JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

ARTICLE

pubs.acs.org/JAFC

Coexpression of the Superoxide Dismutase and the Catalase Provides Remarkable Oxidative Stress Resistance in *Lactobacillus rhamnosus*

Haoran An, Zhengyuan Zhai, Sheng Yin, Yunbo Luo, Beizhong Han, and Yanling Hao*

Key Laboratory of Functional Dairy of Ministry of Education of the People's Republic of China & Municipal Government of Beijing, College of Food Science & Nutritional Engineering, China Agricultural University, 17 Qing Hua East Road, Hai Dian District, Beijing 100083, China

ABSTRACT: Lactic acid bacteria (LAB) are generally sensitive to oxidative stress caused by reactive oxygen species (ROS). Antioxidant enzymes, especially superoxide dismutase (SOD) and catalase (CAT), can protect against ROS by eliminating superoxide and H_2O_2, respectively. *Lactobacillus rhamnosus* is a valuable probiotic starter culture but is deficient in both SOD and CAT, and is thus likely to suffer from oxidative stress in industrial fermentation. To confer high level of oxidative resistance on *L. rhamnosus*, the SOD gene *sodA* from *Streptococcus thermophilus* and CAT gene *katA* from *L. sakei* were coexpressed in *L. rhamnosus* AS 1.2466. The enzyme activities of SOD and CAT were 147.80 ± 1.08 U/mg protein and 2.53 μmol of H_2O_2 /min/10^8 cfu, respectively, in the recombinant *L. rhamnosus* CS. After incubation with 10 mM H_2O_2, the survival ratio of *L. rhamnosus* CS was 400-fold higher than that of *L. rhamnosus* CAT. In long-term aerated conditions, viable cells of *L. rhamnosus* CS remained ~10^6 cfu/mL after incubation for 7 days, while no living cells of the control were detected. These results showed that the cooperation between SOD and CAT could significantly enhance oxidative resistance in *L. rhamnosus*. To our best knowledge, this is the first report of two synergistic antioxidant genes being coexpressed in the same *Lactobacilli*.

KEYWORDS: Superoxide dismutase, catalase, coexpression, *Lactobacillus rhamnosus*, oxidative stress

INTRODUCTION

Lactic acid bacteria (LAB) are widely used in a large variety of food fermentations including dairy, meat, and vegetable products. Their main functions are to produce acid, which lowers the pH and prevents the growth of spoilage bacteria.[1] Meanwhile, their products, such as lactic acid, acetic acid, ethanol, and exopolysaccharide, are beneficial to the development of favorable flavor and texture of the final product.[2] Furthermore, the importance of LAB in human health becomes more popular; some strains can help alleviate gastrointestinal disorders[3] and may be used as a vehicle for the delivery of therapeutic agents into human intestinal tract.[4]

LAB are commonly regarded as facultative anaerobes;[5] they may generate partially reduced reactive oxygen species (ROS) during metabolism in the presence of oxygen. ROS include superoxide (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical ($HO^\bullet$), which can cause damage to proteins, lipids, and nucleotides, leading to the growth arrest and cell death.[6,7] To offset the harmful effects of ROS, most bacteria have evolved defensive systems utilizing antioxidant enzymes such as superoxide dismutase (SOD) and catalase (CAT). SOD catalyzes the conversion of O_2^- to oxygen and H_2O_2, which is subsequently decomposed by CAT. As a result, the cooperation between SOD and CAT can prevent the formation of $HO^\bullet$ via Fenton chemistry.[8]

Superoxide dismutases (EC 1.15.1.1) are metalloenzymes and contain three isoforms that are distinguished by their metal catalytic center: manganese, iron, and copper.[9] In LAB, so far, only manganese superoxide dismutase (MnSOD) has been found in *S. thermophilus*[10] and *Lactococcus lactis*.[11] By now, two distinct families of catalase including the heme-dependent and the manganese-containing catalase have been discovered in some LAB, such as *Lactobacillus*, *Pediococcus*, and *Leuconostoc*.[12−16] *L. rhamnosus* is known as a typical probiotic and is very suitable as starter culture in meat fermentation,[17] but none of them has been found to harbor the SOD gene or CAT gene in the chromosome. Therefore, the lack of endogenous SOD and CAT may lead to high sensitivity of *L. rhamnosus* to oxidative stress in industrial processes.

In recent years, to improve the oxidative stress resistance of LAB, the genes encoding SOD or CAT were expressed in heterologous hosts, which greatly improved the survival of recombinant strains in oxidative stress conditions.[18−20] In our previous study, the catalase gene *katA* from *L. sakei* YSI8 has been expressed in *L. rhamnosus* AS 1.2466 and conferred high-level resistance to oxidative stress on the host.[21] In this work, the superoxide dismutase gene *sodA* from *S. thermophilus* 99241 was further introduced into *L. rhamnosus* AS 1.2466 harboring the *katA*, and the objective of this study was to investigate whether the coexpression of the *sodA* and the *katA* would provide remarkable antioxidant ability in *L. rhamnosus*.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Growth Conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* DH5α was aerobically grown with shaking at 250 rpm in Luria−Bertani (LB) broth at 37 °C. *S. thermophilus* 99241 was grown at 42 °C in M17 medium[23] supplemented with 0.5% glucose (M17G). *Lactobacillus* spp. was cultured in MRS medium at 37 °C.[24] For LAB,

Received: January 19, 2011
Accepted: March 17, 2011
Revised: March 17, 2011
Published: March 18, 2011

Table 1. Bacterial Strains and Plasmids

strain/plasmid	characteristics	source/reference
strains		
E. coli DH5α	F- φ80d *lacZ*ΔM15 Δ(*lacZ*YA-*arg*F) U169 *endA*1 *recA*1 *hsdR*17 (r_k^-,m_k^+) *supE*44λ- *thi*-1 *gyrA*96 *relA*1 *phoA*	TransGen, Beijing, CN
L. sakei YSI8	endogenous production of heme-dependent catalase KatA	21
S. thermophilus 99241	endogenous production of manganese superoxide dismutase MnSOD	this work
L. rhamnosus AS 1.2466	wild-type strain, CAT and SOD negative	this work
L. rhamnosus CK	*L. rhamnosus* AS 1.2466 with pSIPCK	21
L. rhamnosus CAT	*L. rhamnosus* AS 1.2466 with pSIPCAT	21
L. rhamnosus CS	*L. rhamnosus* AS 1.2466 with pSIPCS	this work
plasmids		
pSIP502	Em^r, nis-based expression vector carrying *gusA*, 7.3 kb, NisR/K expression driven by *ermL* read-through	22
pSIPCK	pSIP502 where the *gusA* is cut by *Nco*I and *Hind*III, then self-ligated, 5.4 kb	21
pSIPCAT	pSIP502 where the *gusA* is replaced by the *katA* gene, under the control of P_{nisA}	21
pSIPCS	pSIPCAT containing *sodA* gene behind *katA*	this work

aerated cultures (50 mL) were performed in 250 mL Erlenmeyer flasks with shaking at 200 rpm, and nonaerated cultures (10 mL) were grown in 25 mL closed tubes. When required, erythromycin (Em) was added at 5 μg/mL for *L. rhamnosus* or 300 μg/mL for *E. coli*. For enumeration, appropriate dilutions of samples (prepared in 0.8% physiological saline) were plated on MRS medium and incubated at 37 °C for 48 h. To test the activity of catalase, 30 μM hematin (Sigma) was added to the medium. For long-term aerated growth, *L. rhamnosus* strains were cultured in glucose-limited MRS (1.25 g/L).

DNA Manipulation Techniques. Mini-prep isolation of *E. coli* plasmids was performed using the QIAprep spin kit according to the manufacturer's instructions (Qiagen Inc., Valencia, CA). Plasmids from lactobacilli were isolated by the alkaline lysis method with some modifications.[25] Before plasmid extraction, cultures at OD_{600} of 0.8−1.0 were collected and washed in TES buffer (50 mM Tris-HCl, 1 mM EDTA, 25% sucrose; pH 8.0). Lysozyme was then added at a final concentration of 30 mg/mL, and the suspension was incubated at 37 °C for 1 h. Total DNA from *L. sakei* and *S. thermophilus* was prepared as described by te Riele et al.[26] Plasmids were introduced into *E. coli* DH5α using standard heat shock transformation,[27] and electroporation was used for plasmid transfer into lactobacilli as previously described.[28] Restriction endonuclease digestions were conducted according to the supplier's instructions (Takara, Beijing, CN). DNA ligation was performed using the DNA Ligation kit (Tiangen, Beijing, CN) according to the manufacturer's instructions. DNA sequencing was determined with the Bigdye Terminator cycle sequencing kit (Sangon, Beijing, CN).

Cloning of *S. thermophilus sodA* and Coexpression Vector Construction. Standard PCR was carried out using *Ex Taq* polymerase according to the manufacturer's instructions (Takara, Beijing, CN). The structural gene of *sodA* and its own promoter P_{sodA} were amplified by PCR from the chromosomal DNA of *S. thermophilus* 99241 using the primers 5′-CCGCTCGAGCAAGATTTTGTAAG and 3′-GGGGTACCTGAGGATGATTCTAGAC, which were designed according to the DNA sequence (GenBank accession no. AF538722).[10] Restriction sites used for subsequent cloning are underlined: *Xho*I and *Kpn*I for the 5′ and 3′ primers, respectively. The PCR product was digested by *Xho*I and *Kpn*I, and then inserted into the expression vector pSIPCAT at the downstream of the catalase gene *katA*. The recombinant coexpression vector harboring *katA* and *sodA*, designated as pSIPCS, was sequenced and further analyzed with the DNAMAN software package. The plasmid pSIPCS was then transformed by electroporation into the heterologous host *L. rhamnosus* AS 1.2466 using Bio-Rad Gene Pulser Xcell (Bio-Rad, Richmond, Calif.) in a 0.2 cm cuvette with the field strength of 1.5 kV/cm.

SDS-PAGE Analysis of Recombinant MnSOD Protein. Single-colony transformants were inoculated into MRS medium containing 5 μg/mL Em and grown at 37 °C. The cultures of *L. rhamnosus* containing pSIPCAT or pSIPCS were harvested in the exponential growth phase (OD_{600} of ∼0.5) by centrifugation at 6000*g* for 15 min at 4 °C. The cells were washed in 0.05 M phosphate buffer containing 10^{-4} M EDTA (KPi-EDTA buffer, pH 7.8), pelleted by centrifugation, and resuspended in the same buffer. The cell suspensions were disrupted by sonication in an ice bath. Cellular debris was removed by centrifugation at 12 000*g* for 15 min at 4 °C. The cell-free extracts (CFEs) were dialyzed for 24 h at 4 °C against three changes of the KPi-EDTA buffer. The recombinant MnSOD protein in the CFEs of *L. rhamnosus* CS was then detected by SDS-PAGE analysis as previously described.[29] The CFEs of *L. rhamnosus* CAT were used as a negative control.

Detection of Catalase and Superoxide Dismutase in Recombinant *L. rhamnosus* Strain. The activity of catalase in *L. rhamnosus* AS 1.2466 with plasmid pSIPCS was determined as previously described.[30] Briefly, exponentially growing cells were harvested and resuspended in phosphate buffer (0.1 M, pH 7.0), and then mixed with 0.8 mmol H_2O_2. H_2O_2 concentration was determined every minute as follows: an aliquot was mixed with 3 volumes of a solution of dichromate in acetic acid (1/3 dipotassium chromate 50 g/L, 2/3 glacial acetic acid). Next, the samples were boiled and centrifuged to remove cells, and the absorbance was measured at 570 nm. Dialyzed CFEs were assayed for protein concentration by the Lowry method,[31] using bovine serum albumin as the standard. Specific activity of SOD in the CFEs was then assayed using the pyrogallol autoxidation method.[32]

Survival after Short-Term H_2O_2 Exposure. To estimate the level of H_2O_2 resistance of *L. rhamnosus* AS 1.2466 harboring plasmid pSIPCAT or pSIPCS, exponential growth phase (OD_{600} of ∼0.5) cultures were centrifuged at 6000*g* for 15 min and resuspended in fresh MRS medium containing 1, 10, 15, 30, and 45 mM H_2O_2, respectively, and then incubated at 37 °C. After 1 h, H_2O_2 was eliminated using bovine liver catalase (10 U/mL, Sigma) and viable cells were counted by plating appropriate dilutions on MRS medium. Cultures incubated for 1 h without H_2O_2 treatment were used as a reference to calculate survival ratio. The results presented correspond to the mean of three different assays.

Growth Situation of *L. rhamnosus* under Long-Term Oxidative Stress Conditions. Aerated cultures (50 mL) were carried out with shaking at 200 rpm in 250 mL Erlenmeyer flasks at 37 °C. Overnight grown cultures of *L. rhamnosus* CAT or CS were diluted 1000-fold in glucose-limited MRS medium supplemented with hematin and Em. Viable bacterial counts were determined by plating on MRS medium every 24 h during 7 days.

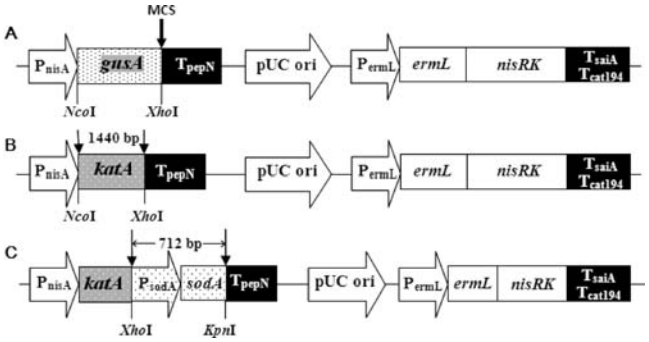

Figure 1. Construction of plasmid pSIPCS based on the lactobacilli expression vector pSIP502. (A) pSIP502. (B) Recombinant plasmid pSIPCAT containing the 1440 bp long *katA* replacing the *gusA*. (C) Recombinant coexpression plasmid pSIPCS with the 712 bp long *sodA* and its promoter P_{sodA} behind the *katA*.

RESULTS

Cloning of Superoxide Dismutase Gene *sodA* and Coexpression Vector Construction. The superoxide dismutase gene *sodA* was obtained by PCR with specific primers, and the chromosomal DNA of *S. thermophilus* 99241 was used as the template. The expected 700 bp PCR product was purified and cloned into the vector pSIPCAT at the downstream of the *katA*. The coexpression vector was designated as pSIPCS (Figure 1). After sequencing, the DNA length was 712 bp, which contained a native promoter and predicted an open reading frame encoding 201 amino acids and a TAA stop codon. The nucleotide sequence of the amplified PCR product showed 99% homology with the superoxide dismutase gene in *Streptococcus thermophilus* LMD-9 (GenBank accession no. CP000419.1), and this sequence showed two mutations at positions 231 (G→A) and 465 (C→T). However, these mutations did not result in changes in amino acid sequence. The coexpression vector pSIPCS was then transformed into *L. rhamnosus* AS 1.2466 by electroporation.

Heterologous Expression of Superoxide Dismutase in *L. rhamnosus*. To remarkably enhance antioxidant ability, the gene *sodA* encoding manganese superoxide dismutase was also introduced into *L. rhamnosus* AS1.2466, which have harbored the catalase gene *katA*. The dialyzed CFEs of the recombinant *L. rhamnosus* CS and *L. rhamnosus* CAT were, respectively, separated by SDS-PAGE and stained using Coomassie brilliant blue. A ~26 kDa migrating band, which was consistent with the expected molecular weight of 25.7 kDa, was only founded in the CFEs of coexpression strains *L. rhamnosus* CS (Figure 2). This result suggested that the gene *sodA* was successfully transcribed and translated in the heterologous host.

Activity of Recombinant Superoxide Dismutase in *L. rhamnosus* CS. The recombinant MnSOD protein had been found in the CFEs of *L. rhamnosus* CS by SDS-PAGE analysis. Furthermore, the specific activity of MnSOD was assayed using the pyrogallol autoxidation method. The high-level activity of MnSOD was detected in *L. rhamnosus* CS, while almost no activity was detected in both *L. rhamnosus* CK and *L. rhamnosus* CAT (Table 2). These results demonstrated that the gene *sodA* from *S. thermophilus* was successfully expressed in heterologous host *L. rhamnosus* CS.

Activity of Catalase KatA in *L. rhamnosus* CS. The activity of heme catalase KatA from *L. sakei* was determined in *L. rhamnosus* CS by two different methods. At first, the presence or absence of activity was judged by the formation of gas (O_2) upon the addition of H_2O_2. Activity was observed in both *L. rhamnosus* CAT and *L. rhamnosus* CS, but no activity was detected in *L. rhamnosus* CK (Figure 3A). Next, quantitative assays showed that the activities of KatA were 2.53 and 2.48 μmol H_2O_2/min/10^8 cfu in *L. rhamnosus* CS and *L. rhamnosus* CAT, respectively (Figure 3B). These results indicated that the coexpression of *katA* and *sodA* did not affect the catalase activity.

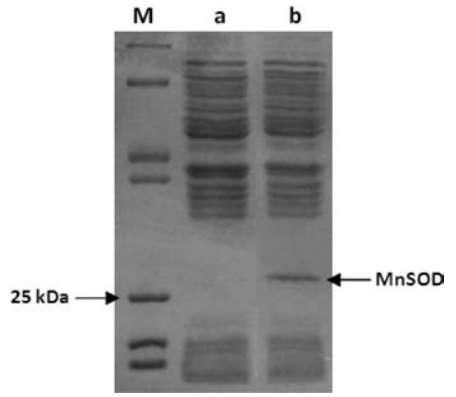

Figure 2. Detection of the recombinant MnSOD protein in *L. rhamnosus* strains. The dialyzed CFEs were separated by SDS-PAGE and stained with Coomassie brilliant blue. M, Protein molecular weight marker; a, *L. rhamnosus* CAT harboring *katA* gene; b, *L. rhamnosus* CS harboring *katA* and *sodA* gene.

Table 2. Specific Activity of MnSOD in *L. rhamnosus* Strains

strains	specific activity (U/mg protein)[a]
L. rhamnosus CK	0
L. rhamnosus CAT	10.84 ± 0.16
L. rhamnosus CS	147.80 ± 1.08

[a] Results are based on three independent experiments, ±SD.

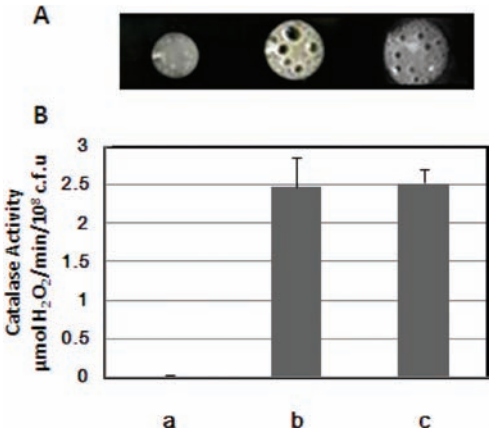

Figure 3. Detection of catalase activity in *L. rhamnosus* strains. (A) Cells resuspended in TES buffer (20 μL) were mixed with 10 μL of 8 M H_2O_2. The bubble formation represented catalase activity resulting from the transformation of H_2O_2 to H_2O and O_2. a, *L. rhamnosus* CK; b, *L. rhamnosus* CAT; c, *L. rhamnosus* CS. (B) Catalase activity in cell suspensions. a, *L. rhamnosus* CK; b, *L. rhamnosus* CAT; c, *L. rhamnosus* CS. Activity was calculated as micromoles H_2O_2 degraded per minute per 10^8 cfu. These results correspond to the mean of three parallel assays. Error bars correspond to the values of standard errors of the means (SEM).

dx.doi.org/10.1021/jf200251k |*J. Agric. Food Chem.* 2011, 59, 3851–3856

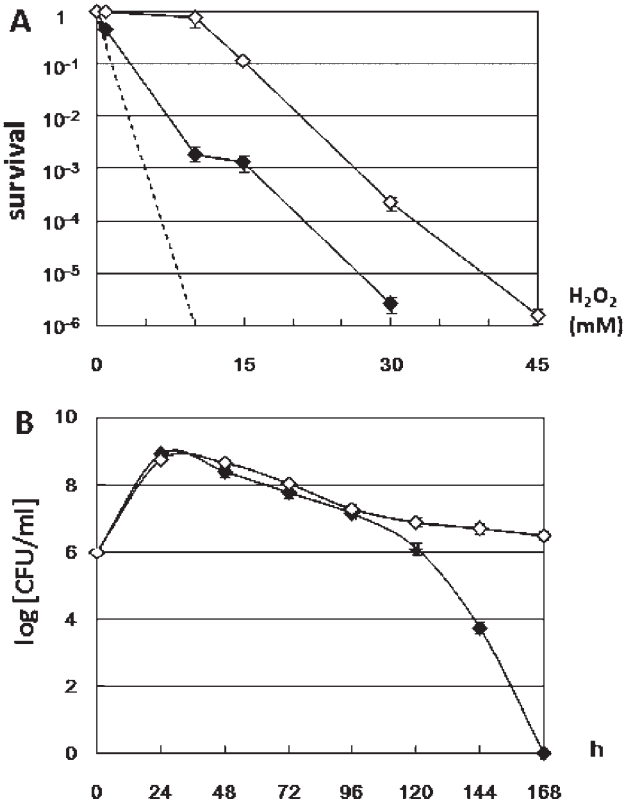

Figure 4. Survival of *L. rhamnosus* strains under oxidative stress conditions. (A) Survival after short-term exposure to different concentrations of H_2O_2 in exponential growth phase. Dotted line indicated survival of *L. rhamnosus* CK. (B) Long time growth conditions in aerated limited-glucose medium. Viable cells were counted every 24 h in 1 week. Symbols: (◇) *L. rhamnosus* CS; (◆) *L. rhamnosus* CAT. Error bars correspond to the SEM.

Sensitivity of *L. rhamnosus* Strains Harboring KatA and MnSOD to Oxidative Stress. The effect of the coexpression of KatA and MnSOD in *L. rhamnosus* on the antioxidant ability was evaluated under oxidative stress induced by both H_2O_2 and aeration. At first, the survivals of *L. rhamnosus* CAT and *L. rhamnosus* CS under different H_2O_2 concentrations were investigated. After incubation with 10 mM H_2O_2, the survival ratio of *L. rhamnosus* CS was 400-fold higher than that of *L. rhamnosus* CAT, and in the presence of higher concentrations of H_2O_2 (e.g., 15 and 30 mM), *L. rhamnosus* CS displayed about 100-fold better viability than the control strains, and the coexpression strain can survive even after 45 mM H_2O_2 challenge (Figure 4A). To investigate whether coexpression recombinant strains of *L. rhamnosus* display higher resistance to aerobic stress, both *L. rhamnosus* CAT and *L. rhamnosus* CS were grown in aerated glucose-limited MRS (1.25 g/L). Significant difference in survivals of the two strains was observed after 5 days. One week later, the viable cells of *L. rhamnosus* CS remained $\sim 10^6$ cfu/mL, while no living cells of *L. rhamnosus* CAT were detected (Figure 4B). The above results suggested that the coexpression of KatA and MnSOD can provide further protection of *L. rhamnosus* AS 1.2466 against oxidative stress.

DISCUSSION

The food-associated LAB are facultative anaerobic microorganisms that reduce pyruvate to lactate to regenerate NAD^+. Oxygen is generally associated with toxicity in these bacteria that cannot use oxygen as a terminal electron acceptor.[33] Much research has demonstrated that antioxidant enzymes, especially SOD and CAT, can protect cells from oxidative stress. The reason is that they can scavenge O_2^- and H_2O_2, respectively, and thus prevent the formation of $HO^\bullet$ via the Fenton chemistry reaction.[8] In a previous study, *L. sakei* catalase gene *katA* and *S. thermophilus* MnSOD gene *sodA* were, respectively, introduced into *L. rhamnosus* AS 1.2466. The expression of the *katA* gene in *L. rhamnosus* conferred enhanced oxidative resistance on the host. The survival ratios of *L. rhamnosus* CAT after 6 and 10 mM H_2O_2 challenge for 1 h were 600- and 10^4-fold higher than the negative control at exponential and stationary phase, respectively.[21] However, the expression of the *sodA* gene in *L. rhamnosus* represented even a little lower oxidative resistance than the control strain *L. rhamnosus* CK. For example, after 5 mM H_2O_2 exposure for 1 h at exponential phase, the survival ratio of *L. rhamnosus* CK was $\sim 4 \times 10^{-4}$, while no viable cells were detected for *L. rhamnosus* with *sodA*. The paradoxical result has been mentioned previously by Scott et al.[34] The main reason may be that the heterologous SOD leads to intracellular H_2O_2 accumulation, while *L. rhamnosus* AS 1.2466 has no native catalase to deal with the H_2O_2 toxicity. These results demonstrated that a single gene *sodA* expression cannot enhance antioxidative ability in *L. rhamnosus* AS 1.2466. In this work, the gene *sodA* encoding MnSOD from *S. thermophilus* was introduced into *L. rhamnosus* CAT, and we investigated whether the coexpression of *katA* and *sodA* can obviously enhance the antioxidant ability in the host.

It has been known that ferrous ion [Fe(II)] can react with H_2O_2 to catalyze the production of the highly reactive $HO^\bullet$ in organisms.[8] Fe(II) could be released from labile iron−sulfur $[4Fe{-}4S]^{2+}$ clusters by the action of O_2^-.[35,36] Thus, the protection against generation of $HO^\bullet$ can be accomplished by elimination of O_2^- and H_2O_2. Superoxide dismutase can decompose O_2^- to generate H_2O_2 and O_2, and then H_2O_2 can be eliminated by catalase. So, we speculated that the synergy between SOD and CAT would exist in response to oxidative stress. In this study, the results clearly demonstrated that the coexpression of SOD and CAT in *L. rhamnosus* could provide much higher resistance to oxidative stress than expression of a single CAT. In recent years, much research has revealed that other enzymes and nonenzymatic factors, such as superoxide reductase,[37] thioredoxin reductase,[38] divalent cations,[39] and glutathione,[40] were involved in the oxidative stress response in bacteria. Therefore, it is worthwhile to attempt to simultaneously express two or more antioxidant-related genes in heterologous host to remarkably improve oxidative stress resistance of LAB.

In conclusion, because O_2^- was the direct substrate of MnSOD, the effect of O_2^- generator, such as menadione, on survivals of *L. rhamnosus* CS and CAT was first investigated in this study, but even 100 mM menadione could not inhibit the cell growth (data not shown). Similar results have been obtained in previous studies.[10,41] The main reason was that superoxide generators, such as paraquat and menadione, cannot be transported through the cell membrane of gram-positive bacteria; moreover, the MnSOD is cytoplasmic enzyme and not secreted into the culture medium.[42] As a result, superoxide generators cannot be used to evaluate the antioxidant efficiency of MnSOD in *L. rhamnosus* CS. Although H_2O_2 is not a substrate of SOD, *L. rhamnosus* CS showed 100-fold better viability than did *L. rhamnosus* CAT at high concentration of H_2O_2. The reason

was that SOD can protect bacteria against hydrogen peroxide stress by preventing the accumulation of Fe(II).[43] In addition, under glucose-limited aerobic conditions, intracellular O_2^- and H_2O_2 may be simultaneously generated, the viable cells of *L. rhamnosus* CS remained ~10^6 cfu/mL after 1 week of incubation, while no living cells of *L. rhamnosus* CAT were detected. These results sufficiently indicated the coexpression of KatA and MnSOD can provide further protection of *L. rhamnosus* AS 1.2466 against oxidative stress.

AUTHOR INFORMATION

Corresponding Author

*Tel.: +86 10 62738670. E-mail: haoyl@cau.edu.cn.

Funding Sources

This work was supported by National High-Tech R&D Program Grants (2007AA10Z354 and 2006AA10Z317) from the Ministry of Science and Technology of the People's Republic of China.

ACKNOWLEDGMENT

We thank Professor Xinghua Guo (Institute of Microbiology, Chinese Academy of Sciences) for providing *Lactobacillus rhamnosus* AS 1.2466 and Elisabeth Sørvig (Agricultural University of Norway) for the gift of plasmid pSIP502.

REFERENCES

(1) McKay, L. L.; Balwin, K. A. Applications for biotechnology: present and future improvements in lactic acid bacteria. *FEMS Microbiol. Rev.* **1990**, *87*, 3–14.

(2) Leroy, F.; De Vuyst, L. Lactic acid bacteria as functional starter cultures for the food fermentation industry. *Trends Food Sci. Technol.* **2004**, *15*, 67–78.

(3) Reuter, G. The *Lactobacillus* and *Bifidobacterium* microflora of the human intestine: composition and succession. *Curr. Issues Intest. Microbiol.* **2001**, *2*, 43–53.

(4) Kaur, I. P.; Chopra, K.; Saini, A. Probiotics: potential pharmaceutical applications. *Eur. J. Pharm. Sci.* **2002**, *15*, 1–9.

(5) Axelsson, L. Lactic acid bacteria: classification and physiology. In *Lactic Acid Bacteria: Microbiology and Functional Aspects*; Salminen, S., von Wright, A., Eds.; Marcel Dekker: New York, 1998; pp 1−72.

(6) Berlett, B. S.; Stadtman, E. R. Protein oxidation in aging, disease, and oxidative stress. *J. Biol. Chem.* **1997**, *272*, 20313–20316.

(7) Imlay, J. A. Pathways of oxidative damage. *Annu. Rev. Microbiol.* **2003**, *57*, 395–418.

(8) Fenton, H. J. H. Oxidation of tartaric acid in the presence of iron. *J. Chem. Soc. Trans.* **1894**, *65*, 899–910.

(9) Hassan, H. M. Cytotoxicity of oxyradicals and the evolution of superoxide dismutases. In *Oxygen, Gene Expression and Cellular Function*; Massaro, D., Clerch, L., Eds.; Marcel Dekker: New York, 1997; pp 27−45.

(10) Andrus, J. M.; Bowen, S. W.; Klaenhammer, T. R.; Hassan, H. M. Molecular characterization and functional analysis of the manganese-containing superoxide dismutase gene (*sodA*) from *Streptococcus thermophilus* AO54. *Arch. Biochem. Biophys.* **2003**, *420*, 103–113.

(11) Sanders, J. W.; Leenhouts, K. J.; Haandrikman, A. J.; Venema, G.; Kok, J. Stress response in *Lactococcus lactis*: cloning, expression analysis, and mutation of the lactococcal superoxide dismutase gene. *J. Bacteriol.* **1995**, *177*, 5254–5460.

(12) Abriouel, H.; Herrmann, A.; Stärke, J.; Yousif, N. M.; Wijaya, A.; Tauscher, B.; Holzapfel, W.; Franz, C. M. Cloning and heterologous expression of hematin-dependent catalase produced by *Lactobacillus plantarum* CNRZ 1228. *Appl. Environ. Microbiol.* **2004**, *70*, 603–606.

(13) Delwiche, E. A. Catalase of *Pediococcus cerevisiae*. *J. Bacteriol.* **1961**, *81*, 416–418.

(14) Igarashi, T.; Kono, Y.; Tanaka, K. Molecular cloning of manganese catalase from *Lactobacillus plantarum*. *J. Biol. Chem.* **1996**, *271*, 29521–29524.

(15) Knauf, H. J.; Vogel, R. F.; Hammes, W. P. Cloning, sequence, and phenotypic expression of *katA*, which encodes the catalase of *Lactobacillus sake* LTH677. *Appl. Environ. Microbiol.* **1992**, *58*, 832–839.

(16) Wolf, G.; Strahl, A.; Meisel, J.; Hammes, W. P. Heme-dependent catalase activity of lactobacilli. *Int. J. Food Microbiol.* **1991**, *12*, 133–140.

(17) Erkkilä, S.; Suihko, M. L.; Eerola, S.; Petäjä, E.; Mattila-Sandholm, T. Dry sausage fermented by *Lactobacillus rhamnosus* strains. *Int. J. Food Microbiol.* **2001**, *64*, 205–210.

(18) Noonpakdee, W.; Sitthimonchai, S.; Panyim, S.; Lertsiri, S. Expression of the catalase gene *katA* in starter culture *Lactobacillus plantarum* TISTR850 tolerates oxidative stress and reduces lipid oxidation in fermented meat product. *Int. J. Food Microbiol.* **2004**, *95*, 127–135.

(19) Rochat, T.; Miyoshi, A.; Gratadoux, J. J.; Duwat, P.; Sourice, S.; Azevedo, V.; Langella, P. High-level resistance to oxidative stress in *Lactococcus lactis* conferred by *Bacillus subtilis* catalase KatE. *Microbiology* **2005**, *151*, 3011–3018.

(20) Rochat, T.; Gratadoux, J. J.; Gruss, A.; Corthier, G.; Maguin, E.; Langella, P.; van de Guchte, M. Production of a heterologous nonheme catalase by *Lactobacillus casei*: an efficient tool for removal of H_2O_2 and protection of *Lactobacillus bulgaricus* from oxidative stress in milk. *Appl. Environ. Microbiol.* **2006**, *72*, 5143–5149.

(21) An, H. R.; Zhou, H.; Huang, Y.; Wang, G. H.; Luan, C. G.; Mou, J.; Luo, Y. B.; Hao, Y. L. High-level expression of heme-dependent catalase gene *katA* from *Lactobacillus sakei* protects *Lactobacillus rhamnosus* from oxidative stress. *Mol. Biotechnol.* **2010**, *45*, 155–160.

(22) Sørvig, E.; Grönqvist, S.; Naterstad, K.; Mathiesen, G.; Eijsink, V.; Axelsson, L. Construction of vectors for inducible gene expression in *Lactobacillus sakei* and *L. plantarum*. *FEMS Microbiol. Lett.* **2003**, *229*, 119–126.

(23) Terzaghi, B. E.; Sandine, W. E. Improved medium for lactic streptococci and their bacteriophages. *Appl. Microbiol.* **1975**, *29*, 807–813.

(24) de Man, J. C.; Rogosa, M.; Sharpe, M. E. A medium for the cultivation of lactobacilli. *J. Appl. Bacteriol.* **1960**, *23*, 130–135.

(25) van de Guchte, M.; van der Vossen, J. M.; Kok, J.; Venema, G. Construction of a lactococcal expression vector: expression of hen egg white lysozyme in *Lactococcus lactis* subsp. *lactis*. *Appl. Environ. Microbiol.* **1989**, *55*, 224–228.

(26) te Riele, H.; Michel, B.; Ehrlich, S. D. Single-stranded plasmid DNA in *Bacillus subtilis* and *Staphylococcus aureus*. *Proc. Natl. Acad. Sci. U.S.A.* **1986**, *83*, 2541–2545.

(27) Sambrook, J.; Russell, D. W. *Molecular Cloning: a laboratory manual*, 3rd ed.; Cold Spring Harbor Laboratory Press, Cold Spring Harbor: New York, 2001.

(28) Thompson, K.; Collins, M. A. Improvement in electroporation efficiency for *Lactobacillus plantarum* by the inclusion of high concentrations of glycine in the growth medium. *J. Microbiol. Methods* **1996**, *26*, 73–79.

(29) Le Loir, Y.; Gruss, A.; Ehrlich, S. D.; Langella, P. A nine-residue synthetic propeptide enhances secretion efficiency of heterologous proteins in *Lactococcus lactis*. *J. Bacteriol.* **1998**, *180*, 1895–1903.

(30) Sinha, A. K. Colorimetric assay of catalase. *Anal. Biochem.* **1972**, *47*, 389–394.

(31) Lowry, O. H.; Rosebrough, N. J.; Farr, A. L.; Randall, R. J. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **1951**, *193*, 265–275.

(32) Marklund, S.; Marklund, G. Involvement of the superoxide anion radical in the autoxidation of pyrogallol and a convenient assay for superoxide dismutase. *Eur. J. Biochem.* **1974**, *47*, 469–474.

(33) van de Guchte, M.; Serror, P.; Chervaux, C.; Smokvina, T.; Ehrlich, S. D.; Maguin, E. Stress responses in lactic acid bacteria. *Antonie Van Leeuwenhoek* **2002**, *82*, 187–216.

(34) Scott, M. D.; Meshnick, S. R.; Eaton, J. W. Superoxide dismutase-rich bacteria: paradoxical increase in oxidant toxicity. *J. Biol. Chem.* **1986**, *262*, 3640–3645.

(35) Flint, D. H.; Tuminello, J. F.; Emptage, M. H. The inactivation of Fe-S clusters containing hydro-lyases by superoxide. *J. Biol. Chem.* **1993**, *268*, 22369–22376.

(36) Keyer, K.; Imlay, J. A. Superoxide accelerates DNA damage by elevating free iron levels. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*, 13635–13640.

(37) Jenney, F. E.; Verhagen, M. F. J. M.; Cui, X. Y.; Adams, M. W. W. Anaerobic microbes: oxygen detoxification without superoxide dismutase. *Science* **1999**, *286*, 306–309.

(38) Stewart, E. J.; Aslund, F.; Beckwith, J. Disulfide bond formation in the *Escherichia coli* cytoplasm: an in vivo role reversal for the thioredoxins. *EMBO J.* **1998**, *17*, 5543–5550.

(39) Archibald, F. S.; Fridovich, I. Manganese, superoxide dismutase, and oxygen tolerance in some lactic acid bacteria. *J. Bacteriol.* **1981**, *146*, 928–936.

(40) Fu, R. Y.; Bongers, R. S.; van Swam, I. I.; Chen, J.; Molenaar, D.; Kleerebezem, M.; Hugenholtz, J.; Li, Y. Introducing glutathione biosynthetic capability into *Lactococcus lactis* subsp. *cremoris* NZ9000 improves the oxidative-stress resistance of the host. *Metab. Eng.* **2006**, *8*, 662–671.

(41) Bruno-Bárcena, J. M.; Azcarate-Peril, M. A.; Klaenhammer, T. R.; Hassan, H. M. Marker-free chromosomal integration of the manganese superoxide dismutase gene (*sodA*) from *Streptococcus thermophilus* into *Lactobacillus gasseri*. *FEMS Microbiol. Lett.* **2005**, *246*, 91–101.

(42) Britton, L.; Fridovich, I. Intracellular localization of the superoxide dismutases of *Escherichia coli*: a reevaluation. *J. Bacteriol.* **1977**, *131*, 815–820.

(43) Bruno-Bárcena, J. M.; Andrus, J. M.; Libby, S. L.; Klaenhammer, T. R.; Hassan, H. M. Expression of a heterologous manganese superoxide diamutase gene in intestinal lactobacilli provides protection against hydrogen peroxide toxicity. *Appl. Environ. Microbiol.* **2004**, *70*, 4702–4710.