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INCREASE OF STRESS RESISTANCE IN *LACTOCOCCUS LACTIS* VIA A NOVEL FOOD-GRADE VECTOR EXPRESSING A *SHSP* GENE FROM *STREPTOCOCCUS THERMOPHILUS*

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ABSTRACT

The effects of the expression of a small heat shock protein (*shsp*) gene from *Streptococcus thermophilus* on stress resistance in *Lactococcus lactis* under different environmental stresses were investigated in this study. pMG36e-shsp, an expression vector, was first constructed by inserting a *shsp* open reading frame (ORF) cloned from *S. thermophilus* strain St-QC into pMG36e. Then, a food-grade expression vector, pMG-shsp, was generated by deleting the erythromycin resistance gene from pMG36e-shsp. The transformation rate of pMG-shsp was comparable to that of pMG36e-shsp when each of these two vectors was introduced into *L. lactis*. These results demonstrated that the *shsp* ORF could successfully used as a food-grade selection marker in both pMG-shsp and pMG36e-shsp. Furthermore, the growth characteristics were almost the same between *L. lactis* ML23 transformants harboring pMG36e or pMG-shsp. The survival rate of *L. lactis* ML23 expressing the *shsp* ORF were increased to 0.032%, 0.006%, 0.0027%, 0.03%, and 0.16% under the following environmental stresses: heat, acid, ethanol, bile salt and H_2O_2, respectively. These results indicated that the expression of the *shsp* gene in the food-grade vector pMG-shsp conferred resistance to environmental stresses without affecting the growth characteristics of *L. lactis* ML23.

Key words: *shsp* gene, *Streptococcus thermophilus*, *Lactococcus lactis*, food-grade vector, stress resistance

INTRODUCTION

The fermented food industry based on lactic acid bacteria (LAB) is one of the fastest-growing sectors in the food industry in China. However, LAB are sensitive to environmental conditions when commercial starters or fermentation products are being processed, transported and stored. To increase resistance of LAB to environmental stresses, one strategy is to construct a transgenic strain of LAB expressing *shsp* gene that can improve the stress-resistance of the host. The expression vector that harbors the *shsp* gene in the host could be a food-grade vector to ensure food safety.

sHSPs are a group of heat shock proteins (HSPs, or stress proteins) with low molecular mass ranging from 14 to 43 kDa (7, 8). sHSPs are induced by different stresses such as high temperature and low pH. They can improve the thermo- and acid-

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resistance ability of LAB (15, 18). Composed of a stress-inducible group of molecular chaperones that can bind to denatured proteins, sHSPs prevent the latter irreversible aggregation and maintain it in a refolding state under stress conditions (9, 14). The *S. thermophilus* parent strain carrying plasmid-encoded *shsp* gene is significantly more resistant to temperature shifting from 42 °C to 62 °C compared with its variant strain lacking plasmid-encoding *shsp* (15). Additionally, the *S. thermophilus* strains harboring plasmid carrying the *shsp* gene showed significantly higher cell survival rate than the strains harboring plasmid eliminating *shsp* gene under heat shock condition (16). Therefore, introducing and expressing a *shsp* gene in LAB can improve their survival ability to stresses.

Presently, β-galactosidase of *S. thermophilus* (10), *shsp* genes (3, 4), as well as genes associated with auxotrophy (2, 6), bacteriocins (13, 19) and new metabolic pathways (1) have been used in constructing food-grade vectors as selection markers. The *shsp* genes from the plasmid of *S. thermophilus* are ideal selection marker because sHSP can elevate resistance to environmental stresses (3). Based on the plasmid-encoded *shsp* gene as a selection marker, a food-grade cloning system (pHMR1) for *S. thermophilus* was developed (3) and a two-plasmid system (pST04 and a help plasmid) was constructed and successfully applied to yogurt fermentation (4).

We previously cloned a *shsp* gene with a 429-bp open reading frame encoding a 142-amino acid polypeptide from *S. thermophilus* strain St-QC (20). Here, we report the construction of a food-grade vector carrying the *shsp* gene successfully expressed in mesophilic *L. lactis* which is an important main strain for manufacturing cheese. The engineered *L. lactis* ML23 exhibited enhanced heat resistance. The function of the sHSP in different environmental stresses and the potential of the *shsp* gene as a food-grade selection marker are presented in this study. Our findings will provide basis for research on the function of *shsp* gene from *S. thermophilus* and its application in the dairy industry to improve the stress resistance of starters.

MATERIALS AND METHODS

Bacterial strains and plasmids

S. thermophilus St-QC (20) and *L. lactis* ML23 (original strain name was *L. lactis* ML0230 subsp. *lactis* and provided by Dr. Xing-hua Guo) were cultured in M17 medium supplemented with 20 g lactose-1 (LM17) for 16 h at 42 °C and 30 °C, respectively. *Escherichia coli* DH5α was purchased from TIANGEN and grown in LB broth medium or on LB agar plate at 37 °C. Plasmid pMG36e, which was originally generated by van de Guchte et al. (21), was provided by Dr. Xing-hua Guo.

Cloning a *shsp* ORF by overlapping PCR

To obtain the complete sequence of the p32 promoter (5) followed by the full length *shsp* ORF, four different polymerase chain reactions (PCR) were performed in a PCR thermal cycler (Hybaid Limited, Britain) in a 20 μl reaction mixture containing 2 μl of the template, 20 mM of each deoxynucleoside triphosphate, 0.75 mM of each primer, 1.25 U of Taq polymerase, and 2 μl of 10X buffer. The PCR was run following this program: pre-denaturation 5 min at 94 °C followed by 30 cycles of 1 min at 94 °C, 30 s at 60 °C, 30 s at 72 °C, and finally 10 min at 72° C. Templates and primers used in PCR are listed in Table 1.

Table 1. Templates, sequence of primer pairs, and amplicon size of different PCR products

Primer	Sequence*	Template Amplicon no. ** (size)
SHSP11F	5'-TTCACTAGTGGTCCTCGGGATATGATAACA-3'	pMG36
SHSP11R	5'-CGTGGTTGAATCTTATTTAACATTTCAAAATTCCTCCGAATATTTTTTT-3'	Amplicon 1, (209 bp)
SHSP12F	5'-ATGTTAAATAAGATTCAACCACG-3'	Plasmid of strain St-QC
SHSP12R	5'-TTACTTTAAGAATACCATCTGAAT-3'	Amplicon 2, (376 bp)
SHSP13F	5'-ATTCAGATGGTATTCTTAAAGTAA-3'	Plasmid of strain St-QC
SHSP13R	5'-CTGCAGACCTTGTGGTGGCTCTCCTA-3'	Amplicon 3, (299 bp)
SHSP1FF	5'-CCGGAATTCACTAGTGGTCCTCGGGA-3'	Amplicons 1, 2 and 3
SHSP1RR	5'-GCATGCCTGCAGACCTTGTGGTGGC-3'	Amplicon 4, (836 bp)

*: Underlined sequences are the restriction endonuclease sites of *Spe*I in primer SHSP11F, *Eco*RI in primer SHSP1FF, and *Pst*I in primer SHSP1RR.
**: (i) Amplicon 1: Contains the p32 promoter of pMG36e; (ii) Amplicon 2: From the start codon of *shsp* gene to restriction endonuclease *Eco*RI site; (iii) Amplicon 3: From the *Eco*RI site of *shsp* gene to stop codon; (iv) Amplicon 4: Using a mixture of amplicon 1-3 as templates and SHSP1FF/SHSP1RR as primers, a 836-bp fragment containing a p32 promoter and a complete *shsp* gene was amplified.

Construction, transformation, and transformation efficiency

The *shsp* ORF cloning and *E. coli* transformation were performed according to the methods of Sambrook *et al*. (17). pMG36e-shsp was constructed by the procedure shown in Fig. 1. The amplicon 4 (Table 1) was digested with *Pst*I/*Eco*RI and then inserted into the same restriction sites of plasmid pMG36e followed by transformation into *E. coli* DH5α competent cells. The resultant plasmid pMG36e-shsp was isolated using EasyPure plasmid MiniPrep Kit (Beijing TransGen Biotech Co., Ltd.) and was transformed into *L. lactis* ML23 by electroporation at 2.5 kV/cm, 200 Ω, and 25 μF in a Bio-Rad GenePulser, and the cells were then diluted with 1 ml of regeneration medium (LM17 medium plus 0.5 M sucrose, 20 mM $CaCl_2$, and 20 mM $MgCl_2$). The competent *L. lactis* ML23 cells were prepared followed the protocol below. *L. lactis* ML23 cells were cultured in GM17 medium (M17 medium supplemented with 0.5% glucose) at 30 °C overnight. 100 ml of SGM17 medium (GM17 medium supplemented with 2% glycine and 0.5 M sucrose) was inoculated with 5% of the overnight culture. When cells grew to an OD600 of 0.3-0.4 at 30 °C, cells were harvested by centrifugation at 5,500 x g and 4°C for 15 min. The pellets (cells) were washed with ice-cold electroporation buffer (0.5 M sucrose and 100 $g.ml^{-1}$ glycerol) twice and were then resuspended in the same buffer at a final OD600 of 2. Cells were frozen in aliquots of 100 μl in liquid nitrogen and stored at -80°C.

After electroporation, two procedures were followed to determine the transformation efficiency of pMG36e-shsp. (I) When erythromycin was taken as the selection marker, the cells were incubated at 30 °C for 3 h and plated on LM17 medium plus 5 $\mu g.ml^{-1}$ of erythromycin and cultivated for 48 h. (II) When the *shsp* gene was used as the selection marker, the cells were plated on LM17 plates and incubated for one day at 41 °C followed by 24h incubation at 37 °C. The number of the positive colony-forming units (CFU) was counted and the transformation rate ($CFU.\mu g^{-1}$) was calculated based on the results of three experiments.

A food-grade vector, pMG-shsp, was constructed based on pMG36e-shsp (Fig. 1). After digestion with *Spe*I and *Nhe*I to remove the erythromycin resistance gene (Em^r), the large fragment of pMG36e-shsp was self-linked leading to the generation of pMG-shsp. The transformation and determination of transformation rate were performed according to the protocol described.

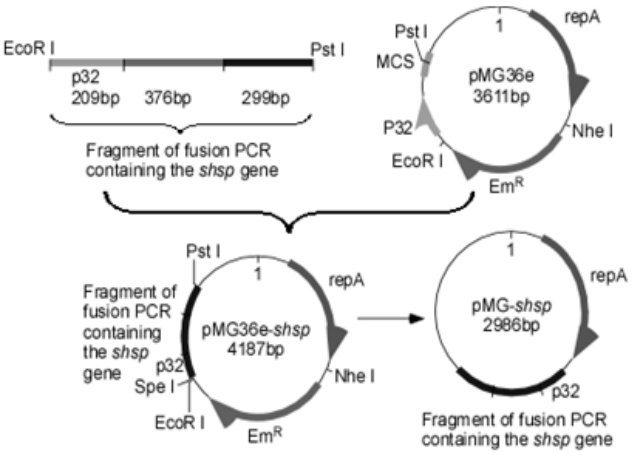

Figure 1. The schematic strategy for construction of the expression vector pMG36e-shsp and the food-grade vector pMG-shsp. Briefly, the 209 bp long fragment was amplified by PCR using pMG36e as template. The 376 bp and 299 bp long fragments were obtained through PCR using plasmid pQC2 (20) as template. Then, to generate the fusion fragment containing the p32 promoter and the full length *shsp* ORF with a *Eco*R I cutting site on the 5' terminus and a *Pst* I cutting site on the 3' terminus, an overlapping PCR was performed, using these three PCR products as templates and the primers SHSP1FF/SHSP1RR. All primers used in these procedures were listed in Table 1. Then pMG36e-shsp was constructed by replacing the *Eco*R I/*Pst* I fragment of pMG36e with the overlapping PCR product. Finally, the food-grade expression vector pMG-shsp was generated by deleting the Em^r gene from pMG36e-shsp.

sHSP expression analysis by SDS-PAGE

Cells of *L. lactis* strain ML23 (pMG-shsp) were washed twice in 50 mM sodium phosphate buffer (pH 7.0), frozen and thawed thrice, and finally centrifuged to collect the soluble protein fraction in the supernatant. The soluble protein samples containing sHSP were dissolved in a sample buffer (25 mM Tris-HCl 2% (w/v) SDS, 47 mM 2-mercaptoethanol and 10% (v/v) glycerol pH 6.8) and run in 12.5% SDS-PAGE gel (11).

Growth studies

L. lactis strains ML23, ML23 (pMG36e), and ML23 (pMG-shsp) were inoculated (2%, v/v) in 100 ml of LM17 medium and incubated at 37 °C. The growth of the strains was monitored turbidimetrically at 600 nm at 2 h intervals using a UV-2102 PC spectrum (Unicol Shanghai Co. LTD).

Stress challenge

Cells of *L. lactis* strain ML23 (pMG-shsp) and ML23 (pMG36e) were cultured in LM17 medium. Cells in the exponential growth phase were harvested at an OD600 of 0.4–0.5 (corresponding to about 2 X 10^8 CFU.ml^{-1}), washed with 0.9% NaCl and suspended in fresh LM17 medium to an OD600 of 1.0. Each culture (10 ml) received one of the following treatments: (a) 42 °C, (b) pH 3.0, (c) 0.3% bile salt (Bovine, Shanghai Solarbio Bioscience & Technology Co., LTD) (d) 2 mM H_2O_2, and (e) 10% (v/v) ethanol. The temperature for treatments (b) through (e) was 30 °C. Every 30 min, 100 µl of samples was collected and diluted to 10^{-2}–10^{-7} and then plated on LM17 agar. The survival rate of cells was monitored by counting CFU after incubation at 30 °C for 48 h. All experiments were carried out in triplicate.

RESULTS AND DISCUSSION

Construction of the expression vectors, pMG36e-shsp and pMG-shsp.

As Fig. 1 shown, an overlapping PCR fragment containing both the p32 promoter and the *shsp* ORF was inserted into pMG36e by replacing the corresponding fragment between the *Eco*R I/*Pst* I cleavage sites to construct the expression vector pMG36e-shsp. This strategy allows the inserted *shsp* ORF to express as an independent protein instead of a fusion protein containing the small peptide (Fig. 1). Furthermore, a food-grade expression vector pMG-shsp was constructed by deleting the *Em*^{r} gene from pMG36e-shsp (Fig. 1). The genetically engineered strain (*L. lactis* ML23 (pMG-shsp)) and the control strain *L. lactis* ML23 were cultured and the soluble protein fraction prepared from their cells were subjected to SDS-PAGE analysis. As shown in Fig. 2, compared with the pattern of soluble proteins from *L. lactis* ML23 (Fig. 2, lane 2), an extra protein band (Fig. 2, lane 1, labeled with sHSP) between the standard 14.3 kDa and 20.1 kDa proteins was present in the *L. lactis* ML23 (pMG-shsp) sample. Its size was consistent with the molecular weight of the sHSP (16.4 kDa) encoded by a 429-bp long *shsp* ORF which was inserted into pMG-shsp. This result indicated that the sHSP encoded by the food grade vector pMG-shsp was successfully induced by the p32 promoter and expressed in mesophilic *L. lactis* ML23. Moreover, the growth of *L. lactis* ML23 (pMG-shsp) at 41 °C for one day implied that the sHSP conferred resistance to high temperature stress. On the other hand, the growth curves of transformant ML23 (pMG-shsp), transformant ML23 (pMG36e) and strain ML23 (shown in Fig. 3A) revealed that the expression of the *shsp* ORF and the deletion of *Em*^{r} gene didn't affect the characteristic of growth of transformant ML23 (pMG-shsp) cultured at the normal temperature.

Constructing a food-grade selection vector is important to the fermented food industry. The *shsp* gene from *S. thermophilus* has its own merits being both a selection marker and a target expression gene. Firstly, sHSP encoding by a *shsp* gene cloned from *S. thermophilus* is believed safe to food since its host is usually used in the fermented food industry. Secondly, sHSP can elevate microbial tolerance to different severe stresses and help them survive under stresses to which LAB are sensitive. Finally, screening for positive transformants is made simple and easy by increasing the culture temperature to 41-42 °C. These are why the *shsp* gene was chosen as a selection marker in constructing a food-grade vector in this study. These considerations have been shared with previous research that the *shsp* gene from pST04 plasmid was used as a food-grade selection marker in *S. thermophilus* and *L. lactis* (3).

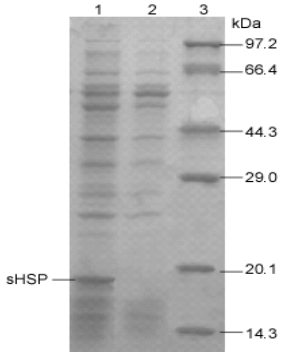

Figure 2. The expression of the *shsp* ORF. Lane 1: The soluble proteins from ML23 (pMG-shsp), Lane 2: The soluble proteins from ML23 (control strain); Lane 3: Protein standard marker

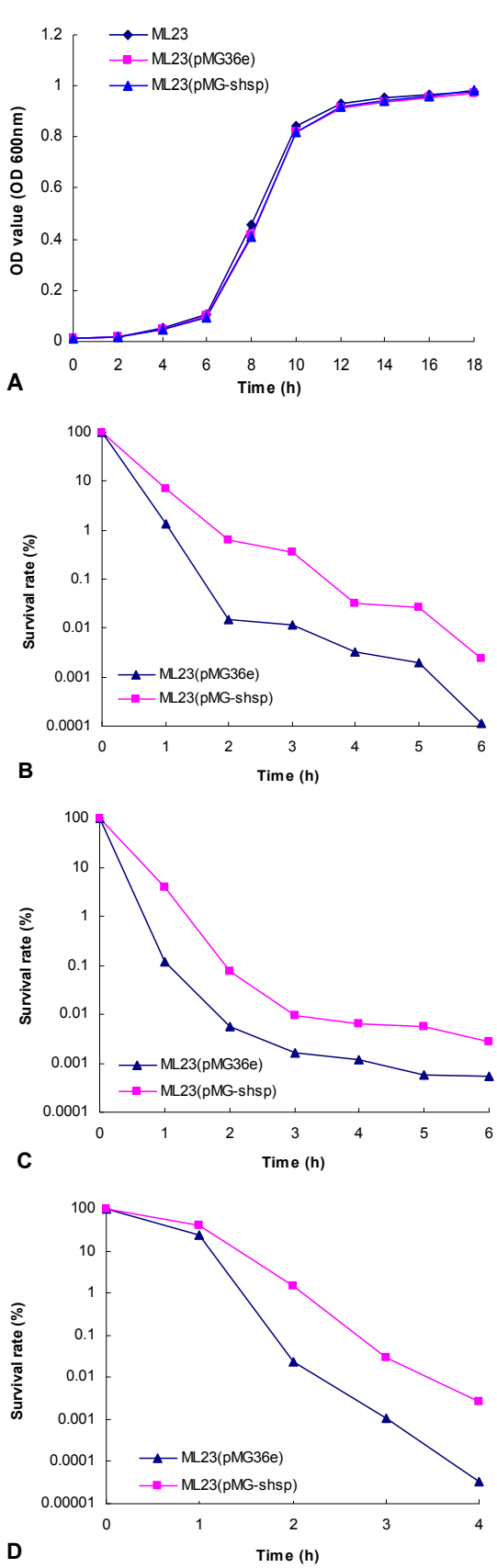

Figure 3. The growth curve (A) and the survival rate (B-F) of ML23 (pMG36e), ML23 (pMG-shsp) under normal condition and under the severe stresses: A. Normal culture condition; B. Heat stress (42 °C); C. Acid stress (pH 3.0); D. Ethanol stress (10% ethanol); E. Bile-salt stress (0.3%); F. 2 mM H_2O_2.

Determination of transformation efficiency of pMG36e-shsp and pMG-shsp

The transformation efficiency of pMG36e-shsp was determined by Em^r-based selection system and shsp-based selection system. The transformation efficiency of pMG36e-shsp for mesophilic *L. lactis* ML23 in Em^r-based selection system was 1.22 X 10^4 CFU per μg DNA with a standard deviation of 1.92 X 10^3 CFU.μg^{-1} DNA. The average transformation rate of pMG36e-shsp in the shsp-based selection system was 1.15 X 10^4 CFU.μg^{-1} with a standard deviation of 2.18 X 10^3 CFU.μg^{-1} DNA of pMG36e-shsp, which was comparable to those obtained with the Em^r selection

method. When the shsp-based selection system was applied to the pMG-shsp, the transformation rate increased to 1.32 X10^4 CFU.μg^{-1} with a standard deviation of 2.02 X 10^3 CFU.μg^{-1}. These data indicated that the temperature selection method based on the expression of the *shsp* gene in *L. lactis* could be applied to positive transformant screening with high efficiency that the antibiotics selection method had. This is consistent with the conclusions of El Demerdash *et al.* (3). Moreover, this research may have two potential merits. (I) Two restriction enzymes in multiple cloning sites were left on the vector pMG36e-shsp and pMG-shsp that will allow the exogenous gene to be inserted. (II) Although the transformation rate obtained in this study is lower than what El Demerdash reported, that the size of pMG-shsp (2.986 kb) is much smaller than that of pHMR1 (6.4 kb) implies that the transformation rates of pMG-shsp can be increased at least up to that of pHMR1 obtained by El Demerdash (3) and may be increased further if the transformation conditions are optimized.

The transformant efficiency of both new constructed vectors indicated that both expression vector pMG36e-shsp and food-grade vector pMG-shsp could be successfully used as a selection vector to generate transgenically engineered *s*train used in the fermentation industry. Furthermore, we could also impart new features of stress-resistance to LAB by introducing pMG-shsp harboring the corresponding gene to improve their fermentation ability.

The expression of the *shsp* ORF increased survival rate of *L. lactis* under environmental stresses

After determining the effect of new vectors containing the *shsp* ORF on the transformation efficiency of *L. lactis*, the expression of the *shsp* ORF on the growth of *L. lactis* under different conditions was studied. Under normal growth condition, the growth curves of genetically engineered strain ML23 (pMG-shsp) was similar to that of transformed strain ML23 (pMG36e) as well as the control strain ML23 (Fig. 3A). Lag phase and logarithmic phase lasted about 6 h. Then, all strains entered the stationary phase of growth. These results demonstrate that expressing the *shsp* gene with or without expressing *Em^r* gene in *L. lactis* had no negative effects on its growth.

The growth of *L. lactis* expressing the *shsp* ORF under different stress conditions was detected. We demonstrated that the expression of the exogenous *shsp* ORF in *L. lactis* could help the positive transformants tolerate moderately high temperature stress (incubating and screening at 41 °C). sHSP over-expressed in the engineered strain *L. lactis* ML23 (pMG-shsp) might have enhanced tolerance to other stresses in addition to high temperature. In these experiments, each of the stresses applied was defined as extremely lethal because it was able to kill above 90% of the cells in the first hour of exposure. After 4 h exposure to different stresses, significant differences in the survival rate against the stresses between *shsp* transgenic *L. lactis* strain and its control strain were observed. The survival rate of ML23 (pMG-shsp) was significantly higher than that of ML23 (pMG36e) under high temperature 42 °C, pH 3.0, 10% ethanol, 0.3% bile-salt, or 2 mM H_2O_2 condition as shown in Figs. 3B, C, D, E, and F. At the treatment time of 4 h, the survival ratios of *L. lactis* ML23 (pMG-shsp) vs. *L. lactis* ML23 (pMG36e) were 0.032% vs. 0.003% (Fig. 3B), 0.006% vs. 0.001% (Fig. 3C), 0.0027% vs. 0.000033% (Fig. 3D), 0.03% vs. 0.000024% (Fig. 3E), or 0.16% vs. 0.00023% (Fig. 3F). Therefore, the survival rate of ML23 (pMG-shsp) was 10.7, 6, 81.8, 1250, or 695.7 times higher than that of ML23 (pMG36e). These data indicated that the resistance of LAB to the environmental stresses, especially to ethanol, bile salt, and H_2O_2, could be conferred by the expression of the *shsp* ORF.

Previous studies have shown significant differences in cell survival rate between the *S. thermophilus* strains harboring plasmid carrying *shsp* gene and plasmid eliminating *shsp* gene under heat shock condition (16). By introducing pMG-shsp into *L. lactis* strain ML23, the expression of the *shsp* ORF significantly increased the survival rate of ML23 (pMG-shsp) compared to the transformants harboring the control vector

pMG36e (ML23 (pMG36e)) under each of the stresses. The data are consistent with the conclusions that the *shsp* gene from *S. thermophilus* could protect cells in different environmental stresses (3), and reveals that the sHSP may help to increase the survival rate of cells under bile salt and H_2O_2 stresses, which had not been reported before. These findings imply that sHSP can normally be expressed in *L. lactis* and possibly act as a chaperone to protect cells. The process of sHSP as a chaperone might be accomplished by binding denatured cell proteins and thereby suppressing inappropriate interactions leading to the precipitation of aggregates (9, 14). They continue to maintain the damaged proteins in a "folded-competent state" (12).

In summary, an expression vector, pMG36e-shsp and a food-grade vector, pMG-shsp, were constructed based on the *shsp* ORF cloned from *S. thermophilus*. The expression of the *shsp* ORF in *L. lactis* could not only be used as a food-grade selection marker, but also to improve the survival rate of *L. lactis* against different severe stresses, especially bile salt and H_2O_2 stress that first reported here.

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