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Molecular cloning and characterization of bile salt hydrolase in *Lactobacillus casei* Zhang

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Abstract - Bile salt hydrolase (BSH) is one of the genes relevant to bile tolerance. Biochemical assay showed that *Lactobacillus casei* Zhang was able to deconjugate sodium taurocholate during growth. To explore the possible relationship between BSH activity and bile tolerant ability, we cloned and determined full-length DNA sequence of the BSH gene in *L. casei* Zhang, and monitored its expression pattern under the stress of bile salts. The DNA sequence consists of 1181 nucleotides; the putative protein includes 338 amino acids. The sequences alignment and homology studies illustrated a great diversity between species. Further real-time PCR analysis revealed that expression of the BSH gene was up-regulated when there presented bile salts in the growth medium.

Key words: bile salt hydrolase; *Lactobacillus casei* Zhang; clone; expression.

INTRODUCTION

Lactobacillus genus includes several species which have a long history use in food fermentations. In order to exert their beneficial effects, the ability of resistance to bile salt is required for transiting the intestinal flora (Gueimonde and Salminen, 2006). *Lactobacillus* strains have been studied extensively, and actually, some of the probiotic strains showed a high survival rate in the presence of bile salts (Succi *et al*., 2005). However, molecular mechanisms of these bacteria to tolerate bile remain unclear. Several genes including those for lipopolysaccharid biosynthesis, bile salt deconjugation, and sugar transporting were reported to help bacteria keep alive under bile stress (Van De Guchte *et al*., 2002). Among them, bile salt hydrolase (BSH) appeared to be more important for probiotic strains (Bron *et al*., 2006). Recently, the ability to hydrolyze bile salts has been included in the criteria for probiotic strain selection (Pineiro and Stanton, 2007).

Bile salt hydrolases (BSH; EC 3.5.1.24) are in the linear amide C-N hydrolases chologlycine hydrolase family that also contains penicillin amidases. Intestinal bacteria encoded the BSH protein are involved in transformation reactions, catalyzing hydrolysis of the amino acid taurine or glycine from the C-24 position of conjugated bile salts (Ridlon *et al*., 2006). It can be commonly detected in many bacteria including several members of genera *Lactobacillus* and *Bifidobacterium*, such as *Lactobacillus acidophilus* (Mcauliffe *et al*., 2005), *Bifidobacterium longum* (Grill *et al*., 1995), *Bifidobacterium adolescentis* (Kim *et al*., 2005), *Lactobacillus johnsonii* (Elkins *et al*., 2001), *Lactobacillus reuteri* (De Boever *et al*., 2000), *Bifidobacterium bifidum* (Kim *et al*., 2004), *Lactobacillus plantarum* (Christiaens *et al*., 1992) and many other gastrointestinal autochthonous organisms (Jones *et al*., 2008), some of which have been cloned and characterized. Some homologues of BSH genes, if any, have also been identified through microbial genome-wide analysis. Of the dairy-related *Lactobacillus casei* strains, limited information can be obtained about its BSH activity until now.

Lactobacillus casei Zhang was isolated from home-made koumiss in Inner Mongolia of China, which showed high resistance to bile salts (Wu *et al*., 2009). In this context, we first evaluated BSH activity of *L. casei* Zhang, then cloned, characterized, and examined the expression pattern of the BSH gene under the stress of bile salts. We aim to explore the association of the BSH gene with bile salt tolerance in *L. casei* Zhang.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *Lactobacillus casei* Zhang was obtained from Key Laboratory of Dairy Biotechnology and Engineering Ministry of Education, Inner Mongolia Agricultural University, China. It was propagated at 37 °C in de Man, Rogosa, and Sharpe broth (MRS; Difco, Detroit, USA). Gene transcriptional expression studies of BSH were done in MRS broth supplemented with 0.5% and 1.5% (w/v) bile salts (Sigma, St. Louis, MO, USA).

Assay of BSH activity. BSH activity was tested by deconjugation of sodium taurocholate in MRS-THIO broth (MRS sup-

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plemented with 0.2% sodium thioglycolate) (Sigma). The broth media were inoculated with 1% strain culture and incubated at 37 °C for 24 h, and then the release of free cholic acid (Sigma) was analyzed as described by Walker and Gilliland (1993). An un-inoculated sample served as control. Each test was carried out in triplicate.

Primer design. The primers were designed based on the published genome sequences of *Lactobacillus casei* ATCC 334 and *Lactobacillus casei* BL23 (GenBank accession number: NC_008526, NC_010999) (Table 1). A primer pair (BSH-F, BSH-R) that flanked the BSH sequence was designed to amplify the full gene. For real-time PCR analysis, primers of BSH (BSHQ_F, BSHQ_R) and glyceraldehyde-3-phosphate (GAPDH, GAPDH_F, GAPDH_R) were designed with an amplicon size less than 200 bp.

PCR amplification. Total DNA was extracted by using EZNA bacterial DNA kit (Omega Bio-tek, GA, USA) according to the manufacturer's directions. PCR reactions were carried out in a 25 µl reaction mixture containing 0.2 µl Taq polymerase (5 U/µl, Takara, Tokyo, Japan), 2.5 µl 10X PCR Buffer (Mg^{2+} free), 2 µl dNTPs (2.5 mM each), 2 µl $MgCl_2$ (25 mM), 0.2 µl forward primer (50 pM), 0.2 µl reverse primer (50 pM), 1 µl genomic DNA (100 ng/µl), and 16.9 µl ddH_2O. Amplifications were performed on a thermal cycler (Bio-Rad, California, USA) using following program: 94 °C for 5 min, 94 °C for 30 s, 48 °C for 30 s, 72 °C for 40 s, 45 cycles, then 72 °C for 10 min, and 4 °C for 30 min.

Sequencing and sequence analysis. The PCR products were separated by agarose (1%) gel electrophoresis using a Huashun Gel Extraction Kit (Huashun, Shang Hai, China). The extracted PCR product was ligated to pMD 18-T Vector (Takara) by using T4 DNA ligase and incubated at 16 °C overnight, and then transformed into *Escherichia coli* DH5α competent cells. Positive clones were identified using blue-white color selection on agar plates containing ampicillin, X-gal and IPTG. The recombinant plasmids were cut by Hind III and BamH I (Takara) and, before sequencing, the expected inserts were run on the gel to verify their size.

The BSH gene sequence was analyzed for start and stop codon with ORF Finder (http://www.ncbi.nlm.nih.gov) and was translated into putative amino acid sequence. BLAST was used for homolog searching (Altschul *et al*., 1997). Domain characterization was carried out by using Interpro database (Mulder *et al*., 2005). Multiple amino acid sequence alignments were performed using the ClustalW software package (Thompson *et al*., 1994).

RNA isolation and cDNA synthesis. Before being used in experiment, stock cultures were subcultured twice in MRS broth. The third subcultures, when the cells went into the exponential-phase, were harvested by centrifugation at 4,000 *x g* for 5 min at 4 °C. Total RNA were extracted by using EZNA bacterial RNA kit (Omega Bio-tek, GA, USA) and were digested with the RNase-free DNase-I (Omega Bio-tek) to remove the genomic DNA.

cDNA was synthesized by using the PrimeScript™ RT reagent Kit (Takara). Reverse transcriptions were performed with 0.2 µl of total RNA (1 µg/µl), 0.5 µl PrimeScript™ RT Enzyme Mix™, 2 µl 5 × PrimeScript™ Buffer, 0.5 µl Random 6 mers (50 µM), and 6.8 µl RNase Free ddH_2O. The RT reaction condition was as follows: 37 °C for 15 min, 85 °C for 5 s.

Real-time PCR. The $2^{-\Delta\Delta Ct}$ method was used to calculate the relative changes in gene expression (Livak and Schmittgen, 2001). GAPDH gene was selected as an internal control for normalizing the amount of RNA added to the reaction of reverse transcription. For validation of the ΔΔCt calculation, PCR efficiency was measured for each primer pairs using serial dilutions of cDNA. Primer dimer formation was checked by agarose gel electrophoresis and melting curve analysis.

TABLE 1 - PCR primers

Primer	Sequence (5′-3′)	Amplicon size (bp)
BSH_F	GGATCGAGCCATTAAGCAA	1181
BSH_R	ACACTGAACCCCAAAACCA	
BSHQ_F	ATAGTCCTGAATACGGGTGG	166
BSHQ_R	GATGGTGATGTGTAATCGCC	
GAPDH_F	GGCTATCGGTTTGGTTATCC	164
GAPDH_R	TGTTTTCGGTGTGCTTCTTG	

Real-time PCR was performed in the Mastercycler ep realplex system (Eppendorf, Hamburg, Germany), using the following thermo-cycler program: initial denaturation at 95 °C for 5 min, followed by 40 cycles of amplification at 94 °C for 30 s, at 63 °C for 30 s, and at 72 °C for 30 s. The reaction mixture containing 1 µl cDNA template, 2.5 µl 10X PCR Buffer (Mg^{2+} plus), 1 µl dNTP mix (2.5 mM each), 1 µl forward primer (10 pM), 1 µl reverse primer (10 pM), 0.7 µl 0.6×SYBR Green-I dye (Generay Biotech, Shang Hai, China), 0.3 µl Taq polymerase (5 U/µl; Takara), and 16.5 µl ddH_2O. Individual real-time PCR reactions were carried out in triplicate for each gene.

RESULTS

Detection of the BSH activity

The deconjugation of sodium taurocholate by *L. casei* Zhang after incubation at 37 °C for 24 h is measured in terms of the amount of cholic acid liberated, which was 2.84 ± 0.13 µmol/ml. This could indicate that *L. casei* Zhang is capable of deconjugating bile salts during growth by producing certain BSH.

Cloning and sequencing of the BSH gene

To identify the gene encoded BSH enzyme, PCR analysis was performed and the products had been sequenced. After sequencing, full-length DNA sequence of the BSH of *L. casei* Zhang was obtained. It is composed of 1181 bases including one partial ORF and one complete ORF of BSH gene. The complete ORF of 1017 bp encoded a putative 338 amino acids protein with a conjugated bile acid hydrolase (CBAH) domain. The translation start codon and stop codon were capitalized and emphasized by boxes in Fig. 1. The complete sequence has already been deposited in GenBank database under the accession number of EU599213.

Sequence comparison of the BSH gene

Using BLAST program, BSH sequence of *L. casei* Zhang was aligned with other BSHs from GenBank database. The results showed that it displayed 86, 46, 44, 37, and 33% identities with BSH-related proteins from *Lactobacillus rhamnosus* HN001, *Pediococcus pentosaceus* ATCC25745, *Enterococcus faecalis* V583, *Clostridium perfringens* D str. JGS1721, and *Lactobacillus brevis* ATCC367, respectively. Subsequent multiple alignments with these sequences identified 5 putative active cites at Cys, Asp, Asn, Asn, and Arg amino acids residues (Fig. 2). Moreover, two conserved amino acid motifs, namely DGXNXXGL and TSPXRXXR around the active cites, were discovered during the analysis process.

Transcriptional expression of the BSH gene

Real-time PCRs were first optimized for each gene by

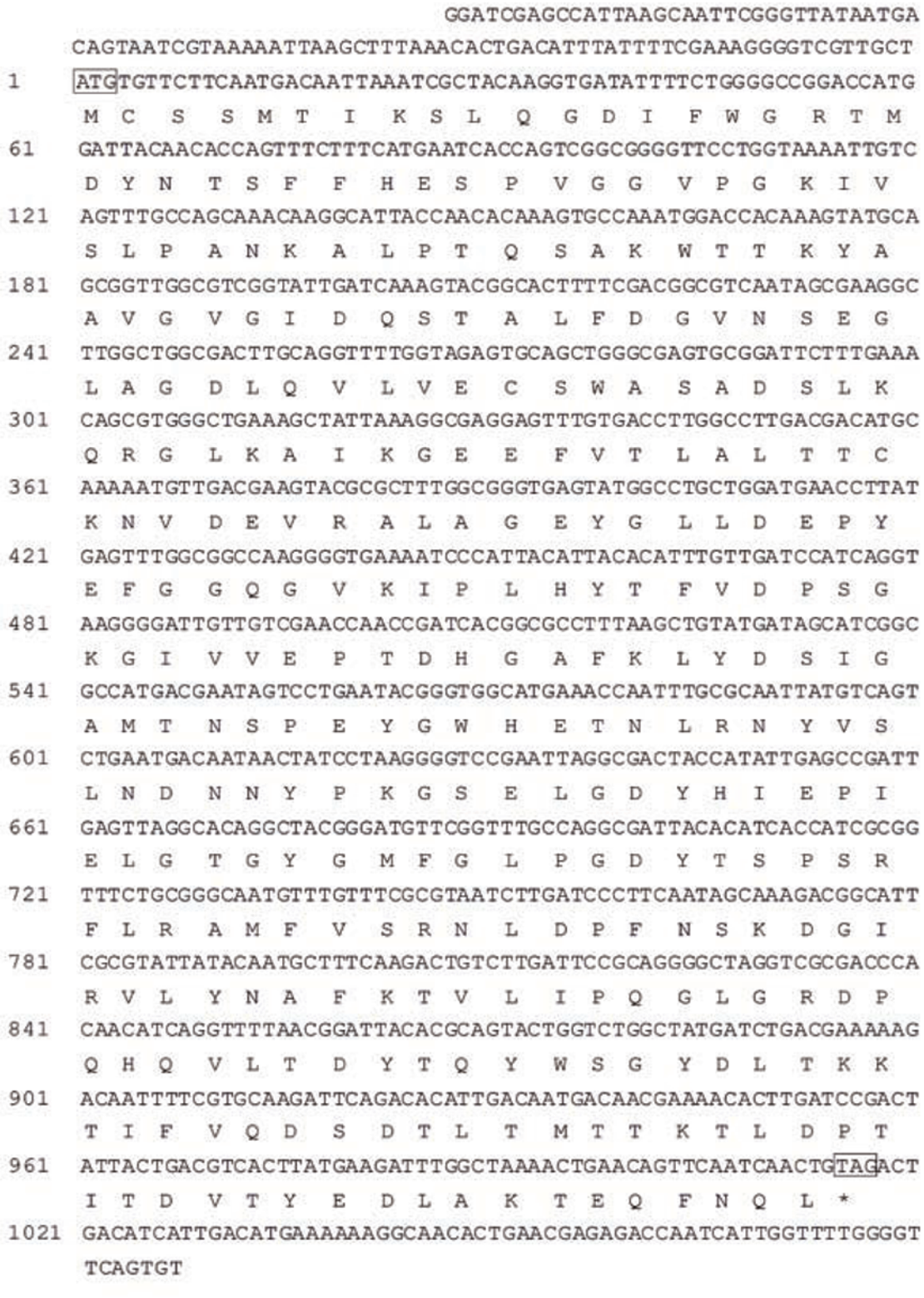

FIG. 1 - Nucleotide sequence and deduced amino acid sequence of the BSH gene from *Lactobacillus casei* Zhang.

varying both primer and PCR reagent. After several rounds of optimization, the real-time efficiencies were close to 100% in all test conditions (data not shown). A single product-specific melting curve was obtained as well, which suggested the feasibility of further experiment. The fold changes in gene expression are depicted in Fig. 3.

The above results showed that the BSH gene was up-regulated in an approximately 3.12 fold in the 1.5% bile salts treatment medium, more than which propagated in the 0.5% bile salts. Thus, we postulate here that there must be some relationship between BSH activity and bile salt tolerance in *L. casei* Zhang.

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Lcase  MCSSMTIKSLQGDIFWGRTMDYNTSFF-HESPVG-GVPGKIVSLPANKALPTQSAKWTTK 58
Lrham  MCSSMTIKSLQGDIFWGRTMDYNTSFF-HESPAG-GVPGKIVSLPANQTLPAQTATWKTK 58
Efaec  MCTGIKIISKTNDIFYGRTMDFTFDFFGNEDPIAPKIPTLIAQFPKGTVLNSQLNPWTAK 60
Ppent  MCTNIEITAKNGEHFWGRTMDLALPMFGEDPEHDLGARGMITTIPAGVDIDSQLANWQAD 60
Cperf  MCTHIHISSNKNNVYWGRTLDTSFNPF--------DIGSKIVIVPKNFMLKTQSEPWKTK 52
Lbrev  MCTSLTYENSRGDHFLARTMDFAFELG----------GQPIFMPRQQEISGDAGTFTTK 49
       **: :      .: : .**:*                      .*    :  :   : :.

Lcase  YAAVG---VGIDQ--------STALFDGVNSEGLAGDLQVLVECSWASADSLKQRGLKAI 107
Lrham  YAAVG---VGVDQ--------SVALFDGVNSEGLAGDLQVLVECSWASAESLAKRNLKPI 107
Efaec  YAFMGLAMSGTDQPANDGKTVSLAITDGINEAGLSGDIQYLMESSTAPAESLADRGLTPH 120
Ppent  YAAMG---IGIGG--------THILFDGINEHGLAGDLQVLFESTADTLENIQQRQQVPV 109
Cperf  YSFLG---ISLSE--------STLFFDGVNEKGLAGGLLFLNACTWDKKENIEKEGLIAI 101
Lbrev  YGFIG---AGRNLS-------HYIFVDGVNEFGLG--AAALYFRGYAKYQQSAPADKLAI 97
       *. :*    . .              : **:*. **.     *        :.      .

Lcase  KGEEFVTLALTTCKNVDEVRALAGEYGLLDEPYEFGGQG---VKIPLHYTFVDPSGKGIV 164
Lrham  KGEEFVTLALTTCKNVTEVRALASQYGLLDEPFQFGGQG---VKIPLHYTFVDPSGAGLV 164
Efaec  IAEEVLAYILSNFESVDEVKVAFEKIGLLDQKFQLDSLGE--VHFTLHWTINDKNNNSIV 178
Ppent  MNTEFVTYVLTHFKSVKEIRENYQNFAVVDQPTMLNGHS---ISFPLHYSFVDESGDGVV 166
Cperf  NSGEIVPWILSNFESVDDIKENISKVAVTGDDISSLGELGKGNPITAHYTFTDKMGNSVV 161
Lbrev  APHDVVAWALGNAQSVADLRELVKHIQILDVPVSLLGLT-----TPLHFIFSDPTGDTAV 152
          :.:.  *   :.* :::    .  : .     .         . *: : *  .   *

Lcase  VEPTDHGAFKLYD-SIGAMTNSPEYGWHETNLRNYVSLNDNNYPKGSELG-DYHIEPIEL 222
Lrham  IEPTDHGAFKLYD-SVGAMTNSPEYGWHTTNLRNYVSLNDRNYPEGADLG-DQHLEPIEL 222
Efaec  LQPTDNGAFVIYD-SIGVVTNSPEYNYHLTNARNYIGMRNYAIKEPYTLKSGATLDPIEG 237
Ppent  LEPVDNGSFKLYD-SVGVVTNSPEYNWHTVNLRNYIALNELNIKQPKTYKNGVTLNPIEG 225
Cperf  LEPTNNGRFKVFNNTVGVMANDPTFDWHMTNLSNYIQVQGFNKNQ-KTLNKNTVITPISN 220
Lbrev  LETTN-ADLHLIDDPVGVMANSPELNWHLQNLSTYGTLQAAERPLQDRLG----YQLPTQ 207
       ::..: . : : : .:*.::*.*   .:*  *  .*  :.

Lcase  GTGYGMFGLPGDYTSPSRFLRAMFVSRNLDPFNSK-DGIRVLYNAFKTVLIPQGLGRDPQ 281
Lrham  GTGYGMFGLPGDYTSPSRFVRAMFVSRNLDPFNSN-EGIRVLYNAFKTVLIPQGLGRDPK 281
Efaec  GTSYGLLGIPGDFTSPSRFIRALYYSDNLQEFDSS-EGIMQLYRAFQTVMIPRGIGHLGQ 296
Ppent  GTGYGLTGLPGTYTSPDRLVRSFMVANLMDDFEAE-DGIAQLYAAFRPVIIPRGMERNTS 284
Cperf  GT--GMLGLPGDYTSPSRFVRATYLRNFIGDISDE-EAPVCLFSLLNSVWVPKGVERFNK 277
Lbrev  GPGTGALGLPGDYTSPSRFVRTVFNKHYSEAAADTPSTLTTLQHLLDGVTIPKGVKLMAD 267
       *.  *  *:** :***.*::*:                .    *   :  * :*:*:    .

Lcase  HQVLTDYTQYWSGYDLTKKTIFVQDSDTLTMTTKTLDPTIT----DVTYEDLAKTEQFNQ 337
Lrham  HSILTDYTQYWSGYDLSKRAIFVQDANTLTMTTKTLDPNLT----EVTYDDLVKTEQFNQ 337
Efaec  SNSLSDFTHYWSGYDVTNLTMYVQPESTTSFTKYTLDPALT----EVTTFAVSNELLLTD 352
Ppent  EDILSDYTRYWSGYDLKQRKVYIQTGRGLAITAKCLNQTAT----TISFDEVDTGNYIHE 340
Cperf  DKDDSDFSSYMCAYDQNLGKLYLRTFNKINTMEFSLKNGKD----DKLVTYSI------- 326
Lbrev  G--TSDYTQYRGYACLDDRVYYMEPYDNQELQGIRLTDEMLNDWDTPVEYPLDHTPHVKH 325
           :*:: *            ::.           *

Lcase  L-- 338
Lrham  L-- 338
Efaec  LNQ 355
Ppent  I-- 341
Cperf  --- 326
Lbrev  LN- 327
```

FIG. 2 - Amino acid sequences comparison of BSH proteins. The introduction of gaps is indicated by dashes. Identical amino acids are indicated by asterisks. Five putative active sites (C, D, N, N, R) are highlighted with shadows. Lcase, *Lactobacillus casei* Zhang (GenBank Accession Number: EU599213); Lrham, *Lactobacillus rhamnosus* HN001 (GenBank Accession Number: EDY98569); Efaec, *Enterococcus faecalis* V583 (GenBank Accession Number: AAO82690); Ppent, *Pediococcus pentosaceus* ATCC25745 (GenBank Accession Number: ABJ67168); Cperf, *Clostridium perfringens* D str. JGS1721 (GenBank Accession Number: EDT71741).

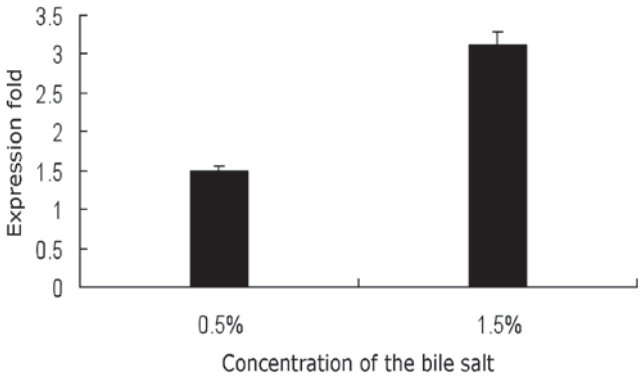

FIG. 3 - Trendgraph of BSH gene transcriptional expression under different concentrations of bile salts in *Lactobacillus casei* Zhang.

DISCUSSION

BSH plays an important role for bacterial hosts, especially for their persistent survival and colonization in vivo (Begley *et al*., 2005a). Up to now, a number of BSHs have been cloned and characterized from *Lactobacillus* strains. However, little information correlated with *L. casei* BSH was reported except some initial screening tests (Tanaka *et al*., 1999). In this study, we detected the BSH activity of *L. casei* Zhang at first, and then cloned, characterized, and monitored the expression pattern of the BSH gene under the stress of bile salts.

Since various degrees of BSH activity have been reported, it is convincible to give a scientific validation of the test strain. Biochemical assay indicated that *L. casei* Zhang deconjugated sodium taurocholate during the experiment, which also exhibited considerable activity. Similarly, Brashears *et al*. (1998) found that the isolate of *L. casei* had the BSH activity both on sodium taurocholate and sodium glycocholate, but differed from each other.

On the basis of the published BSH homologs encoded by *L. casei* ATCC334 and *L. casei* BL23, we obtained the full sequence of BSH gene in *L. casei* Zhang. Compared with others, *L. casei* Zhang BSH shared a high amino acid sequence similarity with it from *L. rhamnosus* HN001, but relative lower with it from *P. pentosaceus* ATCC25745, *E. faecalis* V583, *C. perfringens* D str. JGS1721, and *L. brevis* ATCC367, which suggested the biodiversity of BSHs either at inter-genera level or yet determined in *Lactobacillus*. However, all of the five proposed active sites at Cys, Asp, Asn, Asn, and Arg amino acids residues are highly conserved (Oh *et al*., 2008), which have been elucidated as catalytically important (Suresh *et al*., 1999). This strongly implies that the cloned gene encodes BSH gene in *L. casei* Zhang.

BSH activity may confer an advantage for bacteria to tolerate bile salts, whereas the contribution is still somewhat controversial (Begley *et al*., 2006). Under different concentrations of bile salts, transcriptional expression of the BSH gene was highly up-regulated. This data is well consistent with the findings as shown for *Lactobacillus plantarum* WCSF1 during the microarray analysis of bile-responsive genes (Bron *et al*., 2006). Identical results were also obtained when *Listeria monocytogenes* was exposed to bile salts (Begley *et al*., 2005b). Therefore we tentatively considered that the BSH activity was associated with bile salt tolerant ability of *L. casei* Zhang.

In conclusion, *L. casei* Zhang showed the detectable BSH activity towards bile salts, we focused our research on the BSH homologue encoded by all published *L. casei* genomes and had it cloned. In addition to BSH biodiversity among different bacteria, we found the predicted BSH significantly up-regulated under the stress of bile salts. And last but not least, the extent to which BSH will benefit *L. casei* hosts in the presence of bile salts needs to be proved by other molecular techniques.

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