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Molecular Cloning and Characterization of a Bile Salt Hydrolase from *Lactobacillus acidophilus* PF01

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Phenotypic screening for bile salt hydrolase (BSH) activity was performed on *Lactobacillus acidophilus* PF01 isolated from piglet feces. A gene encoding BSH was identified and cloned from the genomic library of *L. acidophilus* PF01. The *bsh* gene and surrounding regions were characterized by nucleotide sequence analysis and were found to contain a single open reading frame (ORF) of 951 nucleotides encoding a 316 amino acid protein. The potential *bsh* promoter region was located upstream of the start codon. The protein deduced from the complete ORF had high similarity with other BSHs, and four amino acid motifs located around the active site, FGRNXD, AGLNF, VLTNXP, and GXGXGXXGXPGD, were highly conserved. The *bsh* gene was cloned into the pET21b expression vector and expressed in *Escherichia coli* BLR(DE3) by induction with 0.1 mM of isopropylthiogalactopyranoside. The BSH enzyme was purified with apparent homogeneity using a Ni^{2+}-NTA agarose column and characterized. The overexpressed recombinant BSH enzyme of *L. acidophilus* PF01 exhibited hydrolase activity against tauroconjugated bile salts, but not glycoconjugated bile salts. It showed the highest activity against taurocholic acid. The maximum BSH activity occurred at approximately 40°C. The enzyme maintained approximately 70% of its maximum activity even at 60°C, whereas its activity rapidly decreased at below 37°C. The optimum pH was 6, and BSH activity was rapidly inactivated below pH 5 and above pH 7.

Keywords: Lactic acid bacteria, *Lactobacillus acidophilus*, bile salt hydrolase

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Probiotics are defined as "living microorganisms, which upon ingestion in certain numbers exert health benefits on the host beyond inherent basic nutrition" [17]. The selection of probiotic strains has generally been based on *in vitro* tolerance of physically relevant stresses; *e.g.*, low pH, bile, elevated osmolarity, and functionality [38, 42, 52]. The ability of probiotic strains to hydrolyze bile salts is one of the important properties considered for probiotic strain selection [2]. Bile acids synthesized in the liver are important end products of cholesterol metabolism in mammals, and are secreted in bile after conjugation with taurine or glycine [14, 22]. Upon dietary lipid intake, they are mixed and emulsified with lipids for absorption in the duodenum [2].

Recently, increased attention has been given to the possibility of using bile-salt conjugation, carried out by indigenous microflora in the mammalian intestine, to lower serum cholesterol levels in hypercholesterolemic humans and to prevent hypercholesterolemia in humans with normal cholesterol levels [30]. The control of cholesterol by intestinal bacteria is based on the observation that intestinal bacteria can significantly reduce serum cholesterol levels *via* the hydrolysis of conjugated bile salts by bile salt hydrolase (BSH) [49]. BSH produced by intestinal bacteria catalyzes the hydrolysis of the amide bond between either glycine or taurine and cholesterol-based bile acids [2, 33]. The deconjugated bile acids have low water solubility and are easily excreted in the feces. Fecal loss of bile acids and the decreased amount of bile salts returned to the liver may result in the increased conversion of cholesterol to bile salts to maintain bile salt homeostasis [53]. The oral administration of lactobacilli with BSH activity lowers blood cholesterol levels in humans [9] and other animals [18, 36, 39].

A number of BSH genes have been cloned and characterized from lactic acid bacteria such as *Lactobacillus plantarum* [5, 19], *L. johnsonii* [12], *Bifidobacterium longum* [47], and *B. bifidum* [23]. Most BSH activity is found in strains isolated from the gastrointestinal tract or feces of mammals from environments rich in conjugated and free bile acids [25]. *Lactobacillus acidophilus* can reportedly survive in the gastrointestinal tract [43], adhere to host epithelial cells *in vitro* [1, 16], modulate the host immune response [54], inhibit pathogenic bacteria such as *Helicobacter pylori* [31], and provide a hepatoprotective effect in mice [21]. Bile-tolerant *L. acidophilus* strains have been isolated [3, 7, 20]. Although *L. acidophilus* plays a biologically important role in the mammalian intestine, BSH enzyme from *L. acidophilus* has not yet been characterized. *L. acidophilus* strain PF01 was previously isolated from healthy piglet's feces; this strain had high bile resistance activity *in vitro* and adhered specifically to piglet duodenal and jejunal epithelium cells [1]. We cloned the *bsh* gene of *L. acidophilus* PF01 and characterized the recombinant BSH enzyme overexpressed in *Escherichia coli*.

Materials and Methods

Materials, Bacterial Strains, and Vectors

E. coli strains DH5α (Takara Biomedical, Japan) and BLR (DE3) (Novagen, U.S.A.) were used as hosts for cloning and gene expression, respectively. For the isolation of chromosomal DNA, *L. acidophilus* PF01 was cultured at 37°C in deMan-Rogosa-Sharpe (MRS) broth (Difco, U.S.A.). The plasmid pUC119 (Life Technology, U.S.A.) was used as a vector for cloning, and pET21b (Novagen) was used for gene expression. Restriction enzymes and modifying enzymes were purchased from Takara Biomedical and used according to the supplier's recommendations. The preparation of DNA was carried out using the alkaline lysis method [41]. *E. coli* cells were grown in Luria-Bertani (LB) medium at 37°C, and ampicillin (50 μg/ml) was added, if necessary. Other recombinant DNA techniques were used as described by Sambrook and Russell [41]. Sodium salts of bile acids were purchased from Sigma (U.S.A.).

Cloning of *bsh* from *L. acidophilus* PF01

Chromosomal DNA was prepared from *L. acidophilus* PF01 cultured overnight in MRS broth, as described previously [13]. Total DNA was digested with HindIII and ligated into pUC119 digested with HindIII, and dephosphorylated. The genomic library was then transformed into *E. coli* DH5α competent cells. Ampicillin-resistant clones were tested for bile salt-hydrolyzing activity. The transformants were grown on LB agar plates containing bile salts and incubated at 37°C to identify bile-salt hydrolyzing colonies. The screening medium contained 0.5% sodium taurocholate.

Sequence Analysis of *bsh*

The nucleotide sequence of the insert was determined using a BigDye-terminator sequencing kit and ABI PRISM 377 sequencer (Perkin-Elmer, U.S.A.), according to the manufacturer's instructions. The BLAST2 program from the National Center for Biotechnology Information (NCBI) was used for nucleotide sequence analysis and amino acid sequence deduction. Protein sequences were aligned using the ClustalW software package [51].

Expression of *bsh* in *E. coli*

bsh was amplified and then subcloned into a BamHI/XhoI-digested pET21b plasmid (Novagen), resulting in the pET-bsh where *bsh* was fused with a vector sequence encoding $(His)_6$. The pET-bsh expression plasmid was introduced into *E. coli* BLR(DE3) cells by electroporation. The resulting transformants were grown in LB liquid medium in the presence of ampicillin (50 μg/ml) at 30°C to an OD_{600} of 0.5–0.6. The expression of *bsh* was induced by the addition of isopropylthiogalactopyranoside (IPTG) to give a final concentration of 0.1 mM. The cells were harvested by centrifugation at 12,000 rpm for 5 min. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using 10% (w/v) polyacrylamide gels containing 0.1% (w/v) SDS, as described by Laemmli [29], to confirm the production of the BSH enzyme. Protein bands were detected using Coomassie brilliant blue R250 staining. The protein concentration was measured by the Bradford method (Bio-Rad, U.S.A.), using bovine serum albumin as a standard.

Purification of the BSH Enzyme

To purify the recombinant BSH, the cell pellet was washed with and resuspended in lysis buffer (300 mM NaCl, 50 mM Na-Pi, pH 7.0) and then disrupted by sonication (5×20 s, 0.5 cycle, 50% amplitude). The mixture was centrifuged at 12,000 rpm for 20 min at 4°C to separate the soluble supernatant and the insoluble pellet fraction containing inclusion bodies. The soluble fraction containing BSH was applied to a Ni^{2+}-NTA agarose column (Qiagen, Germany) that had been equilibrated with lysis buffer. The column was washed twice with lysis buffer containing 20 mM imidazole, and BSH bound to the resin was eluted with elution buffer (lysis buffer containing 250 mM imidazole).

Substrate Specificity

The substrate specificity of the BSH was determined using eight different bile salts. We used the methods of Tanaka *et al.* [46], with some modifications. Specifically, the purified BSH was mixed with 10 mM conjugated bile acid and 100 mM sodium phosphate (pH 6.0) in a total volume of 0.2 ml. The reactions were run for 30 min at 37°C and stopped with 15% trichloroacetic acid. The amount of amino acid released from conjugated bile acids was measured in a reaction involving 0.5 ml of 1% ninhydrin, 1.2 ml of glycerol, and 0.2 ml of 0.5 M citrate (pH 5.5). The substrates used were glyco- and tauro-conjugated bile salts (Sigma). A_{570} was measured using a UV-1601PC spectrophotometer (Shimadzu, Japan).

Nucleotide Sequence Accession Numbers

The *bsh* nucleotide sequence for *L. acidophilus* PF01 was deposited in the GenBank database under Accession No. EF536029.

Results

Cloning of *bsh* from *L. acidophilus*

We previously isolated *L. acidophilus* PF01, which exhibited high bile tolerance, from the feces of healthy

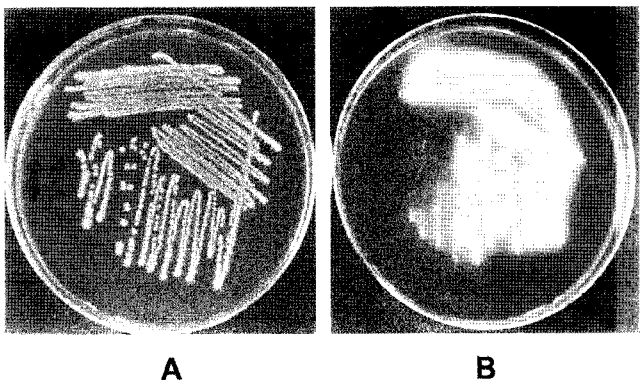

Fig. 1. Manifestation of *Lactobacillus acidophilus* PF01 BSH activity on solid MRS medium.
Plates were incubated anaerobically at 37°C for 72 h. **A**. Control plate; **B**. Plate containing 0.5% sodium taurocholate.

piglets [1]. This strain has high bile-salt-hydrolyzing activity (Fig. 1B), which was observed as cloudiness in MRS agar containing 0.5% bile salts. To isolate *bsh* from *L. acidophilus* PF01, a HindIII-digested genomic library of *L. acidophilus* PF01 was constructed in *E. coli* DH5α using pUC119, as described in Materials and Methods. Ampicillin-resistant clones were transferred onto LB agar plates containing 0.5% bile salts. After incubation at 37°C for between 3 and 7 days, two colonies were surrounded by a halo of precipitated free bile acids because of hydrolysis of bile salts. These were selected (Fig. 2) and designated pLBSH1 and pLBSH2, respectively.

Genetic Characterization of *L. acidophilus bsh*

The selected clones were further characterized. Preliminary restriction enzyme analysis and sequence analysis revealed that the two clones contained the same 1.5-kb insert in the same direction (data not shown). The entire 1.5-kb insert

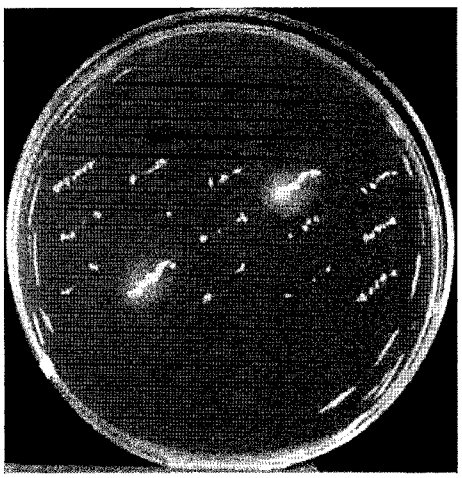

Fig. 2. Detection of the BSH activity of *Escherichia coli* transformants.
BSH-active colonies produce copious amounts of cholic acid.

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                                        -35                 -10
AAGCTTTACAGTTGCTTGGTACTTTATGATTGCCTTTGTTGCAATTGGTTTTATCATTT      60
AGTATTTGCCGTTATCCAAATTAAGAGATACCAAAAGAAATACATTGCAGAGCAAGCAGC     120
AAAAGCTAATGCTAAATAAGGAGGTTTTTTGGAAATGTGTACTGGTTTAAGATTCACAGA     180
                                    M  C  T  G  L  R  F  T  D       9
TGATCAAGGAAATTTATACTTTGGCCGTAATCTAGATGTTGGACAGGATTATGGCGAAGG     240
  D  Q  G  N  L  Y  F  G  R  N  L  D  V  G  Q  D  Y  G  E  G      29
CGTTATTATTACGCCGGGTAATTATCCTCTTCCATATAAGTTCTTAGATAACACCACTAC     300
  V  I  I  T  P  G  N  Y  P  L  P  Y  K  F  L  D  N  T  T  T      49
TAAAAAGGCTGTTATTGGAATGGGAATTGTGGTTGATGGCTATCCATCATACTTTGACTG     360
  K  K  A  V  I  G  M  G  I  V  V  D  G  Y  P  S  Y  F  D  C      69
CTATAACGAAGATGGATTAGGCATTGCAGGTTTAAACTTCCCACATTTTGCTAAATTTAG     420
  Y  N  E  D  G  L  G  I  A  G  L  N  F  P  H  F  A  K  F  S      89
TGATGGTCCTATTGACGGTAAAATCAACTTAGCTTCTTACGAAATTATGCTCTGGGTTAC     480
  D  G  P  I  D  G  K  I  N  L  A  S  Y  E  I  M  L  W  V  T     109
TCAAAACTTTACTCATGTTAGTGAAGTAAAGGAAGCGTTAAAGAATGTTAACTTAGTGAA     540
  Q  N  F  T  H  V  S  E  V  K  E  A  L  K  N  V  N  L  V  N     129
TGAAGCTATTAACACATCATTTGCGGTTGCCCCTCTTCACTGGATCATTAGTGATAGTGA     600
  E  A  I  N  T  S  F  A  V  A  P  L  H  W  I  I  S  D  S  D     149
CGAAGCCATTATTGTTGAAGTTTCAAAACAATATGGAATGAAAGTCTTTGATGATAAAGT     660
  E  A  I  I  V  E  V  S  K  Q  Y  G  M  K  V  F  D  D  K  V     169
TGGCGTTTTAACTAATAGCCCTGACTTTAACTGGCACCTTACTAACCTTGGTAACTATAC     720
  G  V  L  T  N  S  P  D  F  N  W  H  L  T  N  L  G  N  Y  T     189
TGGTTTAAATCCACATGAGGCTACAGCCCAAAGCTGGAACGGTCAAAAAGTTGCTCCTTG     780
  G  L  N  P  H  D  A  T  A  Q  S  W  N  G  Q  K  V  A  P  W     209
GGGTGTAGGAACTGGTAGTTTAGGTCTGCCTGGTGACAGCATTCCAGCCGACCGTTTTGT     840
  G  V  G  T  G  S  L  G  L  P  G  D  S  I  P  A  D  R  F  V     229
TAAAGCTGCTTACTTAAACGTAAACTATCCAACTGCTAAAGGTGAAAAAGCAAACGTCGC     900
  K  A  A  Y  L  N  V  N  Y  P  T  A  K  G  E  K  A  N  V  A     249
TAAATTCTTTAACATCTTAAAGTCTGTTGCCATGATCAAAGGCAGTGTAGTCAACGATCA     960
  K  F  F  N  I  L  K  S  V  A  M  I  K  G  S  V  V  N  D  Q     269
AGGCAAGGACGAATATACTGTTTATACTGCATGCTACTCTTCTGGAAGCAAGACTTACTA    1020
  G  K  D  E  Y  T  V  Y  T  A  C  Y  S  S  G  S  K  T  Y  Y     289
CTGTAATTTTGAAGATGATTTTGAATTAAAGACTTATAAACTAGATGATCACACGATGAA    1080
  C  N  F  E  D  D  F  E  L  K  T  Y  K  L  D  D  H  T  M  N     309
TTCAACCAGTCTTGTGACTTACTAGTTAAATTTATATCCTATATTCCCAACAAAAAAAGC    1140
  S  T  S  L  V  T  Y  *                                         316
CACCTGAAACAGGTGGCTTTTGTCGTATACAAGTGTTTAATTTTAGAAAGGACTATTATA    1200
GCCTGATATAGCAATACCGTCAACCGCTCGATATCCTTTTTATTAATTAATCTTCTTCTG    1260
ATCTAATTACTTTTTTAGAGCTAGCATCAATTTCAACTTCATGTTTTGTGCTACCGGATT    1320
CTACTGTTACCTCCCAAATAGCCTTACCATCTTCCATTTTTAAGTCCCATTCCTTGGCAA    1380
CACTATTTTTTACTTCTTTTTCTGCTATTTCACTAGCTTCTTTACGCGAAATTGTTTTAT    1440
CTAAATCTAAACCTTTTTGTAGGTGTTCATCTAGATCAAGCTT                     1483
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Fig. 3. Nucleotide sequence and deduced amino acid sequence of the *Lactobacillus acidophilus* PF01 *bsh* gene.
Putative promoter elements are boxed, and a possible ribosome-binding site is underlined.

of pLBSH1 was sequenced on both strands. The fragment contained a single ORF of 951 nucleotides flanked by a methionine start codon (ATG) and a translational termination codon (TAG), and encoded a 316 amino acid protein (Fig. 3). The assigned ATG initiation codon was preceded by a typical Shine-Dalgarno sequence (5'-AGGAGG-3') 10 bp upstream of the start codon. Potential -35 (TTGCCT) and -10 (TATCAT) regions of the *bsh* promoter were also located upstream of the start codon.

The protein deduced from the complete ORF had a theoretical molecular mass of 34,856 Da and a pI of 4.88. It showed high homology to BSHs from other lactic acid bacteria, which strongly implies that the cloned gene encodes the *L. acidophilus* BSH; it was therefore designated the *bsh* gene. The deduced amino acid sequence of the *L. acidophilus* BSH was aligned with the reported sequences of BSHs from other bacteria (Fig. 4). Overall, the deduced amino acid sequence of the *L. acidophilus* BSH shared 98%, 38%, 43%, 43%, and 38% identities with BSH from *L. johnsonii* [12], *L. plantarum* [5], *B. bifidum* [23], *B. longum* [47], and *Clostridium perfringens* (CBAH, conjugated bile acid hydrolase) [6], respectively. Multiple amino acid sequence alignments identified a highly conserved

Fig. 4. Alignment of the amino acid sequence of BSH proteins. Lacid, *Lactobacillus acidophilus* PF01 (GenBank Accession No. ABQ01980); Ljohn, *L. johnsonii* (GenBank Accession No. AAC34381); Lplan, *L. plantarum* (GenBank Accession No. AAA25233); Bbifi; *Bifidobacterium bifidum* (GenBank Accession No. AAR39435); Blong, *B. longum* (GenBank Accession No. AAF67801); Cperf, *Clostridium perfringens* (GenBank Accession No. AAC43454). Identical amino acids are indicated by asterisks. Five proposed active sites (C, D, N, N, R) are highlighted in white on a black background. The dashes indicate gaps in the alignments.

domain. Recently, the crystal structure of CBAH from *C. perfringens* was elucidated [40], and the residues Cys-2, Asp-21, Asn-82, Asn-175, and Arg-228 have been identified as catalytically important. A comparison of the deduced amino acid sequences of *L. acidophilus* BSH and other BSHs with the deduced amino acid sequences of the CABH showed that all of these amino acids are conserved (Fig. 5). In addition, four conserved amino acid motifs located around the active site, FGRNXD, AGLNF, VLTNXP, and GXGXGXXGXPGD, were highly conserved [25].

Heterologous Expression of *L. acidophilus bsh* in *E. coli*

The insert containing the *bsh* gene was cloned into a BamHI/XhoI-digested pET21d expression vector and expressed in *E. coli* BLR(DE3) by induction with 0.1 mM IPTG. The transformants grew normally after the induction of fusion peptide expression, indicating that the expression

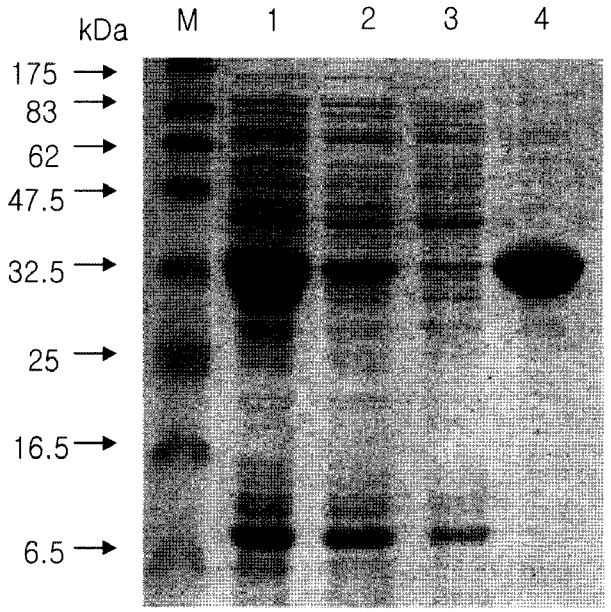

Fig. 5. Expression and purification of *Lactobacillus acidophilus* BSH enzyme.
Lane M, prestain protein marker (New England Biolab, U.K.); lane 1, total cell lysate; lane 2, flow-through fraction; lane 3, washing fraction; lane 4, eluted fraction.

of BSH enzyme is not toxic to the host. A product with a molecular mass of approximately 34 kDa was observed as a prominent band and accounted for as much as approximately 30% of the total cellular protein (Fig. 5). This is the predicted M_r for the product specified by the *bsh* gene from *L. acidophilus*. There was no significant difference in the expression level of the fusion peptide according to induction time (data not shown).

For the purification of the recombinant BSH enzyme, the cell pellet was disrupted and the soluble fraction containing BSH enzyme was applied to an Ni^{2+}-NTA agarose column. The purified BSH enzyme showed apparent homogeneity.

Substrate Specificity of the *L. acidophilus* BSH Enzyme

The substrate specificity of the BSH enzyme was determined in enzyme assays using the eight major human bile salts. The recombinant BSH enzyme exhibited hydrolyzing activity against tauroconjugated bile salts, but not glycoconjugated bile salts (Fig. 6). Most BSHs, especially those from lactic acid bacteria, are more efficient at hydrolyzing glycoconjugated bile salts than tauroconjugated bile salts [6, 24, 47]. The purified BSH enzyme from *L. plantarum* CK 102 had broad substrate specificity and exhibited higher affinity for glycoconjugated bile acids than for tauroconjugated bile acids [19]. The enzymes from *L. plantarum* [5], *Lactobacillus* sp. 100-100 [32], *C. perfringens* [15], and *Bacteroides fragilis* [45] are generally more active on glycoconjugated bile salts, whereas those from *Bacteroides vulgatus* preferentially hydrolyze tauroconjugated bile salts.

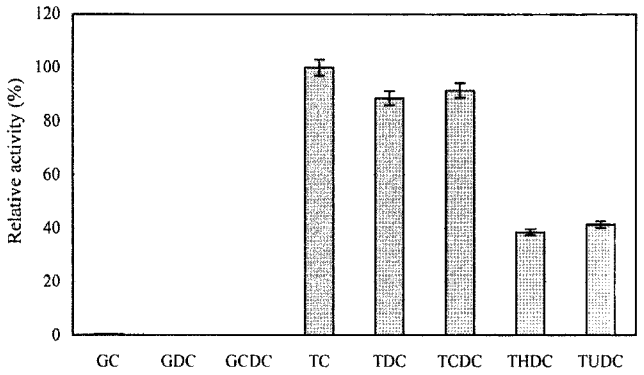

Fig. 6. Substrate specificity of the purified BSH enzyme.
Eight major bile salts are shown: glycocholic acid (GC), glycodeoxycholic acid (GDC), glycochenodeoxycholic acid (GCDC), taurocholic acid (TC), taurodeoxycholic acid (TDC), taurochenodeoxycholic acid (TCDC), taurohyodeoxycholic acid (THDC), and tauroursodeoxycholic acid (TUDC). The relative activity was calculated using TC as a standard at 100%.

Effect of pH and Temperature on *L. acidophilus* BSH Activity

The maximum BSH activity occurred at approximately 40°C (Fig. 7A). The enzyme maintained approximately 70% of its maximum activity even at 60°C, whereas its activity rapidly decreased below 37°C. The optimum pH was 6, and BSH activity rapidly declined below pH 5 and above pH 7 (Fig. 7B). Tanaka *et al.* [47] reported that the optimum pH of *B. longum* BSH was between 5 and 7, and its maximal activity occurred at 40–45°C. The *L. acidophilus* BSH was similar to the *B. longum* BSH in optimum pH and temperature, but *L. acidophilus* BSH was stable at higher temperatures.

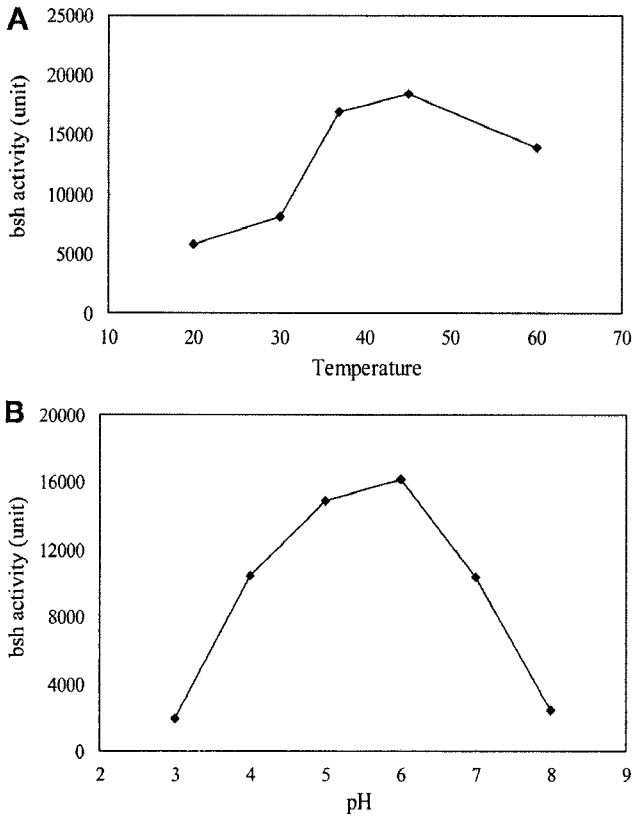

Fig. 7. Effects of temperature (**A**) and pH (**B**) on BSH activity.
BSH activity at different temperatures was measured in 100 mM sodium phosphate buffer (pH 6.0). BSH activity at various pHs was measured in 100 mM sodium acetate buffer at pH 3.0–5.0 and 100 mM sodium phosphate buffer at pH 6.0–8.0.

DISCUSSION

Reports that a reduction in the level of serum cholesterol is associated with the presence of BSH-producing lactic acid bacteria has led to increased interests in the possible application for hypercholesterolemic individuals or to prevent elevated cholesterol levels in individuals with normal cholesterol status [9, 18]. In this aspect, bacterial cell therapy by the administration of a bile-hydrolyzing bacterial strain to control serum cholesterol levels seems promising. To date, a number of BSH genes have been cloned and characterized from lactic acid bacteria. However, data on the BSH enzyme from *L. acidophilus*, which plays an important role in human and animal health by stabilizing the normal intestinal microflora and lowering the serum cholesterol level [3, 39, 43], are very limited, although recently McAuliffe *et al.* [34] identified two bile-salt-hydrolyzing genes in *L. acidophilus* NCFM.

We cloned, characterized, and examined the expression of a *bsh* gene from *L. acidophilus* PF01, which was isolated from piglet feces. Based on the homology of the deduced protein to previously known protein sequences, *L. acidophilus* BSH shares high amino acid sequence similarity with other BSHs. Based on its structural similarity to CBAH from *C. perfringens* [40] and PVA from *B. sphaericus* [37], a cysteine residue serves as a nucleophile and a proton donor in the catalytic process that is common to all members of the N-terminal nucleophile (Ntn) hydrolase superfamily [26]. A comparison of the deduced amino acid sequences of *L. acidophilus* BSH and other BSHs with the deduced amino acid sequences of CABH showed that all of these amino acids are conserved (Fig. 4). In addition, four amino acid motifs located around the active site, FGRNXD, AGLNF, VLTNXP, and GXGXGXXGXPGD, are highly conserved [23]. Sequence alignments indicate that, whereas residues of the active site are strictly conserved in BSHs, the residues for substrate recognition are not particularly conserved. These observations indicate that there are a variety of BSH enzymes in nature that have distinct substrate-hydrolyzing capabilities. Future structural studies of BSHs that have different substrate specificities will elucidate the key residues of the substrate-binding pocket and provide information on the substrate selectivity of BSH enzymes. [26].

The *bsh* promoter sequence was predicted to reside approximately 130 bp upstream of the ATG start codon. This sequence contains all of the conserved promoter elements, including -35 and -10 hexamers, a TG motif immediately upstream of the -10 region, and a spacer of 16 nucleotides between the -35 and -10 regions; they are all conserved relative to those of their *E. coli* promoter counterparts [4]. It has been proposed that the -35 hexamer, -10 hexamer, and TG motifs are significant determinants of promoter strength in lactobacilli [35]. Therefore, this promoter sequence could be a good candidate for the *E. coli*-lactobacilli shuttle vector construction, as well as for improving the expression of heterologous genes in lactobacilli by modifying some of these core promoter elements to enhance transcription initiation. The *bsh* gene of *L. acidophilus* PF01, including the predicted promoter sequence characterized here, is highly conserved in *L. johnsonii* strain 100-100 [12]. However, the DNA sequence similarity of these two strains ends immediately downstream of the *bsh* gene. This fact implies that the *bsh* gene might be acquired by horizontal gene transfer (HGT) possibly because BSH activity is important at some level for colonization in the gastrointestinal tract (GIT) of humans as well as pigs. Bile salt resistance is one of the most important requirements for probiotic strains to survive and colonize in the GIT and exhibit their health-promoting effect on the host. This is also the case for some pathogenic bacteria to survive in the GIT and to show the virulence *in vivo*. In some pathogenic bacteria, BSH activity confers the ability to resist the antimicrobial actions of bile salts and may contribute to a pathogen's ability to infect the host through the oral route [10, 11].

We expressed the *L. acidophilus* PF01 BSH enzyme in *E. coli* and characterized it; to our knowledge, this is the first report on *L. acidophilus* BSH. The substrate specificity of *L. acidophilus* PF01 BSH was different from those of the majority of known BSHs. It showed hydrolase activity against tauroconjugated bile salts, but not against glycoconjugated bile salts, with the highest activity being against taurocholic acid. This is very interesting, because BSHs from intestinal lactic acid bacteria generally have higher affinity for glucoconjugated bile salts [6, 24, 47]. McAuliffe *et al.* [34] identified two *bsh* genes in the genome sequence of *L. acidophilus* NCFM; targeted inactivation of these genes demonstrated that the two enzymes have different specificities. The possibility of another *bsh* gene that has different substrate specificity in *L. acidophilus* PF01 cannot be excluded and should be examined in the future.

It has been proposed that the BSH activity of enteric bacteria might have an important role for persistent colonization in the gastrointestinal tract [12]. Interestingly, lactobacilli contribute 74% and 86% of total bile acid hydrolase activity in the cecum and ileum, respectively, in mice [48]. *Lactobacillus* species colonize the gastrointestinal tract of mammals and birds to high population levels [44]. *L. acidophilus* PF01 adheres specifically to the duodenal and jejunal epithelium cells of piglets [1]. However, it is somewhat controversial whether the BSH activity of lactic acid bacteria is beneficial or detrimental to the host [8, 11] because the hydrolytic products of BSH, free bile acids, are toxic to cells and can damage the membranes of mammalian cells [50]. Cholic acid, the main free bile acid produced by BSH activity in the intestine, can accumulate inside lactobacilli and bifidobacterial cells in the intestine, as long as the bacteria are energized [27, 28]. The entrapment of free bile acids by bifidobacteria can contribute to a decrease in free bile acids. Whether *L. acidophilus* PF01 can accumulate free bile acids inside the cell needs to be determined. In addition, the construction of a BSH-negative mutant of *L. acidophilus* PF01 would be interesting because it could be used to investigate whether the bacterium still adheres to piglet intestinal epithelial cells and lowers blood cholesterol levels in humans and animals.

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References

1. Ahn, Y. T., K. L. Lim, J. C. Ryu, D. K. Kang, J. S. Ham, Y. H. Jang, and H. U. Kim. 2002. Characterization of *Lactobacillus acidophilus* isolated from piglets and chicken. *Asian Aust. J. Anim. Sci.* **15:** 1790–1797.
2. Begley, M., C. Hill, and C. G. Gahan. 2006. Bile salt hydrolase activity in probiotics. *Appl. Environ. Microbiol.* **72:** 1729–1738.
3. Buck, L. M. and S. E. Gilliland. 1994. Comparisons of freshly isolated strains of *Lactobacillus acidophilus* of human intestinal origin for ability to assimilate cholesterol during growth. *J. Dairy Sci.* **77:** 2925–2933.
4. Burr, T., J. Mitchell, A. Kolb, S. Minchin, and S. Busby. 2000. DNA sequence elements located immediately upstream of the -10 hexamer in *Escherichia coli* promoters: A systematic study. *Nucleic Acids Res.* **28:** 1864–1870.
5. Christiaens, H., R. J. Leer, P. H. Pouwels, and W. Verstraete. 1992. Cloning and expression of a conjugated bile acid hydrolase gene from *Lactobacillus plantarum* by using a direct plate assay. *Appl. Environ. Microbiol.* **58:** 3792–3798.
6. Coleman, J. P. and L. L. Hudson. 1995. Cloning and characterization of a conjugated bile acid hydrolase gene from *Clostridium perfringens*. *Appl. Environ. Microbiol.* **61:** 2514–2520.
7. Corzo, G. and S. E. Gilliland. 1999. Bile salt hydrolase activity of three strains of *Lactobacillus acidophilus*. *J. Dairy Sci.* **82:** 472–480.

8. De Boever, P. and W. Verstraete. 1999. Bile salt deconjugation by *Lactobacillus plantarum* 80 and its implication for bacterial toxicity. *J. Appl. Microbiol.* **87:** 345–352.
9. De Smet, I., L. Van Hoorde, N. De Saeyer, M. Vande Woestyne, and W. Verstraete. 1994. *In vitro* study of bile salt hydrolase (BSH) activity of BSH isogenic *Lactobacillus plantarum* 80 strains and estimation of cholesterol lowering through enhanced BSH activity. *Microb. Ecol. Health Dis.* **7:** 315–329.
10. Delpino, M. V., M. I. Marchesini, S. M. Estein, D. J. Comerci, J. Cassataro, C. A. Fossati, and P. C. Baldi. 2007. A bile salt hydrolase of *Brucella abortus* contributes to the establishment of a successful infection through the oral route in mice. *Infect. Immun.* **75:** 299–305.
11. Dussurget, O., D. Cabanes, P. Dehoux, M. Lecuit, C. Buchrieser, P. Glaser, and P. Cossart. 2002. European *Listeria* genome consortium. *Listeria monocytogenes* bile salt hydrolase is a PrfA-regulated virulence factor involved in the intestinal and hepatic phases of listeriosis. *Mol. Microbiol.* **45:** 1095–1106.
12. Elkins, C. A., S. A. Moser, and D. C. Savage. 2001. Genes encoding bile salt hydrolases and conjugated bile salt transporters in *Lactobacillus johnsonii* 100-100 and other *Lactobacillus* species. *Microbiology* **147:** 3403–3412.
13. El-Osta, Y. G., A. J. Hillier, and M. Dobos. 2005. Construction of a combined physical and genetic map of the chromosome of *Lactobacillus acidophilus* ATCC 4356 and characterization of the rRNA operons. *Microbiology* **151:** 875–892.
14. Eyssen, H. 1973. Role of the gut microflora in metabolism of lipids and sterols. *Proc. Nutr. Soc.* **32:** 59–63.
15. Gopal-Srivastava, R. and P. B. Hylemon. 1988. Purification and characterization of bile salt hydrolase from *Clostridium perfringens*. *J. Lipid Res.* **29:** 1079–1085.
16. Greene, J. D. and T. R. Klaenhammer. 1994. Factors involved in adherence of lactobacilli to human Caco-2 cells. *Appl. Environ. Microbiol.* **60:** 4487–4494.
17. Guarner, F. and G. J. Schaafsma. 1998. Probiotics. *Int. J. Food Microbiol.* **39:** 237–238.
18. Ha, C.-G., J.-K. Cho, C.-H. Lee, Y.-G. Chai, Y.-A. Ha, and S. H. Shin. 2006. Cholesterol lowering effect of *Lactobacillus plantarum* isolated from human feces. *J. Microbiol. Biotechnol.* **16:** 1201–1209.
19. Ha, C.-G., J.-K. Cho, Y.-G. Chai, Y.-A. Ha, and S. H. Shin. 2006. Purification and characterization of bile salt hydrolase from *Lactobacillus plantarum* CK 102. *J. Microbiol. Biotechnol.* **16:** 1047–1052.
20. Han, K. S., Y. Kim, S. Choi, S. Oh, S. Park, S. H. Kim, and K. Y. Whang. 2005. Rapid identification of *Lactobacillus acidophilus* by restriction analysis of the 16S-23S rRNA intergenic spacer region and flanking 23S rRNA gene. *Biotechnol. Lett.* **27:** 1183–1188.
21. Han, S. Y., C. S. Huh, Y. T. Ahn, K. S. Lim, Y. J. Baek, and D. H. Kim. 2005. Hepatoprotective effect of lactic acid bacteria. *J. Microbiol. Biotechnol.* **15:** 887–890.
22. Hoffman, N. E. and A. F. Hofmann. 1977. Metabolism of steroid and amino acid moieties of conjugated bile acids in man. V. Equations for the perturbed enterohepatic circulation and their application. *Gastroenterology* **72:** 141–148.
23. Kim, G.-B., C. M. Miyamoto, E. A. Meighen, and B. H. Lee. 2004. Cloning and characterization of the bile salt hydrolase genes (*bsh*) from *Bifidobacterium bifidum* strains. *Appl. Environ. Microbiol.* **70:** 5603–5612.
24. Kim, G.-B., S. H. Yi, and B. H. Lee. 2004. Purification and characterization of three different types of bile salt hydrolases from *Bifidobacterium* strains. *J. Dairy Sci.* **87:** 258–266.
25. Kim, G.-B. and B. H. Lee. 2005. Biochemical and molecular insights into bile salt hydrolase in the gastrointestinal microflora: A Review. *Asian Aust. J. Anim. Sci.* **18:** 1505–1512.
26. Kumar, R. S., J. A. Brannigan, A. A. Prabhune, A. V. Pundle, G. G. Dodson, E. J. Dodson, and C. G. Suresh. 2006. Structural and functional analysis of a conjugated bile salt hydrolase from *Bifidobacterium longum* reveals an evolutionary relationship with penicillin V acylase. *J. Biol. Chem.* **281:** 32516–32525.
27. Kurdi, P., H. W. van Veen, H. Tanaka, I. Mierau, K. N. Konings, G. W. Tannock, F. Tomita, and A. Yokota. 2000. Cholic acid is accumulated spontaneously, driven by membrane delta pH, in many lactobacilli. *J. Bacteriol.* **182:** 6525–6528.
28. Kurdi, P., H. Tanaka, H. W. van Veen, K. Asano, F. Tomita, and A. Yokota. 2003. Cholic acid accumulation and its diminution by short-chain fatty acids in bifidobacteria. *Microbiology* **149:** 2031–2037.
29. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227:** 680–685.
30. Larsen, C. T., C. Sorum, J. F. Hansen, H. A. Jensen, and V. Rasmussen. 2000. Blood pressure level and relation to other cardiovascular risk factors in male hypertensive patients without clinical evidence of ischemic heart disease. *Blood Press* **9:** 91–97.
31. Lee, Y. H., E. J. Shin, J. H. Lee, and J. H. Park. 1999. *Lactobacillus acidophilus* inhibits the *Helicobacter pylori* adherence. *J. Microbiol. Biotechnol.* **9:** 794–797.
32. Lundeen, S. G. and D. C. Savage. 1990. Characterization and purification of bile salt hydrolase from *Lactobacillus* sp. strain 100-100. *J. Bacteriol.* **172:** 4171–4177.
33. Masui, T. and E. Staple. 1966. The formation of bile acids from cholesterol. The conversion of 5-beta-cholestane-3-alpha, 7-alpha-triol-26-oic acid to cholic acid *via* 5-beta-cholestane-3-alpha, 7-alpha,12-alpha, 24-xi-tetraol-26-oic acid I by rat liver. *J. Biol. Chem.* **241:** 3889–3893.
34. McAuliffe, O., R. J. Cano, and T. R. Klaenhammer. 2005. Genetic analysis of two bile salt hydrolase activities in *Lactobacillus acidophilus* NCFM. *Appl. Environ. Microbiol.* **71:** 4925–4929.
35. McCracken, A. and P. Timms. 1999. Efficiency of transcription from promoter sequence variants in *Lactobacillus* is both strain and context dependent. *J. Bacteriol.* **181:** 6569–6572.
36. Nguyen, T. D. T., J. H. Kang, and M. S. Lee. 2007. Characterization of *Lactobacillus plantarum* PH04, a potential probiotic bacterium with cholesterol-lowering effects. *Int. J. Food Microbiol.* **113:** 358–361.
37. Olsson, A. and U. Uhlen. 1986. Sequencing and heterologous expression of the gene encoding penicillin V amidase from *Bacillus sphaericus*. *Gene* **45:** 175–181.
38. Park, S. Y., Y. T. Ko, H. K. Jeong, J. O. Yang, H. S. Chung, Y. B. Kim, and G. E. Ji. 1996. Effect of various lactic acid bacteria on the serum cholesterol levels in rats and resistance to acid, bile and antibiotics. *Kor. J. Microbiol. Biotechnol.* **24:** 304–310.

39. Park, Y. H., J. G. Kim, Y. W. Shin, S. H. Kim, and K. Y. Hwang. 2007. Effect of dietary inclusion of *Lactobacillus acidophilus* ATCC 43121 on cholesterol metabolism in rats. *J. Microbiol. Biotechnol.* **17:** 655–662.
40. Rossocha, M., R. Schultz-Heienbrok, H. von Moeller, J. P. Coleman, and W. Saenger. 2005. Conjugated bile acid hydrolase is a tetrameric N-terminal thiol hydrolase with specific recognition of its cholyl but not of its tauryl product. *Biochemistry* **44:** 5739–5748.
41. Sambrook, J. and D. W. Russell. 2001. *Gene Cloning. A Laboratory Manual*, 3rd Ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
42. Saarela, M., G. Mogensen, R. Fondén, J. Mättö, and T. Mattila-Sandholm. 2000. Probiotic bacteria: Safety, functional and technological properties. *J. Biotechnol.* **84:** 197–215.
43. Sanders, M. E. and T. R. Klaenhammer. 2001. Invited review: The scientific basis of *Lactobacillus acidophilus* NCFM functionality as a probiotic. *J. Dairy Sci.* **84:** 319–331.
44. Savage, D. C. 1977. Microbial ecology of the gastrointestinal tract. *Annu. Rev. Microbiol.* **31:** 107–133.
45. Stellwag, E. J. and P. B. Hylemon. 1976. Purification and characterization of bile salt hydrolase from *Bacteroides fragilis* subsp. *fragilis*. *Biochim. Biophys. Acta* **452:** 165–176.
46. Tanaka, H., K. Doesburg, T. Iwasaki, and I. Mierau. 1999. Screening of lactic acid bacteria for bile salt hydrolase activity. *J. Dairy Sci.* **82:** 2530–2535.
47. Tanaka, H., H. Hashiba, J. Kok, and I. Mierau. 2000. Bile salt hydrolase of *Bifidobacterium longum*: Biochemical and genetic characterization. *Appl. Environ. Microbiol.* **66:** 2502–2512.
48. Tannock, G. W., A. Tangerman, A. Van Schaik, and M. A. McConnell. 1994. Deconjugation of bile acids by lactobacilli in the mouse small bowel. *Appl. Environ. Microbiol.* **60:** 3419–3420.
49. Taranto, M. P., M. Medici, G. Perdigon, A. P. Ruiz Holgado, and G. F. Valdez. 2000. Effect of *Lactobacillus reuteri* on the prevention of hypercholesterolemia in mice. *J. Dairy Sci.* **83:** 401–403.
50. Thomas, L. A., M. J. Veysey, T. Bathgate, A. King, G. French, N. C. Smeeton, G. M. Murphy, and R. H. Dowling. 2000. Mechanism for the transit-induced increase in colonic deoxycholic acid formation in cholesterol cholelithiasis. *Gastroenterology* **119:** 806–815.
51. Thompson, J. D., D. G. Higgins, T. J. Gibson, and W. Clustal. 1994. Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res.* **22:** 4673–4680.
52. Tuomola, E., R. Crittenden, M. Playne, E. Isolauri, and S. Salminen. 2001. Quality assurance criteria for probiotic bacteria. *Am. J. Clin. Nutr.* **73:** 393S–398S.
53. Turley, S. D. and J. M. Dietschy. 1988. The metabolism and excretion of cholesterol by the liver, pp. 617–641. *In* I. M. Arias, W. B. Jakoby, H. Popper, D. Schachter, and D. A. Shafzitz (eds.), *The Liver Biology and Pathobiology*, 2nd Ed. Raven Press, New York.
54. Varcoe, J. J., G. Krejcarek, F. Busta, and L. Brady. 2003. Prophylactic feeding of *Lactobacillus acidophilus* NCFM to mice attenuates overt colonic hyperplasia. *J. Food Prot.* **66:** 457–465.