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ORIGINAL PAPER

Coexpression of bile salt hydrolase gene and catalase gene remarkably improves oxidative stress and bile salt resistance in *Lactobacillus casei*

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Abstract Lactic acid bacteria (LAB) encounter various types of stress during industrial processes and gastrointestinal transit. Catalase (CAT) and bile salt hydrolase (BSH) can protect bacteria from oxidative stress or damage caused by bile salts by decomposing hydrogen peroxide (H_2O_2) or deconjugating the bile salts, respectively. *Lactobacillus casei* is a valuable probiotic strain and is often deficient in both CAT and BSH. In order to improve the resistance of *L. casei* to both oxidative and bile salts stress, the catalase gene *katA* from *L. sakei* and the bile salt hydrolase gene *bsh1* from *L. plantarum* were coexpressed in *L. casei* HX01. The enzyme activities of CAT and BSH were 2.41 μmol H_2O_2/min/10^8 colony-forming units (CFU) and 2.11 μmol glycine/min/ml in the recombinant *L. casei* CB, respectively. After incubation with 8 mM H_2O_2, survival ratio of *L. casei* CB was 40-fold higher than that of *L. casei* CK. Treatment of *L. casei* CB with various concentrations of sodium glycodeoxycholate (GDCA) showed that $\sim 10^5$ CFU/ml cells survived after incubation with 0.5% GDCA, whereas almost all the *L. casei* CK cells were killed when treaded with 0.4% GDCA. These results indicate that the coexpression of CAT and BSH confers high-level resistance to both oxidative and bile salts stress conditions in *L. casei* HX01.

Keywords Bile salt hydrolase · Catalase · Coexpression · *Lactobacillus casei* · Stress resistance

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Introduction

Lactobacillus casei strains have been widely used in the food industry for centuries. They are commonly used as acid-producing starter cultures during the fermentation of milk and as adjunct cultures for intensifying and accelerating flavor development in ripening cheese [5]. Furthermore, some strains of *L. casei* confer general health benefits by acting as probiotics. They decrease incidences of gastrointestinal diseases caused by pathogenic bacteria [10], modulate immunity, and reduce the risk of bladder cancer of the host [18, 23]. However, probiotic strains have to survive a number of stresses during industrial processes and gastrointestinal transit to exert their activities.

In industrial processes, lactic acid bacteria (LAB) often suffer from oxidative stress when reactive oxygen species (ROS) accumulate in the cells. ROS include superoxide (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical (HO·), which can cause damage to proteins, lipids, and nucleotides, leading to the arrest of cell growth and cell death [4, 11]. Catalase plays an important role in reducing oxidative stress by decomposing H_2O_2 to water and oxygen. Recently, genes encoding catalase have been successfully expressed in heterologous hosts to improve the oxidative stress resistance of LAB strains [1, 17, 20].

After ingestion, LAB strains are subjected to harsh conditions, such as the low pH of the stomach and bile salts present in the small intestine. Bile salts play an essential role in emulsification of lipids and have also been shown to have antimicrobial activity [3]. A common mechanism of resistance against bile employed by bacteria is the production of bile salt hydrolase (BSH). BSH catalyses the hydrolysis of glycine- or taurine-conjugated bile salts into amino acid residues and unconjugated bile acids. The unconjugated bile acids may be precipitated and excreted

in the feces. Furthermore, the unconjugated bile acids are far less efficient at solubilization of lipids and intestinal reabsorption, leading to an increase in demand for cholesterol for the synthesis of new bile salts. This results in a decrease in the serum cholesterol level of the host [8].

L. casei strains are important probiotic starter cultures in milk fermentation. However, the lack of the CAT or BSH gene in their genomes may lead to high sensitivity of *L. casei* to oxidative or bile salts stress [6, 14]. In our laboratory, the catalase gene, *katA*, has been successfully expressed in *L. rhamnosus* AS 1.2466 using Nisin-induced expression vector pSIP502 [2]. In this work, the *bsh1* gene from *L. plantarum* WQ0815 was further introduced into the recombinant vector downstream of the *katA* gene, and the effects of the coexpression of CAT and BSH on the growth of recombinant *L. casei* under oxidative or bile salt stress were simultaneously investigated.

Materials and methods

Bacterial strains, plasmids, and growth conditions

Bacterial strains and plasmids used in this study are listed in Table 1. Lactobacilli were cultured under anaerobic conditions at 37°C in de Man, Rogosa and Sharpe (MRS) medium [7]. *Escherichia coli* DH5α was aerobically grown at 37°C in Luria-Bertani (LB) broth with shaking at 250 rpm. For plates, liquid medium was solidified with 1.5% agar. When needed, erythromycin was added at 300 μg/ml for *E. coli* and 5 μg/ml for *L. casei*, respectively. MRS medium with sodium glycodeoxycholate (GDCA) (Sigma) was used to detect the activity of bile salt hydrolase. To test the activity of catalase, 30 μM hematin (Sigma) was added to the MRS medium.

DNA manipulations and transformation

Isolation of plasmid DNA from *E. coli* was performed using the QIAGEN Mini Spin isolation kit according to the manufacturer's instructions (Qiagen Inc, Valencia, CA, USA). Plasmids from lactobacilli were isolated by the alkaline lysis method, with some modifications [19]. Chromosomal DNA of *L. plantarum* was extracted using TIANamp Bacteria DNA kit according to the manufacturer's instructions (TianGen, Beijing, PRC). Plasmids were introduced into *E. coli* DH5α by standard heat-shock transformation [21], and electroporation was used for plasmid transfer into lactobacilli, as previously described [25]. Restriction endonuclease digestions and DNA ligation were performed according to the supplier's instructions (Takara, Beijing, PRC). DNA sequencing was performed with the Bigdye Terminator cycle sequencing kit (Sangon, Beijing, PRC).

Cloning of *L. plantarum bsh1* and coexpression vector construction

Standard polymerase chain reaction (PCR) was carried out using *Ex Taq* polymerase according to the manufacturer's instructions (Takara, Beijing, PRC). PCR primers (bshF 5′-CGGAATTCTATATCGGTTATAAGGG-3′, bshR 5′-GGGGTACCTTAGTTAACTGCATAGT-3′) were designed according to the *bsh1* gene of *L. plantarum* WCFS1 (GenBank Accession No. AL935262.1). Restriction sites used for subsequent cloning are underlined: *Eco*R I and

Table 1 Bacterial strains and plasmids used in this study

Strain/plasmid	Characteristics	Source/reference
Strains		
Escherichia 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, PRC
Lactobacillus plantarum WQ0815	Endogenous production of bile salt hydrolase	Laboratory collection
L. casei HX01	Host strain isolated from fermented milk, catalase and bile salt hydrolase negative	Laboratory collection
L. casei CK	*L. casei* HX01 with pSIPCK	This work
L. casei catalase (CAT)	*L. casei* HX01 with pSIPCAT	This work
L. casei CB	*L. casei* HX01 with pSIPCB	This work
Plasmids		
pSIPCK	Em^r, Nis-based expression vector, 5.4 kb NisR/K expression driven by *ermL* read-through	[2]
pSIPCAT	pSIPCK with *katA* gene, under the control of P_{nisA}	[2]
pSIPCB	pSIPCAT with *bsh1* gene, under the control of *bsh1* native promoter	This work

Kpn I for bshF and bshR, respectively. The structure gene *bsh1* with its native promoter was amplified from the chromosomal DNA of *L. plantarum* WQ0815. The amplicon obtained was digested with *EcoR* I and *Kpn* I and then inserted into the expression vector pSIPCAT downstream of the catalase gene *katA*. The recombinant coexpression vector harboring *katA* and *bsh1*, designated as pSIPCB, was sequenced and further analyzed with the DNAMAN software package. The plasmid pSIPCB was then transformed by electroporation into the heterologous host *L. casei* HX01 using Bio-Rad Gene Pulser Xcell^{TM} (Bio-Rad, Richmond, CA, USA) in a 0.2-cm cuvette with the field strength of 7.5 kV/cm.

Detection of BSH activity in *L. casei* strains

BSH activity was tested through a direct plate assay developed by Dashkevicz and Feighner [6]. Briefly, overnight cultures were streaked on solid MRS medium supplemented with GDCA (0.05%, w/v) and incubated anaerobically for 72 h at 37°C. The presence of BSH activity leads to the formation of an opaque halo around colonies due to the precipitation of deconjugated bile salts. Furthermore, quantitative assay of BSH activity in cell-free extracts was performed as described by Tanaka, with minor modifications [24]. Overnight cultures were centrifuged at 12,000*g* for 10 min at 4°C. Cells were washed twice with 0.1 M sodium-phosphate buffer (pH 7.0), and then resuspended in the same buffer containing 10 mM dithiothreitol (DTT) to give a cell density of approximately 5 U at 600 nm. Four milliliters of the cell suspension was sonicated for 9 min with constant cooling in ice, followed by centrifugation at 12,000*g* for 10 min at 4°C. The supernatant was stored as cell-free extracts. BSH activity was determined by estimating the amount of free amino acids liberated from conjugated bile salts with a two-step procedure. One unit of BSH activity was defined as the amount of enzyme that releases 1 μmol of amino acids from the substrate per minute per milliliter.

Detection of catalase activity in *L. casei* strains

Overnight cultures were inoculated at a 1:50 dilution in fresh MRS medium with 5 μg/ml erythromycin. Two milliliters of exponentially growing culture was centrifuged and resuspended in 30 μl of TES buffer [50 mM Tris-hydrochloride (HCl), pH 8.0, 1 mM ethylenediaminetetraacetate (EDTA), 25% sucrose]. TES-resuspended cells (20 μl) were mixed with 10 μl of 8 M H_2O_2. The presence of catalase activity leads to the formation of bubbles resulting from the transformation of H_2O_2 to H_2O and O_2. A quantitative assay of catalase activity was performed according to the method of Sinha [22]. Briefly, exponentially growing cells were harvested and the pellet was resuspended in phosphate buffer (0.1 M, pH 7.0) to obtain a concentration of 1.0×10^8 colony-forming units (CFU)/ml and then mixed with 0.8 mM H_2O_2. An aliquot was withdrawn after 10 min and mixed with three volumes of a solution of dichromate in acetic acid (1/3 dipotassium chromate 50 g/l, 2/3 glacial acetic acid). The mixture was boiled, and the absorbance at 570 nm was measured. Catalase activity was defined as μmols of H_2O_2 degraded per min per 10^8 CFU.

Survival after bile salt stress

Bile salt tolerance of *L. casei* CK and *L. casei* CB was performed according to the method of De Smet et al. [9]. Stationary phase cultures were inoculated into MSR containing 0.05–0.5% GDCA to an $OD_{600\ nm}$ of 0.1 then incubated at 37°C for 3 h. Viable bacterial counts were determined by plating appropriate dilutions on MRS medium.

Survival after short H_2O_2 exposure

To estimate the level of H_2O_2 resistance of *L. casei* CK and *L. casei* CB, exponential phase ($OD_{600\ nm}$ of $\sim$0.5) cultures were centrifuged and resuspended in fresh MRS medium containing 8 mM H_2O_2 for 1 h at 37°C. Excess H_2O_2 was removed by the addition of bovine catalase (10 U/ml, Sigma), and viable bacterial counts were determined by plating on MRS medium. Cells incubated for 1 h without H_2O_2 were used as a reference to calculate the survival ratio.

Results

Construction of the *katA* and *bsh1* coexpression vector

The bile salt hydrolase gene *bsh1* was amplified from the chromosomal DNA of *L. plantarum* WQ0815 by PCR using specific primers mentioned in "Materials and methods". The expected 1.2-kb PCR product was purified and cloned into the vector pSIPCAT downstream of the catalase gene *katA*. The coexpression vector was designated as pSIPCB (Fig. 1). DNA sequencing results verified that the 1,157-bp-long stretch of DNA contained a native promoter, an open reading frame encoding 324 amino acids followed by a TAA stop codon. The nucleotide sequence of the amplified PCR product showed 99% homology with the *bsh1* gene from *L. plantarum* WCFS1 (lp_3536) (GenBank Accession No. AL935262.1). Five mutations were detected in the sequence we amplified, resulting in three amino acid changes at the following positions: 199 (Lys → Arg), 236

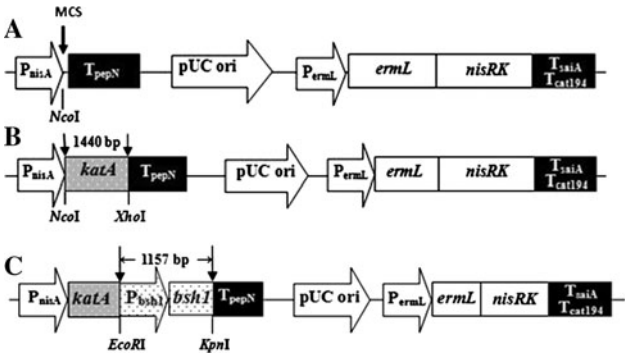

Fig. 1 Construction of plasmid pSIPCB based on the lactobacilli expression vector pSIPCK containing the 1,440-bp-long *katA*. **a** pSIPCK. **b** Recombinant plasmid pSIPCAT containing the 1,440-bp-long *katA* under the control of P_{nisA}. **c** Recombinant coexpression plasmid pSIPCB with the 1,157-bp-long *bsh1* and its promoter P_{bsh1} behind the *katA*

(Ser → Pro), and 290 (Asp → Glu). The coexpression vector pSIPCB was transformed into *L. casei* HX01 by electroporation.

Detection of BSH activity in *L. casei* strains

Initial studies on BSH activity in *L. casei* were performed by the plate assay method. *L. casei* strains grown overnight were streaked onto MRS agar supplemented with 0.05% GDCA and incubated anaerobically for 72 h. Only *L. casei* CB displayed detectable BSH activity, with white precipitates around colonies. However, no activity was observed for the wild *L. casei* HX01 or *L. casei* CK, and the growth of these bsh-negative strains was retarded in the presence of GDCA (Fig. 2a). Quantitative assays revealed that *L. casei* CB produced 2.11 U/ml of BSH under conditions mentioned in "Materials and methods", which was higher than that of control strains grown under identical conditions (Fig. 2b). These results indicate that the *bsh1* gene was successfully expressed in *L. casei* HX01.

Activity of catalase KatA in *L. casei* strains

The activity of heme catalase KatA from *L. sakei* was determined in *L. casei* CB by two methods. At first, the presence of activity was judged by the formation of oxygen gas (O_2) upon the addition of H_2O_2. Activity was observed only in *L. casei* CB, whereas no activity was detected in the wild-type *L. casei* HX01 or *L. casei* CK (Fig. 3a). Quantitative assays showed that *L. casei* CB produced 2.41 μmol H_2O_2/min/10^8 CFU of catalase activity, whereas almost no activity was detected in supernatant samples of the control strains (Fig. 3b). These results indicate that the gene *katA* from *L. sakei* was successfully expressed at a high level in the heterologous host *L. casei* HX01.

Effect of BSH on bile salt resistance of *L. casei* strains

When stationary-phase cultures were incubated for 3 h in MRS containing 0.05–0.5% GDCA, significant growth inhibition was observed at 0.1% GDCA in *L. casei* CK. However, *L. casei* CB was able to grow in the presence of 0.5% GDCA (Fig. 4). At the concentration of 0.1% GDCA, the survival ratio of *L. casei* CK and *L. casei* CB was

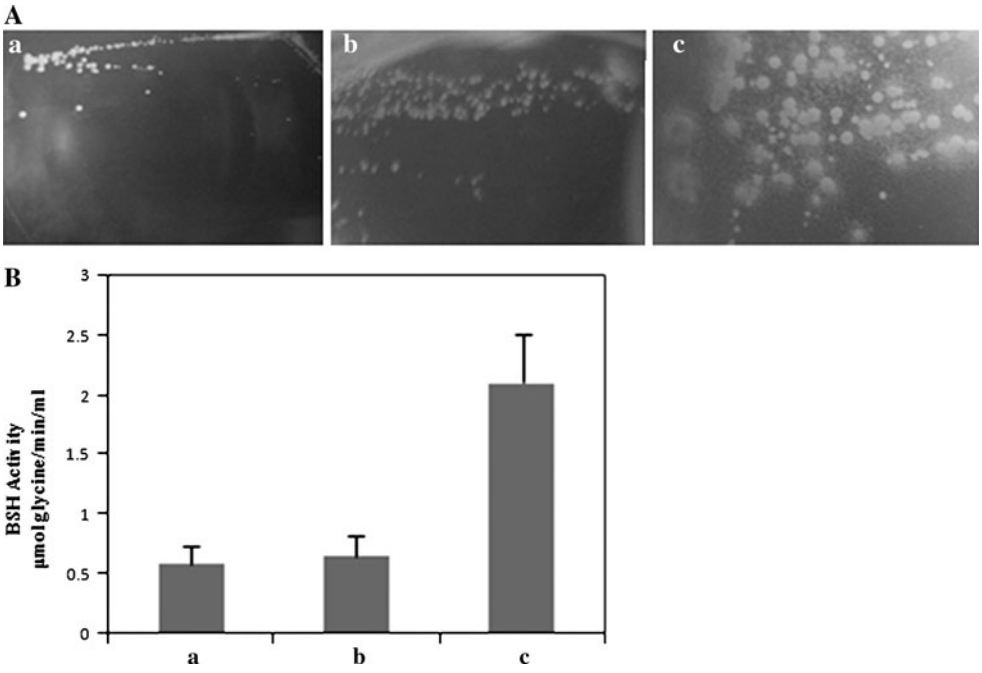

Fig. 2 Detection of bile salt hydrolase (BSH) activity in *Lactobacillus casei* strains. **a** Plate assay for the detection of BSH activity. *Lanes*: *a L. casei* HX01; *b L. casei* CK; *c L. casei* CB. **b** Quantitative assay of BSH activity (μmol glycine/min/ml) in cell-free extracts. *Lanes*: *a L. casei* HX01; *b L. casei* CK; *c L. casei* CB. Each value is the average of three independent assays. *Error bars* correspond to the standard error of the mean (SEM)

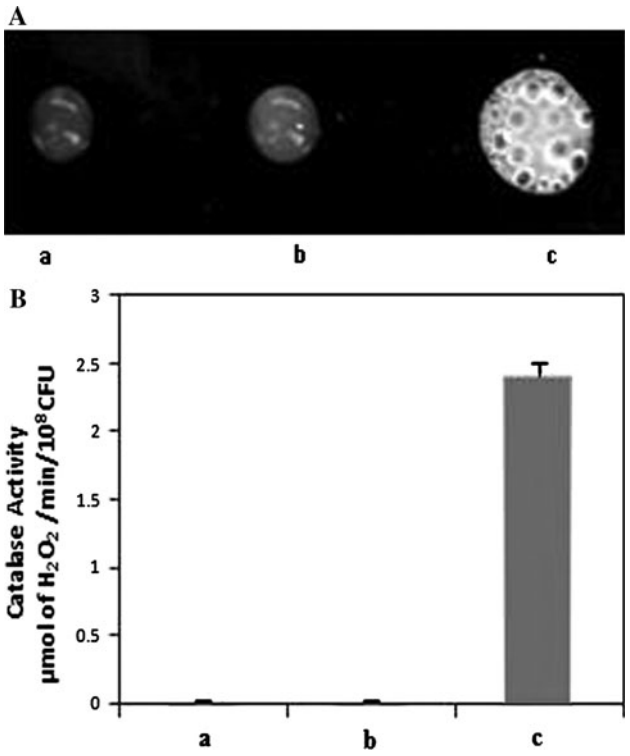

Fig. 3 Detection of catalase activity in *Lactobacillus casei* strains. **a** Cells were resuspended in TES+8 M hydrogen peroxide (H_2O_2). The presence of catalase activity was detected by the formation of oxygen gas (O_2) due to the H_2O_2 added. *Lanes*: *a L. casei* HX01; *b L. casei* CK; *c L. casei* CB. **b** Catalase activity in cell suspensions. *Lanes*: *a L. casei* HX01; *b L. casei* CK; *c L. casei* CB. Each value is the average of three independent assays. *Error bars* correspond to the standard error of the mean (SEM)

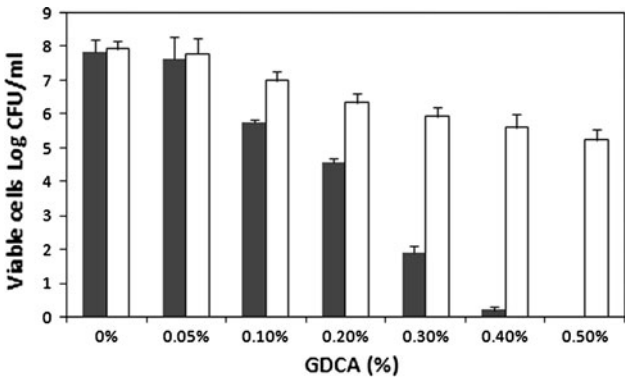

Fig. 4 Survival of *Lactobacillus casei* strains in the presence of glycodeoxycholate (GDCA). *Filled square*, *L. casei* CK; *open square*, *L. casei* CB. Each value is the average of three independent assays. *Error bars* correspond to the standard error of the mean (SEM)

0.79% and 11.38%, respectively. At the concentration of 0.4% GDCA, almost all the *L. casei* CK cells were killed, whereas $\sim 10^5$ CFU/ml cells of *L. casei* CB survived after incubation with 0.5% GDCA. These results demonstrate that the presence of BSH significantly enhanced bile salt tolerance of *L. casei*.

Effect of catalase KatA on oxidative resistance of *L. casei* strains

To examine the effect of catalase KatA on the growth of *L. casei* under oxidative stress conditions, exponential-phase cells were incubated for 1 h in MRS containing 8 mM H_2O_2. The survival ratio of *L. casei* CB reached 63.75%, whereas only 1.53% cells survived for *L. casei* CK. These results suggest that the expression of KatA provided remarkable protection against oxidative stress in *L. casei*.

Discussion

Lactobacillus casei strains are commonly used in food fermentation, and several strains are recognized as putative candidates of probiotics [10]. The host strain, *L. casei* HX01, used in our research was isolated from the Chinese traditional fermented milk product koumiss, which displayed neither CAT nor BSH activity. Therefore, oxidative or bile salts stress encountered in food processing environments or in the gastrointestinal tract could probably cause damage to the cells. In this study, CAT and BSH were successfully coexpressed in *L. casei* HX01, and our results indicate that the recombinant strain shows significantly improved survival rate when subjected to both oxidative and bile salts stress, suggesting a promising strategy to improve the resistance of probiotic strains toward multiple stresses.

BSH activity was measured using two different methods: High-performance liquid chromatography (HPLC) was used to measure the disappearance of conjugated bile acid [13]; the ninhydrin reaction was used to determine the amount of glycine or taurine released from the conjugated bile acid [24]. The latter method was used in this work to determine the BSH activity in cell-free extracts; however, results revealed that BSH-negative strains also showed a slight activity of 0.58 μmol/min/ml (Fig. 2b). The possible reason could be that the ninhydrin method is based on the reaction between ninhydrin and alpha-amino acids, resulting in the formation of a purple-colored product, which absorbs maximally at 570 nm. It is a simple and sensitive method routinely used to quantify free alpha-amino acids [15]. Therefore, amino acids or peptides containing alpha-amino groups in the culture medium and the host strain can contribute to the basal concentration in the negative strains.

BSH activity in LAB is considered to be strongly correlated with natural habitat, and most BSH-positive strains

were isolated from gastrointestinal tract or feces of mammals. The metagenomic study also suggested that BSH activity was an ancient adaption to the gut environment [12]. The presence of BSH activity in strains not only contributes to their survival by the detoxification of bile salts but also exerts probiotic effects on the hosts, such as lowing the cholesterol level and reducing the risk of obesity and arthrosclerosis. Although some reports disagree on the relationship between bile salts tolerance and BSH activity in lactobacilli [16], the heterologous expression studies of *bsh1* gene in this work support and confirm the importance of BSH activity in the survivability of strains under bile salts stress.

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