any band in the supernatant fraction of this strain which could correspond to secreted mature form of KatE suggests either an absence of maturation or a very low maturation rate of SPUsp:KatE. We checked that the net global charge of the first ten amino acid residues of the N-terminal end of mature KatE (~3) is compatible with an efficient secretion (Le Loir et al., 1998). This weak processing of the precursor did not abolish KatE activity, as an efficient catalase activity was detected in the nisin-induced L. lactis NZ(pSEC:KatE) strain (Fig. 1).

**KatE allows better survival of L. lactis in oxidative stress conditions**

We previously determined the viability of L. lactis subsp. cremoris when challenged with a range of H2O2 concentrations (Rochat et al., 2005). Here, we asked whether the production of active KatE contributes to a higher survival rate of L. lactis. The levels of oxidative stress resistance of control and KatE-producing strains were compared after H2O2 exposure. In contrast to strain MG(pILN12 : KatE), which did not survive better under H2O2 stress than the control (both control and KatE+ strains showed a decrease in viability of 2×10^5 c.f.u. ml⁻¹ and 3×10^4 c.f.u. ml⁻¹ after incubation with 2 and 4 mM of H2O2 respectively; data not shown), strain NZ(pSEC : KatE) showed significantly better H2O2 resistance than the control strain. Survival was 300- and 800-fold higher than the control strain after 1 h incubation with 2 and 4 mM H2O2, respectively (Fig. 3). We tested whether KatE activity produced by the engineered KatE+ strain in co-culture could have a beneficial effect on the survival of the wild-type strain after incubation in the presence of H2O2 by measuring the survival of an oxygen-sensitive strain [MG(pIL253), Em+] incubated with either NZ(pSEC:KatE) or NZ(pVE3655). We did not observe a significant increase of the survival of MG(pIL253) in the presence of NZ(pSEC:KatE), suggesting that the production level of catalase is not sufficient to protect another strain in co-culture. L. lactis is also known to accumulate H2O2 during its growth in the presence of O2 (Rezaï et al., 2004). We therefore also compared the long-term survival of NZ(pSEC:KatE) during aerated growth. Since KatE requires exogenous haem for activity, these aerated cultures supplemented with haemin will lead to respiratory metabolism. This metabolic shift results in a good long-term survival, a higher biomass, a weaker acidification and a shift in end-products (Duwat et al., 2001). Thus, these tests were performed with a NZ cydA mutant strain unable to respire. Addition of nisin and haemin had no effect on control strain survival (data not shown). In aerated cultures, the NZ cydA(pSEC:KatE) strain survived around 160-fold better than the NZ cydA(pVE3655) control strain after 3 days in stationary phase (Fig. 4). The slower growth of NZ(pSEC:KatE) may be due to high katE expression (1 ng nisin ml⁻¹ results in high-level induction of the 79 kDa KatE protein). These results show that, compared to the constitutive expression of KatE, we improved the production of the heterologous haem-catalase KatE by fusion to the nisin-inducible promoter and a lactococcal signal peptide (SPUsp45). This expression cassette leads to protection against oxidative stress generated by H2O2 or by aerated growth in L. lactis.
DNA damage generated by oxidative stress is reduced in the KatE-producing L. lactis strain

The involvement of oxidative stress in DNA degradation has been previously described (Rezaïki et al., 2004). In L. lactis, aerated growth provokes DNA degradation in stationary-phase cultures, which is visible for the DNA repair-deficient MG1363 recA mutant (Duwat et al., 1995). As L. lactis KatE+ survives better in oxidative stress conditions, we tested whether KatE activity could maintain DNA integrity in the recA context. Cultures of strain NZ cydAl/recA containing either pVE3655 or pSEC:KatE were grown under nonaerated or aerated conditions, and with or without KatE induction; whole-cell DNA was extracted each 24 h for 7 days. Degradation was evaluated by comparing chromosomal DNA migration in agarose gels (Fig. 5). After 2 days, no significant difference in the patterns of the KatE− and KatE+ strains was observed. After 5 and 7 days of aerations, DNA extracted from the KatE− strain showed greater degradation than DNA from the KatE+ strain. The recA context reveals the involvement of KatE in protection mechanisms of L. lactis against oxidative stress. Efficient catalase activity thus protects cells from oxidative damage and limits DNA degradation in long-term aerated cultures of the recA mutant. This observation could suggest that even in a wild-type strain, KatE could contribute to limiting this DNA degradation.

DISCUSSION

We previously isolated spontaneous oxidative stress mutants of L. lactis to improve their survival in oxidative stress conditions like oxygenated milk or co-cultures with H2O2-producing lactobacilli (Rochat et al., 2005). Here, we evaluated whether the sensitivity of L. lactis to oxygen was due in part to the absence of catalases in this bacterium. Catalase addition is currently used in numerous industrial applications to remove H2O2 from milk and avoid variable lag phase growth of starter cultures due to the presence of oxygen in the medium (Pifferi et al., 1993). It was previously shown that addition of purified catalase to the medium protects L. lactis from oxidative stress (Duwat et al., 1995; Rezaïki et al., 2004) and also that L. lactis is able to produce an active catalase (Gaudu et al., 2003). Thus catalase-producing strains of lactic acid bacteria could be useful tools to improve the reproducibility of industrial fermentation processes. Such strains have already been screened to obtain improved starters for meat fermentation (Mares et al., 1994). In this study, we evaluated the effects of the production of the B. subtilis haem-catalase KatE on the oxidative stress resistance of L. lactis.

We initially introduced the katE gene into L. lactis under control of a constitutive lactococcal promoter. The resulting recombinant KatE+ L. lactis strain possessed catalase activity but no increased H2O2 resistance was measured. Combination of an inducible promoter and fusion with a signal peptide led to production of an active KatE able to confer resistance to oxidative stress generated either by the presence of high levels of H2O2 or by aerated conditions. The quantity of KatE expressed under control of the constitutive promoter is probably too low to confer a resistance phenotype at 2 mM H2O2, and this was confirmed by the catalase activity assay. In the case of KatE expression under P_niaA, the major form detected in the cellular protein fraction corresponds to the precursor form SP_Usp45:KatE (Fig. 2). No mature form was observed in the supernatant fraction by Coomassie staining of SDS-PAGE gels. This was confirmed by the absence of detectable catalase activity in culture supernatants. Interestingly, precursors of naturally secreted proteins are in a partially unfolded conformation which is translocation-competent, and they are generally considered as inactive (Simonen & Palva, 1993). At present, we cannot determine whether the observed KatE activity is attributable to SP_Usp45: KatE, which might be properly conformed into the cytoplasm, or to weak processing of the precursor that would lead to release of active mature forms in the supernatant. The lack of anti-KatE antibodies and our inability to visualize catalase activity on non-denaturing gels (data not shown) hamper the confirmation of this hypothesis via determination of the cellular distribution of KatE.

The significant increase in the survival rate of the KatE-producing L. lactis strain in aerated conditions supports the use of the present strategy for improving antioxidant properties by cloning heterologous catalases. We observed that the presence of KatE protects cells and DNA from oxidative damage, not only upon exposure to H2O2, but also under conditions of aerated growth. The effects of KatE were observed in a DNA repair-defective strain. Prevention of DNA damage could be one of the main reasons for the better survival rate of the KatE-producing L. lactis strain.

This KatE-producing strain might also eliminate H2O2 from the environment and thus could be used as new antioxidant strain to deliver this antioxidant enzyme in vivo at the mucosal level. Mucosal tissue damage and dysfunction in chronic inflammatory bowel diseases or in radio-induced inflammation are partly caused by an exposure to...
excessive amounts of ROS, which can destroy biomolecules (Kruidenier et al., 2003a, b, c). In healthy individuals, the harmful effects of ROS are counteracted in the intestinal mucosa by an extensive system of antioxidants. A previous study established that the production of an active superoxide dismutase by two strains of lactobacilli protected them efficiently against oxidative stress by removing $O_2^-$ ions (Bruno-Barcena et al., 2004). The supply of antioxidant enzymes like catalase, alone or combined with superoxide dismutase, into the intestinal mucosa via ingestion of lactic acid bacteria could eliminate ROS and offer a promising new therapeutic strategy.

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REFERENCES


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