APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Sept. 2004, p. 5603–5612 Vol. 70, No. 9
0099-2240/04/$08.00+0 DOI: 10.1128/AEM.70.9.5603–5612.2004

Cloning and Characterization of the Bile Salt Hydrolase Genes (*bsh*) from *Bifidobacterium bifidum* Strains

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Received 19 December 2003/Accepted 16 May 2004

Biochemical characterization of the purified bile salt hydrolase (BSH) from *Bifidobacterium bifidum* ATCC 11863 revealed some distinct characteristics not observed in other species of *Bifidobacterium*. The *bsh* gene was cloned from *B. bifidum*, and the DNA flanking the *bsh* gene was sequenced. Comparison of the deduced amino acid sequence of the cloned gene with previously known sequences revealed high homology with BSH enzymes from several microorganisms and penicillin V amidase (PVA) of *Bacillus sphaericus*. The proposed active sites of PVA were highly conserved, including that of the Cys-1 residue. The importance of the SH group in the N-terminal cysteine was confirmed by substitution of Cys with chemically and structurally similar residues, Ser or Thr, both of which resulted in an inactive enzyme. The transcriptional start point of the *bsh* gene has been determined by primer extension analysis. Unlike *Bifidobacterium longum bsh*, *B. bifidum bsh* was transcribed as a monocistronic unit, which was confirmed by Northern blot analysis. PCR amplification with the type-specific primer set revealed the high level of sequence homology in their *bsh* genes within the species of *B. bifidum*.

Bifidobacteria are one of the major constituents of the human gastrointestinal (GI) microflora (15, 41, 43), and they have attracted particular attention due to their potential health-promoting properties (3). In the GI tracts of humans and animals, most intestinal bacteria encounter significant amounts of bile salts, which are continuously present via enterohepatic circulation (2). Bile salts are synthesized mainly from cholesterol, conjugated with glycine or taurine in the liver, stored in the gall bladder, and released into the duodenum in response to the ingestion of fatty food. In addition to their function in the intestine as natural emulsifiers, bile salts possess some detergent-like antimicrobial properties. Some bacterial species have developed mechanisms to resist the detergent action of bile salts and have evolved to transform bile salts biochemically. Among the biochemical modifications of bile salts that are exhibited by many GI microorganisms, hydrolysis of the conjugated bile salts is considered the primary metabolic activity because bile salts need to be deconjugated before further sterol transformations take place (4). The enzyme responsible, bile salt hydrolase (BSH) (EC 3.5.1.24), has been widely studied in *Bacteroides fragilis* subsp. *fragilis* (44), *Clostridium perfringens* (16), *Enterococcus* spp. (23), *Xanthomonas maltophilia* (9), *Listeria monocytogenes* (11), *Lactobacillus* spp. (26, 36, 47, 50), and *Bifidobacterium* spp. (17, 47). In particular, the genus *Bifidobacterium* has been reported to possess higher BSH activity than other bacterial groups. In previous work (22), BSH enzymes were purified from five strains of bifidobacteria, and three different types of enzyme were identified based on the biochemical characteristics. To date, the *bsh* genes of *C. perfringens* (7), *Lactobacillus plantarum* (5), *Lactobacillus johnsonii* (12), *Bifidobacterium longum* (48), and *Listeria monocytogenes* (11) have been cloned and characterized. With the advent of the genomics era, many microbial genome-sequencing projects are providing several homologous *bsh* gene sequences.

Bifidobacteria are among the most common genera in the human colon and have been considered as key commensals in promoting host health, but very little is known about the genetics of the genus *Bifidobacterium*. Among the 32 species of bifidobacteria (21), *B. longum* strains have been the most studied. *Bifidobacterium bifidum* is one of the major bifidobacterial species commonly detected in human feces (29), and it was proposed that a high level of *B. bifidum* was an indication of the typical *Bifidobacterium* flora in healthy infants as opposed to low levels in allergic infants (19). Attempts have been made in recent years to define some beneficial effects of *B. bifidum* strains, including antibacterial activities (1, 39), immunostimulating activity (24, 35), antioxidative properties (20), production of bacteriocins (53), improvement of the microbial balance (6), and reduction of inflammation in broiler chickens (14).

For the identification and detection of the bifidobacterial species, genus- and species-specific primers have been developed, mostly on the basis of 16S rRNA sequences (29). Since BSH activity is commonly detected in almost all species of *Bifidobacterium* (47), further investigation of the conserved and variable regions of the *bsh* genes from various species could be useful for the development of alternative phylogenetic markers for the genus *Bifidobacterium*.

In this report, we describe the molecular cloning, sequencing, and characterization of a BSH enzyme from *B. bifidum* ATCC 11863. The putative *bsh* promoter sequence was analyzed by primer extension to determine the transcriptional start point (TSP). The *bsh* gene sequences were obtained by PCR cloning of *B. bifidum* ATCC 15696 and ATCC 29521, and the

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Origin or relevant characteristics	Source or reference
Bacterial strains		
B. bifidum ATCC 11863	Wild type for cloning	ATCC
B. bifidum ATCC 35914	Human feces	ATCC
B. bifidum ATCC 15696	Infant intestine	ATCC
B. bifidum ATCC 29521	Type strain; infant feces	ATCC
B. bifidum KL 301	Dairy isolate	This study
B. bifidum KL 306	Dairy isolate	This study
B. adolescentis ATCC 15703	Type strain; adult intestine	ATCC
B. longum ATCC 15707	Type strain; adult intestine	ATCC
B. infantis ATCC 15697	Type strain; infant intestine	ATCC
B. breve ATCC 15707	Type strain; infant intestine	ATCC
E. coli DH5α	F^- ϕ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)*U169 endA1 recA1 hsdR17* (r_K^- m_K^+) *deoR*	GIBCO-BRL
E. coli BL21(DE3)	F^- *ompT hsdS*$_B$ (r_B^- m_B^-) *gal dcm lacY1* (DE3)	Novagen
Plasmids		
pBR322	Ap^r; cloning vector	Invitrogen
pUC19	Ap^r; cloning vector	Invitrogen
pDrive	Ap^r Kan^r; U-overhang PCR cloning vector	QIAGEN
pET36b(+)	Kan^r; *E. coli* expression vector	Novagen
pBSH14	pBR322 with 3.5-kb *B. bifidum* ATCC 11863 chromosomal insert; BSH^+	This study
pBSH27	pBR322 with 7.5-kb *B. bifidum* ATCC 11863 chromosomal insert; BSH^+	This study
pBSH274	pUC19 with 4.0-kb *Pst*I insert from pBSH27; BSH^+	This study
pDB150	pDrive with 1.5-kb PCR product; BSH^+	This study
pUCB150	pUC19 with 1.5-kb *Hind*III & *Kpn*I insert from pDB150; BSH^+	This study
pDB095	pDrive with 950-bp PCR product; BSH^+	This study
pUCB095	pUC19 with 950-bp *Hind*III & *Kpn*I insert from pDB095; BSH^-	This study
pBSH36b	pET36b(+) with 950-bp *Nde*I & *Hind*III insert from pDB095; BSH^+	This study
pBCS	pDB095 with Cys-1–Ser mutation; BSH^-	This study
pBCT	pDB095 with Cys-1–Thr mutation; BSH^-	This study

sequence comparison revealed the high level of homology (more than 98% nucleotide sequence identity) within the species of *B. bifidum*.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. All *Bifidobacterium* strains were verified for genus and species by 16S rRNA gene sequence typing according to the method of Kaufmann et al. (21). Bifidobacteria were propagated anaerobically at 37°C in MRS medium (Difco, Detroit, Mich.) supplemented with 0.05% (wt/vol) cysteine HCl. *Escherichia coli* cells were propagated at 37°C in Luria-Bertani (LB) broth with vigorous shaking or on LB medium solidified with 1.5% agar. When appropriate, ampicillin (200 μg/ml) and kanamycin (40 μg/ml) were added.

Enzyme purification. Anion-exchange chromatography (Mono Q HR 5/5; Amersham Pharmacia Biotech, Inc., Baie d'Urfé, Québec, Canada) was performed as described by Kim et al. (22). Cell extracts were applied to a column that was equilibrated with buffer A (50 mM bis-Tris propane buffer [pH 6.5]). Proteins were eluted by a linear gradient of 1 M sodium chloride in buffer A at a flow rate of 0.5 ml/min. Fractions (each, 1 ml) were collected and assayed for BSH activity. Hydrophobic interaction chromatography (HIC) was carried out with a HiTrap Butyl FF column (Amersham Pharmacia Biotech, Inc.). The active BSH fractions from the Mono Q column were pooled, desalted, and further concentrated with the Ultrafree-15 centrifugal filter unit (30 molecular weight cutoff; Millipore, Bedford, Mass.). Portions (each, 2 ml) of concentrated sample were applied to the HIC columns and eluted at a flow rate of 0.5 ml/min by a decreasing linear ammonium sulfate gradient (0.8 to 0 M) in 50 mM sodium phosphate buffer (pH 7.0). Fractions exhibiting BSH activity were pooled and assayed for protein and enzymatic activity as described below.

Determination of N-terminal amino acid sequence. The resulting active fractions were pooled, concentrated, and applied to a mini-electrophoresis unit (Bio-Rad, Hercules, Calif). The purity of the pooled sample was evaluated after the sample was stained with Coomassie brilliant blue R-250. N-terminal amino acid sequencing of the purified BSH was carried out with a Procise protein sequencing system (Applied Biosystems, Foster City, Calif.).

Enzyme assays. For bifidobacteria, BSH activity in cell extracts was measured by the hydrolysis of sodium taurocholate and/or sodium glycocholate (Sigma, St. Louis, Mo.) at 37°C in sodium phosphate buffer (0.1 M; pH 6.5). The amounts of the amino acids released from conjugated bile salts were measured by the ninhydrin assay (22). One unit of BSH activity was defined as the amount of enzyme that liberated 1 μmol of amino acids from the substrate per min. Specific activity was defined as the number of units per milligram of protein. Protein concentrations were determined by the Bio-Rad (Mississauga, Ontario, Canada) protein assay with bovine serum albumin as a standard. For the measurement of BSH activity in *E. coli* cells, assays were performed similarly, except that whole cells were disrupted with B-PER reagent (Pierce, Rockford, Ill.) according to the manufacturer's protocol.

Activity staining of polyacrylamide gel. The BSH activity staining was carried out with a nondenaturing 10% (wt/vol) acrylamide gel by the method of Kim et al. (22). BSH activity band in the gel was visualized by the formation of a white precipitate of deoxycholic acid at the position of the enzyme after the gel was incubated in the reaction mixture containing 10 mM sodium taurodeoxycholate as a substrate for the enzyme.

DNA isolation, manipulation, and transformation. *Bifidobacterium* genomic DNA was isolated according to the method of Meile et al. (31), with some modifications. Briefly, cells were harvested by centrifugation from 100 ml of an early-stationary-phase culture in MRS, washed with GTE (50 mM glucose, 25 mM Tris [pH 8.0], 10 mM EDTA) and incubated in lysis buffer (GTE containing 15 mg of lysozyme, 1 kU of mutanolysin, and 50 μg of RNase per ml) at 37°C for 1 h. Cells were lysed with 5% sodium dodecyl sulfate (SDS) at 65°C for 15 min. The cell lysates were extracted first with phenol and then with phenol-chloroform (1:1 [vol/vol]) and chloroform. DNA was precipitated by isopropanol, washed with 70% ethanol, and dissolved in TE buffer (10 mM Tris–1 mM EDTA [pH 8]). Small-scale *E. coli* plasmid preparations were performed with the QIAprep Spin Miniprep kit (QIAGEN). All of the DNA manipulations in this study were performed according to standard procedures as described previously (40). T4 DNA ligase and other DNA modifying enzymes were purchased from New England Biolabs, Inc., Invitrogen Life Technologies, or Amersham Pharmacia Biotech, Inc., and used according to the manufacturers' specifications. Electroporation was performed with a Gene-Pulser II electroporation apparatus (Bio-Rad) according to the manufacturer's specifications.

TABLE 2. Oligonucleotide primers used in this study

Primer	Nucleotide sequence (5′–3′)[a]	Location[b]	Use
BSH-F	AGTCCATATGTGCACTGGTGTCCGTTTCTCC	1–24	Cloning
BSH-R	AGTCAAGCTTCAATCGGCGGTGATCAGCTCG	951–931	Cloning
BF-SER	AGTCCATATG**TCA**ACTGGTGTCCGTTTCTCC	1–24	Cys-1–Ser mutation primer
BF-THR	AGTCCATATG**ACT**ACTGGTGTCCGTTTCTCC	1–24	Cys-1–Thr mutation primer
BBI-F	CTATGAGTATGGGGCCGAAG	120–139	Screening
BBI-R	GTTCCGCCTTGCCCCAAGTG	613–594	Screening
PEA-F	GCGGCATGTACTACGAGGAG	−555 to −536	Primer extension sequencing
PEA-1	ATCGTCGGAGAAACGGACAC	30–11	Primer extension

[a] Engineered NdeI and HindIII sites are underlined. Changed nucleotide sequences for the site-directed mutagenesis are in boldface.
[b] Based on the ATG start codon as a starting point (position 1).

Construction and screening of *B. bifidum* BSH genomic library. The isolated chromosomal DNA from *B. bifidum* ATCC 11863 was partially digested with the restriction enzyme Sau3A and analyzed by DNA electrophoresis. DNA fragments ranging from 2.0 to 8.0 kb were isolated with the QIAquick gel extraction kit (QIAGEN) and then ligated to the pBR322 vector that had been restricted with BamHI and dephosphorylated. The ligation mixture was transformed into *E. coli* DH5α-competent cells to create a plasmid library. The transformed *E. coli* was plated on LBGCT medium (LB agar plates containing 1% glucose, 0.035% calcium chloride, and 3 mM taurodeoxycholic acid), and BSH-positive clones were detected based on the formation of deoxycholate precipitate around the colony (7).

PCR. The PCRs were performed with genomic DNA or plasmid DNA from BSH-positive clones as templates for amplifying the target genes. When appropriate, restriction sites were designed at the 5′ end of the primers to facilitate future cloning steps. Template DNA and primers were added to 50 μl of PCR mixture containing 200 μM each deoxynucleoside triphosphate, PCR buffer, and 2.5 U of HotStarTaq DNA polymerase (QIAGEN). The PCR was conducted in a Perkin Elmer GeneAmp system with an initial activation step at 95°C for 15 min, followed by 35 cycles each consisting of a denaturation step at 94°C for 1 min, an annealing step at 55 to 65°C for 30 s, and an extension step at 72°C for 1 min. A final extension step of 10 min at 72°C was performed to ensure complete amplification of all DNA fragments. PCR products were analyzed by electrophoresis in agarose gels containing ethidium bromide (1 μg/ml) and purified with the QIAquick PCR purification kit (QIAGEN).

DNA sequencing and sequence analysis. The nucleotide sequences were determined by AmpliTaq FS DNA polymerase fluorescent dye terminator reactions with an Applied Biosystems 373 stretch automated sequencer. Assembly and analysis of DNA sequences were performed with DNASIS for Windows (Hitachi Software). Protein homology searches were performed with the Basic Local Alignment Search Tool (BLAST) available at the website of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/).

RNA manipulation. RNA was isolated from *B. bifidum* with the RNeasy kit (QIAGEN), starting with digestion of the bacterial cell wall in diethyl pyrocarbonate-treated TE buffer containing 15 mg of lysozyme and 1 kU of mutanolysin per ml for 15 min at 37°C. Purified RNA was treated with RNase-free DNase according to the manufacturer's instructions (QIAGEN) and resuspended in RNase-free water. The absence of any significant DNA contamination of the RNA was confirmed by PCR. The RNA was quantified by spectrophotometric measurement at an optical density of 260 nm and stored at −70°C.

Identification of *bsh* promoter sequence. Promoter sequences were predicted by using the Neural Network Promoter Prediction program (NNPP), version 2.2 (http://www.fruitfly.org/seq_tools/promoter.html). To verify the predicted promoter sequence and to determine the TSP of the mRNA, primer extension analysis was performed under conditions described by Swartzman et al. (46). The reverse primer PEA-1 (Table 2), corresponding to nucleotide positions 11 to 30 of the *bsh* gene, was 5′-end labeled with [γ-^{32}P]ATP by using T4 polynucleotide kinase (Boehringer Mannheim). The purified labeled primer was sealed in a glass microcapillary tube with total RNA (20 μg) in a total volume of 10 μl containing piperazine-*N,N*′-bis(2-ethanesulfonic acid) (PIPES) at pH 6.6 and 0.4 M NaCl. Hybridization was carried out at 55°C for 12 to 16 h. The primer was extended using Moloney murine leukemia virus reverse transcriptase (Invitrogen). At the end of the reaction, 2 μl of EDTA (0.5 M) and 1 μl of RNase (10 mg/ml) were added. The labeled cDNAs were purified, heat denatured in 50% formamide and 5 mM EDTA (pH 7.5), and electrophoresed on a sequencing gel (8% [wt/vol] acrylamide–7 M urea). The same primer and a PCR product corresponding to the region from the *bsh* gene, containing the putative TSP, were used to generate a sequencing ladder by the dideoxy-chain termination method with a T7 sequencing kit (USB Corp., Cleveland, Ohio). The resultant gel was dried and exposed to BioMax MS film (Eastman Kodak, Rochester, N.Y.).

Northern blotting. RNA (20 μg) was separated on formaldehyde agarose gels prepared as described previously (40). After the transfer, RNAs were cross-linked to the nylon membrane by UV irradiation. PCR amplicons obtained with primers BBI-F and BBI-R were radiolabeled with [α-^{32}P]dCTP (Amersham) in a Klenow reaction mixture (NEB) according to the manufacturer's specifications. DNA-RNA hybridization was performed as described by Miyamoto et al. (32). The sizes of the transcripts were estimated by direct comparison to a molecular RNA ladder (Invitrogen).

Expression of *bsh* in *E. coli*. To create the plasmid pBSH36b, the *bsh* gene was amplified with the primers BSH-F and BSH-R (Table 2). An NdeI site was designed in primer BSH-F and a HindIII site was created in primer BSH-R to include the start codon sequence and the stop codon (TGA) sequence, respectively. Cloning into the NdeI-HindIII sites of pET36b resulted in the translational fusion of the *bsh* gene to the T7 promoter and *E. coli* ribosome binding site (RBS) of the plasmid. Plasmid pBSH36b was created in *E. coli* DH5α and transformed into *E. coli* BL21(DE3) to perform the overexpression studies. For the overexpression, cells (optical density at 600 nm, 0.6) were induced with 1.0 mM isopropyl-β-D-thiogalactopyranoside (IPTG) at 37°C for 3 h. Samples were taken at 1-h intervals to measure growth and BSH activity. For the construction of the mutant by PCR-based site-directed mutagenesis, two forward primers (BF-SER and BF-THR) (Table 2) were designed to replace Cys-1 with Ser and Thr, respectively.

Nucleotide sequence accession numbers. The *bsh* nucleotide sequences for *B. bifidum* strains have been deposited in the GenBank database under accession numbers AY506536, AY604516, and AY604517.

RESULTS

Strain selection and BSH purification. Among many strains of bifidobacteria (Table 1), *B. bifidum* ATCC 11863 was selected on the basis of the high level of BSH activity as well as the distinct biochemical characteristics that are different from those of *B. longum* and *Bifidobacterium infantis* strains (22). Three different types of BSH enzyme (types A, B, and C) from the genus *Bifidobacterium* have previously been proposed (22), in which *B. bifidum* BSH belongs to type B.

For the purification of the BSH enzyme, the conditions were optimized with anion-exchange and HIC columns. Although BSH activity was eluted from a Mono Q column at an NaCl concentration of between 0.35 and 0.38 M linear gradients, conditions were further optimized by a stepwise gradient using the same molarity to improve selectivity of this column for the BSH enzyme. The established conditions with a Mono Q column were fairly effective in purifying the active BSH from the cell extracts (Fig. 1B, lane 3). When the cell extracts were applied to a Hi-Trap Butyl-FF HIC column, the selectivity profile on the column was very different from that of the Mono Q column. The BSH enzyme could thus be purified to electro-

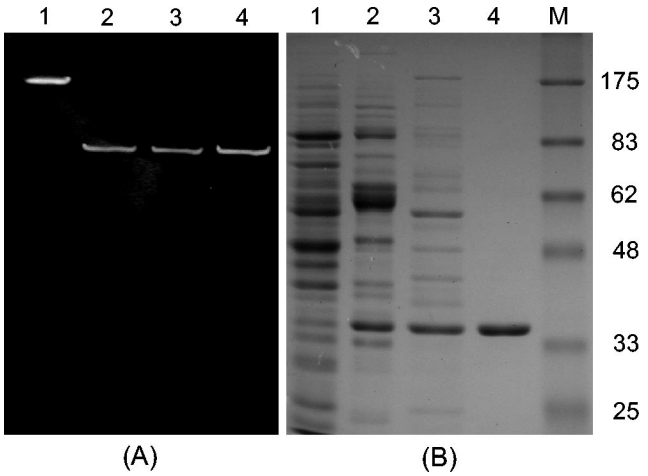

FIG. 1. (A) Activity staining on a nondenaturing polyacrylamide gel. Lane 1, BSH enzyme from *L. acidophilus* ATCC 53546 (as a positive control); lane 2, soluble fraction of *B. bifidum* ATCC 11863 from sonication treatment; lane 3, purified native BSH; lane 4, soluble fraction of IPTG-induced *E. coli* BL21(DE3) containing pBSH36b from B-PER treatment. (B) SDS-PAGE analysis of cell extracts at each purification step. Lanes 1 through 4: active fractions from *B. bifidum* ATCC 11863 (lane 1, cell extract; lane 2, HIC column; lane 3, anion-exchange column; lane 4, anion-exchange column plus HIC); lane M, marker proteins (molecular masses in kilodaltons are indicated on the right).

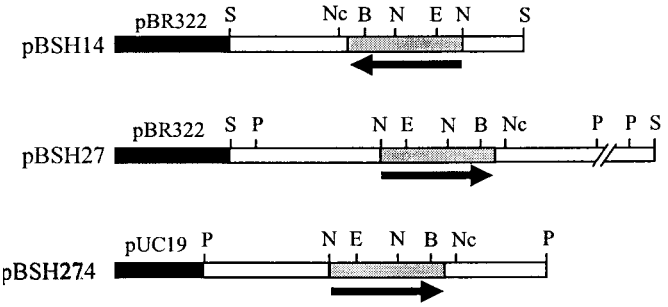

FIG. 2. Map of plasmids pBSH14, pBSH27, and pBSH274. Black boxes show cloning vectors pBR322 and pUC19, grey boxes indicate the position of the *bsh* gene, and white boxes are the cloned sequences outside the *bsh* gene. The arrows indicate the direction of the *bsh* gene. Restriction enzyme sites used for mapping the clones are shown above the maps (S, Sau3A; Nc, NcoI; B, BamHI; N, NdeI; E, EcoRI; and P, PstI).

phoretic homogeneity by combining these two chromatographic steps (Fig. 1B, lane 4). The enzyme was purified 33-fold over the crude extract, and the yield was about 29%. The subunit molecular mass was estimated to be 35 kDa by SDS-polyacrylamide gel electrophoresis (PAGE), and the native mass was estimated to be about 150 kDa by gel filtration chromatography.

The BSH activity staining on a native PAGE gel revealed that the cell extract showed only one active band with the same R_f value as that of the purified and recombinant BSH enzyme (Fig. 1A).

The N-terminal amino acid sequence of the purified enzyme is XTGVRFSDDEGNMYFGRNLD. A protein sequence comparison showed that this sequence was homologous to the N-terminal amino acid sequences of the BSH enzymes from many enteric bacteria, with the highest identity to that of the *B. longum* BSH enzyme (42, 48). Only one difference between the amino acids, Thr^{13} of *B. longum* BSH and Met^{13} from *B. bifidum* BSH, was found.

Cloning and sequence analysis. To identify the gene(s) encoding BSH activity in *B. bifidum* ATCC 11863, an Sau3AI-digested genomic library was created in pBR322 and screened in *E. coli* DH5α cells. Two positive clones were identified by the formation of white precipitate around the colonies on the selective medium (LBGCT) supplemented with 200 μg of ampicillin/ml and were designated pBSH14 and pBSH27. Preliminary restriction enzyme analysis revealed that pBSH14 contained a 3.5-kb insert and that pBSH27 contained a 7.5-kb insert. With the PstI site of the insert in pBSH27, two subclones, pBSH272 (2.0-kb insert) and pBSH274 (4.0-kb insert), were constructed in pUC19 (Fig. 2), with pBSH274 showing BSH activity. Sequence analysis of the 4.0-kb insert revealed that it included the overlapping fragment of pBSH14. In this sequence, one complete open reading frame (ORF) was detected, and this ORF encoded a 316-amino-acid protein (Fig. 3). The N-terminal amino acid sequence of the deduced protein was identical to that of the purified BSH enzyme. The deduced protein had a theoretical molecular mass of 35,144 Da and a pI of 4.48. The theoretical data derived from the deduced protein were in good agreement with the measured biochemical data obtained from the purified enzyme.

Comparison of the predicted amino acid sequence to the database by BLAST analysis revealed highest similarities to the BSH of *B. longum* strains (91% identity and 95% similarity) (42, 48). Significant similarities were also found to the BSH enzymes of several *Lactobacillus* strains (35 to 43% identity) (GenBank accession numbers AF054971, AF305888, AF091248, AF297873, and M96175), as well as to BSH enzymes of *Enterococcus* strains (36 to 37%) (GenBank accession numbers AY032999 and AY260046), *L. monocytogenes* (37%) (11), *C. perfringens* (36%) (7), and penicillin V amidase (PVA) of *Bacillus sphaericus* (30%) (GenBank accession number M15660) (Fig. 4).

Characterization of *bsh* transcript and *bsh* promoter. The *bsh* TSP was identified by primer extension with total RNA isolated from log-phase *B. bifidum* ATCC 11863 cells. The first base of the transcript was identified as the cytosine located 42 bp upstream of the proposed *bsh* ATG start codon (Fig. 5). The experimentally verified TSP of +1 was in accordance with the position predicted by NNPP software, version 2.2. The relatively low score value (0.39) (Fig. 5C) of the predicted promoter sequence was partly due to the potential −35 (TTAAGA) and −10 (TACCAT) sequences that are different from those of the consensus hexamer counterparts. A putative rho-independent type transcription terminator sequence ($\Delta G = -75.5$ kcal/mol at 25°C) could be recognized 75 nucleotides downstream of the stop codon, suggesting that the *bsh* gene is transcribed monocistronically. Furthermore, hybridizations of total RNA from *B. bifidum* cells with the internal probe revealed a distinct signal of an approximately 1.2-kb mRNA transcript (Fig. 5), indicating that the *bsh* gene is not cotranscribed with other adjacent genes.

In the region preceding the ATG start codon of *B. bifidum*

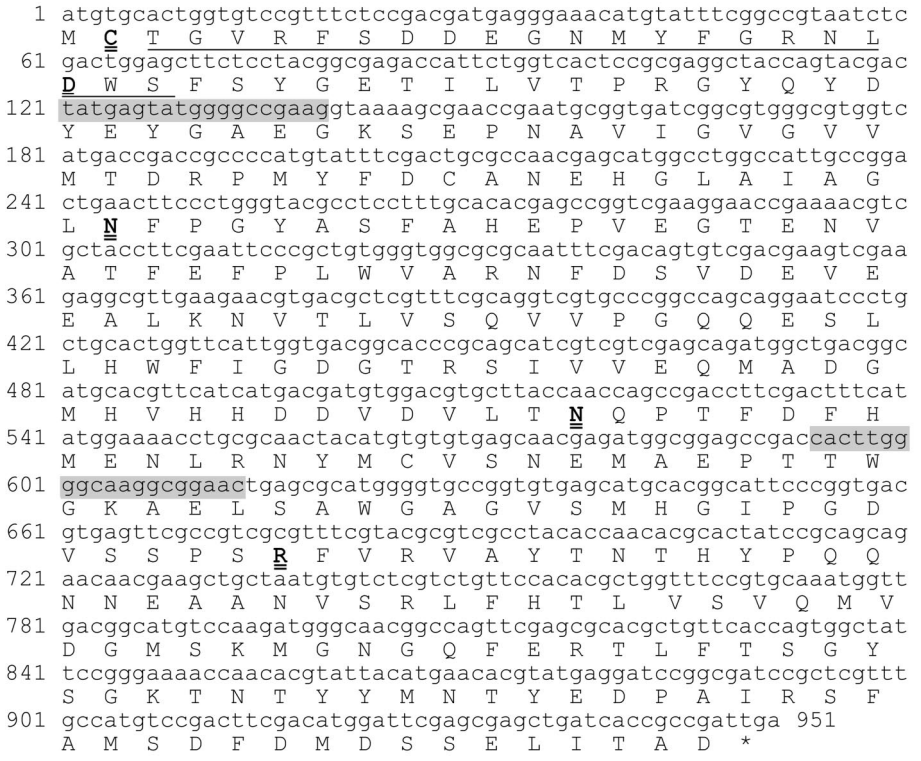

FIG. 3. Nucleotide sequence of the *bsh* gene. The N-terminal amino acid sequence determined from the purified BSH is underlined. The amino acid sequences of the proposed active site residues (C-1, D-20, N-81, N-172, and R-225) are in double-underlined boldface type. Variable regions used for the *B. bifidum*-specific primer set are shaded.

bsh, a putative RBS, a purine-rich region (AAAGGA), was found; this sequence is complementary to the 3′ end of *B. bifidum* 16S rRNA (3′-UCUUUCCUCC-5′) (Table 3). This potential base-pairing region between mRNA and 16S rRNA is also observed in many other genes of bifidobacteria (33), and the sequence of the 3′ end of 16S rRNA is well conserved in other species of bifidobacteria (Table 3).

Heterologous expression of *B. bifidum* BSH in *E. coli*. The *Bifidobacterium* BSH genes on plasmids pBSH14, pBSH27, and pBSH274 were constitutively expressed in *E. coli*, which was the case in the *Bifidobacterium* host. No significant difference in their expression levels among the cells containing those plasmids was observed, regardless of the different orientation of the inserts and the different vectors. This finding suggested that the *bsh* gene on these plasmids could be transcribed by its own promoter sequence and that the initiation of translation might be facilitated by the putative *E. coli* RBS (AGGA) located directly upstream of the *Bifidobacterium bsh* gene (Fig. 5). To confirm this hypothesis, PCR products with and without the 550-bp upstream region of the *bsh* gene were cloned into a PCR cloning vector (pDrive) and screened for BSH activity in *E. coli* DH5α cells. Colonies from those cells harboring pDB150 and pDB095 (Fig. 6) showed BSH activity when grown on the selective medium, LBGCT. However, when they were cloned in the direction opposite that of the *lac* promoter in pUC19, BSH activity was observed only from the clone containing the upstream region of the *bsh* gene in pUC150 (Fig. 6), suggesting that the putative *Bifidobacterium bsh* promoter and RBS are recognized by *E. coli* cells.

To facilitate purification and characterization of the enzyme, the BSH was overexpressed in *E. coli* by using the overexpression vector pET36b(+). The recombinant enzyme showed characteristics similar to those of the native enzyme during the chromatographic steps, and the same migration pattern was observed with native PAGE followed by activity staining (Fig. 1A). Using PCR-based site-directed mutagenesis, we constructed two *B. bifidum bsh* mutants in which Cys-1 was replaced by other nucleophilic amino acids, Ser (pBCS) or Thr (pBST), which have an OH group instead of an SH group at this position in the original BSH. The resulting mutations did not exhibit BSH activity, while the protein band was still present when they were overexpressed in *E. coli* with pET36(b) (data not shown). The *bsh* gene in the mutant plasmids was sequenced to examine unexpected mutations during the PCR cloning experiments, but no such changes were detected.

Substrate specificity of BSH. The substrate specificity of the BSH was determined in enzyme assays with the six major human bile salts (22). The BSH enzyme from *B. bifidum* ATCC 11863 showed the highest enzyme activity with glycocholic acid (defined as 100% activity). The enzymes exhibited a preference for glycine-conjugated bile salts over taurine-conjugated forms, and no difference was observed with di- or trihydroxyconjugated bile salts. (Fig. 7). Recombinant and native enzymes showed similar characteristics in their preference for bile salt substrates (data not shown). The substrate specificity of BSH from *B. bifidum* was more similar to that of type A than type C (22).

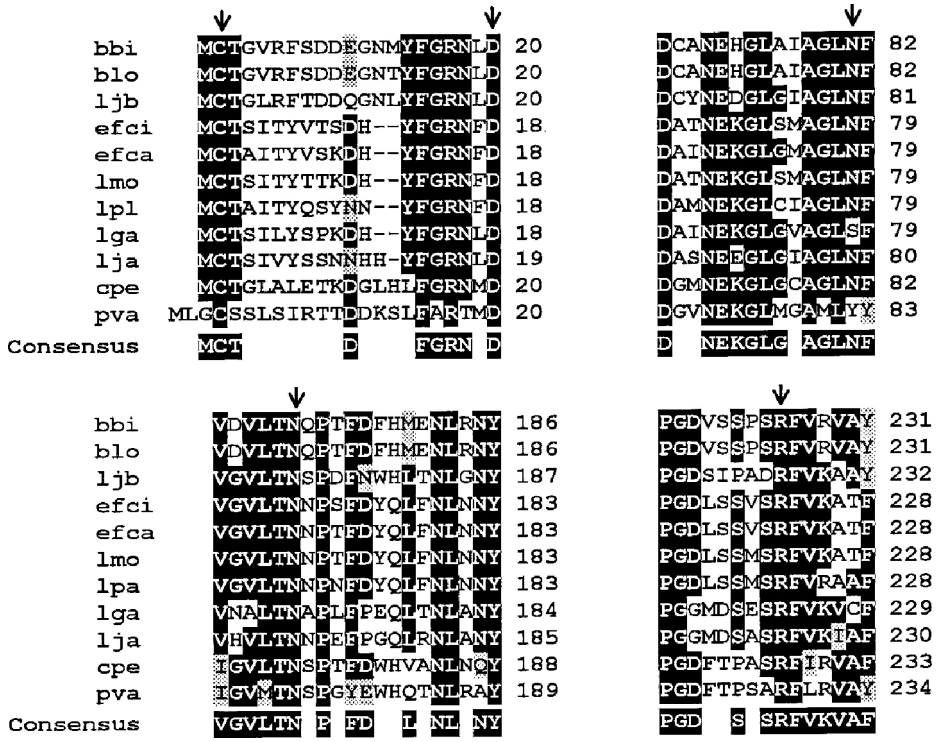

FIG. 4. Multiple alignment of BSHs of various bacteria and PVAs of *B. sphaericus* based on the sequences around the proposed active sites of *B. sphaericus* PVA. Abbreviations for BSHs: bbi, *B. bifidum*; blo, *B. longum*; ljb, *L. johnsonii* BSH-β; efci, *Enterococcus faecium*; efca, *Enterococcus faecalis*; lmo, *L. monocytogenes*; lpa, *L. plantarum*; lga, *Lactobacillus gasseri*; lja, *L. johnsonii* BSH-α; cpe, *C. perfringens*; pva, *B. sphaericus* PVA. Conserved amino acids are highlighted in black boxes, and similar amino acids are in grey boxes. Arrows indicate the amino acids proposed to be the key residues in the active sites of *B. sphaericus* PVA, including C-1, D-20, Y-82, N-175, and R-228. Positions are based on the PVA of *B. sphaericus*, starting with the Cys residues at the N-terminal end of mature proteins (BSH and PVA).

PCR screening for *B. bifidum* strains. All of the *B. bifidum* strains screened in this study showed BSH activity towards the conjugated bile salts. To examine the distribution of the *bsh* locus, genomic DNA from each strain was screened by standard PCR with two primers, BSH-F and BSH-R. All the *B. bifidum* strains produced the same size (970 bp) of amplicon (Fig. 8). From the PCR cloning of this amplicon into the pDrive vector and screening in *E. coli* DH5α cells, BSH activity was detected with the amplicons from all the strains tested, suggesting that the *bsh* gene was selectively amplified from total genomic DNA with the primer set used. Another set of internal primers (BBI-F and BBI-R) was designed to produce a 495-bp amplicon targeting the variable region of the *bsh* sequence that is different from those of *B. longum* strains (42, 48). All of the *B. bifidum* strains and *Bifidobacterium adolescentis* ATCC 15703 produced the expected size of amplicon. However, the other BSH positive strains, including *Bifidobacterium breve*, *B. infantis*, and *B. longum*, were all negative in the PCR screening with the primer set developed in this study (Fig. 8), implying that these three species might have *bsh* gene sequences different from those of *B. bifidum* strains.

DISCUSSION

In this paper, we present the cloning and transcriptional analysis of a new BSH gene from *B. bifidum* ATCC 11863. In a previous report, Kim et al. (22) described the purification of three different types of BSH that are found in the genus *Bifidobacterium*, and their study reported some minor differences in their biochemical characteristics. In this study, supporting evidence for such differences is provided by molecular characterization; to our knowledge, this is the first report of a transcriptional analysis of a bifidobacterial *bsh* gene.

Purified *B. bifidum* BSH enzyme exhibited extensive similarities to *B. longum* BSH (48) with respect to the major characteristics such as subunit size (35 kDa) and homotetrameric structure (4×35 kDa = 140 kDa). The N-terminal amino acid sequence of the purified enzyme was the same as that of *B. longum* BSH, with only one difference. As observed in previous reports (13, 22, 48), the first amino acid was not resolved under experimental conditions. However, the cysteine can be deduced from the genetic data, and this residue is highly conserved in all BSH enzymes. Based on its striking structural similarity to PVA, the BSH enzyme has been proposed as a new member of the N-terminal nucleophile (designated Ntn) hydrolase superfamily with a Cys at the N-terminal end. This Cys residue serves as the nucleophile and proton donor in the catalytic process (45). The absence of a Met residue at the N-terminal end could be explained by the fact that the Cys-1 residue becomes a catalytic center after removal of the initiation formyl methionine by an autoproteolytic process, which is one of the common features of the Ntn hydrolase superfamily (34). The importance of the SH group in the N-terminal cysteine was confirmed by the fact that replacing Cys with other

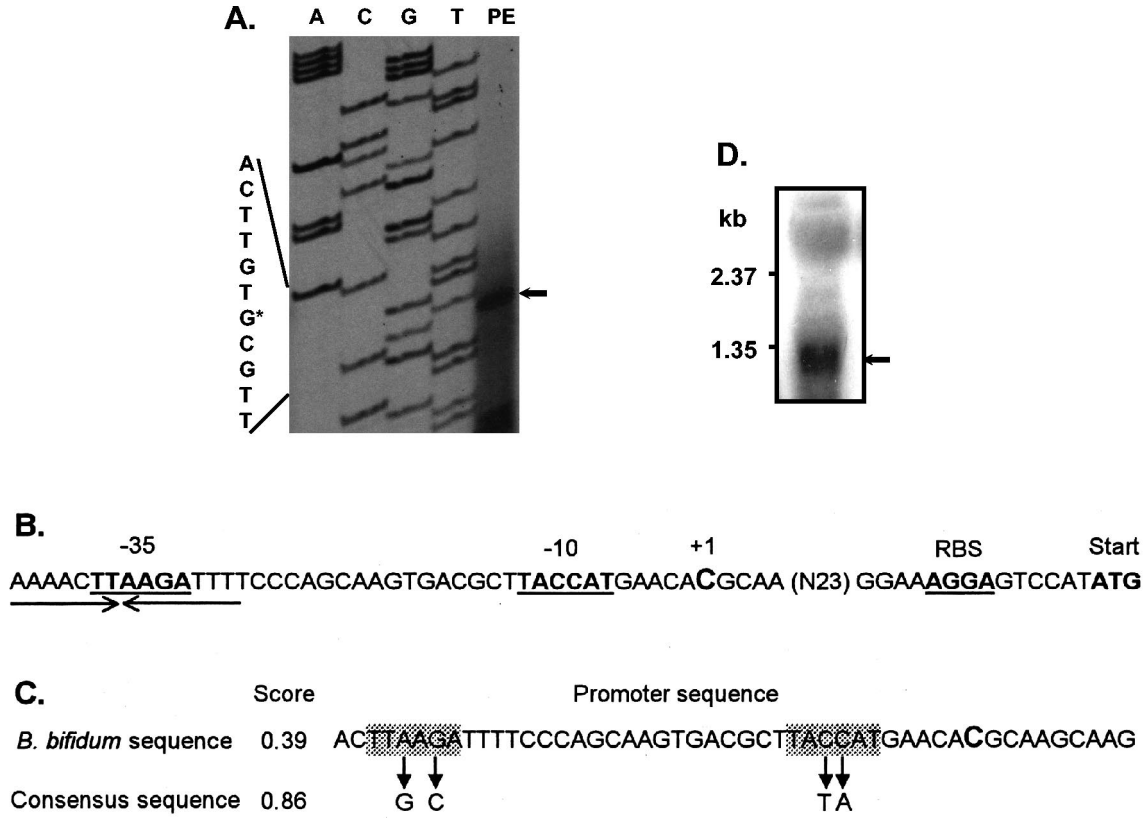

FIG. 5. Characterization of the *bsh* transcript and *bsh* promoter. (A) Identification of the 5′ terminus of the *bsh* transcript by primer extension. The TSP (+1; indicated by an arrow) is shown in the right lane. The DNA sequence is shown on the left and +1 is indicated by an asterisk. (B) Schematic organization of the *bsh* promoter. The putative −10 and −35 motifs of the *bsh* promoter are underlined, and the proposed RBS and start codon (ATG) of *bsh* are indicated in boldface. The TSP (+1) is enlarged. An inverted repeat sequence in the −35 region is indicated by arrows. (C) A promoter sequence predicted with NNPP software, version 2.2. The consensus −35 (TTGACA) and −10 (TATAAT) sequences are presented under the arrows, and the proposed TSP is shown in enlarged type. Score, the fitness value to the consensus promoter sequences. (D) Northern hybridization of total RNA with the probe prepared with the primers BBI-F and BBI-R. The 1.2-kb transcript corresponding to the *bsh* transcript is indicated by an arrow.

TABLE 3. Alignments of the region immediately upstream of the ATG start colon in *Bifidobacterium* genes and *B. bifidum bsh* with the 3′-terminal consensus sequence of 16S RNA from the genus *Bifidobacterium*

Organism or sequence	Gene or gene encoding:	Sequence upstream from start codon[a]	Δ*G* (kcal/mol)[b]	Distance (nt)[c]
B. breve D88311[d]	β-Glucosidase	ACT**AG**AAAGGAATCACCG **ATG**	−16.2	7
B. adolescentis AF124596	α-Galactosidase	CAAGA**AAAGGA**T**G**CTGCA **ATG**	−11.8	7
B. adolescentis AF213175	β-Galactosidase	AAA**AC**AAAGGA**G**TGGAT **ATG**	−14.0	6
B. bifidum AJ224435	β-Galactosidase	ATGAA**G**AAGGAACGTTT **ATG**	−10.6	6
B. infantis AF192265	β-Galactosidase	GAC**AG**AAAG**C**A**GG**AGAAC **ATG**	−16.2	7
B. animalis AJ293946	F6PPK	GGAGT**A**CAGGA**G**CACAC **ATG**	−11.6	6
B. longum AE014718	F6PPK	GGAGT**A**CAGGA**G**TACAC **ATG**	−11.6	6
B. longum AF148138	*bsh*	GATG**G**AA**G**GGA**G**TCCGTT **ATG**	−12.8	7
B. bifidum	*bsh*	CATG**G**AAAGGA**G**TCCAT **ATG**	−16.2	6
3′-End sequence of bifidobacterial 16S rRNA[e]		3′-**TCTTTCCTCC**ACTAGG-5′		

[a] Potential base pairing between the purine-rich region of the genes and the complementary region near the 3′ end of 16S rRNA sequence is in boldface italic type. Double underlining indicates a highly conserved area from listed sequences. The central AGGA motif of the SD region in bifidobacteria is indicated in single-underlined boldface type and the ATG start codon is in boldface.

[b] Free energy of complementarity of the SD and the 3′ end of 16S rRNA.

[c] Distance in nucleotides (nt) between ATG and the core AGGA sequence in the SD region.

[d] GenBank nucleotide sequence accession numbers are shown.

[e] This sequence is highly conserved in many strains of the genus *Bifidobacterium*, including *B. animalis* DSM10140^T (X89513), *B. bifidum* DSM20456^T (S83624), *B. infantis* ATCC 15697^T (U09792), *B. breve* ATCC 15698 (U09518), and *B. adolescentis* C1P6461 (U09514) (numbers in parentheses are GenBank accession numbers).

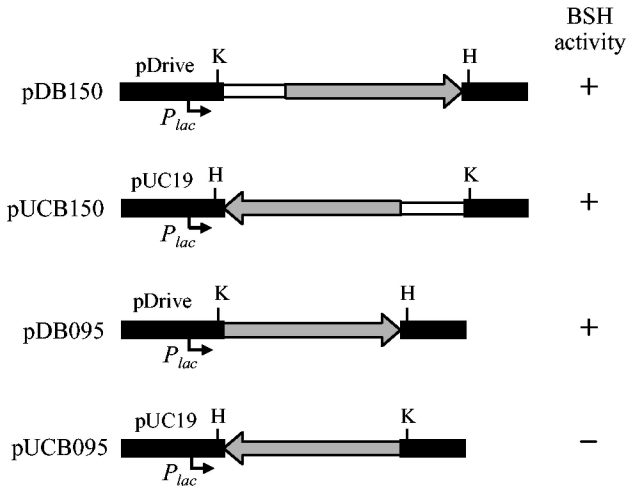

FIG. 6. Map of plasmids pDB150, pUCB150, pDB095, and pUCB095. Black boxes indicate the vectors pDrive and pUC19, grey arrows indicate the position and orientation of the *bsh* gene (950 bp), and white boxes are for the upstream region (550 bp) of the *bsh* gene. P_{lac}, *lac* promoter in the vectors. Restriction enzyme sites used for the cloning in the opposite direction are HindIII (H) and KpnI (K).

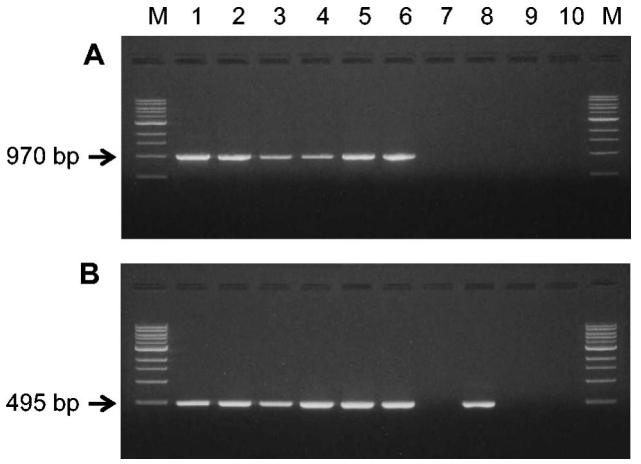

FIG. 8. PCR products of *B. bifidum* strains with primers BSH-F and BSH-R (A) and BIF-F and BIF-R (B). Lanes: 1, *B. bifidum* ATCC 11863; 2, *B. bifidum* ATCC 35914; 3, *B. bifidum* ATCC 15696; 4, *B. bifidum* ATCC 29521; 5, *B. bifidum* KL 301; 6, *B. bifidum* KL 306; 7, *B. longum* ATCC 15707; 8, *B. adolescentis* ATCC 15703; 9, *B. infantis* ATCC 15697; and 10, *B. breve* ATCC 15700; M, 1-kb DNA ladder (New England Biolabs). PCR amplicons of 970 bp (A) were obtained with DNA extracted from *B. bifidum* strains, and amplicons of 495 bp (B) were obtained with DNA extracted from *B. bifidum* and *B. adolescentis* strains.

potential nucleophilic residues, Ser or Thr, results in an inactive enzyme.

From the homology comparison of the deduced protein to previously known sequences, *B. bifidum* BSH shares the highest amino acid sequence similarity with the BSH from *B. longum* strains (42, 48). The predicted ORF of 951 bp displayed 83% DNA sequence identity and 95% deduced amino acid sequence similarity to BSH from *B. longum* NCC 2705 (42). Such a discrepancy is due to different codon usage, mostly at the third position. However, DNA sequences flanking the *bsh* gene were not conserved in these two species. Partial ORFs flanking the *bsh* gene that have been identified by BLAST do not display significant sequence similarity to any known genes, indicating that they are either specific to *B. bifidum* or yet to be determined in other genomes.

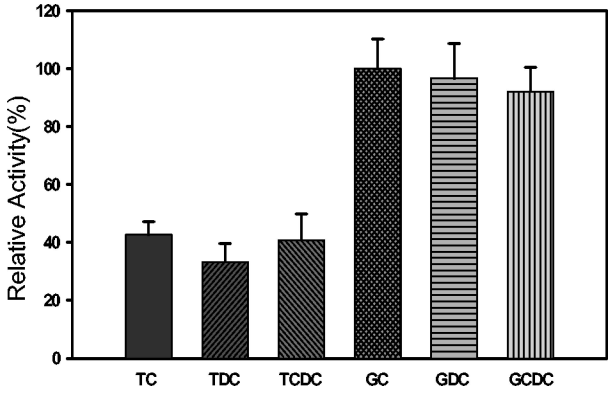

FIG. 7. Substrate specificity of *B. bifidum* ATCC 11863 BSH. Six major human bile salts are shown: taurocholic acid (TC), taurodeoxycholic acid (TDC), taurochenodeoxycholic acid (TCDC) glycocholic acid (GC), glycodeoxycholic acid (GDC), and glycochenodeoxycholic acid (GCDC). The relative activity was calculated using GC as a standard at 100%.

Although the TSP of the *bsh* gene has been identified in *L. monocytogenes* (11), that of *Bifidobacterium* spp. has only now been determined by primer extension analysis. The putative *bsh* promoter sequence was different from the consensus promoter sequences in the consensus −35 (TTGACA) and −10 (TATAAT) hexamer sequences and in the absence of a TG motif upstream of the −10 hexamer. In addition, the spacer (20 nucleotides) between the region spanning −35 to −10 was longer than that of the consensus promoter sequences, which were reported to be 17 ± 1 nucleotides (18, 30). Interestingly, a palindromic sequence in the −35 region was identified (Fig. 5B), which was also found in the *bsh* promoter of *L. monocytogenes* (11). The spacer between the palindromic sequence and the −10 position, which was 16 nucleotides in *B. bifidum* and 18 nucleotides in *L. monocytogenes*, is close to the spacer of the consensus promoter sequences. Further experiments will be required to establish how this palindromic sequence works as an RNA polymerase recognition site in *B. bifidum*. Since little is known about the the vegetative RNA polymerase in *Bifidobacterium* species compared to those of other bacterial species, we could not exclude the possibility that the sequence investigated in this study is not representative of typical bifidobacterial −35 and −10 hexamers. Until now, there have been only a few reports of primer extension analysis being used to determine a TSP of genes from the genus *Bifidobacterium* (27, 38).

The putative rho-independent transcription terminator sequence located 75 nucleotides downstream of the stop codon of the *bsh* gene and the 1.2-kb transcript size shown by Northern hydridization indicates that the *bsh* gene of *B. bifidum* is transcribed as a monocistronic unit. Monocistronic *bsh* transcripts in *L. plantarum* (5) and *L. monocytogenes* (11) have been previously reported. On the contrary, Tanaka et al. (48)

proposed that the *bsh* gene of *B. longum* SBT2928 is part of an operon in which the *bsh* gene is transcribed with at least one more gene, the glutamine synthetase gene (*glnE*). The same genetic organization was revealed from the recently completed genomic projects with *B. longum* NCC 2705 (42) and the ongoing project with *B. longum* DJO10A (www.jgi.doe.gov /JGI_microbial/html). Directly upstream of the *bsh* gene in both strains, a gene with high homology to the 5,10-methylenetetrahydrofolate reductase gene (*metF*) was found. If these three genes, *metF*, *bsh*, and *glnE*, were cotranscribed in one operon as reported by Tanaka et al. (48) it would be interesting to find out the functional relationships between the three enzymes they encode. To date, the extent of the operon in *B. longum* strains has not been revealed. Another polycistronic *bsh* transcript has been reported by Elkins et al. (13). They identified a complete BSH operon from *L. johnsonii* 100-100 and *L. acidophilus* KS-13, in which the *bsh* gene is transcribed with another gene for a putative conjugated bile salt transporter.

It has been proposed that acquisition of the *bsh* gene occurs by horizontal (lateral) gene transfer in lactobacilli (13) and in *L. monocytogenes* (11). Whether the *bsh* gene in the genus *Bifidobacterium* is duplicated or acquired by horizontal transfer is not known at this time. However, the following four facts contradict the hypothesis that the *bsh* gene in *B. bifidum* is acquired by horizontal gene transfer from other enteric bacteria. (i) The G+C content (58%) of the *bsh* gene reflects the overall G+C content (57 to 64%) of the *Bifidobacterium* genus chromosome. (ii) The *bsh* gene sequence was highly conserved within all the *B. bifidum* strains tested in this study. For the similarity comparison, two more *bsh* genes obtained by PCR amplification from *B. bifidum* ATCC 15696 and ATCC 29521 were sequenced, and they showed more than 98% nucleotide sequence similarity to the genes of *B. bifidum* ATCC 11863. Furthermore, three *B. longum bsh* genes available from GenBank showed a high level of similarity. (iii) There are no reports of BSH activity or the *bsh* gene from any G+C-rich gram-positive bacteria other than the *Bifidobacterium* species. (iv) In the genus *Bifidobacterium*, the BSH phenotype is not strain specific and is rather widespread among almost all the strains within the genus. This indicates that the *bsh* gene in the bifidobacterial genome could be a paralogous gene, not an orthologous gene acquired by lateral gene transfer. Given these facts, it appears that BSH activity is important at some level for bifidobacteria to respond efficiently to bile salts and to colonize at the lower part of the large intestine of human and animal.

Finally, it remains to be determined whether the BSH activity of the probiotics, including many commercially available *Bifidobacterium* strains, is beneficial or detrimental to the host. While the potential positive aspects of BSH activity of the probiotics have been previously discussed (10, 36, 37, 49), other possible negative concerns about BSH activity have also been raised (11, 28, 51). Recently, Kurdi et al. (25) proposed one possible beneficial consequence of BSH activity in bifidobacteria upon investigation of the cholic acid transport and accumulation in some intestinal *Bifidobacterium* strains, including two *B. bifidum* strains. They observed that cholic acid, the main free bile acid produced by BSH activity in the intestine, could accumulate inside the bacterial cell in the intestine so long as the bacteria were energized. The entrapment of free bile acids by bifidobacteria can contribute to the decreased production of secondary bile acids, which are considered cytotoxic and precarcinogenic. The enzyme responsible for this undesirable reaction, 7α-dehydroxylase, has been found in *Clostridium* and *Eubacterium* species (8, 52) but not in lactic acid bacteria or bifidobacteria (25). Since the hydrolytic products of BSH have properties toxic to cells and can damage the membrane of mammalian cells (51), the accumulation of free bile acids within BSH-producing bacteria could counteract the toxic nature of deconjugated bile salts. On the other hand, it has yet to be determined what advantage BSH activity provides *Bifidobacterium* strains. So far, there is no report of the ability of bifidobacteria to produce energy from bile salts or of any additional catabolic pathways of the steroid ring structure of bile salts in bifidobacteria. Further experiments will be needed to unveil the physiological significance of BSH activity for the enzyme-producing bacterial cells as well as for the mammalian hosts.

ACKNOWLEDGMENTS

This research was supported by a research operating grant from Natural Sciences and Engineering Research Council of Canada (NSERC-222026) and by Medical Research Grant MT-7672. G.-B.K. is the recipient of a Fonds pour la Formation de Chercheurs et l'Aide a la Recherche (FCAR) scholarship from the Government of Quebec.

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