Expression of a Heterologous Manganese Superoxide Dismutase Gene in Intestinal Lactobacilli Provides Protection against Hydrogen Peroxide Toxicity

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In living organisms, exposure to oxygen provokes oxidative stress. A widespread mechanism for protection against oxidative stress is provided by the antioxidant enzymes: superoxide dismutases (SODs) and hydroperoxidases. Generally, these enzymes are not present in *Lactobacillus* spp. In this study, we examined the potential advantages of providing a heterologous SOD to some of the intestinal lactobacilli. Thus, the gene encoding the manganese-containing SOD (*sodA*) was cloned from *Streptococcus thermophilus* AO54 and expressed in four intestinal lactobacilli. A 1.2-kb PCR product containing the *sodA* gene was cloned into the shuttle vector pTRK563, to yield pSodA, which was functionally expressed and complemented an *Escherichia coli* strain deficient in Mn and FeSODs. The plasmid, pSodA, was subsequently introduced and expressed in *Lactobacillus gasseri* NCK334, *Lactobacillus johnsonii* NCK89, *Lactobacillus acidophilus* NCK56, and *Lactobacillus reuteri* NCK932. Molecular and biochemical analyses confirmed the presence of the gene (*sodA*) and the expression of an active gene product (MnSOD) in these strains of lactobacilli. The specific activities of MnSOD were 6.7, 3.8, 5.8, and 60.7 U/mg of protein for *L. gasseri*, *L. johnsonii*, *L. acidophilus*, and *L. reuteri*, respectively. The expression of *S. thermophilus* MnSOD in *L. gasseri* and *L. acidophilus* provided protection against hydrogen peroxide stress. The data show that MnSOD protects cells against hydrogen peroxide by removing O₂^{•−} and preventing the redox cycling of iron. To our best knowledge, this is the first report of a *sodA* from *S. thermophilus* being expressed in other lactic acid bacteria.

Lactic acid bacteria (LAB) constitute a commercially important group of microorganisms extensively utilized in the production of fermented foods. The importance of LAB in human health is becoming more popular, since they are considered as beneficial microorganisms and are used as dietary adjuncts. Studies in the area of probiotics have shown that several strains of LAB can help alleviate gastrointestinal disorders (11, 48, 53). Furthermore, LAB may be used as a vehicle for the delivery of therapeutic agents (pharmaceuticals or nutraceuticals) into the intestinal tract of humans and animals (31).

LAB are typically regarded as aerotolerant anaerobes (5); they can grow in the presence of oxygen (13, 19, 29, 34, 54) and generate partially reduced reactive oxygen species (ROS). ROS include the superoxide radical (O₂^{•−}), hydrogen peroxide (H₂O₂), and the hydroxyl radical (HO[•]). ROS have been demonstrated to be both cytotoxic (25, 26) and mutagenic (16, 42). To offset the harmful effects of ROS, cells have evolved protective mechanisms that utilize antioxidant enzymes such as superoxide dismutases (SODs) and hydroperoxidases, which scavenge superoxide radicals and hydrogen peroxide, respec-

tively, and thus prevent the formation of HO[•] via the Fenton chemistry (17).

Superoxide dismutases (SODs) (EC 1.15.1.1) are metalloenzymes that catalyze the conversion of the superoxide anion into hydrogen peroxide and dioxygen (38). There are three forms of the enzyme that are distinguished by their metal center: manganese, copper-zinc, or iron (25). These enzymes are found across a broad range of organisms, which can use one, two, or all three enzymes to meet their antioxidant needs (25). For example, *Escherichia coli* possesses all three isoforms (7, 32, 60). In most *Streptococcus* and *Lactococcus* spp., elimination of ROS conform to this general antioxidant defense system since they both possess MnSOD (46, 52). Previously, our group has identified, characterized, and cloned the gene (*sodA*) encoding the manganese-containing SOD from *Streptococcus thermophilus* AO54 (1, 12). Unlike most *sodA* genes, *S. thermophilus* *sodA* is expressed under both anaerobic and aerobic conditions and is not induced by the redox cycling compound, paraquat (12). This antioxidant enzyme was shown to be essential for the growth of *S. thermophilus* under aerobic conditions (1). However, most lactobacilli lack this general defense system (SODs). *Lactobacillus plantarum* developed an alternative nonenzymatic defense system that involves the accumulation of high intracellular concentrations of manganese ions, which can scavenge O₂^{•−} (2).

The lack of endogenous SODs (49) and catalase may account for the high sensitivity of most species of *Lactobacillus* to oxidative stress. In this study, we report the expression of the *sodA* from *S. thermophilus* in four lactobacilli: *L. gasseri*

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TABLE 1. Bacterial strains, plasmids, and phage used in this study^a

Strain, plasmid, or phage	Relevant characteristic(s)	Source and/or reference
Strains		
<i>Escherichia coli</i>		
DH5 α	rec cloning strain	Stratagene
QC774	F ⁻ Δ lacU169 (<i>sodA-lacZ</i>)49 Lac ⁺ RpsL ⁻ (<i>sodB-Kan</i>)1- Δ_2 Cm ^r Km ^r	56
BL21 (DE3)pLysS	Protein expression strain	Novagen
MC1061	λ^- F ⁻ Δ (<i>araA-leu</i>)7697 <i>araD139</i> Δ (<i>codB-lac</i>)3= Δ lac74 <i>galK16 galE15 mcrA0 relA1 rpsL150 spoT1 mcrB9999 hsdR2</i> (Str ^r)	Stratagene (10)
MCKO	As MC1061, but (<i>sodA-lacZ</i>)49 Lac ⁺ (<i>sodB-Kan</i>)1- Δ_2 Cm ^r Km ^r	This study
<i>Streptococcus thermophilus</i> AO54	Wild-type industrial strain	40
<i>Lactobacillus gasseri</i>		
NCK 334	Type strain, human isolate ATCC 33323	T. Klaenhammer (collection stock of NC)
NC1500	NCK334 harboring pTRK563	This study
NC1501	NCK334 harboring pSodA	This study
<i>Lactobacillus johnsonii</i>		
NCK 89	Derivate of NCK88 VPI 11088 ATCC 11506 Lac ⁻ <i>laf 1 str-6 rif-7</i> (pPM4) (pPM27)	43
NC1510	NCK89 harboring pTRK563	This study
NC1511	NCK89 harboring pSodA	This study
<i>Lactobacillus acidophilus</i>		
NCK 56	Human intestinal isolate, NCFM Rhodia, Madison, Wis.	T. Klaenhammer (collection stock of NC)
NC1520	NCFM harboring pTRK563	This study
NC1521	NCFM harboring pSodA	This study
<i>Lactobacillus reuteri</i>		
NCK 932	Type strain, human intestinal isolate DSM20016, ATCC 23272	T. Klaenhammer (collection stock of NC)
NC1530	NCK932 harboring pTRK563	This study
NC1531	NCK932 harboring pSodA	This study
Plasmids		
pET16b		Novagen
pGEM-T Easy		Promega
pTRK563	Em ^r ; Δ cat derivative of pGK12 with <i>lacZ</i> from pBluescript II KS(+)	50
pSodX-1	1.2-kb <i>sodA</i> PCR amplicon from <i>S. thermophilus</i> cloned into pGEM-T Easy	This study
pSodA	1.2-kb <i>sodA</i> fragment from pSODX-1 cloned into pTRK563	This study
pETSodA	0.63-kb <i>sodA</i> PCR amplicon from <i>S. thermophilus</i> cloned into pET16b	This study
Phage		
P1		Collection stock of NC

^a NC and NCK, culture collection at North Carolina State University, Raleigh; ATCC, American Type Culture Collection; VPI, Virginia Polytechnic Institute; DSM, Deutsche Sammlung von Mikroorganismen.

NCK334, *L. johnsonii* NCK89, *L. acidophilus* NCK56, and *L. reuteri* NCK932. Under the conditions used in this study, the enzyme provided a clear protection against the inhibitory effect of H₂O₂ on the growth of *L. gasseri* and *L. acidophilus*.

MATERIALS AND METHODS

Bacterial strains and media. The bacterial strains, phage, and plasmids used in this study are listed in Table 1. *E. coli* strains were grown either at 37°C in Luria-Bertani medium or M-9 minimal medium (51) supplemented with the appropriate antibiotics. The antibiotics used were chloramphenicol (20 μ g/ml), kanamycin (50 μ g/ml), tetracycline (20 μ g/ml), and erythromycin (200 μ g/ml). *S. thermophilus* AO54 (40) was grown at 42°C in M17 (55) medium supplemented with 0.5% glucose (M17G). *Lactobacillus* spp. were grown at 37°C in MRS (14) or APT broth (15). Solid media for plating were prepared by adding 1.5% agar to the appropriate liquid media. When required, erythromycin (2 or 0.5 μ g/ml) was added to LAB cultures. For anaerobic growth, cells were grown in a Coy anaerobic chamber (Coy Laboratory Products, Ann Arbor, Mich.).

Sources of chemicals and enzymes. Cytochrome *c*³⁺, xanthine, xanthine oxidase, riboflavin, nitroblue tetrazolium, lysozyme, proteinase K, phenol, chloroform, 2, 2'-dipyridyl (DIP), methyl viologen (paraquat), and all antibiotics used

were purchased from Sigma (St. Louis, Mo.). All other chemicals as well as bacteriological media were purchased from Fisher Scientific (Pittsburgh, Pa.). Restriction enzymes, DNA ligase, *Taq* polymerase, and Klenow fragment were purchased from Promega (Madison, Wis.), New England BioLabs (Beverly, Mass.), Qiagen (Valencia, Calif.), or Roche (Indianapolis, Ind.). All radiochemicals were purchased from ICN (Irvine, Calif.).

P1 phage transduction. P1 phage transduction was carried out as previously described (41).

DNA isolation and manipulation. Isolation of plasmid DNA from *E. coli* was performed by using the QIAGEN Mini Spin isolation kit (Qiagen) as per the supplier's recommendation. Chromosomal DNA isolation was carried out as previously described (4). Restriction enzymes, T4 ligase, and other DNA-modifying enzymes were used as per the instructions of the respective suppliers. DNA isolation from agarose gels was carried out with either GeneCleanII (Bio101, La Jolla, Calif.) or a Qiagen QiaQuick isolation kit, as per the respective supplier's recommendations.

Southern blot hybridization and DNA probes. DNA probes were made by random hexamer nucleotide labeling (4). Unincorporated nucleotides were removed by using a ProbeQuant G-50 Micro Column (Amersham Pharmacia Biotech, Inc., Piscataway, N.J.). Prior to use, probes were denatured by heating at 95°C for 10 min and quickly chilled on ice for 5 min. Southern blot analysis was

carried out according to Sambrook et al. (51). The blot was then probed with a DNA probe labeled with either ^{32}P or digoxigenin (DIG) (Roche) and detected by using the respective protocols (51).

PCR. Standard PCR was carried out with QIAGEN *Taq* polymerase (QIAGEN) as per the manufacturer's instructions. Primers STSODF (5'-GAGAGGACAGATTCAAGATG-3') and STSODR (5'-GTTTGGCGGCTCC-3') (Integrated DNA Technologies, Coralville, Iowa) were used to amplify a 1.2-kb DNA fragment containing the structural gene of *sodA* from *S. thermophilus* and flanking sequences, in particular the upstream region of the gene, to include any promoter elements. Primers KOF (5'-GGAATTCCTTCCTTACGCTTACGA TGTTGG-3') and KOR (5'-GGAATTCCTTCAGCAACTTATTTC-3') (Integrated DNA Technologies) were used to amplify an approximately 500-bp DNA fragment consisting of the internal region of the *S. thermophilus sodA*, which was used as the probe in the Southern hybridization analysis. In both instances, chromosomal DNA isolated from *S. thermophilus* served as the template. All PCRs were carried out with a Perkin-Elmer Biosystems GeneAmp 2400 PCR system (Boston, Mass.).

Bacterial transformations. *E. coli* strains were transformed via electroporation using a Bio-Rad Gene Pulser (Bio-Rad, Richmond, Calif.) according to the manufacturer's instructions. The strains of *Lactobacillus* were transformed as previously described (58).

Preparation of dialyzed CFEs. Cultures of *S. thermophilus*, *E. coli*, and *Lactobacillus* spp. containing pSodA were grown aerobically with shaking at 200 rpm at 37°C. The cultures were harvested in the exponential growth phase (optical density at 600 nm [OD₆₀₀] of 0.2 to 0.4) by centrifugation at 5,000 × g 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, and resuspended in the same buffer at 1/40 the original volume. The cells were disrupted by bead beating the cell suspension with a MiniBeadbeater-8 (Biospec Products, Bartlesville, Okla.) with six 1-min intervals for a total of 6 min. Overheating was prevented by placing the tubes on ice for 3 min between treatments. Cellular debris was removed by centrifugation at 14,000 × g at 4°C for 30 min. The supernatants (cell-free extracts [CFEs]) were dialyzed at 4°C for 24 h against four changes of the phosphate-EDTA buffer.

Biochemical assays. Dialyzed cell extracts were assayed for protein concentration by the Lowry method (36), using bovine serum albumin as the standard. The proteins were separated by electrophoresis on a 10% nondenaturing polyacrylamide gel and stained for SOD activity using nitroblue tetrazolium (6). Specific activity of SOD in the cell extracts was assayed by using the cytochrome *c*³⁺ method (38).

Cloning of *S. thermophilus sodA* in pET16b and its expression in *E. coli*. The pET16b bacterial expression vector, based on the T7 promoter-driven system and containing a His tag, was purchased from Novagen (Madison, Wis.). Promoterless *sodA* was amplified by PCR from *S. thermophilus* chromosomal DNA, using primers containing the NdeI and BamHI restriction sites. The primers start-SODF 5'-GGACCTTTCATATGGCTATTATCC-3' and stopSODR 5'-TCAA GACTGAGGATCCTTCTAGAC-3' were used. The PCR product was digested and directionally cloned into an NdeI-BamHI site of pET16b. The plasmid containing the insert (pETsodA) was identified and used for transformation of competent BL21 (DE3)pLysS cells (Novagen).

Affinity purification of *S. thermophilus* MnSOD from *E. coli* harboring pETsodA. *E. coli* BL21 (DE3)pLysS/pETsodA was grown at 37°C at 200 rpm in LB medium supplemented with 1 mM MnCl₂. Isopropyl-β-D-thiogalactopyranoside (IPTG) (5 mM) was added at an OD₆₀₀ of 1.0, and growth was continued for another 3 h to allow for the maximum expression of the His-tagged fusion protein (His-tag SodA). The fusion protein was further purified by published procedures (Qiagen) using His-tagged Ni-nitrilotriacetic acid resin for chromatography. The purified SodA protein was lyophilized and stored at -80°C.

Preparation of antibodies against *S. thermophilus* MnSOD and Western blot hybridization. Antiserum containing polyclonal antibodies against the purified SodA protein (anti-SodA) was prepared by standard rabbit immunization protocols (Scantibodies, Inc., Ramona, Calif.). For the detection of SodA in cell extracts, samples were subjected to electrophoresis on 4 to 15% gradient SDS-polyacrylamide minigels before electrotransfer to nitrocellulose membranes or were dot blotted onto Hybond-ECL nitrocellulose membranes (Amersham Bioscience, Piscataway, N.J.). Membranes were blocked with 5% (wt/vol) nonfat milk in TBS-T (Tris-buffered saline containing 0.1% [vol/vol] Tween 20) for 2 h at room temperature and subsequently incubated for another 2 h with the specific primary antibodies (anti-SodA) diluted 1:5,000. The membranes were washed three times with TBS-T and incubated for 2 h at room temperature with the secondary antibodies (goat anti-rabbit immunoglobulin G [IgG]) conjugated with horseradish peroxidase (Sigma) diluted 1:10,000. Signal detection was realized

with SuperSignal reagents (Pierce, Rockford, Ill.) followed by exposure to X-ray film.

RTQ-PCR. One-step real-time quantitative PCR (RTQ-PCR) was used for the detection and quantification of 16S rRNA and *sodA* mRNA in *S. thermophilus* and all species of *Lactobacillus* used in this study. The ribosomal DNA (rDNA) primers used included RLact16SF (5'-GTAGGGAATCTTCCACAATG-3'), RLact16SR (5'-TAGTTAGCCGTGACTTTCTG-3'), and *sodA* primers KOF (as above) and RTSodAR (5'-GCAACTTACGTGGCGAATG-3') (Integrated DNA Technologies). The 16S rRNA primers amplified 157-bp products with a melting temperature (*T*_m) ranging from 83 to 86°C as a function of the species analyzed. The *sodA* primers amplified a 232-bp product with a *T*_m of 84°C consisting of the internal region of the *S. thermophilus sodA*.

Total RNA from cultures in the log phase of growth were extracted with the RNeasy Mini kit and checked for quality by electrophoresis on 1.2% agarose gel. DNase treatment and SYBR Green I RTQ-PCR were carried out as described by the manufacturer (Qiagen). In brief, RTQ-PCR amplification mixtures (20 μl) contained a 50-ng template of total mRNA, 2× SYBR Green I Master Mix buffer (10 μl) (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 of polymerase activation at 95°C, and 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 duplicate) a standard curve of four serial dilution points of *sodA* DNA (ranging from 50 ng to 50 pg), a no-template control, and a template control without reverse transcriptase added. PCR efficiencies were obtained by using Sequence Detection iCycler iQ optical system software (version 3.0 a) (Bio-Rad), and the values always were above 95%. 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 as the absolute copy number of mRNA of each molecule.

Effect of hydrogen peroxide on growth. The maximum specific growth rate (μ_{max}) per hour of each strain was determined in media containing different concentrations of hydrogen peroxide by monitoring changes in OD₆₀₀ as a function of time.

Changes in OD₆₀₀ as function of time. Overnight (16 h) cultures (harboring either pTRK563 or pSodA) growing in APT medium containing erythromycin (2 μg/ml) were used to prepare cells in the exponential phase (OD₆₀₀ = 0.2 to 0.4). Cells from the overnight cultures or from the exponential-phase cultures were harvested via centrifugation, washed in sterile medium, and resuspended in APT medium containing erythromycin (0.5 μg/ml) and used as the inocula. Standardized inocula were used to inoculate fresh APT (200 μl) containing erythromycin (0.5 μg/ml) plus different concentrations of hydrogen peroxide, placed in 100-well microtiter plates, to an initial OD₆₀₀ of ca. 0.1. The cultures in the microtiter plates were incubated at 37°C with continuous shaking, and growth was automatically monitored by measuring the changes in OD₆₀₀ as a function of time using a Bioscreen-C microbiological analyzer (Labsystems, Frankfurt, Germany). For each culture, the μ_{max} per hour was calculated from the slope by fitting to a linear regression of the exponential-growth-phase data with a correlation coefficients, *r*², of 0.99. Each point represents the mean of five independent assays.

Effect of DIP on the toxicity of H₂O₂. The effect of different concentrations of DIP on the μ_{max} (per hour) of *L. gasseri* in the presence of 0, 1, and 2.5 M H₂O₂ was tested by using a Bioscreen-C microbiological analyzer, as outlined above.

RESULTS

In order to assess the expression of *sodA* from genetic constructs, an *E. coli* host deficient in *sodA* and *sodB* was required. We transferred the *sodA sodB* mutation from *E. coli* QC774 (56) into MC1061 (RecA⁺) via P1 phage transduction. The new strain was named MCKO. Transductants [MCKO (Δ *sodA sodB*)] were verified for the lack of *sodA sodB* products (i.e., MnSOD and FeSOD, respectively), where no SOD activity was detected. The levels of SOD specific activity for MC1061 (wild type) and MCKO(pSodA) were 24.2 ± 1.87 and 42.5 ± 2.25 U/mg of protein, respectively (mean ± standard deviation for results from three independent experiments).

Construction of pSodA. The cloning strategy of pSodA is shown in Fig. 1. A 1.2-kb fragment containing the *sodA* from *S. thermophilus* AO54 (1) was amplified with primers STSODF

FIG. 1. Construction of pSodA plasmid. pSodX-1 containing the *sodA* gene and flanking sequence from *S. thermophilus* in pGEM-T Easy cloning vector (Promega) was digested with EcoRI. The 1.2-kb fragment was gel purified and ligated into the EcoRI-digested shuttle vector, pTRK563. The new plasmid construct was termed pSodA.

and STSODR and cloned into the PCR cloning vector pGEMT-Easy (Promega), creating the construct pSodX-1. This construct was digested with EcoRI to reveal the expected 1.2-kb fragment encoding the *sodA*. The isolated *sodA* fragment was then cloned into the pTRK563 shuttle vector (50). The newly formed plasmid, pSodA, was transformed into the *E. coli* MCKO strain. Positive transformants were recovered from M-9 selective minimal medium containing 10^{-6} M paraquat. To confirm that the *sodA* was correctly expressed in the Sod-deficient strain of *E. coli*, SOD activity gel (not shown) and assays for SOD in the cell extracts were used. The specific activity of *S. thermophilus* MnSOD in cell extracts from *E. coli* MCKO containing pSodA was 42.5 ± 2.25 U/mg of protein. The presence of pSodA was confirmed by plasmid extraction and digestion, Southern blot hybridization (data not shown), and sequencing. These data indicated that the 1.2-kb fragment containing the MnSOD gene from *S. thermophilus* AO54 was functionally expressed in *E. coli* MCKO.

Transformation of four *Lactobacillus* species with pSodA and activity of MnSOD in the transformants. The plasmids pSodA and pTRK563 vector (i.e., without *sodA*) were transformed into four *Lactobacillus* spp. via electroporation. The

presence of the pSodA plasmid was verified by PCR. Only the strains containing pSodA amplified the expected 1.2-kb fragment representing the *sodA* of *S. thermophilus* AO54 (Fig. 2), and nondenaturing polyacrylamide gel electrophoresis (PAGE) SOD activity gels of the positive clones revealed the presence of an active MnSOD that migrated to the same position as that of *S. thermophilus* AO54 (data not shown). The specific activities of MnSOD in CFEs prepared from the transformants are presented in Fig. 3A. These results indicate that the *sodA* from *S. thermophilus* is functionally expressed in the four species of *Lactobacillus* tested.

CFEs from the transformants (up to a maximum of 100 μ g of total protein) were subjected to electrophoresis on 4 to 15% gradient sodium dodecyl sulfate (SDS)-polyacrylamide minigels, electrotransferred to nitrocellulose membranes, and subjected to Western analysis with SodA antibodies. No cross-reacting material was detected in the controls (i.e., cells transformed with pTRK563) even after extended exposure times (~16 h), whereas a single band was detected when CFEs from cells transformed with pSodA were used. Because these data indicated absence of cross-reacting material or degraded SodA protein, the samples were subjected to dot blotting to avoid electrotransfer of artifacts from SDS-PAGE to the nitrocellulose membranes. The results revealed the presence of different levels of SodA protein that closely correlated with the level of *sodA* transcripts from the different strains (Fig. 3B and C), while the specific activity of MnSOD in the transformants did not correlate with the concentration of SodA protein observed (Fig. 3A and B).

Effect of *S. thermophilus* *sodA* product on the $\mu_{\max}$ (per hour) of *Lactobacillus* spp. The effect of *sodA* expression on the $\mu_{\max}$ of the different strains was examined to determine its impact on the host strains. Standardized inocula from stationary- and exponential-phase cultures of *Lactobacillus* harboring either pTRK563 or pSodA were grown aerobically in APT broth. The effects of the expression of SodA on $\mu_{\max}$ per hour in the different strains are shown in Table 2. The data showed

FIG. 2. PCR confirmation of *sodA* in the transformants. *Lactobacillus* spp. were grown at 37°C in MRS medium supplemented with 2% glucose. *S. thermophilus* AO54 was grown at 42°C in M17 medium supplemented with 0.5% glucose. Primers STSODF and STSODR and cells from presumptive transformants or *S. thermophilus* were used for PCRs. Lanes: 1, 1-kb ladder; 2, pSodA; 3, negative control; 4, *L. gasseri* NC1501; 5, *L. johnsonii* NC1511; 6, *L. acidophilus* NC1521; 7, *L. reuteri* NC1531; 8, *S. thermophilus* AO54.

FIG. 3. Transcription/translation of *sodA* and MnSOD activity in the recombinant *Lactobacillus* spp. (A) *Lactobacillus* spp. and SOD activity (units per milligram of protein). (B) Western blots (dot blots) showing the levels of SodA protein in 15 µg of protein of CFEs from *S. thermophilus* AO54 and *Lactobacillus* harboring pSodA. (C) Results from RTQ-PCR showing the levels of *sodA* mRNA and 16S mRNA and their ratios for the recombinant *Lactobacillus* spp. and *S. thermophilus* AO54.

that the expression of SodA caused a 15 to 28% reduction in the $\mu_{\max}$ of *L. gasseri* and *L. johnsonii*, but had no significant effect on the growth rates of *L. acidophilus* and *L. reuteri*. Strains harboring pSodA grew marginally more slowly than strains harboring the vector only (pTRK563), indicating that the expression of the gene encoding the MnSOD was most likely responsible for the slight reduction in the growth rate.

Sensitivity of the *Lactobacillus* strains harboring pSodA to oxidative stress. We exposed all of the strains and their wild types to extreme concentrations of paraquat (up to 0.5 M): no growth inhibition was seen, suggesting either the lack of transport into the cells or lack of enzymes needed for the redox cycling of paraquat. Previous studies have demonstrated that paraquat and quinones are transported into *E. coli* by an active transport system, and mutants deficient in this transport system are resistant to these redox recycling compounds (30). Studies of gram-positive bacteria suggested that paraquat and quinones may not be actively transported. Thus, *S. thermophilus* was shown to be resistant to paraquat and to other redox cycling compounds (1, 11), and in *L. fermentum*, paraquat can be internalized only when the membrane is modified (57). We

concluded, therefore, that we cannot use paraquat or quinones as superoxide generators (i.e., oxidative stress agents) in the organisms used in this study.

Previous studies demonstrated that *E. coli* cells deficient in both the manganese- and iron-containing SODs are hypersensitive to hydrogen peroxide (H_2O_2) when grown in minimal medium (9). In order to examine the ability of the cloned SOD to protect against H_2O_2 , we examined the sensitivity of the parental strains to this oxidant. We found that *L. gasseri* and *L. acidophilus* were highly sensitive to H_2O_2 (Fig. 4, broken lines), while *L. johnsonii* and *L. reuteri* were resistant to hydrogen peroxide (data not shown). Therefore, we reasoned that the expression of *sodA* in *L. gasseri* and *L. acidophilus* may provide protection against H_2O_2 . To test this hypothesis, the effects of different concentrations of H_2O_2 on the $\mu_{\max}$ (per hour) of the test organisms containing either the pSodA or the vector (pTRK563) were analyzed. APT broth was used because it is devoid of heme-containing compounds that could potentially react with and inactivate the added hydrogen peroxide. The data (Fig. 4) showed that the expression of *S. thermophilus* *sodA* provided protection against H_2O_2 in *L. gasseri* and *L. acidophilus*.

Effect of DIP on the toxicity of H_2O_2 . *L. gasseri* was used to test the possible involvement of cellular free iron in the toxicity of H_2O_2 . The iron-chelator DIP was able to reverse the inhibitory effect of H_2O_2 on $\mu_{\max}$ per hour in a concentration-dependent fashion (Fig. 5).

DISCUSSION

The SOD gene (*sodA*) from *S. thermophilus* AO54 was cloned in a shuttle vector and successfully expressed in four species of *Lactobacillus*, and its impact on the physiology of the host organism was examined. The expression of *sodA* in the

TABLE 2. Effect of expression of SodA on the $\mu_{\max}$ of different *Lactobacillus* spp.^a

Species	$\mu_{\max}$ h ⁻¹	
	pTRK563	pSodA
<i>L. gasseri</i>	0.82 ± 0.03	0.71 ± 0.05
<i>L. johnsonii</i>	0.52 ± 0.01	0.44 ± 0.001
<i>L. acidophilus</i>	0.40 ± 0.04	0.47 ± 0.02
<i>L. reuteri</i>	0.93 ± 0.02	0.96 ± 0.03

^a Logarithmic cells from cultures of the different species of lactobacilli with pSodA or pTRK563 were grown aerobically in APT broth at 37°C. Results are based on five independent experiments and are expressed as means ± standard deviations.

FIG. 4. Sensitivity to hydrogen peroxide. Exponentially growing cells from *L. gasseri* and *L. acidophilus* harboring pSodA (continuous lines, solid symbols) or pTRK563 (dotted lines, open symbols) were tested for growth in the presence of increasing concentrations of hydrogen peroxide. The entire procedure was carried out in APT broth under aerobic conditions at 37°C as described in Materials and Methods.

different lactobacilli slightly decreased the μ_{max} (i.e., increased the doubling time) of *L. gasseri* (28%) and *L. johnsonii* (15%) as compared to controls with the empty plasmid pTRK563 (Table 2). This is most likely due to the energetic and metabolic cost required for expressing the foreign protein.

What is special about *sodA* from *S. thermophilus* AO54? Previous studies (49) attempted to express the *sodA* from *E. coli* in a variety of LAB. They successfully cloned and expressed the *sodA* from *E. coli* in *Lactococcus lactis* and *L. gasseri* at low levels, but they were unable to express the gene in other species. In this study, however, we were successful in expressing *sodA* from *S. thermophilus* AO54 in four different lactobacilli at significantly high levels of activity. The *sodA* from this organism has proven to be more suited for expression in other LAB because *S. thermophilus* is a gram-positive, non-spore-forming organism and has a low G+C content that is more close to the *Lactobacillus* group than is *E. coli*. Furthermore, the *sodA* of *S. thermophilus* is constitutively expressed (12) and does not appear to have a complex promoter region (1). This appears to promote recognition by the *Lactobacillus* transcription and translation machinery and allows constitutive production of the enzyme in the different hosts.

Transcription and translation of *sodA* and posttranslation activation of SodA. The ratios of *sodA* to 16S mRNAs were different among the individual species of *Lactobacillus* studied

(Fig. 3C), which may be due to differences in the plasmid copy number, levels of expression, or stability of *sodA* mRNAs. However, there was a positive correlation between the level of *sodA* mRNA and the amount of SodA protein in each strain, except for *L. johnsonii* (Fig. 3B and C). The exact reason for this discrepancy is not clear at the present time, but it could be due to the greater instability of *sodA* mRNA or the higher efficiency of its translation in *L. johnsonii*. Finally, the levels of active MnSOD did not correlate with the levels of SodA protein among the different *Lactobacillus* species (Fig. 3A and B). The data showed that *S. thermophilus* and *L. reuteri* were most efficient at producing active MnSOD relative to the total SodA present in the cells. The posttranslation modification of SodA to MnSOD requires the availability of sufficient manganese ions inside the cells. Therefore, we hypothesize that strains with a high capacity to accumulate manganese (3, 21) or with a high rate of active manganese uptake would be able to produce more active MnSOD than strains lacking these properties. In accordance with this, preliminary experiments showed that *L. plantarum* and *Leuconostoc mesenteroides*, known to possess putative Nramp (natural resistance-associated macrophage protein) superfamily transporters (GenBank accession no. AF416710 and ZP_00063650), expressed high levels of active MnSOD when transformed with pSodA (data not shown). Also, the genomes of *S. agalactiae*, a species related to *S. thermophilus*, and *L. reuteri* contain a Mn(II)/Fe(II) transporter (GenBank accession no. AE014283) and a putative proton-dependent manganese transporter (GenBank accession no. AY267207), respectively. Both transporters belong to the MntH or Nramp superfamily. On the other hand, *L. gasseri* (GenBank accession no. ZP_00046560) and the closely related species *L. acidophilus* (E. Altermann and T. Klaenhammer, personal communication) and *L. johnsonii* (47) possess a putative cadmium/manganese-transporting P-type ATPase homologous to MntA of *L. plantarum* (24), which belongs to the MntP manganese transport group. Thus, the fundamental differences in the systems of manganese acquisition and their

FIG. 5. Protection against hydrogen peroxide toxicity by DIP. Exponentially growing cells of *L. gasseri* were tested for growth in the presence of increasing concentrations of H_2O_2 and DIP. The entire procedure was carried out in APT broth under aerobic conditions at 37°C as described in Materials and Methods.

FIG. 6. A schematic presentation showing how SODs, hydroperoxidases, iron chelators, iron-binding proteins, protectors of [Fe-S] clusters, and/or DNA and cellular repair mechanisms could protect against H_2O_2 toxicity. Reaction 1 shows the oxidation of labile iron-sulfur clusters by O_2^- , reaction 2 shows the generation of $\text{HO}^\cdot$ by Fenton chemistry, reaction 3 shows the regeneration of Fe(II) from Fe(III) by O_2^- (the sum of reactions 2 and 3 is also known as the Haber-Weiss reaction), and reaction 4 shows the deleterious effects of $\text{HO}^\cdot$ and the generation of damaged DNA and damaged cellular components. Protective molecules/mechanisms are shown in boxes: SODs inhibit reactions 1 and 3, hydroperoxidases inhibit reaction 2, iron-binding proteins (Dpr and Dps) and iron chelators (DIP) inhibit reaction 2, protectors of [Fe-S] clusters (YggX and FeSII) inhibit reaction 1, and DNA repair and other cellular repair mechanisms repair the damage caused by reaction 4.

possible growth phase and/or gene regulation may explain the difference in the values of active MnSOD observed (Fig. 3).

MnSOD and protection against hydrogen peroxide. Data in Fig. 4 showed that the expression of *S. thermophilus* MnSOD in *L. gasseri* and *L. acidophilus* provided protection against H_2O_2 . Figure 6 explains how SOD could provide such protection even though H_2O_2 is not a substrate for this enzyme. It is known that the deleterious effects of H_2O_2 on cell growth and survival are largely dependent on the availability of "free" soluble iron [Fe(II)]. Free iron could be released from labile iron-sulfur $[\text{4Fe-4S}]^{2+}$ clusters by the action of superoxide radicals (Fig. 6, reaction 1) (18, 33) or could come from other intracellular sources. Ferrous iron reacts with H_2O_2 to catalyze the production of the highly reactive hydroxyl radical ($\text{HO}^\cdot$) via the Fenton chemistry (17) (Fig. 6, reaction 2). The involvement of Fe(II) in the toxicity of H_2O_2 in *L. gasseri* and *L. acidophilus* is supported by the protective effect of DIP (Fig. 5). The continuous generation of $\text{HO}^\cdot$ requires a continuous supply of Fe(II), which can be provided by reaction 1 or via the reduction of Fe(III) by O_2^- (reaction 3, Fig. 6). The sum of reactions 2 and 3 is also known as the Haber-Weiss reaction (22, 37). The reduction of Fe(III) could also be accomplished by other intracellular reductants: e.g., glutathione, thioredoxin, flavoenzymes, NAD(P)H-dependent enzymes, etc. (23, 28, 45). Figure 6 shows that the cells have redundant mechanisms for protection against the toxicity of H_2O_2 . Thus, the protection against H_2O_2 and against the generation of $\text{HO}^\cdot$ can be accomplished by (i) elimination of O_2^- by SODs to inhibit reactions 1 and 3; (ii) elimination of H_2O_2 by hydroperoxidases to inhibit reaction 2; (iii) chelation of free iron by iron-chelator or by specific DNA-binding proteins (e.g., Dpr and Dps in gram-positive and gram-negative organisms, respectively) (27, 59) to inhibit reaction 2; (iv) use of specific [Fe-S] cluster protectors—e.g.,

YggX in *Salmonella enterica* (20) and the Shetna protein (FeSII) in *Azotobacter vinelandii* (35) to inhibit reaction 1; or (v) the repair of damaged DNA and other macromolecules (8). Data in Fig. 4 clearly showed that SOD provided significant protection against H_2O_2 in *L. gasseri* and *L. acidophilus* by presumably interfering with reactions 1 and 3 (Fig. 6). Previous studies have shown that *E. coli* cells deficient in *sodA* and *sodB* are more sensitive to hydrogen peroxide (9). Also, recent studies have shown that the endogenous SOD levels control the iron-dependent $\text{HO}^\cdot$ formation in *E. coli* cells exposed to hydrogen peroxide (39). Our data and those of others clearly demonstrate the benefits of expressing SODs in order for the cells to withstand the challenges of oxidative stress. Since most of the *Lactobacillus* spp. lack endogenous SODs and hydroperoxidases, it is tenable that strains expressing a cloned SOD would be more resistant to hydrogen peroxide and oxidative stress.

Conclusion. This research has shown the expression of the *S. thermophilus* *sodA* into different species of *Lactobacillus*, demonstrating that SOD can increase tolerance to oxidative stress in *L. acidophilus* and *L. gasseri*. The physiological advantages of the cloned SOD in *L. reuteri* and *L. johnsonii* remain to be determined, but further work is needed to define the appropriate test parameters. Nevertheless, strains of *Lactobacillus* producing heterologous MnSOD may offer some benefits to the intestinal tract of the host if provided as probiotics. Oral administration of probiotics has clear effects on the numbers and activities of intestinal and fecal bacteria (11, 44, 53). It may be possible to use these SOD-rich species in biotherapy (i.e., as vehicles for the delivery of MnSOD in the gastrointestinal tracts of humans and farm animals). Delivery of MnSOD-rich cells may provide a substantial amount of this antioxidant enzyme in vivo to the gastrointestinal tract for the treatment of

peptic ulcers or ulcerative colitis. Future work will involve examining the possible added benefit that these organisms could provide for the treatment of gastrointestinal disorders, as well as the prospect of enhancing the probiotic properties of *Lactobacillus*. Work is currently under way to integrate and express the *sodA* from *S. thermophilus* AO54 into the chromosome of these probiotic organisms.

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