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Full Length Research Paper

Cloning and heterologous expression of the plasmid-encoded *shsp* gene of *Streptococcus thermophilus* isolated from Chinese dairy

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We first tested 12 strains of *Streptococcus thermophilus* isolated from China yogurt or its starter culture for their plasmid content. Two strains were found to harbor two plasmids, identified as pQC1 and pQC2, from the *S. thermophilus* ST-QC, as well as pHS1 and pHS2 from the *S. thermophilus* ST-HS. Agarose gel electrophoresis profiles indicated that the molecular size of about 4.5 kb for the two bigger plasmids (pQC1 and pHS1) was nearly identical, same with the molecular size of approximately 3.5 kb for the two smaller plasmids (pQC2 and pHS2). A 765 bp DNA fragment, including a 429 bp open reading frame of the *shsp* gene of the smaller plasmid pQC2 from *S. thermophilus* St-QC, was successfully cloned and sequenced. Multiple sequence alignment revealed that the nucleotide sequence of the coding region of the *shsp* gene or the deduced amino acid sequence of the sHSP protein shared a high degree of identity (> 86.67 or 81.33% identity) with the *shsp* genes or the sHSP proteins described from other *S. thermophilus* plasmids. In addition, 222 bp nucleotide sequences upstream and 114 bp nucleotide sequences downstream belonging to the *shsp* gene coding region were also analyzed. The separation of SDS-PAGE and the analysis of Western blotting for the soluble cell proteins showed that the s*hsp* gene of plasmid pQC2 of *S. thermophilus* St-QC was successfully expressed in mesophilic *Escherichia coli*. In addition to strong heat and acid tolerance, recombinant *E. coli* cells overexpressing the *S. thermophilus* St-QC *shsp* gene had significantly higher resistance to ethanol stress, which is the first physiological function found to be linked to the *S. thermophilus* plasmid-borne *shsp* gene. This study will provide a basis for the cloning and expression of the s*hsp* genes from a thermophilic microorganism in the mesophilic LAB or yeast and for further development of stress-resistant microorganism strains used in dairy fermentation and brewing wine.

Key words: *Streptococcus thermophilus*, plasmid, small heat shock protein gene (*shsp* gene), cloning, expression, abiotic stresses, *Escherichia coli*.

INTRODUCTION

Heat shock proteins (HSPs), also called stress proteins, are a set of proteins preferentially synthesized when cells are exposed to high or low temperature, anoxic environment, heavy metal, microorganism infection and other stress conditions (Cooper and Ho, 1983; Qian and Yu, 1989). Small heat shock proteins (sHSPs) (Haslbeck et al., 2005) with a molecular mass range of 12～43 kDa are demonstrated by a great number of experiments that they comprise a stress-inducible group of molecular chape-rones that can bind to the denatured proteins to prevent the latter irreversible aggregation and maintain them in a refolding state (Geis et al., 2003; O'Sullivan et al., 1999).

The plasmid containing a sHSP (HSP16.4) with a

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Abbreviations: HSPs, Heat shock proteins; **LAB,** lactic acid bacteria; **SDS-PAGE,** sodium dodecyl sulfate; **PCR,** polymerase chain reaction.

potential role in temperature-regulated functions was first isolated from dairy fermentation of *Streptococcus thermophilus* (*S. thermophilus*) in 1998 (Somkuti et al., 1998). Up until recently, all *shsp* genes described in *S. thermophilus* are localized in plasmids (O'Sullivan et al., 1999; Somkuti and Steinberg, 1999; Solow and Somkuti, 2000; Su et al., 2002; Geis et al., 2003; El Demerdash et al., 2003; Makarova et al., 2006), although only less than 20% of *S. thermophilus* have plasmids in contrast to the mesophilic dairy *lactococci* which are believed widely to contain plasmids (Janzen et al., 1992). Among the plasmid-bearing strains of *S. thermophilus*, most of them generally contain one plasmid (Sudhamani et al., 2008; O'Sullivan et al., 1999; Su et al., 2002; Geis et al., 2003; El Demerdash et al., 2003; Petrova et al., 2003), whereas only a few strains carry two plasmids (Somkuti and Steinberg, 1986; Somkuti et al., 1998; Somkuti and Steinberg, 1999; Janzen et al., 1992; Geis et al., 2003; Makarova et al., 2006). Although the role of plasmid DNA in *S. thermophilus* has not yet been fully understood, some of them were found to carry genes encoding replication (Rep) proteins and genes encoding sHSPs (Somkuti et al., 1998; Derré et al., 1999; Solow and Somkuti, 2000; El Demerdash et al., 2003) or enzymes of restriction-modification systems (O'Sullivan et al., 1999; Janzen et al., 1992). More than half of *S. thermophilus* plasmids encode only sHSPs and replicative proteins (Petrova and Gouliamova, 2006).

Expression and some physiological functions of genes encoding sHSPs from *S. thermophilus* plasmids have been reported. The expression of *shsp* in cells was increased when *S. thermophilus* strain containing plasmid encoding *shsp* was subjected to heat or acid stress (Somkuti et al., 1998; Solow and Somkuti, 2000). The *S. thermophilus* parent strain carrying plasmid-encoding *shsp* was significantly more resistant to a temperature shift from 42 to 62°C compared to its variant strain lacking plasmid-encoding *shsp* (O'Sullivan et al., 1999). By heat shock (52°C, 30 min) or acid shock (pH 4.5, 30 min) treatment, *S. thermophilus* cells carrying plasmid-encoding *shsp* significantly increased their thermo- and acid resistance (Geis et al., 2003). *S. thermophilus* strains cured from the *shsp* plasmids showed significantly reduced heat and acid resistance as well as lower maximal growth temperature (El Demerdash et al., 2003). The transformation of the cloned *shsp* gene into the *S. thermophilus* strain lacking plasmid-encoding *shsp* resulted in an increased resistance to incubation at 60°C or pH 3.5 and in the ability to grow at 52°C (El Demerdash et al., 2003). Significant differences in cell survival rates between carrying *shsp* plasmid and eliminating *shsp* plasmid of the *S. thermophilus* strain under heat shock condition have also been observed (Petrova and Gouliamova, 2006). *Escherichia coli* (*E. coli*) cells overexpressing foreign *shsp* genes displayed greater resistance to heat (Yeh et al., 1997; Fernando and Heikkila, 2000; Satoru, 2006; Guo et al., 2005; Yan et al., 2007), cold (Soto et al., 1999; Guo et al., 2005) and acid (Jobin et al., 1999) when compared with wild-type cells. The transgenic *Saccharomyces cerevisiae* cells expressing the *Trichoderma harzianum hsp24* gene had significantly higher resistance to salt, drought and heat stresses (Yang et al., 2008). However, very little is known about the expression and physiological functions of the s*hsp* genes from thermophilic microorganisms (for example *S. thermophilus*) in mesophilic microbacterium (such as *E. coli* and dairy lactococci, among others). Meanwhile, although the *shsp* gene of *S. thermophilus* plasmid pSt04 was successfully expressed in *Lactobacillus casei*, the physiological functions of the heterologous expression of the *shsp* gene from *S. thermophilus* plasmid pSt04 was not described (Sudhamani et al., 2008).

During the recent decade, the fermented dairy industry has been developing rapidly in China, whose output of fermented dairy products was increased by 20 ~ 30%. To explore whether *S. thermophilus* strains from China yogurt or its starter culture contains plasmids, especially those that carry genes encoding sHSPs and furthermore, to investigate more functions of sHSP of *S. thermophilus*. In this study, we examined 12 *S. thermophilus* strains isolated from China yogurt or its starter culture for their plasmid content and found that two of them have plasmids containing *shsp* gene. The *shsp* gene from the *S. thermophilus* plasmid was cloned and identified before being expressed in mesophilic *E. coli*. The physiological functions, including resistance to heat, acid and ethanol stress, of recombinant *E. coli* cells overexpressing the *shsp* gene from the *S. thermophilus* plasmid were further investigated. This study will provide a basis for the cloning and expression of s*hsp* genes from thermophilic microorganism in mesophilic LAB or yeast, leading to the further development of stress-resistant microorganism strains used in dairy fermentation and brewing wine.

MATERIALS AND METHODS

Bacterial strains, culture conditions, plasmids, enzymes and chemicals

S. thermophilus strains ST-99201, ST-QC, ST-2, ST-3, ST-G, ST-LDY, ST-TX, ST-SY519, ST-HS, ST-JY, ST-LT and ST-MH, all of which were identified and deposited in our laboratory, were isolated from yogurt, or its commercial starter culture, locally produced in China. *S. thermophilus* cells were grown at 37°C in an M17 medium (Terzaghi and Sandine, 1975) supplemented with 5 g/l lactose sterilized by filtration (LM17) and adjusted to pH 6.5 or in an MRS broth (pH 6.8) (Deman et al., 1960) without shaking. Competent *E. coli* DH5 and competent *E. coli* BL21 (DE3) (TIANGEN Biotechnology Company Limited, Beijing, China) were used as host for cloning and expressing the *shsp* gene, respectively. *E. coli* cells were cultivated at 37°C in a Luria-Bertani (LB) medium (Laigret et al., 1996) supplemented with 100 mg/ml ampicillin for selecting DH5α transformants and 100 mg/ml kanamycin for screening BL21 transformants under aerobic conditions. X-Gal and IPTG were utilized at concentrations of 40 and 0.16 mmol/l (40 µg/ml), respectively. When used as agar plates, all media were solidified

with 1.5% agar (Difco). Vector pGM-T (TIANGEN Biotechnology Company Limited, Beijing, China) and pET-30a (Novagen, Germany) were used for cloning and expressing the *shsp* gene, respectively. Pyrobest DNA Polymerase and common Taq DNA Polymerase (TaKaRa, Dalian, China) were used for the PCR amplification of the *shsp* gene. Restriction endonucleases BamHI and HindIII (TaKaRa, Dalian, China) were used for double enzymes restriction digests of DNA fragments and vector pET-30a. T4 DNA ligase (TIANGEN Biotechnology Company Limited, Beijing, China) was used for the ligation of DNA fragments into vectors. All other chemicals were of analytical grade and obtained from local commercial resources.

Isolation and survey of plasmids from *S. thermophilus* strains

Plasmids from *S. thermophilus* were isolated from 18 h old cultures by a standard procedure of Somkuti and Steinberg (1986). Plasmid DNA was examined through 8 g/L agarose gel electrophoresis (AGE) in TAE buffer [40 mmol/l Tris-base, 1.14 ml/l (19.8 mmol/l) ice acetic acid and 1 mmol/L Na-EDTA (pH 8.0)] at a setting of 60 to 80 V (1 to 5 V/cm) for approximately 20 min at room temperature, after which it was visualized by UV light (254 nm). A supercoiled DNA ladder marker was purchased from TaKaRa (Dalian, China) and was used to estimate the size of plasmids in kilobases (kb). *L. lactis* MG1614 carrying the pLEB590 plasmid, which we used as a positive control, was kindly provided by Professor Saris (Department of Applied Chemistry and Microbiology, Division of Microbiology, University of Helsinki, Finland).

Cloning and sequencing of the shsp gene containing its 5′ and 3′ flanking regions from the *S. thermophilus* plasmid

To amplify a 765 bp DNA fragment, including a 429 bp open reading frame of *shsp* gene of the smaller plasmid pQC2 from *S. thermophilus* St-QC, upstream primer1 (5'-AGGTTTGTTTGTTCGT TTTGCG-3') and downstream primer2 (5'-GGGCTTTTTAGAGGG CTTTAG-3') were designed on the basis of the reported *shsp* gene regions' plasmid nucleotide sequences (GenBank accession numbers AJ242476 and NC008501) from other *S. thermophilus*. For the PCR experiments, template DNA, plasmid pQC2 from *S. thermophilus* St-QC, was separated through 8 g/L agarose gel electrophoresis and purified using the common agarose gel DNA midi purification kit (TIANGEN Biotechnology Company Limited, Beijing, China) before having it spectro photometrically quantified. Using a PCR thermal cycler (Hybaid Limited, Britain), PCR amplification was carried out in a 20 µl reaction system containing 2 µl 10×Pyrobest DNA Polymerase PCR Buffer, 2 µl dNTP (2.5 mmol/L, each), 1 µL primer1 (10 µmol/L, synthesized by Shanghai Biotechnology Company, Ltd., Beijing Filiale, China), 1 µl primer2 (10 µmol/l, synthesized by Shanghai Biotechnology Company, Ltd., Beijing Filiale, China), 3 µl template DNA (about 30 ng) and 0.5 µL Pyrobest DNA Polymerase (5 U/µL) to which ddH_2O was added. A cooling manipulation method at 0℃ was used in the initiation for PCR amplification. A polymerase chain reaction (PCR) program comprised an initial denaturation at 95℃ for 5 min, followed by 30 cycles each of denaturation carried out at 94℃ for 30 s, annealing at 60℃ for 30 s, primer extension at 72℃ for 1 min and a final extension at 72℃ for 10 min.

Afterwards, the PCR fragment with blunt ends was separated through 12 g/l agarose gel electrophoresis and purified using a common agarose gel DNA midi purification kit. This was further amplified by PCR using Taq DNA Polymerase for 20 min at 72℃ in order to carry adenine nucleotide on their 3′ ends. Amplified fragment was separated and purified as described above, before being ligated into cloning vector pGM-T with T4 DNA ligase overnight at 4℃ as recommended by the manufacturer. Ligation mixtures were transformed into competent *E. coli* DH5α by a 90 s heat shock at 42℃, in accordance with the suppliers' instructions. Transformed cells were then spread on Luria-Bertani (LB) agar plates supplemented with 100 mg/ml ampicillin, 40 µg/ml X-gal and 0.16 mmol/l (40 µg/ml) IPTG. These were then cultivated at 37℃ for 16 to 24 h. Positive clones of *E. coli* DH5α containing recombinant plasmid were screened by blue/white plaques selection and then grown overnight in an LB medium containing 100 mg/ml ampicillin at 37℃ with shaking (200 r/min). Recombinant plasmids from *E. coli* DH5α were also isolated by an alkaline lysis protocol (Sambrook and Russell, 2001) before being purified as described above. These were then sent to Shanghai Biotechnology Company, Ltd. (Beijing Filiale, China), where the sequencing reactions were performed.

Sequence analysis

Designing primers and searching for restriction endonuclease cleavage sites for nucleotide sequence were carried out using the Primer 5.0 software. Sequence similarity searches and comparesons with other known DNA sequences or DNA-derived protein sequences were made with BLAST N and BLAST P programs. Multiple sequence alignments were performed using the DNAman software, while open reading frame and molecular mass were predicted using the DNAman program. The theoretical isoelectric point of protein was calculated through the Gene Runner software.

Recombinant expression of the shsp gene in *E. coli* and preparation of cell soluble proteins

The coding region for the *shsp* gene was amplified from the smaller plasmid pQC2 of *S. thermophilus* St-QC by PCR using upstream primer3 (5'-CGGGATCCATGTTAAATAAGATTCAACC-3') and downstream primer4 (5'-CCCAAGCTTGCTCAATTGGAATAGA TTTT-3') with BamHI and HindIII restriction sites (underlined) on their 5′ ends, respectively. Afterwards, the PCR product was separated and purified as described above.

Amplified product and expression vector pET-30a (Novagen, Germany) were digested by BamHI and HindIII at 37℃ for 3 h and then separated and purified as described above. Double enzyme-digested PCR product was ligated into the BamHI and HindIII restriction sites of the expression vector pET-30a using T4 DNA ligase overnight at 4℃. Ligation mixtures were transformed into competent *E. coli* DH5α by a 90 s heat shock at 42℃. The transformed cells were spread on Luria-Bertani (LB) agar plates supplemented with 100 mg/ml kanamycin, 40 µg/ml X-gal and 0.16 mmol/l (40 µg/ml) isopropylthiogalactoside (IPTG). These were then cultivated at 37℃ for 16 to 24 h. Positive clones of the *E. coli* DH5α containing recombinant plasmid were screened by blue/white plaques selection and grown overnight in an LB medium containing 100 mg/ml kanamycin at 37℃ with shaking (200 r/min). Recombinant plasmids from *E. coli* DH5α were isolated and purified before being verified by DNA sequencing as described above. This recombinant plasmid was designated as pET-*shsp*, which contained the *shsp* coding region with the hexahistidine tag under the control of a T7 promoter as regulated by an IPTG inducible operator sequence.

Recombinant plasmid pET-*shsp* and control plasmid pET-30a were transformed into competent *E. coli* BL21 (DE3) by a 90 s heat shock at 42℃, in accordance with the suppliers' protocol. Transformed cells were spreaded on Luria-Bertani (LB) agar plates supplemented with 100 mg/ml kanamycin, 40 µg/ml X-gal and 0.16 mmol/l (40 µg/ml) IPTG. These were then cultivated at 37℃ for 16 to 24 h. The positive *E. coli* BL21 (DE3) clones containing recombinant plasmid pET-*shsp* and control plasmid pET-30a were screened by blue/white plaques selection, after which they were

grown overnight in an LB medium containing 100 mg/ml kanamycin at 37℃ with shaking (200 r/min). These were then identified by PCR using primer3 and primer4, as well as double enzyme digestion using BamHI and HindIII.

E. coli BL21 (DE3) containing recombinant plasmid pET-*shsp* and control plasmid pET-30a were grown overnight in an LB medium containing 100 mg/ml kanamycin at 37℃ with shaking (200 r/min). These were diluted 50 times with the same medium and continuatively grown to the OD600 of 0.5 ~ 0.8 under the same conditions. Expression of recombinant protein was induced by adding IPTG to a final concentration of 1.0 mmol/L at 30℃ for 3 h.

After inducing the expression of recombinant protein, *E. coli* BL21 (DE3) cells from 50 ml cultures were harvested by centrifugation at 7,000 r/min for 5 min at 4℃, washed twice with 10 ml of chilled PBS buffer (8 g/l NaCl, 0.2 g/l KCl, 1.44 g/l Na_2HPO_4, 0.24 g/l KH_2PO_4 and pH 7.4) and then resuspended in the same buffer to a final volume of 2 ml. Cells were maintained in an ice bath and disrupted by sonication using the micro tip at 400 W for 10 pulses of 10 s, with 10 s intervals after each pulse. The soluble protein fraction of the supernatant was obtained after centrifugation at 9,000 r/min for 5 min at 4℃, after which protein content was determined by the method of Bradford (1976) (Bradford, 1976) .

SDS-PAGE

Protein samples were mixed with equal volumes of 2 × loading buffer (0.765% Tris-base, 10% glycerol, 2% SDS, 5%β-mercaptoethanol and 0.1% bromophenol blue), boiled for 5 min and then centrifuged at 9,000 r/min for 1 min. Protein samples mixed with the loading buffer (20 µl) were separated by SDS-PAGE according to the method of Laemmli (1970) (Laemmli, 1970). Molecular masses of protein were estimated with blue plus protein marker (purchased from TransGen Biotechnology Company, Ltd., Beijing, China). Protein samples expressed in *E. coli* BL21 (DE3) containing plasmid pET-30a by the induction of IPTG were used as the control.

Western blotting

Protein samples separated by SDS-PAGE were analyzed by Western blotting as recently described by Sudhamani et al. (2008). Mouse anti-histidine antibody (purchased from TIANGEN Biotechnology Company Limited, Beijing, China) was diluted at 1:2,000 as primary antibody. Alkaline phosphatase-conjugated horse anti-mouse-IgG antibody (purchased from ZhongShan-JinQiao Biotechnology Company, Ltd., Beijing, China) was used as a secondary antibody at a dilution of 1:1,000. Alkaline phosphatase was detected using a visualized kit of BCIP (5-bromo-4-chloro-3-indolyl-phosphate)/NBT (nitroblue tetrazolium) (purchased from TIANGEN Biotechnology Company Limited, Beijing, China).

Growth studies

Growth characteristics of *E. coli* BL21, *E. coli* BL21 (pET-30a) and *E. coli* BL21 (pET-*shsp*) cultures in LB media were examined. Cultures were sub-cultured twice in this medium, inoculated (2%) into 100 ml of the same medium and then grown at 37℃. Growth was monitored turbidimetrically at 600 nm at 1 h intervals.

Resistance to abiotic stresses of shsp gene in transgenic *E. coli* cells

To test whether the *shsp* gene confers stress resistance on the transgenic *E. coli* cells, *E. coli* BL21 (pET-*shsp*) and control strain *E. coli* BL21 (pET-30a) were cultured in an LB medium containing 100 mg/ml kanamycin at 37℃ with shaking (200 r/min). Cells were grown to the OD600 of 0.5 ~ 0.8; afterwards, IPTG was added to the final concentration of 1.0 mmol/l, further inducing the cells at 30℃ for 3 h. The induced cells were havested by centrifugation at 9000 r/min for 5 min, washed twice with 0.9% NaCl and then resuspended in 10 ml of 0.9% NaCl with different treatments for stress experiments, to the OD600 of 1.0, respectively. For heat stress, cells resuspended in 10 mL of 0.9% NaCl were exposed to 52℃ in a water bath for 4 h. Acid stress was imposed by resuspending cells in 10 ml 0.9% NaCl adjusted to pH 3.5 with lactic acid, after which they were incubated at 37℃ for 4 h. For ethanol stress, cells were resuspended in 10 mL of 0.9% NaCl, to which 12% (v/v) absolute ethanol was added and then incubated at 37℃ for 4 h. Every 1 h, 100 µl samples were taken, diluted to 10^{-2} to 10^{-7} and then spread on LB agar plates. The percentage survival of cells was monitored by counting the colony-forming units (CFU) after incubation at 37℃ for 48 h. All experiments were done in triplicate and the mean values were identified.

RESULTS

Examination and analysis of plasmids from the *S. thermophilus* strains

In this study, 12 strains of *S. thermophilus* isolated from locally produced yogurt in China or its commercial starter culture were examined for their plasmid content. Among them, only two strains (ST-QC and ST-HS) were found to carry two plasmids, identified as pQC1 and pQC2 for the *S. thermophilus* ST-QC, as well as pHS1 and pHS2 for the *S.thermophilus* ST-HS, respectively. The two larger plasmids (pQC1 and pHS1) with almost the same size of 4.5 kb and the two small ones (pQC2 and pHS2) with the similar size of 3.5 kb, were identified using agarose gel electrophoresis as shown in Figure 1. To determine whether the electrophoretic mobilities of the two bigger plasmids were identical, plasmids pQC1 and pHS1 were mixed and electrophoresed in 12 g/l agarose gel. As results, only a single band was detectable which was suggested that the mixture contained the plasmids of the same size. The same was done for the mixture of pQC2 and pHS2 and the results also indicated they had the same size (data not shown). Based on their molecular sizes, two smaller plasmids (pQC2 and pHS2) and two bigger ones (pQC1 and pHS1) belong to homology group I and II of the *S. thermophilus* plasmids, respectively, according to the classification proposed by Janzen et al., (1992).

Cloning of the shsp gene containing its 5′ and 3′ flanking regions from *S. thermophilus* plasmid pQC2 and sequence analysis

The 765 bp DNA fragment including the coding region of the *shsp* gene was amplified from the smaller plasmid pQC2 of *S. thermophilus* St-QC by PCR using upstream primer1 and downstream primer2. The amplified DNA fragments were then cloned and sequenced. The amplified DNA fragments included a 429 bp open reading

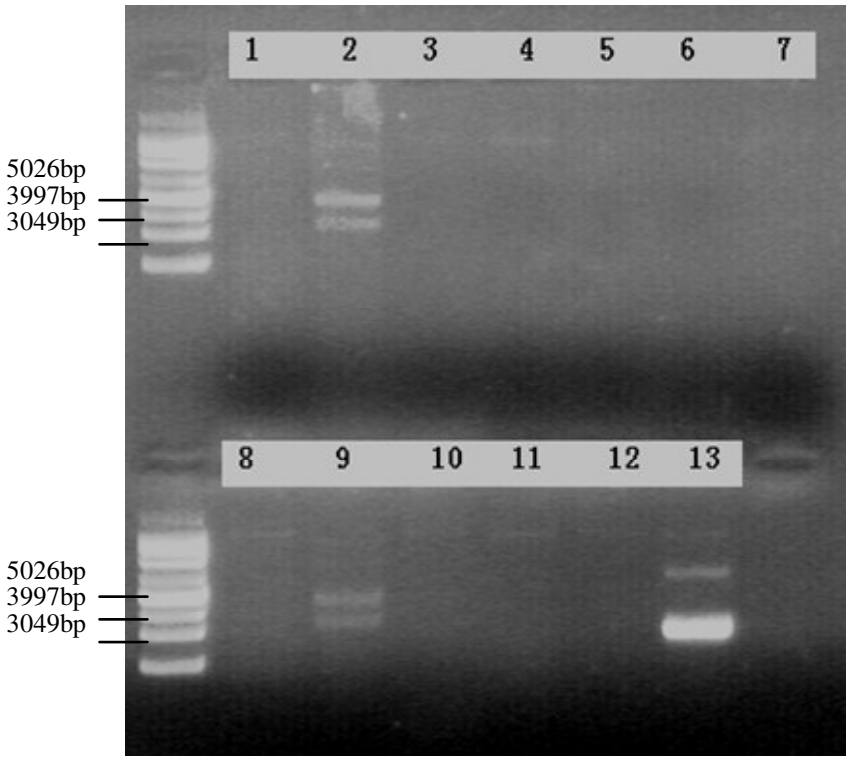

Figure 1. Agarose gel electrophoresis profiles of the plasmid DNAs of *S. thermophilus* isolated from China yogurt or its commercial starter culture. Lane 1, ST-99201; lane 2, ST-QC; lane 3, ST-2; lane 4, ST-3; lane 5, ST-G; lane 6, ST-LDY; lane 7, ST-TX; lane 8, ST-SY519; lane 9, ST-HS; lane 10, ST-JY; lane 11, ST-LT and lane 12, ST-MH. Lane 13, plasmid pLEB590 presented in *Lactococcus lactis* MG1614 is used as a positive control. Less intense bands in each lane are open circular forms of plasmid DNAs. The sizes of the standard supercoiled DNA ladder markers are in kilobases (kb).

frame of the *shsp* gene with an ATG translation initiation codon and a TAG translation termination codon, indicating that it was translated. The putative *shsp* gene product was a 142-amino acid polypeptide with a calculated molecular mass of 16.433 kDa and a predicted p*I* of 4.77.

The nucleotide sequence of the *shsp* gene coding region and the deduced amino acid sequence of the sHSP protein were further aligned with sequences available from the GenBank databases using the BLAST program. A comparison of the nucleotide sequences revealed that the 765 bp DNA fragment showed high homology with the region containing the *shsp* gene of pER1-1 (99.48% identity, pER1-1 GenBank accession number AJ242476) and plasmid2 (100% identity, plasmid2 GenBank accession number NC008501) (Figure 2). It also showed that a 429 bp open reading frame of the *shsp* gene was quite identical between pQC2 and pER1-1 or plasmid2 (Figure 2). Meanwhile, multiple sequence alignment revealed that the nucleotide sequence of the *shsp* gene coding region and its deduced amino acid sequence shared a high degree of identity with the *shsp* genes and the sHSP proteins, respectively, of other *S. thermophilus* plasmids (> 86.67% or > 81.33% identity), such as pER341 (ORF4: 93.47 or 87.23% identity with the nucleotide sequence or the amino acid sequence) (Somkuti et al., 1998), pCI65st (ORF4: 94.17 or 88.73% identity with the meaning as described above); (ORF2: 88.74 or 81.33% identity) (O'Sullivan et al., 1999), pER16 (93.94 or 88.73% identity), pER35 (86.67 or 81.73% identity), pER36 (96.75 or 94.37% identity) (Solow and Somkuti, 2000), pND103 (93.71 or 89.44% identity) (Su et al., 2002), pS04 (85.81 or 80.52% identity) (Geis et al., 2003), pt38 (82.68 or 89.44% identity) (Petrova et al., 2003), pt39 (93.47 or 89.44% identity), p2980 (94.17 or 89.44% identity), p2991 (94.17 or 89.44% identity), and p2992 (93.71 or 88.73% identity) (Petrova and Gouliamova, 2006) (data not shown). All of these indicate that the sequences of the *shsp* genes or the sHSP proteins from different *S. thermophilus* plasmids were highly conserved. The nucleotide sequence of the *shsp* gene coding region or the deduced amino acid sequence of the sHSP protein also share homology (approximately 40 or 30% identity) with the *shsp* genes or the sHSP proteins found in other prokaryotes and especially to those reported from *Enterococcus faecalis* plasmids such

Figure 2. Comparison of the 765bp nucleotide sequence including the 429 bp open reading frame of the *shsp* gene between plasmid pQC2 and pER1-1 or plasmid2 from different *S. thermophilus* strains.

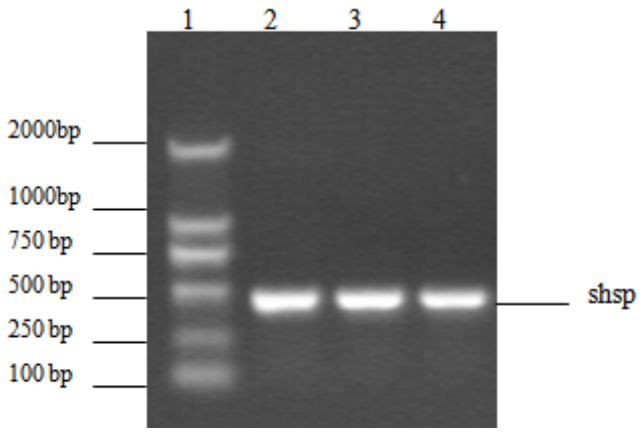

Figure 3. Agarose gel electrophoresis profiles of the identification of the *shsp* gene by PCR using primer3 and primer4 from recombinant plasmid pET-*shsp* in *E. coli* BL21 (DE3) transformants. Lane 1: sizes of standard DL2000 DNA marker are in base pair (bp) and Lanes 2, 3 and 4: identification results from three transformants containing recombinant plasmid pET-*shsp*.

as pSJ33 and pEFR (GenBank accession number EU370687 and AF511037, up to 93 or 85.21% identity with the nucleotide sequence or the amino acid sequence) (data not shown).

As illustrated in Figure 3, the 765 bp DNA fragment amplified by PCR contained not only the 429 bp entire coding region, but also 222 bp sequences upstream and 114 bp sequences downstream of the *shsp* gene. Analysis of 222 bp nucleotide sequences immediately upstream of the *shsp* gene coding region revealed that a putative ribosomal binding site (AAAGGAG) located 8 bp upstream from the starting codon of the *shsp* gene is preceded by a 5′-TTGAAA (16 bp) TATAAT-3′ promoter region (Figure 2) that bears similarity to consensus promoter sequences of the *shsp* genes found in most of the *S. thermophilus* plasmids, such as pER341 (Somkuti et al., 1998), pCI65st (O'Sullivan et al., 1999), pER16, pER35 (Solow and Somkuti, 2000), pND103 (Su et al., 2002), pS04, pER1-1 (Geis et al., 2003), pt38 (Petrova et al., 2003) and plasmid2 (Makarova et al., 2006) (data not shown), as well as to those reported for most of the LAB strains (Koivula et al., 1991; Constable and Mollet, 1994). These indicate that the sequences of the promoter region of the *shsp* genes from most of the *S. thermophilus* plasmids or the most of the LAB strains were very similar. An analysis of 114 bp nucleotide sequences immediately downstream of the *shsp* gene stop codon did not find a typical inverted repeat sequence, but did find a series of T residues (Figure 2). The inverted repeat sequence followed by a series of T residues may form a stem-loop structure in mRNA and may function as a rho-independent transcriptional terminator for the *shsp* genes (Somkuti et al., 1998; Solow and Somkuti, 2000; Petrova et al., 2003; Petrova and Gouliamova, 2006). A comparison of the nucleotide sequences showed that the 114 bp sequence containing the transcription terminal region of the *shsp* gene was completely identical between pQC2 and pER1-1 (Figure 2), indicating that the 114 bp sequence may play a possible role in the transcription termination of the *shsp* gene.

In summary, these results suggested that the 765 bp DNA fragment containing a complete transcriptional unit of *shsp* gene in the smaller pQC2 plasmid from *S. thermophilus* St-QC.

Recombinant expression of shsp gene in *E.coli*

Before the inducible expression in *E. coli* BL21 (DE3), the *shsp* fragment cloned into a recombinant plasmid pET-*shsp* was identified by DNA sequencing, PCR, as well as double enzyme digestion of recombinant plasmid using BamHI and HindIII. According to the result of the DNA sequencing, the coding region sequence of the *shsp* gene cloned into recombinant plasmid pET-*shsp* was accurate and had no mutation (data not shown). Thus the recombinant plasmid pET-*shsp* was transformed into *E.coli* BL21 (DE3). The PCR analysis of the recombinant plasmid pET-*shsp* showed a band of approximately 429 bp and this result is consistent with the size of the *shsp* gene coding region from the smaller plasmid pQC2 (Figure 3). By double enzyme digestion of recombinant plasmid using BamHI and HindIII, we also found that there were two bands at the 5244 and 429 bp positions which represented expression vector pET-30a and the *shsp* gene, respectively; in contrast, the control vector pET-30a did not have this product (Figure 4). These results imply that the *shsp* gene coding region was correctly inserted into recombinant vector pET-*shsp* which was then successfully transformed into *E. coli* BL21 (DE3).

To prevent recombinant fused protein from forming a great deal of intracellular inclusion bodies, *E. coli* BL21 (DE3) transformants containing recombinant pET-*shsp* plasmid were expressed by the induction of IPTG at 30°C which is below its normal growth temperature of 37°C. Separation via SDS-PAGE and analysis via Western blotting of the soluble cell proteins showed that there was a band of approximately 25 kDa in transformants carrying recombinant plasmid pET-*shsp*, which corresponded to a theorical molecular mass of 24.302 kDa from the recombinant fused protein containing a calculated molecular mass of 16.433 kDa from the recombinant sHSP protein with 142 amino acid residues and a calculated molecular mass of 7.869 kDa from the histidine tag protein with 68 amino acid residues. Meanwhile, no such product was found in the transformants carrying control plasmid pET-30a (Figures 5 and 6). These results suggest that the s*hsp* gene of the small plasmid from *S. thermophilus* St-QC was successfully expressed in mesophilic *E. coli.*

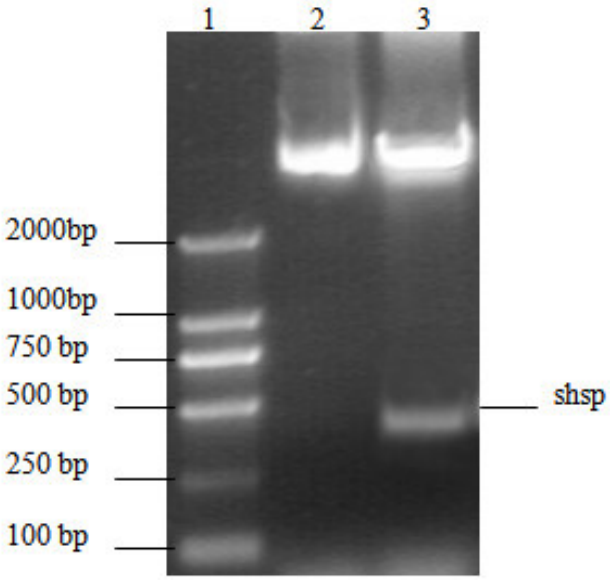

Figure 4. Agarose gel electrophoresis profiles of identification of recombinant plasmid pET-*shsp* from *E. coli* BL21 (DE3) transformants by double enzyme digestion using BamHI and HindIII. Lane 1: sizes of standard DL2000 DNA marker are in base pair (bp); Lane 2: control plasmid pET-30a and Lane 3: recombinant plasmid pET-*shsp*.

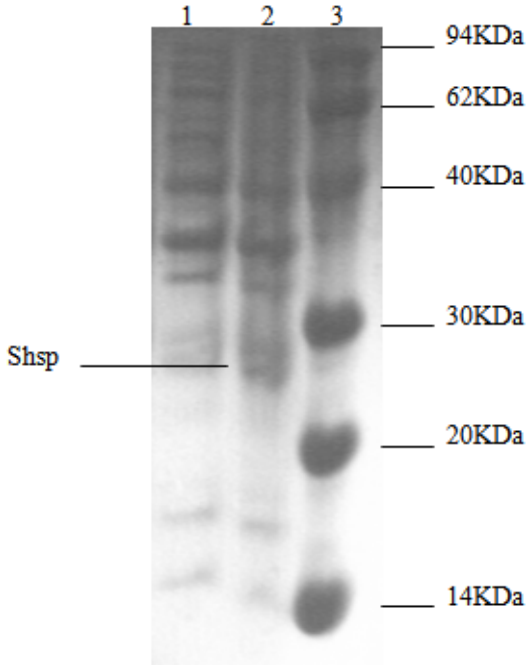

Figure 5. Separation of the sHSP protein in the soluble cell proteins by SDS-PAGE. Lane 1: soluble cell protein samples of *E. coli* BL21 (DE3) transformants containing control plasmid pET-30a induced by IPTG; Lane 2: soluble cell protein samples of *E. coli* BL21 (DE3) transformants carrying recombinant plasmid pET-*shsp* induced by IPTG and Lane 3: the sizes of the blue plus protein marker are in kDa.

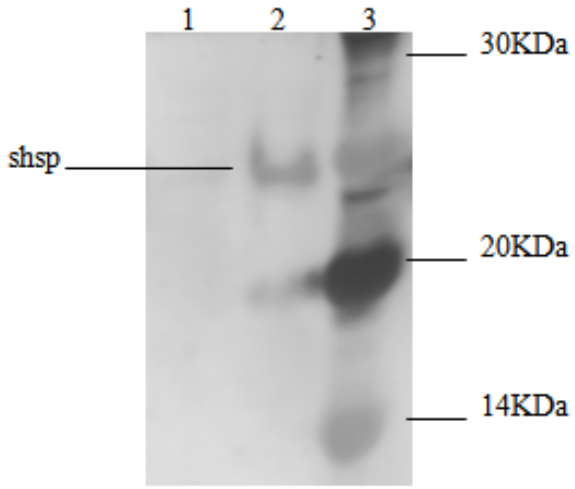

Figure 6. Analysis of the sHSP protein in the soluble cell proteins by Western blotting. Lane 1: soluble cell protein samples of *E. coli* BL21 (DE3) transformants containing control plasmid pET-30a induced by IPTG; Lane 2: soluble cell protein samples of *E. coli* BL21 (DE3) transformants carrying recombinant plasmid pET-*shsp* induced by IPTG and Lane 3: sizes of blue plus protein marker are in kDa.

Biofunctional assay of resistance to abiotic stresses of the shsp gene in transgenic *E. coli* cells

Examination of growth characteristics showed that *E. coli* BL21 (pET-*shsp*) grew at the same rate to the same cell densities as the control strain *E. coli* BL21 (pET-30a) at a normal growth temperature of 37℃. In addition, the wild-type strain *E. coli* BL21 (Figure 7) demonstrated that the *shsp*-bearing pET-30a plasmid transformed into *E. coli* BL21 did not have an adverse effect on bacterial growth. However, further studies revealed that only *E. coli* BL21 (pET-*shsp*) induced by IPTG was able to multiply (data not shown) at 48℃ above the normal growth temperature of 37℃, suggesting that the pET-30a plasmid-borne *shsp* gene was responsible for the growth phenotype at an elevated temperature.

To examine whether the *shsp* gene plays an important role in abiotic stresses for the transgenic *E. coli* cells, several typical stress survival rate data for *E. coli* BL21 (pET-*shsp*) and the *E. coli* BL21 (pET-30a) control strain were obtained and showed in Figures 8A, B and C. Significant differences in the survival rate against elevated temperatures between transgenic *E. coli* and its control strain were observed. Exposure to 52℃ for 1 h resulted in an inactivation of 96.18% of BL21 (pET-30a) cells, while 45.43% of the BL21 (pET-*shsp*) cells survived under this treatment. After 2 h, 7.1% of the BL21 (pET-*shsp*) cells survived, but only 0.053% of the BL21 (pET-30a) cells did so. After 4 h, the survival rates of BL21 (pET-*shsp*) were 114 times higher than that of the control strain (Figure 8A). These data correspond to those

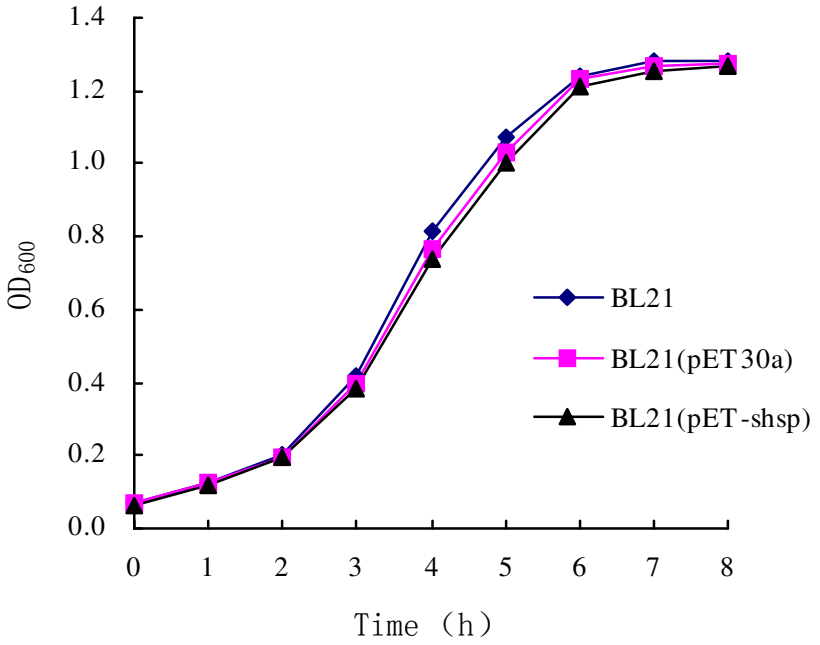

Figure 7. The curves of OD on *E.coli* BL21, BL21 (pET30a) and BL21 (pET-*shsp*) cultivated in 37℃ in an LB.

presented by Fernando et al. (Fernando and Heikkila, 2000), Satoru et al. (2006), Guo et al. (2005) and Yan et al. (2007), whose studies have all demonstrated that the heterologous overexpression of the *shsp* genes conferred thermotolerance against elevated temperatures of 50 or 45℃ on *E. coli* cells.

E. coli cells overexpressing the *shsp* gene also showed strongly improved viability against a low pH. When incubated at a pH level of 3.5 for 1 h, only 2.45% of BL21 (pET-30a) cells were alive, compared to 57.8% of BL21 (pET-*shsp*) cells. After 2 h, 18.5% of BL21 (pET-*shsp*) cells stayed alive, while only 0.14% of BL21 (pET-30a) survived. After 4 h, the survival rates of BL21 (pET-*shsp*) were 22 times higher than those of the control strain (Figure 8B). A previous study indicated that the recombinant strain of *E. coli* expressing the *Oenococcus oeni shsp Lo18* gene improved their acid tolerance (Jobin et al., 1999). In their research on *S. thermophilus* Geis et al. (2003) and Demerdash et al. (2003) showed proof that the cells carrying the plasmid-encoding *shsp* gene and expressing *shsp* gene at a high level displayed greater levels of acid resistance than their variant cells lacking the plasmid-encoding *shsp* gene.

In addition to protection against elevated temperatures and a low pH, the presence of pET-*shsp* and the overexpression of the *shsp* gene proved its ability to protect *E. coli* cells against ethanol stress. Under 12% of ethanol for 1 h, the survival rates of BL21 (pET-*shsp*) decreased to 61.17%; however, the survival rates of BL21 (pET-30a) decreased to 25.51%. After 2 h, 17.85% of BL21 (pET-30a) cells survived, whereas 3.12% of (pET-*shsp*) cells survived. After 4 h, the survival rates of BL21 (pET-*shsp*) were 12 times higher than those of the control strain (Figure 8C). In other studies relevant to the *shsp* genes, Spano et al. (2004) and Francoise et al. (2005) all found that the expression of the *shsp* genes in wine *Lactobacillus plantarum* and *O. oeni* were strongly induced by ethanol stress.

These results support the idea that *E. coli* cells overexpressing the *shsp* gene of the smaller plasmid pQC2 from *S. thermophilus* St-QC significantly increased resistance to heat, acid and ethanol stresses. Thus, it may be more appropriate to address sHSP as a stress protein rather than as a small heat shock protein.

DISCUSSION

As an important factor of endogeneity cytoprotection, sHSPs from different organisms have attracted ever increasing related investigations in recent years. *S. thermophilus*, an important thermophilic microorganism in dairy food fermentation, is widely used as a starter culture for the production of yogurt and certain cheese varieties. Thus far, all the *shsp* genes described in *S. thermophilus* are localized in plasmids (O'Sullivan et al., 1999; Somkuti and Steinberg, 1999; Solow and Somkuti, 2000; Su et al., 2002; Geis et al., 2003; Demerdash et al., 2003; Makarova et al., 2006). Researches on cloning and the expression of the s*hsp* genes from the thermophilic microorganism in mesophilic LAB or yeast and the further development of stress-resistant microorganism strains for dairy fermentation and brewing wine industry both have an important significance in theory and application.

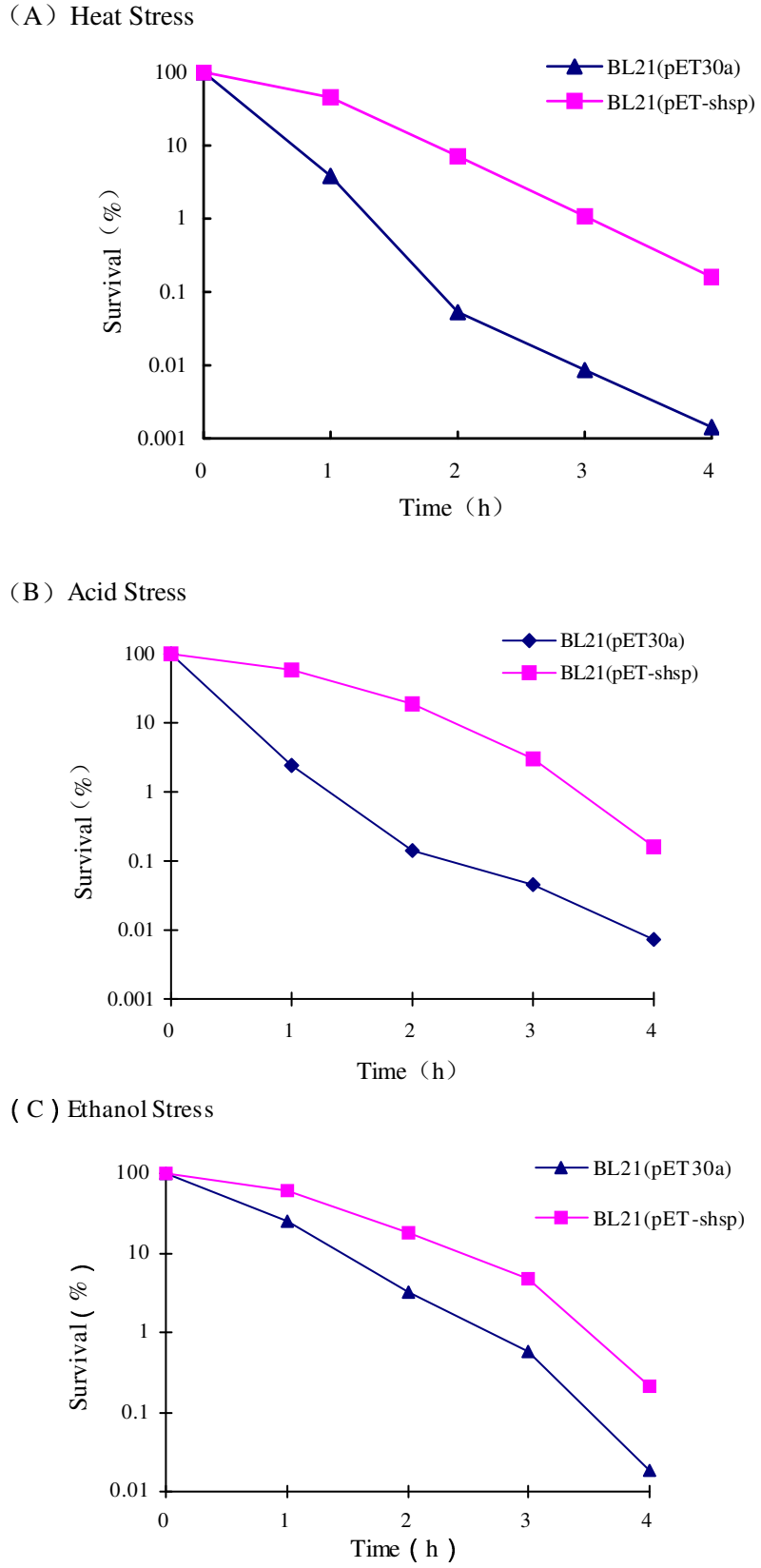

Figure 8. Survival curves of *E. coli* BL21 (pET-*shsp*) and the *E. coli* BL21 (pET-30a) control strain respectively induced by IPTG against several abiotic stresses: (A) heat stress, (B) acid stress and (C) ethanol stress.

Unlike mesophilic dairy lactococci, *S. thermophilus* strains isolated from the starter cultures of yogurt and certain cheese varieties showed low incidence of plasmids (less than 20%) (Janzen et al., 1992; McKay and Baldwin, 1990; O'Sullivan et al., 1999; Somkuti and Steinberg, 1986) and harbored just one or two plasmids (O'Sullivan et al., 1999; Somkuti et al., 1998; Somkuti and Steinberg, 1999; Janzen et al., 1992). These strains contain plasmids ranging in size from 2 to 10 kb (Petrova et al., 2003). Our data that only 16.67% incidence of plasmids was observed in *S. thermophilus* presented new evidence for this. However, the reason behind the paucity of plasmids in *S. thermophilus* as compared with the ubiquity of plasmids in mesophilic dairy lactococci is still unknown. On the other hand, more than half of *S. thermophilus* which contains plasmid were demonstrated to encode sHSP proteins (Petrova and Gouliamova, 2006). Our results in Figure 2, showed that plasmids pQC2 and pHS2 were 3.5 kb long, which is almost the same size with pER1-1 (3,365 bp) or plasmid2 (3,361 bp), implied that these plasmids may have similar genes. Given that plasmids pER1-1 and plasmid2 which both encode *shsp* genes from *S. thermophilus* strains ER1 and LMD-9, separately, we supposed that pQC2 and pHS2 which were from *S. thermophilus* strain St-QC may contain *shsp* gene, too. Thus primers for amplification of the *shsp* gene were disgined accoding to the sequence of the *shsp* gene of (pER1-1 and plasmid2). The successful cloning of *shsp* gene from *S. thermophilus* St-QC and the fact that no difference of the *shsp* gene sequence between plasmid2 and pQC2 provide strong eviedence that demonstrated our surmise. Furthermore, multiple sequence alignment demonstrated that the nucleotide sequence of the coding region of the *shsp* genes or the deduced amino acid sequence of the sHSP proteins from different plasmids of *S. thermophilus* (Somkuti et al., 1998; O'Sullivan et al., 1999; Solow and Somkuti, 2000; Su et al., 2002; Geis et al., 2003; Petrova et al., 2003), which were isolated from very far flung area, even from different continents (Petrova and Gouliamova, 2006), shared a high degree of identity (>86.67% or >81.33%) and were very conserved. However, whether pQC and plasmid2, even the St-QC and LMD-9 strains, could be considered completely identical remains to be determined.

Analysis of 222 bp nucleotide sequences immediately upstream of the *shsp* gene coding region revealed that a putative ribosomal binding site (AAAGGAG) located 8 bp upstream from the start codon of the *shsp* gene is preceded by a 5′-TTGAAA (16 bp) TATAAT-3′ promoter region that bears similarity to consensus promoter sequences of the *shsp* genes found in the plasmids of most *S. thermophilus* and a majority of other LAB strains (Koivula et al., 1991; Constable and Mollet, 1994). However, it should be noted that in a few *S.thermophilus* plas-mids such as pt39 and p2992, expression of the *shsp* genes was directed from a promoter lacking a typical -35 box but containing a consensus "extended -10 motif" (5′-TGTTATAAT-3′) which was usually associated with strong promoters (Petrova and Gouliamova, 2006). This extension in the -10 box was also found in DNAs from *E. coli* and Gram-positive bacteria including *Bacillus subtilis*, *Streptococcus agalactiae*, *Streptococcus pneumoniae*, *Lactococcus lactis* and *Lactobacillus* (Petrova et al., 2003). Moreover, a short sequence (AGTCAAAAAAAGTCAGT) overlapping the promoter-sequence of the *shsp* genes from *S. thermophilus* plasmids pSt04 and pER1-1 (Geis et al., 2003) showed complete sequence similarity to the consensus sequence derived by the alignment of 32 different class three stress gene repressor (CtsR) binding sites (Derré et al., 1999), with the exception of the last two nucleotides (GT for AA). In our study, this short sequence overlapping the promoter-sequence of the *shsp* gene from the *S. thermophilus* plasmid pQC2 was not found.

In the 3′-side non-coding region of the *shsp* genes, a 13 bp downstream from stop codons, an inverted repeat sequence followed by a series of T residues which may form a stem-loop structure in mRNA and may function as a rho-independent transcriptional terminator for the *shsp* genes (Somkuti et al., 1998; Solow and Somkuti, 2000; Petrova et al., 2003; Zhao et al., 2007). In this study, an analysis of 114 bp nucleotide sequences immediately downstream of the *shsp* gene stop codon did not find a typical inverted repeat sequence but found a series of T residues instead. Noticeably, the 114 bp sequence containing the transcription terminal region of the *shsp* gene was completely identical between pQC2 and pER1-1, Geis et al., (2003) proved that the synthesis of the sHSP protein significantly increased and the thermo- and acid-resistance of *S. thermophilus* cells also significantly increased when the *S. thermophilus* ER1 strain carrying plasmid pER1-1 encoding *shsp* gene was subjected to heat or acid stress (52℃ for 30 min or pH 4.5 for 30 min) (Geis et al., 2003). This indicated that the *shsp* gene from the *S. thermophilus* plasmid pER1-1 could be normally transcribed and translated, Thus, we assume that the *S. thermophilus* plasmid pQC2 contained transcriptional terminator for the *shsp* gene; nevertheless, its transcription termination mechanism remains to be elucidated.

Many foreign *shsps* genes (Yeh et al., 1997; Jobin et al., 1999; Soto et al., 1999; Liu and Mariko, 2001; Guo et al., 2005; Yan et al., 2007) have been successfully expressed in *E. coli*. The *shsp* genes from *Trichoderma harzianum* (Yang et al., 2008), *S. thermophilus* (Sudhamani et al., 2008) have been expressed in *Saccharomyces cerevisiae* and *Lactobacillus casei*, respectiveiy. Although the 429 bp open reading frame of the *shsp* gene was quite identical between pQC2 and pER1-1 or plasmid2, heterologous expression of the *shsp* gene from the *S. thermophilus* plasmid pER1-1 or plasmid2 has been not reported. In this study, we tried to express the s*hsp* gene from *S. thermophilus* plasmid pQC2 in *E. coli*. To prevent recombinant fused protein

from forming a great deal of intracellular inclusion bodies, *E. coli* BL21 (DE3) transformants carrying recombinant plasmid pET-*shsp* were expressed by the induction of IPTG at 30°C below its normal growth temperature of 37°C. SDS-PAGE and Western blotting results of the soluble cell proteins showed that the s*hsp* gene of the pQC2 plasmid of *S. thermophilus* St-QC, isolated from China yogurt or its commercial starter culture, was successfully expressed in mesophilic *E. coli* BL21(DE3).

Some physiological functions (resistance to heat, acid, cold, salt, drought and so on) of the homologous expression of the *shsp* genes from *S. thermophilus* plasmids (O'Sullivan et al., 1999; Geis et al., 2003; El Demerdash et al., 2003; Petrova and Gouliamova, 2006) and the heterologous expression of the *shsp* genes from other organisms (Yeh et al., 1997; Fernando and Heikkila, 2000; Soto et al., 1999; Wang et al., 2005; Liu and Mariko, 2001), have been reported. However, very little is known about the physiological functions of the heterologous expression of the *shsp* genes from the *S. thermophilus* plasmids. Although the *shsp* gene of the *S. thermophilus* plasmid pSt04 was successfully expressed in *Lactobacillus case*, the physiological functions of heterologous expression of the *shsp* gene from *S. thermophilus* plasmid pSt04 was not described (Sudhamani et al., 2008). Data from our experiments indicated that *E. coli* cells overexpressing the *shsp* gene of pQC2 significantly increased resistance to heat, acid and ethanol stresses. Heat and acid tolerance is the main advantage of the LAB used in dairy fermentation. Resistance to ethanol is the first biochemical function found to be linked to the *S. thermophilus* plasmid-borne *shsp* gene, which is a good character of yeast or LAB used in brewing wine. Our results imply that the *shsp* gene of the smaller plasmid pQC2 from *S. thermophilus* St-QC is a desirable target gene in genetic engineering for the development of stress-resistant microorganism strains used in dairy fermentation and brewing wine.

In conclusion, we tested 12 strains of *S. thermophilus* isolated from China yogurt or its starter culture for the presence of plasmids. Two strains (ST-QC and ST-HS) were found to harbor two plasmids. The *shsp* gene of the smaller plasmid pQC2 from *S. thermophilus* St-QC was cloned and identified before being successfully expressed in mesophilic *E. coli*. In addition to performing strong heat and acid tolerance, recombinant *E. coli* cells overexpressing the *S. thermophilus* St-QC *shsp* gene showed significantly higher resistance to ethanol stress, which is the first physiological function found to be linked to the *S. thermophilus* plasmid-borne *shsp* gene. This study will provide a basis for cloning and expression of the s*hsps* genes from thermophilic microorganism in mesophilic LAB or yeast and will lead toward the further development of stress-resistant microorganism strains used in dairy fermentation and brewing wine. Heterologous expression of a food grade and the investigation of physiological functions of this *shsp* gene in the LAB are currently in progress.

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