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Bile Salt Hydrolase of *Bifidobacterium longum*—Biochemical and Genetic Characterization

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A bile salt hydrolase (BSH) was isolated from *Bifidobacterium longum* SBT2928, purified, and characterized. Furthermore, we describe for the first time cloning and analysis of the gene encoding BSH (*bsh*) in a member of the genus *Bifidobacterium*. The enzyme has a native molecular weight of 125,000 to 130,000 and a subunit molecular weight of 35,024, as determined from the deduced amino acid sequence, indicating that the enzyme is a tetramer. The pH optimum of *B. longum* BSH is between 5 and 7, and the temperature optimum is 40°C. The enzyme is strongly inhibited by thiol enzyme inhibitors, indicating that a Cys residue is likely to be involved in the catalytic reaction. The BSH of *B. longum* can hydrolyze all six major human bile salts and at least two animal bile salts. A slight preference for glycine-conjugated bile acids was detected based on both the specificity and the K_m values. The nucleotide sequence of *bsh* was determined and used for homology studies, transcript analysis, and construction and analysis of various mutants. The levels of homology with BSH of other bacteria and with penicillin V acylase (PVA) of *Bacillus sphaericus* were high. On the basis of the similarity of BSH and PVA, whose crystal structure has been elucidated, BSH can be classified as an N-terminal nucleophile hydrolase with Cys as the N-terminal amino acid. This classification was confirmed by the fact that a Cys1Ala exchange by site-directed mutagenesis resulted in an inactive protein. Reverse transcription-PCR experiments revealed that *bsh* is part of an operon containing at least two genes, *bsh* and *glnE* (GlnE is glutamine synthetase adenylyltransferase). Two UV-induced BSH-negative mutants and one spontaneous BSH-negative mutant were isolated from *B. longum* SBT2928 cultures and characterized. These mutants had point mutations that inactivated *bsh* by premature termination, frameshift, or amino acid exchange.

Bifidobacteria are important components of the human intestinal microflora, in which they occur at concentrations of 10^9 to 10^{10} cells/g of feces (45), and of fermented milk products, to which they are added mainly because of their supposed health-promoting activities (2, 20). However, molecular genetic research on bifidobacteria is still in its infancy. In addition to many 16S rRNA sequences, only six genes (see Table 6) and two plasmids of members of the genus *Bifidobacterium* have been cloned and sequenced. Only recently has development of cloning vectors and transformation techniques started (19).

One important metabolic activity which is exhibited by almost all bifidobacteria is deconjugation of bile salts, which occurs naturally in the intestines of humans (44). The responsible enzyme, bile salt hydrolase (BSH) (cholylglycine hydrolase; EC 3.5.1.24), catalyzes the hydrolysis of glycine- and/or taurine-conjugated bile salts into amino acid residues and deconjugated bile salts (bile acids). The function of this enzyme in the producing bacterium is not known, although various observations have been made and various theories about its role in the producer have been proposed; these theories include a role in utilization of the liberated amino acid or a role in increased resistance to the toxic levels of bile salts in the gastrointestinal environment (10). A recent screening analysis of more than 300 lactic acid bacterial strains performed in our laboratory showed that BSH activity is found primarily in organisms isolated from the gastrointestinal tracts of mammals (*Bifidobacterium* spp., *Lactobacillus acidophilus*, *Lactobacillus gasseri*, *Lactobacillus johnsonii*, some strains of *Lactobacillus plantarum*), while organisms isolated from fermented milk preparations and vegetables (*Lactococcus lactis*, *Lactobacillus delbrueckii*, *Lactobacillus helveticus*, *Streptococcus thermophilus*) do not exhibit BSH activity (44).

In a mammalian host deconjugation of bile salts takes place in the small and large intestines. The exact location of this process depends on the distribution of the bacterial flora in the host species; for example, in mice a *Lactobacillus* flora is present in the small intestine, while in humans a significant flora starts only at the end of the ileum and is fully developed in the large intestine. This means that bile salt deconjugation in mice starts in the small intestine (47), while significant deconjugation in humans begins at the end of the ileum and is completed in the large bowel (28, 34). The function of bile salt deconjugation in mammalian hosts is also not understood. However, in experiments performed with germfree and conventional mice, it has been observed that the presence of a microflora increases bile acid excretion and decreases serum cholesterol levels (5, 45). More is known about the adverse effects of bile salt metabolism, such as extensive deconjugation of bile salts in the small bowel in humans, which leads to steatorrhoea. Furthermore, enhanced concentrations of secondary bile acids in the colon can have tumor-promoting effects (29).

So far, BSH has been isolated from *Bacteroides fragilis* subsp. *fragilis* (41), *Clostridium* sp. (13), *Lactobacillus* sp. (25), and *Bifidobacterium longum* (15) and characterized. The *bsh* genes of *Clostridium perfringens* (7), *L. plantarum* (6) and *L. johnsonii* (11) have been cloned and sequenced, and BSH-negative mutants of *L. plantarum* (24) and *Lactobacillus* sp. (46) have been constructed.

In recent years the possibility of using bile salt deconjugation by lactic acid bacteria to lower serum cholesterol levels in

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TABLE 1. Strains and plasmids

Strain or plasmid	Properties[a]	Reference or source
Strains		
B. longum SBT2928	Wild type, isolated from human feces	Snow Brand Milk Products Co., Ltd., Kawagoe, Japan
B. longum SBT2928 mutant A3	Spontaneous BSH-negative mutant	This study
B. longum SBT2928 mutant 2001	UV-induced BSH-negative mutant	This study
B. longum SBT2928 mutant 2503	UV-induced BSH-negative mutant	This study
E. coli TOP10	F$^-$ *mcrA* Δ(*mrr-hsdRMS-mcrBC*) ϕ80*lacZ*ΔM15 Δ*lacX74 deoR recA1 araD139* Δ(*ara-leu*)*7697 galU galK rpsL endA1 nupG*	Invitrogen Corp., Carlsbad, Calif.
E. coli NM522	*supE thi* Δ(*lac-proAB*) Δ*hsd5* (r$^-$ m$^-$) [F′ *proAB lacI*^{q}*Z* Δ*M15*]	14
Plasmids		
pUK21	Km^r, multipurpose cloning vector	55
pCR-Blunt	Km^r, blunt end cloning vector	Invitrogen Corp., Carlsbad, Calif.
pBH13	Km^r, 2.7-kb insert of the chromosome of *B. longum* SBT2928, confers BSH activity	This study
pBH16	Km^r, 8-kb insert of the chromosome of *B. longum* SBT2928, confers BSH activity	This study
pBH1322	1.2-kb fragment of pBH13 cloned in pCR-Blunt	This study
pBH1351	1.5-kb fragment of pBH13 cloned in pCR-Blunt, confers BSH activity	This study
pCR-A3	*bsh* region obtained by PCR of *bsh* of *B. longum* SBT2928 mutant A3 cloned into pCR-Blunt	This study
pCR-2001	*bsh* region obtained by PCR of *bsh* of *B. longum* SBT2928 mutant 2001 cloned into pCR-Blunt	This study
pCR-2503	*bsh* region obtained by PCR of *bsh* of *B. longum* SBT2928 mutant 2503 cloned into pCR-Blunt	This study
pCA-1	pBH1351 with Cys-1–to–Ala mutation	This study

[a] Km^r, kanamycin resistance.

hypercholesterolemic humans or to prevent hypercholesterolemia in individuals with normal cholesterol levels has received increased attention (8, 9). This possibility is surrounded by controversy concerning its validity and the mechanisms involved (28). To resolve the questions, a mechanistic approach which works with defined bacterial strains and can be used to investigate the effect of BSH in experimental animals and in humans is necessary. To create a basis for this investigation, we isolated and purified the BSH of *B. longum* SBT2928, a strain which has been isolated from human feces and which exhibits very high BSH activity. We cloned the BSH gene of this strain and selected and characterized a number of mutants, including a spontaneous BSH-negative strain. The latter strain could be used as a negative control in both animal experiments and human studies without the regulatory problems usually encountered with strains constructed by genetic engineering techniques.

MATERIALS AND METHODS

Strains, plasmids, and media. The strains and plasmids used in this study are listed in Table 1. The strains were stored in 20% glycerol at −50°C. *Escherichia coli* was grown in TY medium (37) at 37°C with vigorous agitation or on TY medium solidified with 1.5% agar. For kanamycin resistance selection 50 μg of kanamycin per ml was added to the media. *B. longum* was grown in Briggs Liver Broth (3), which was prepared as follows. Tomato juice was mixed with an equal volume of distilled water and steamed for 1.5 h. Then the pH was adjusted to 7.0 with 2.5 M NaOH, and the preparation was filtered through a paper filter. Liver extract was prepared by using Bacto Liver (Difco Laboratories, Detroit, Mich.). Ten grams of liver powder was extracted with 170 ml of distilled water at 50 to 60°C for 1 h and then heated for 2 to 3 min at 100°C. After the preparation cooled, the pH was adjusted to 7.0, and the resulting extract was filtered through a paper filter. To prepare a 100-ml ascorbate-cysteine solution, 0.2 g of L-cysteine and 34 g of ascorbate were dissolved in water, and the pH was adjusted to 7.0 by adding ca. 11 g of sodium carbonate. The base medium was prepared as described by Briggs (3), and 75 ml of liver extract was added per 1,000 ml of medium. Shortly before the culture was started, 15 ml of the ascorbate-cysteine solution was added per 1,000 ml of medium. The strains were grown in liquid cultures at 37°C under anaerobic conditions (airtight containers with AnaeroGen AN35 bags, [Oxoid, Hampshire, United Kingdom]) for 16 to 20 h and on agar plates for 48 h. Alternatively, especially in the genetic experiments, M17 medium (48) supplemented with 1% glucose and 2.5 mM L-cysteine was used, and cells were grown as described above. To select mutants, M17 agar (1.5% agar) supplemented with 0.5% lactose and 15 ml of the ascorbate-cysteine solution per 1,000 ml of agar was used as the base medium and as the overlay agar for BSH activity detection.

Preparation of cell extracts. Cells in an overnight culture were sedimented by centrifugation for 10 min at 10,000 × *g* and 4°C, washed twice in 0.1 M sodium phosphate buffer (pH 7.0), and resuspended in the same buffer to give a density of approximately 10 to 15 optical density units at 600 nm (path length, 1 cm). To reduce oxidation of the enzyme, 10 mM dithiothreitol (DTT) was routinely added. Seven milliliters of the cell suspension was sonicated (Vibracell; Sonics & Materials, Danbury, Conn.) for 3 min at level 5 by using a 50% duty cycle and constant cooling. To remove residual DNA, 1% streptomycin sulfate and 0.1% polyethylenimine were added, and the suspension was stirred for 15 min. Then the mixture was centrifuged for 10 min at 20,000 × *g* and 4°C. The supernatant was stored as a cell extract at −20°C.

BSH assay and protein assay. A fast BSH assay was carried out as described by Coleman and Hudson (7), with several modifications. Portions (10 to 20 μl) of cell extract or partially purified BSH were added to wells of a 96-well microtiter plate with a flat bottom, and 200 μl of reaction mixture (50 mM sodium phosphate, 10 mM DTT, 1 mM EDTA, 10 mM sodium glycodeoxycholate; pH 5.5) was added. To ensure that the reaction conditions were (semi)anaerobic and to prevent evaporation during longer incubations, the wells were overlaid with 50 μl of light paraffin oil (0.85 g/ml; Merck, Darmstadt, Germany). Precipitation of deoxycholic acid was monitored with a microtiter plate reader (model 3550-UV microplate reader; Bio-Rad, Hercules, Calif.) at 37°C. The time until precipitation was an indication of the enzyme activity in the sample.

BSH activity was determined routinely with a two-step assay by determining the amounts of the amino acids liberated from conjugated bile salts. To 180 μl of reaction buffer (0.1 M sodium phosphate, pH 6.0), 10 μl of appropriately diluted sample and 10 μl of a human bile salt mixture (200 mM) (16, 40) (12% taurocholic acid, 12% taurochenodeoxycholic acid, 8% taurodeoxycholic acid, 23% glycocholic acid, 23% glycochenodeoxycholic acid, 16% glycodeoxycholic acid) were added. The bile salt mixture was used in order to measure the overall activity of BSH with human bile salts. To determine the substrate specificity and K_m values, individual bile salts (final concentration, 10 mM) were used. In some cases 10 mM DTT was added to the reaction mixture. Reactions were carried out

at 37°C. Samples (50 μl) were removed after 10 and 30 min of incubation and mixed immediately with 50 μl of 15% (wt/vol) trichloroacetic acid. Subsequently, the samples were centrifuged at the highest speed with a table top centrifuge (model 5417 C; Eppendorf, Hamburg, Germany) to remove the precipitate. For the second reaction (22), an aliquot of the first reaction mixture was mixed with water to obtain a volume of 100 μl (usually 20 μl of sample and 80 μl of water). To this mixture 1.9 ml of ninhydrin reagent (0.5 ml of 1% [wt/vol] ninhydrin in 0.5 M citrate buffer [pH 5.5], 1.2 ml of glycerol, 0.2 ml of 0.5 M citrate buffer; pH 5.5) was added, and the preparation was thoroughly mixed and boiled for 14 min. Then the tube was cooled for 3 min in tap water, and the absorbance at 570 nm was determined. A standard curve was prepared for each assay by using either glycine or taurine. If DTT was present in the first reaction mixture, then the same amount of DTT was added when the standard curve was prepared. One unit of BSH activity was defined as the amount of enzyme which liberated 1 μmol of amino acids from the substrate per minute.

Protein concentrations were determined with the Bio-Rad Protein Assay; bovine serum albumin was used as the standard.

Chromatography methods. MonoQ anion-exchange chromatography (MonoQ HR5/5; Pharmacia Biotech, Uppsala, Sweden) was carried out as described by Grill et al. (15), with the following modifications. The solvent used was 0.1 M sodium phosphate buffer (pH 7.0), and the elution buffer was 0.1 M sodium phosphate (pH 7.0) to which 1 M NaCl was added, which resulted in a pH of 6.5. After proteins were loaded onto the column, they were eluted with a 0 to 0.5 M NaCl gradient in 20 min by using a flow rate of 1 ml/min. One-milliliter fractions were collected.

MonoS cation-exchange chromatography was performed with a MonoS HR5/5 column (Pharmacia Biotech). The solvent used was 0.05 M sodium acetate (pH 5.0), and the elution buffer was 0.05 M sodium acetate–1 M NaCl (pH 5.0). The flow rate was 1 ml/min. Prior to chromatography the pooled MonoQ fractions that exhibited BSH activity were diluted 1:4 with the solvent, and the pH was adjusted to 5.0.

Gel filtration was carried out with a Superose 12HR 10/30 column (Pharmacia Biotech). The solvent used was 0.1 M sodium phosphate–0.15 M NaCl (pH 7.0), and the flow rate was 0.5 ml/min. For this chromatography analysis the proteins were concentrated, and the buffer was exchanged by using Microcon10 microconcentrators (Amicon, Berkeley, Calif.).

Gel filtration with high-performance liquid chromatography (HPLC) (Pharmacia, Biotech) was carried out by using a SigmaChrom GFC-1300 column (300 by 7.5 mm; Supelco, Bellefonte, Pa.). The mobile phase used was 0.1 M sodium phosphate buffer (pH 7)–0.15 M NaCl, the flow rate was 0.5 ml/min, and chromatography was carried out at 4 or 21°C. A molecular weight standard kit (MW-GF-1000) was obtained from Sigma Chemical Co. (St. Louis, Mo.).

In all chromatography experiments absorption at 280 nm was monitored during elution. To identify BSH activity in the fractions, the microtiter plate assay was used. Quantitative determinations were carried out by using the ninhydrin assay (see above).

Immunostaining of BSH with anti-*C. perfringens* BSH antibodies. Anti-*C. perfringens* BSH antibodies were a kind gift from J. P. Coleman of East Carolina University (7). After a preparation was blotted onto a polyvinylidene difluoride (PVDF) membrane by using the Mini-blot system (Bio-Rad) (49), the membrane was immunostained with a Proto Blot II kit (Promega, Madison, Wis.) by following the manufacturer's instructions. The first antibody was diluted 1:2,000.

Determination of native and subunit molecular weights. The native molecular weight was determined by gel chromatography with HPLC; triplicate preparations were chromatographed at 21 or 4°C. The subunit molecular weight was determined by performing four independent sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) analyses (21) in which low-molecular-weight standard proteins (Bio-Rad) were used.

Determination of the pH and temperature optima for BSH activity. The BSH activity that was purified with consecutive MonoQ and MonoS chromatography steps was determined between pH 3 and pH 9 under standard conditions (see above) without DTT. The following buffers were used: 0.1 M sodium citrate-sodium phosphate buffer (pH 3 to 5); 0.1 M sodium phosphate buffer (pH 5.5 to 8); and 0.1 M sodium citrate-sodium phosphate buffer (pH 9).

The temperature optimum was determined in 0.1 M sodium phosphate buffer at pH 6.0 by using the standard reaction mixture (see above) without DTT.

Determination of the pH and temperature stability of BSH. We examined the stability of BSH at different pH values by using the buffers mentioned above at pH 3 to 10. The pH 10 buffer was prepared with 0.1 M sodium borate. Samples were incubated at the different pH values for 30 min at 37°C, and then the residual activity was determined at pH 6 by using the ninhydrin method without DTT.

The temperature stability of the enzyme was determined at temperatures ranging from 4 to 60°C. BSH was incubated at the different temperatures for 30 min, and the residual activity was determined by the standard assay in the absence of DTT (see above).

Inhibition and activation of the enzyme. Inhibition or activation was studied by using the following compounds: iodoacetate, *N*-ethylmaleimide, periodic acid, EDTA, phenylmethylsulfonyl fluoride (dissolved in 70% ethanol), *para*-chloromercuribenzoic acid (dissolved in dimethyl sulfoxide), $MgCl_2$, $MgSO_4$, $HgCl_2$, $CaCl_2$, $CuCl_2$, and NaCl. Because some of these substances interact with phosphate, 20 mM MES (morpholineethanesulfonic acid) buffer (pH 6) was used for this part of the study. Before the substrate was added, the enzyme was incubated for 30 min at 37°C with the compounds mentioned above. Then the standard enzyme assay was carried out to determine the residual enzyme activity.

Determination of the N-terminal amino acid sequence. BSH was purified by consecutive MonoQ and MonoS chromatography steps. The resulting fractions that exhibited BSH activity were combined, concentrated, and applied to a mini electrophoresis unit (Bio-Rad). After separation, the proteins were electroblotted onto a PVDF membrane (Bio-Rad) and stained with Coomassie brilliant blue R-250 by using the method of Matsudaira (30), and the BSH protein was cut out and used for sequencing. N-terminal amino acid sequencing was performed with a Perkin-Elmer/Applied Biosystems model 476A protein sequencer (Perkin-Elmer/Applied Biosystems, Foster City, Calif.).

General genetic techniques, DNA sequencing, and sequence analysis. *B. longum* DNA was isolated by the method of Leenhouts et al. (23), with several modifications. The cells in 1.5 ml of an overnight culture were sedimented, washed once with distilled water, and resuspended in 20% sucrose–10 mM Tris-HCl (pH 8.1)–10 mM EDTA–50 mM NaCl containing 5 mg of lysozyme per ml. After incubation at 55°C for 10 min, first 20 μl of proteinase K (20 mg/ml) and then 25 μl of 10% SDS were added, and the suspension was mixed gently by inversion. The mixture was incubated at 60°C for at least 1 h to obtain complete lysis. The lysate was cleared by several phenol-chloroform extraction steps, and the DNA was precipitated by using standard methods. After the ethanol was removed without drying, the pellet was dissolved in 200 μl of 10 mM Tris-HCl (pH 7.4)–1 mM EDTA, and 5 μl of RNase (10 mg/ml) was added. The method could easily be scaled up for a culture volume of 15 ml.

A bank of *B. longum* SBT2928 chromosomal DNA fragments was constructed as described by Buist et al. (4) by using the vector pUK21 (55). *E. coli* NM522 (14) was transformed by electroporation as described by Zabarovsky and Winberg (57). BSH-positive clones were detected on TY agar plates containing 2% glucose and 3 mM glycodeoxycholic acid (7). General cloning procedures were performed essentially as described by Sambrook et al. (38).

Nucleotide sequences were determined by using dye primers, the cycle sequencing method (33), and a type RPN 2538 Thermosequenase (43) kit obtained from Amersham-Pharmacia (Uppsala, Sweden). Samples were analyzed with an

TABLE 2. Primers used for PCR

Primer	Sequence (5′ to 3′)	Use
A	CTG CCA CCT GTA TTC GA	Amplification of mutant *bsh* and RT-PCR
B	AGC ACT GGT GTC CGT TTC T	RT-PCR
C	GAC CTC GTC GAC GGA GT	RT-PCR
D	GCC TCG TTC GTC CAC GA	RT-PCR
E	TTG CTC AAT GCC ATA TC	Amplification of mutant *bsh* and RT-PCR
F	CGG ACA CCA GTG GAC AT	RT-PCR
ALA	**GCC** ACT GGT GTC CGT TTC T[a]	Cys-1–to–Ala mutation primer
SAL	GAC CTC **GTC GAC** GGA GT[b]	Primer with *Sal*I site for SDM[d]
ATG	**CAT** AAC GGA CTC CCT TC[c]	Primer with ATG for SDM[d]
FW	GTA AAA CGA CGG CCA G	Standard −20 forward primer

[a] The boldface type indicates nucleotide changes.
[b] The boldface type indicates an *Sal*I site.
[c] The boldface type indicates a complementary sequence for the ATG start codon.
[d] SDM, site-directed mutagenesis.

A.L.F.-Express sequencing robot (Amersham-Pharmacia). The nucleotide sequencing experiments were carried out at Pathology-Laboratory Medicine, Laboratory of Molecular Biology, University of Groningen, Groningen, The Netherlands.

The nucleotide sequence obtained was analyzed with the PC/Gene sequence analysis program (IntelliGenetics, Inc., Geneva, Switzerland) and different Internet online tools as described below.

Plasmid construction. To construct pBH1322 and pBH1351, pBH13 was cut with *Eco*RV and *Asp*718, the overhanging ends were filled in with the Klenow polymerase, and the fragments were separated in and isolated from an agarose gel and then cloned into the blunt end cloning vector pCR-Blunt (Invitrogen, Carlsbad, Calif.). The BSH regions of mutant strains were amplified by PCR performed with PWO polymerase (Roche Diagnostics Nederland b.v., Almere, The Netherlands) and appropriate primers and cloned into pCR-Blunt (Invitrogen).

Cys-1 was exchanged for Ala by using a PCR-based site-directed mutagenesis method and pBH1351 as the template. The first DNA fragment, starting at codon 2 of *bsh*, was amplified with primers ALA and SAL, while the second *bsh* fragment, ending at codon 1, was amplified with primers ATG and FW (Table 2). The high-fidelity PWO polymerase (Roche Diagnostics Nederland) was used in both cases, and the PCR fragments were purified with a High Pure PCR product purification kit (Roche Diagnostics Nederland). After purification, the two PCR products were phosphorylated and cut with *Sal*I and *Xba*I, respectively. Plasmid pBH1351 was cut with *Sal*I and *Xba*I, and the large fragment was purified and ligated with both PCR products. The *bsh* gene in the resulting plasmid, pCA-1, was examined by nucleotide sequencing to determine whether mutations other than those expected (Cys-1-Ala) as a possible result of the PCR were present. No such changes were detected.

PCR and RT-PCR. Table 2 shows the primers which were used in PCR and/or reverse transcription PCR (RT-PCR). Each PCR was carried out by using PWO polymerase as recommended by the manufacturer (Roche Diagnostics Nederland). RNA was isolated by using the macaloid method described by van Asseldonk et al. (50). First-strand cDNA synthesis was carried out with a first-strand cDNA synthesis kit (Roche Diagnostics Nederland) as recommended by the manufacturer by using either primer C or primer E (Table 2 and Fig. 1). Subsequently, PCRs were carried out by using *Taq* polymerase (HT Biotechnology, Ltd., Cambridge, United Kingdom), appropriate primer pairs, and both cDNAs.

Selection of spontaneous and UV-induced BSH-negative mutants. A spontaneous BSH-negative mutant was isolated during development of a selection procedure for detection of UV-induced *bsh* mutants. An M17 agar plate containing *B. longum* SBT2928 colonies was overlaid with M17 agar containing 0.5% lactose, 15 ml of a cysteine-ascorbate solution per liter, and 5 mM glycodeoxycholic acid. One BSH-negative colony which did not produce a precipitation halo was detected, and the organism was purified.

To select UV-induced mutants, a preculture was grown overnight in M17 medium supplemented with 0.5% lactose and 15 ml of a cysteine-ascorbate solution per liter, diluted 100-fold, and grown to a density of about 2×10^8 cells/ml. The cells were washed twice in 10 mM sodium phosphate–0.138 M NaCl (pH 7.4) and resuspended to a density of about 2×10^8 cells/ml. A 10^{-6} dilution was plated to determine the total number of viable cells. Subsequently, 12-ml portions were pipetted into a 140-mm glass petri dish and irradiated from a distance of 25 cm for 1, 1.5, 2, and 2.5 min with 250-nm UV light (model LS-88 lamp; Raytech Industries, Inc., Staffold Springs, Conn.) with shaking (30 rpm). Aliquots were plated directly to calculate survival rates, while 0.5 ml of irradiated cells was transferred into 10 ml of M17 medium and allowed to grow for 2 h. Then the cultures were plated, and approximately 350 to 1,000 colonies per 140-mm plate were obtained. The plates were incubated anaerobically for 48 h and then overlaid with M17 agar containing 2.5 mM glycodeoxycholic acid (see above).

Nucleotide sequence accession number. The *bsh* nucleotide sequence has been deposited in the GenBank database under accession number AF148138.

RESULTS

Isolation and purification of *B. longum* BSH. *B. longum* SBT2928 was selected during a broad screening analysis of lactic acid bacteria (44) because of its high level of BSH activity. Since no major differences in enzyme activity during different growth phases or in the presence and in the absence of a 0.1 mM bile salt mixture (see Materials and Methods) were observed (results not shown), BSH was routinely isolated from overnight cultures grown in Briggs Liver broth.

In preliminary experiments in which cell extracts were used, we observed that adding 10 mM DTT during sonication improved the yield of BSH activity up to 10-fold. To investigate this effect, additional cell suspensions were sonicated with and without 10 mM DTT, after which BSH activity was assayed with and without 10 mM DTT. Table 3 clearly shows that a lack of DTT during both sonication (3 min) and the enzyme assay resulted in ninefold-lower enzyme activity than the activity in a cell extract prepared with DTT and examined in the presence of DTT. If a preparation was sonicated without DTT and DTT was added only during the assay, activity was reduced twofold, indicating that some of the enzyme activity was not lost and could be recovered by adding the thiol reagent. If the length of sonication was extended to 9 min, the amount of residual activity decreased further, and the amount of enzyme activity which could be recovered by adding DTT decreased from 50 to 35%. Because of its stabilizing effect, DTT was added during preparation of cell extracts in all subsequent experiments.

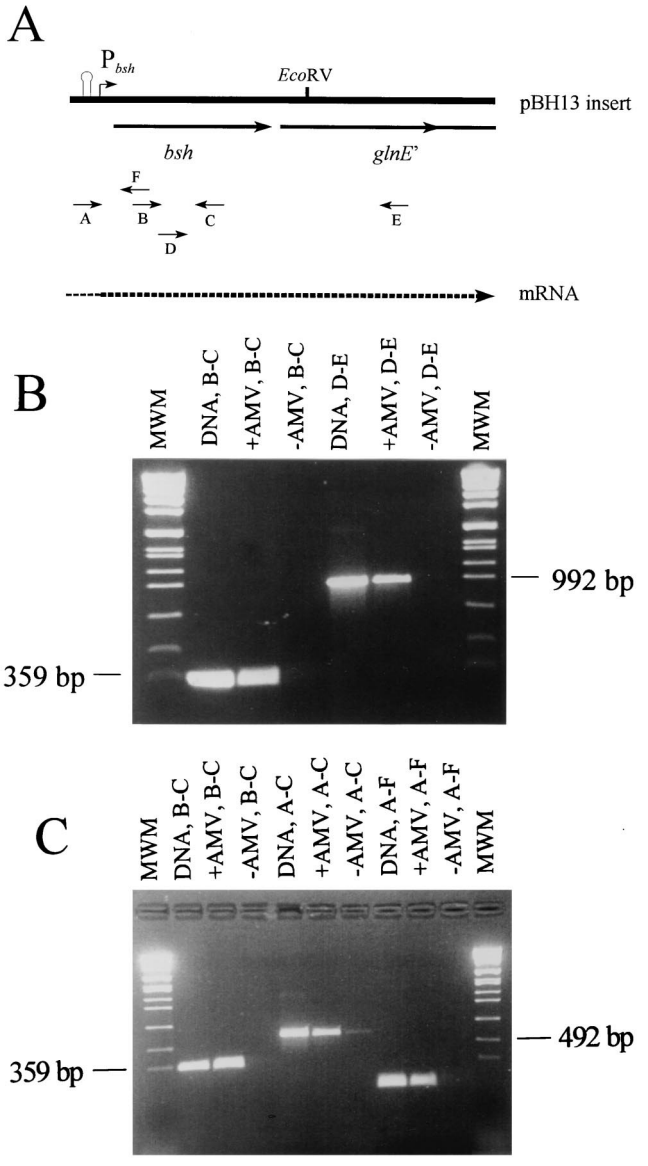

FIG. 1. Genetic and transcriptional organization of the *bsh* site (A) and RT-PCR analysis of the transcript of the *bsh* region (B and C). P_{bsh}, putative *bsh* promoter; A through E, primers A through E used for RT-PCR (Table 2); MWM, molecular weight marker; DNA, control reaction; +AMV, first-strand reaction with AMV; −AMV, first-strand reaction without AMV. (B) First-strand cDNA synthesis with primer E. (C) First-strand cDNA synthesis with primer C. In the lanes in which primers A and C were used and in the lanes in which primers A and F were used three times as much RT-PCR product was applied in order to obtain bands whose intensity was similar to that of the DNA control.

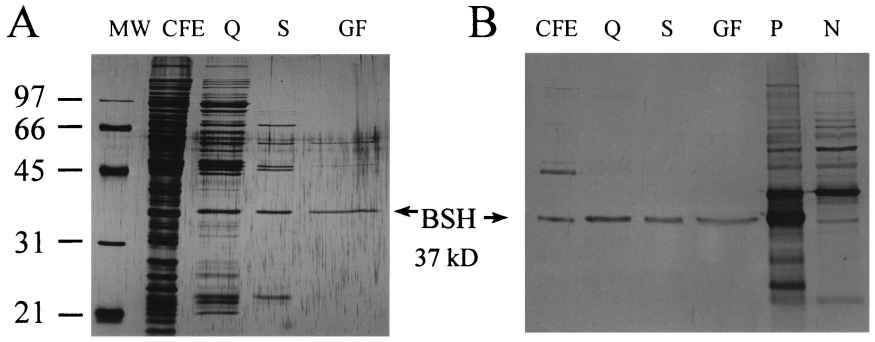

FIG. 2. Purification and immunostaining of *B. longum* BSH. (A) SDS-PAGE (10% polyacrylamide) after silver staining. Lane MW, molecular weight marker; lane CFE, cell extract; lanes Q, S, and GF, BSH active fraction after MonoQ chromatography, MonoS chromatography, and gel filtration, respectively. (B) Immunostaining with anti-*C. perfringens* BSH antibodies. Lane CFE, cell extract; lanes Q, S, and GF, BSH active fraction after MonoQ chromatography, MonoS chromatography, and gel filtration, respectively; lanes P and N, cell extract of *E. coli* with and without *C. perfringens* BSH, respectively.

To purify BSH, a cell extract was first applied to a MonoQ HR column. BSH activity eluted at NaCl concentrations between 0.33 and 0.36 M. Subsequently, the pooled BSH-containing MonoQ fractions were applied to a MonoS HR column. BSH appeared in the breakthrough fraction. This fraction, in which only a few major proteins were still present (Fig. 2A), was used for further characterization. The level of BSH activity in the MonoS fraction was 20.5 U/mg; the enzyme was purified 21-fold, and the yield was 17%.

The BSH protein was identified by using gel filtration (Fig. 2A) combined with immunostaining with anti-*C. perfringens* BSH antibodies (Fig. 2B) and N-terminal amino acid sequencing. An approximately 37-kDa protein was evident throughout the different chromatography steps, and this protein formed the major band after gel filtration. Immunostaining revealed only this band after MonoQ chromatography. N-terminal amino acid sequencing of the protein in this band resulted in the following sequence: XTGVRFSDDEGNTYFGRNLDWS FSYGETIL. A protein homology comparison revealed that this sequence exhibited very high levels of homology to the N-terminal amino acid sequences of *C. perfringens* BSH and the BSH of various lactobacilli (Fig. 3).

Biochemical characterization of *B. longum* BSH. SBT2928 BSH is an enzyme that has a broad substrate range and can hydrolyze not only human bile salts but also tauroursodeoxycholic acid (3α, 7β-OH) and taurohyodeoxycholic acid (3α, 6α-OH) (Table 4). The highest levels of activity were observed with glycochenodeoxycholic acid (defined as 100% activity) and with glycine-conjugated bile acids. Furthermore, the enzyme exhibited a slight preference for conjugated chenodeoxycholic acid over conjugated cholic and deoxycholic acid. The substrate specificity of the purified enzyme and the BSH activity in the cell extract were the same (results not shown). Of the six major human bile salts tested (Table 4), the enzyme exhibited the highest affinity for glycochenodeoxycholic acid and, in general, higher affinities for glycine-conjugated bile acids than for taurine-conjugated bile acids. This is in good agreement with the results of the substrate specificity analysis.

The pH optimum for BSH activity at 37°C was between pH 5 and 7, and maximum activity occurred at pH 6. Maximum enzyme activity occurred at temperatures between 40 and 45°C. The enzyme was stable at pH values from 4 to 8; at pH values above 8 and below 4 it was rapidly inactivated. The enzyme was stable at temperatures from 4 to 37°C. During 30 min of incubation at 40°C, about 20 to 30% of the activity was lost, and at 50°C only 20% of the activity was retained. Therefore, the temperature used for the activity assays was 37°C in order to prevent loss of enzyme activity during the reactions.

The effects of ions and enzyme inhibitors are summarized in Table 5. The enzyme was strongly inhibited by five substances known as SH enzyme inhibitors. Little inhibition was observed with either a metal enzyme inhibitor (EDTA) or a serine enzyme inhibitor (phenylmethylsulfonyl fluoride). Inhibition by $HgCl_2$ could be reversed by adding a sufficient amount of the reducing reagent DTT (data not shown). From these results we concluded that *B. longum* BSH is probably an SH enzyme. Therefore, a cysteine residue(s) with a free SH group should play an important role in the activity of the enzyme (see below). Surprisingly, magnesium sulfate inhibited the enzyme very strongly, while magnesium chloride did not.

The native molecular weight of BSH was determined by gel filtration in triplicate at both 4 and at 21°C and was found to be between 125,000 and 130,000. The subunit molecular weight, as determined in four independent SDS-PAGE analyses, was 37,300 (Fig. 2), which is in good agreement with the genetic data (see below) and indicates that the native enzyme is a tetramer.

Construction of a gene bank for *B. longum* SBT2928 and isolation of the BSH gene. A gene bank of the chromosome of *B. longum* SBT2928 was constructed in plasmid pUK21 and was transferred into *E. coli* NM522 (see Materials and Methods). A total of 30,000 colonies of transformants were plated onto BSH selection agar. Two colonies which produced a white

TABLE 3. Effect of DTT on BSH activity after sonication of *B. longum* SBT2928 cells

Length of sonication (min)	Sonication conditions[a]	Assay conditions[a]	Activity[b]		Protein concn (mg/ml)	Sp act[b]	
			U/ml	%		U/mg	%
3	−DTT	−DTT	1.04	12	0.89	1.17	11
	−DTT	+DTT	4.6	55	0.89	5.2	50
	+DTT	−DTT	9.8	112	0.83	11.8	112
	+DTT	+DTT	8.7	100	0.83	10.5	100
9	−DTT	−DTT	0.47	4	1.61	0.3	4
	−DTT	+DTT	3.9	37	1.61	2.4	35
	+DTT	−DTT	10.8	103	1.57	6.9	103
	+DTT	+DTT	10.5	100	1.57	6.7	100

[a] −DTT, no DTT present; +DTT, 10 mM DTT present.
[b] The level of activity in the presence of 10 mM DTT after sonication in the presence of 10 mM DTT was defined as 100%.

precipitation halo after 2 to 3 days were identified. The plasmids in the cells in these colonies were designated pBH13 and pBH16 and used for further analysis. The inserts of both plasmids were analyzed with various restriction enzymes. pBH13 had a 2.7-kb insert, and pBH16 had a 8-kb insert (Fig. 4). Using the *Eco*RV site of the insert in pBH13, we constructed two subclones, pBH1351 (1.5-kb insert) and pBH1322 (1.2-kb insert) (Fig. 4). As *E. coli*(pBH1351) was positive in BSH activity assays, either most or all of the BSH genetic information was present on the 1.5-kb insert of this clone.

Nucleotide sequencing and analysis. The nucleotide sequence of the insert in pBH13 comprises 2,604 bases. In this sequence one complete open reading frame (ORF) and one partial ORF were detected, and these ORFs could encode a 317-amino-acid protein and a 447-amino-acid partial protein, respectively (Fig. 1A and 5). The complete ORF was present in the pBH1351 insert. The N-terminal amino acid sequence of the deduced 317-amino-acid protein starting from the third amino acid was identical to the N-terminal amino acid sequence determined by using purified BSH (Fig. 5). These data show that the complete ORF encodes BSH, and, accordingly, the gene was designated *bsh*. Taking into account the fact that f-Met was processed, the deduced protein had a molecular weight of 35,024 and a pI of 4.51. The derived molecular weight is in good agreement with the molecular weight obtained from the biochemical characterization of the enzyme. The N terminus of the deduced protein did not contain a typical leader peptide (56) or a membrane-spanning domain.

Screening of the sequence upstream of *bsh* for promoter consensus sequences revealed a −10 sequence in which five of the six bases were conserved (Fig. 5). No −35 sequence was identified, but a number of direct and inverted repeats present in the region around −35 could be involved in transcription initiation (17, 32). Furthermore, a stem-loop structure with a free energy of −24.5 kcal was detected upstream of *bsh*. This structure could have been a rho-independent terminator. However, the canonical T stretch was missing, indicating that the stem-loop structure could also have a different function.

No canonical gram-positive Shine-Dalgarno (SD) sequence (GGAGG [54]) was found upstream of *bsh*. Since very little is known about translation initiation in bifidobacteria, the six genes from members of this genus that have been sequenced (*Bifidobacterium breve* β-D-glu [35]; *B. longum ldh* [31]; all other gene sequences are available only from GenBank [accession numbers are shown in Table 6]) were examined for regions of complementarity upstream of the ATG start codon with the 3′ ends of 16S rRNA of bifidobacteria (as determined from 16S rRNA sequences in the GenBank database [data not shown]). Table 6 shows that the location of a core AGG motive which extended in either the 5′ direction or the 3′ direction could be determined. The free energies of the different SD sequences ranged from −11.8 to −19.4 kcal when single-base gaps were allowed. Using this approach, we identified two possible SD sequences upstream of BSH, and these sequences had free energies of −12.4 and −21.6 kcal (Fig. 5 and Table 6).

Downstream of *bsh* a stem-loop structure which lacked the poly(T) stretch was probably not a rho-independent terminator but could have a different as-yet-unknown function. Furthermore, 55 bases downstream of the stop codon of *bsh* a new putative gene, *glnE* (see below), started. Therefore, *bsh* could be transcriptionally coupled to *glnE* (see below).

The G+C content of the fragment which we sequenced was 62%, which is in good agreement with the G+C range for bifidobacterial DNA (55 to 67%) (39) and with the G+C content reported previously for *B. longum* (62%) (Codon Usage Tabulated from GenBank at http://www.dna.affrc.go.jp/~nakamura/CUTG.html).

Homology studies with *B. longum* BSH. A BlastX search (1) revealed high levels of homology between *B. longum* BSH and BSH of several *Lactobacillus* species (37 to 48% identity) (GenBank accession number AF091248 [6, 11]), as well as *C. perfringens* BSH (36% identity) (7), penicillin V acylase (PVA) of *Bacillus sphaericus* (30% identical amino acids) (36), and a hypothetical protein of *Bacillus subtilis* (29% identity) (GenBank accession number P54948). Recently, the crystal structure of the PVA was elucidated (42). The amino acids of the active site were identified as Cys-1, Asp-20, Tyr-82, Asn-175, and Arg-228. A comparison of the deduced amino acid sequences of *B. longum* BSH and other BSH with the deduced amino acid sequence of the PVA showed that except for Tyr-82 all of these amino acids are conserved (Fig. 3).

The deduced product of the second (partial) ORF exhibited high levels of homology with glutamine synthetase adenylyltransferases (GlnE) of *Mycobacterium tuberculosis* (GenBank accession number Z70692) (40% identical amino acids in domain A and 30% identical amino acids in domain B), *E. coli* (GenBank accession number AE000387) (24% identical amino acids in domain A and 25% identical amino acids in domain B), and various other bacteria.

***bsh* and *glnE* are transcriptionally coupled.** In order to examine whether *bsh* and *glnE* are transcriptionally coupled, total RNA was isolated from *B. longum* SBT2928, and RT-PCR was carried out with several primer pairs that probed

TABLE 4. Relative activity and K_m values of *B. longum* BSH with various bile salts

Substrate (−OH positions)[a]	Relative activity (%)	K_m (mM)
GCA (3α, 7α, 12α)	85	0.16
GDCA (3α, 12α)	91	0.28
GCDCA (3α, 7α)	100	0.13
TCA (3α, 7α, 12α)	43	1.12
TDCA (3α, 12α)	29	0.79
TCDCA (3α, 7α)	51	0.33
THDCA (3α, 6α)	11	ND[b]
TUDCA (3α, 7β)	19	ND

[a] GCA, glycocholic acid; GDCA, glycodeoxycholic acid; GCDCA, glycochenodeoxycholic acid; TCA, taurocholic acid; TDCA, taurodeoxycholic acid; TCDCA, taurochenodeoxycholic acid; THDCA, taurohyodeoxycholic acid; TUDCA, tauroursodeoxycholic acid.
[b] ND, not determined.

TABLE 5. Effects of various ions and inhibitors on BSH activity

Substance[a]	Concn (mM)	% Inhibition
Iodoacetate	3	87
Periodic acid	3	100
N-Ethylmaleimide	1	67
$HgCl_2$	0.1	86
*p*CMBA	0.01	87
$CuCl_2$	5	100
$CaCl_2$	30	94
$MgSO_4$	30	84
$MgCl_2$	30	0
EDTA	30	18
PMSF	0.5	26
NaCl	50	152[b]

[a] *p*CMBA, *para*-chloromercuribenzoic acid; PMSF, phenylmethylsulfonyl fluoride.
[b] Percent activation.

```
BSHBLO    M--CTGVRFSDDEGNTYFGRNLDWSFSYGETILVTPRGYHYDTVPGAG-G   47
BSHLBJ    M--CTGLRFTDDQGNLYFGRNLDVGQDYGEGVIITPRNYPLPYKFLDN-T   47
BSHLBP    M--CTAITY--QSYNNYFGRNFDYEISYNEMVTITPRKYPLVFRKVEN--   44
BSHCLP    M--CTGLALETKDGLHLFGRNMDIEYSFNQSIIFIPRNFK-CVNKSNKKE   47
HYPBSU    M--CTSLTLETADRKHVLARTMDFAPQLGTEVILYPRRYS-WNSEADGRA   47
PENABSP   MLGCSSLSIRTTDDKSLFARTMDFTMEPDSKVIIVPRNYGIRLLEKENVV   50
          *  *:...          ..*..*  .   .   .  ** .      .

BSHBLO    KAKPNAVIGVGVVMADRPMYFDCANEHGLAIAGLNFPGYASFVHEPVEGT   97
BSHLBJ    TTK-KAVIGMGIVVDGYPSYFDCYNEDGLGIAGLNFPHFAKFSDGPIDGK   96
BSHLBP    LDHHYAIIGITADVESYPLYYDAMNEKGLCIAGLNFAGYADYKKYDAD-K   93
BSHCLP    LTTKYAVLGMGTIFDDYPTFADGMNEKGLGCAGLNFPVYVSYSKEDIEGK   97
HYPBSU    HQTQYAFIGMGRKLGNI-LFADAFNESGLSCAALYFPGYAEYEKMIREDT   96
PENABSP   INNSYAFVGMGSTDITSPVLYDGVNEKGLMGAMLYYATFATYADEPKKGT  100
               * .*..    .     *  ** **  * *.. .... .   . .

BSHBLO    ENVATFEFPLWVARNFDSVDEVEETLRNVTLVSQIVPGQ-QESLLHWFIG  146
BSHLBJ    INLASYEIMLWVTQNFTHVSEVKEALKNVNLVNEAINTSFAVAPLHWIIS  146
BSHLBP    VNITPFELIPWLLGQFSSVREVKKNIQKLNLVNINFSEQLPLSPLHWLVA  143
BSHCLP    TNIPVYNFLLWVLANFSSVEEVKEALKNANIVDIPISENIPNTTLHWMIS  147
HYPBSU    VHIVPHEFVTWVLSVCQSLEDVKEKIRSLTIVEKKLDLLDTVLPLHWILS  146
PENABSP   TGINPVYVISQVLGNCVTVDDVIEKLTSYTLLNEANIILGFAPPLHYTFT  150
            .       .      . .* . . . ....          **.  .

BSHBLO    D-GKRSIVVEQMAD-GMHVHHDDVDVLTNQPTFDFHMENLRNYMCVSNEM  194
BSHLBJ    D-SDEAIIVEVSKQYGMKVFDDKVGVLTNSPDFNWHLTNLGNYTGLNPHD  195
BSHLBP    D-KQESIVIESVKE-GLKIYDNPVGVLTNNPNFDYQLFNLNNYRALSNST  191
BSHCLP    DITGKSIVVEQTKE-KLNVFDNNIGVLTNSPTFDWHVANLNQYVGLRYNQ  196
HYPBSU    DRTGRNLTIEPRAD-GLKVYDNQPGVMTNSPDFIWHVTNLQQYTGIRPKQ  195
PENABSP   DASGESIVIEPDKT-GITIHRKTIGVMTNSPGYEWHQTNLRAYIGVTPNP  199
          *    ....*      . .  .  .*.** *.. ..  ** .*  .

BSHBLO    AEPTSWGKASLTAWGAGVGMHGIPGDVSSPSRFVRVAYTNAHYPQQNDEA  244
BSHLBJ    ATAQSWNGQKVAPWGVGTGSLGLPGDSIPADRFVKAAYLNANYPTVKGEK  245
BSHLBP    PQNSFSEKVDLDSYSRGMGGLGLPGDLSSMSRFVRAAFTKLNSLPMQTES  241
BSHCLP    VPEFKLGDQSLTALGQGTGLVGLPGDFTPASRFIRVAFLRDAMIKNDKDS  246
HYPBSU    LESKEMGGLALSAFGQGLGTVGLSGDYTPPSRFVRAVYLKEHLEPAADET  245
PENABSP   PQDIMMGDLDLTPFGQGAGGLGLPGDFTPSARFLRVAYWKKYTEKAKNET  249
             .  ... . * *  *..**  .  .**..... .       .

BSHBLO    ANVSRLFHTLGSVQMVDGMAKMGDGQFERTLFTSGYSSKTNTYYMNTYDD  294
BSHLBJ    ANVAKFFNILKSVAMIKGSVVNDQGSDEYTVYTACYSSGSKTYYCNFEDD  295
BSHLBP    GSVSQFFHILGSVEQQKGLCEVTDGKYEYTIYSSCCDMDKGVYYYRTYDN  291
BSHCLP    IDLIEFFHILNNVAMVRGSTRTVEEKSDLTQYTSCMCLEKGIYYYNTYEN  296
HYPBSU    KGVTAAFQILANMTIPKGAVITEEDEIHYTQYTSVMCNETGNYYFHHYDN  295
PENABSP   EGVTNLFHILSSVNIPKGVVLTNEGKTDYTIYTSAMCAQSKNYYFKLYDN  299
           ..   *..* ..    *     ..  . * ...     .  ** .  ..

BSHBLO    PAIRSYAMADYDMDSSELISVA----------------R   317
BSHLBJ    FELKTYKLDDHTMNSTSLVTY------------------   316
BSHLBP    SQINSVNLNHEHLDTTELISYPLRSEA------QYYAVN   324
BSHCLP    NQINAIDMNKENLDGNEIKTYKYNKTLSINHVN------   329
HYPBSU    RQIQKVNLFHEDLDCLEPKVFSAKAEESIHELN------   328
PENABSP   SRISAVSLMAENLNSQDLITFEWDRKQDIKQLNQVNVMS   338
            .    .    ..  .
```

FIG. 3. Alignment of BSH of various bacteria, of a hypothetical protein of *Bacillus subtilis*, and PVA of *Bacillus sphaericus*. Asterisks, identical amino acids; dots, similar amino acids. The boxes indicate amino acids involved in the active site of PVA, including C-1, D-20, Y-82, N-175, and R-228 (positions based on the *B. sphaericus* DNA sequence). The amino acid sequence which was determined with purified BSH is indicated by boldface type. The enzymes examined were the BSH of *B. longum* (BSHBLO), *L. johnsonii* BSH (BSHLBJ), *L. plantarum* (BSHLBP), and *C. perfringens* (BSHCLP); the hypothetical protein of *Bacillus subtilis* (HYPBSU); and *Bacillus sphaericus* PVA (PENABSP).

three different regions (Fig. 1A). Primers B and C were used for an internal control, primers D and E were used to detect transcripts running from *bsh* to *glnE*, and primers A and F and primers A and C were used to detect whether mRNA extended upstream beyond the possible transcription start site of *bsh*. First-strand cDNA synthesis was carried out both with primer E and with primer C. The results were the same for both cDNAs (Fig. 1B and C). In addition to the positive DNA control, a clear signal was obtained with primers D and E, which showed that *bsh* and *glnE* were present on the same transcript. Products were also obtained with primers A and F and with primers A and C, but the yields were lower. This indicated that a transcript extended upstream from *bsh*, but it may have been less abundant than the *bsh-glnE* transcript. Apparently, the stem-loop structure upstream of *bsh* (see above) could not eliminate transcription completely.

Selection, construction, and analysis of mutants with mutations in the BSH gene. BSH-negative mutants were selected

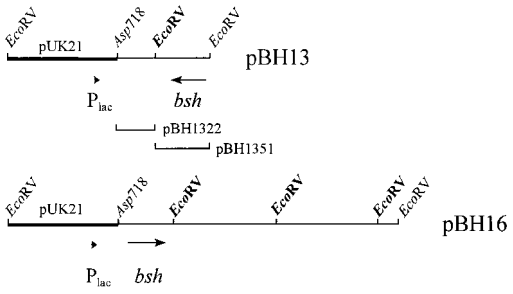

FIG. 4. Plasmid maps of plasmids pBH13 and pBH16, which confer BSH activity, and two subclones. Heavy lines, plasmid pUK21; boldface restriction sites, sites present in the insert; P_{lac}, *E. coli lacZ* promoter.

after spontaneous mutation or after UV treatment. One spontaneous mutant (A3) and two UV-induced mutants (2001 and 2503) were obtained. The *bsh* genes of the mutants were amplified with a high-fidelity polymerase by using primers A and E (Table 2 and Fig. 1) and were sequenced. Table 7 shows the mutations in *bsh* (Fig. 5) and some of the properties of the mutants. The mutation in A3 resulted in a stop codon that resulted in deletion of 117 amino acids. The frameshift mutation in 2001 replaced 50 amino acids at the C terminus with 25 amino acids of reading frame +1. Both truncated forms of BSH were inactive, demonstrating that the C terminus is important for enzyme activity. Surprisingly, the PCR-amplified mutated *bsh* gene of A3 was active in *E. coli*, and the complete protein was detected in this organism (Table 7) (results not shown). In mutant 2503 Leu-80 was replaced by Ser. This amino acid was immediately upstream of the putative active site residue Asn-81. In cell extracts of this *B. longum* mutant and of *E. coli* harboring this recloned mutated *bsh* gene, no activity was detected, but the protein was still present.

Biochemical characterization showed that BSH is a thiol enzyme. Furthermore, homology studies showed that only one Cys residue (Cys-1) is conserved in all BSH enzymes, in PVA, and in a hypothetical protein of *Bacillus subtilis* (Fig. 3). The crystal structure of PVA shows that Cys-1 plays an essential role in the catalytic mechanism of the enzyme. Using site-directed mutagenesis, we constructed a mutant of *bsh* of *B. longum* in which Cys-1 was replaced by Ala (pCA-1), which removed only the sulfur atom at this position in BSH. The resulting mutant did not exhibit BSH activity, while the protein was still present (Table 7); this proved that Cys-1 is essential for BSH enzyme activity.

DISCUSSION

Characterization of *B. longum* SBT2928 BSH showed that this enzyme is similar to the *B. longum* BB536 enzyme described by Grill et al. (15), but the two enzymes may differ in minor characteristics. However, Grill et al. did not provide any genetic data about *B. longum* BSH; such data are provided in this paper.

BSH is known to be very oxygen sensitive (41). The optimized isolation conditions described here, in which 10 mM DTT is included during sonication, allow workers to isolate highly active BSH under normal atmospheric conditions. Optimization of the sonication conditions is crucial since even in the presence of DTT, BSH activity is lost after extended sonication (Table 3).

The reported native molecular weights of BSH of different species vary greatly. Using HPLC, which is the most accurate method, we obtained a value for *B. longum* SBT2928 BSH of

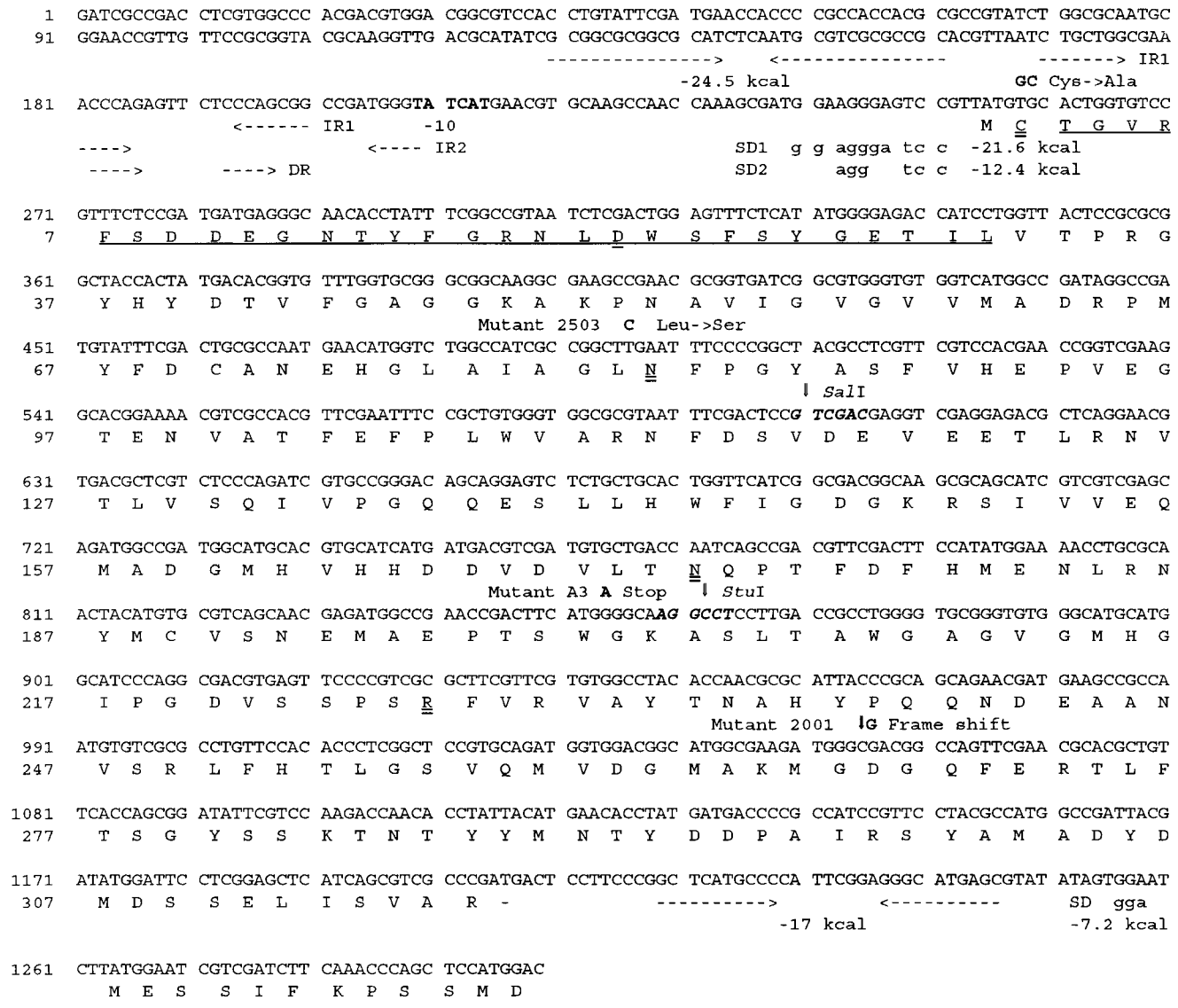

FIG. 5. Nucleotide sequence of the *bsh* gene region of *B. longum*. IR, inverted repeat; DR, direct repeat; −10, putative −10 promoter region; SD, putative SD sequence. The N-terminal amino acid sequence determined for purified BSH is underlined. Mutations are indicated above the nucleotide sequence by boldface letters which indicate the exchanged or inserted nucleotides. The amino acids of the supposed active site (C-1, D-20, N-81, N-172, and R-225) are double underlined.

between 125,000 and 130,000, while Grill et al. (15) reported a value of 250,000 for strain *B. longum* BB536 BSH. Native molecular weights of 250,000 have also been reported for *C. perfringens* and *Bacteroides fragilis* enzymes (13, 41). Another *C. perfringens* strain has a native molecular weight of 147,000 (7). Interestingly, the molecular weight of the enzyme of *Lactobacillus* sp. strain 100/100 (now *L. johnsonii* [11]) is only 105,000 or 115,000. The subunit molecular weights of BSH have been reported to be between about 32,000 and 56,000. Consequently, native enzymes are octamers (*Bacteroides fragilis* [41]), hexamers (*B. longum* BB536 [15]), tetramers (*C. perfringens* [7, 13]), or trimers (*L. johnsonii* [25]). Supporting evidence that the BSHs of *B. longum* and perhaps also all other bacteria are tetramers comes from the high levels of homology of all BSHs whose primary amino acid sequences are known with PVA (42), which is a tetramer. Additionally, four of the five putative active-site amino acids of PVA are conserved in all BSHs, suggesting that these enzymes are also related in their tertiary and quaternary structures (Fig. 3). How this conjecture can be reconciled with the previously described heterotrimeric structure of the well-characterized BSH of *L. johnsonii* 100/100 (25) is not clear, but the problem could be approached by molecular modelling in which the three-dimensional structure of PVA is used as a template (see below).

In summary, biochemical characterization of *B. longum* SBT2928 BSH has shown that this enzyme has a tetrameric structure, a molecular weight of about 130,000, and broad substrate specificity, is active under pH and temperature conditions that occur in the human body, and requires a free —SH group for catalytic activity.

The following facts prove that the BSH encoded by the gene cloned in this work and the enzyme which we purified and biochemically characterized are identical: (i) the 1.5-kb insert conferring BSH activity to *E. coli* contains an ORF for a protein whose deduced N-terminal amino acid sequence from the third amino acid onward is identical to the deduced N-terminal

TABLE 6. Alignment of the regions immediately upstream of the ATG start codons of six *Bifidobacterium* genes and *B. longum* BSH with the 16S rRNA 3′-terminal consensus sequence

Organism	Gene	Sequence around the start codon[a]	Free energy (kcal)	Distance[b]
B. breve (E05040)[c]	β-Galactosidase gene	ATATTTATTCATT**AAAGGA**ACAGC**ATC**ATC *ATGGAACATCGCGAAT* 3′ tctttcctccac **tagg** 5′	−12.4	12
B. breve	β-D-Glucosidase gene	CGGCGCCAACT**AGAAAGGA**ATCACCG *ATGACGATGATCTTCC* 3′ **tctttcct**ccactagg 5′	−16.2	8
B. adolescentis (AF124596)	α-Galactosidase gene	AAGAATCGCAAGA**AAAGGA**TGCTGCA *ATGACGCTCATTCAGA* 3′ tctttcctccactagg 5′	−11.8	8
B. breve (AF094756)	*recA*	CATACGACTTCGAT**AAGGAG**AA**GATC** *ATGGCACTTGAGACCA* 3′ tctttcctccactagg 5′	−18.2	8
B. asteroides (Y11549)	*recA*	GGCCGATCACTGA**AAAGGAG TGAT**TAGCA *ATGACCGATTCTACCG* 3′ tctttcctccactagg 5′	−19.4	11
B. longum	*ldh*	ACCGCCTTT**GAA**GA**GAGGT**TC**ATCC**ATC *ATGGCGGAAACTACCG* 3′ tctttcctccac **tagg** 5′	−17.2	10
B. longum	*bsh*	AACCAAAGCGATGGA**AGG**GAG**TCC**GTT *ATGTGCACTGGTGTCC* 3′ tctttcctccact**agg** 5′[d]	−12.4	9
B. longum	*bsh*	AACCAAAGCGAT**GGAAGG GAGTCC**GTT *ATGTGCACTGGTGTCC* 3′ tctttcc tccact **agg** 5′[d]	−21.6	9

[a] The sequence of the 3′ end of *Bifidobacterium* 16S ribosomal DNA is 3′ tcttcctccactagg 5′, shown in lowercase letters under each SD region. The box indicates the central AGG motif of the SD region in bifidobacteria.
[b] Distance (in nucleotides) between ATG and the core AGG sequence in the SD region.
[c] The numbers in parentheses are GenBank nucleotide sequence accession numbers.
[d] Possible alignment of the 3′ end of the 16S ribosomal DNA with the upstream region of *bsh*.

amino acid sequence of purified BSH, (ii) during MonoQ chromatography BSH activity produced in *E. coli* elutes at the same salt concentration as the BSH isolated from *B. longum* (results not shown), (iii) BSH activity isolated from *E. coli*, like *B. longum* BSH activity, cross-reacts with polyclonal antibodies raised against *C. perfringens* BSH, and (iv) various BSH-negative strains of *B. longum* SBT2928, obtained by UV-induced or spontaneous mutagenesis, have mutations in the *bsh* gene. The mutant *bsh* genes lead to a loss of BSH activity in *E. coli* (except for mutant A3 [see below]).

From the genetic data it is clear that BSH is an intracellular enzyme. This is consistent with the observation that no enzyme activity was present in the supernatants of overnight cultures, while activity was released either by sonication or other cell disruption methods or by lysis in assays performed with whole cells due to the lytic properties of the bile salts (data not shown).

In RT-PCR experiments we obtained products which contained almost the entire known sequence upstream of *bsh*, although the yield was less. This could indicate that *bsh* is transcribed both by its own promoter and to a lesser degree from an upstream region. Another piece of supporting evidence that *bsh* could have its own promoter comes from the fact that BSH activity is found in pBH13 and related constructs in which *bsh* is cloned in the direction opposite that of the *lac* promoter in these plasmids. Primer extension experiments have failed so far and need to be carried out to definitely establish whether *bsh* has a promoter directly upstream of its coding sequence.

Alignment of the 3′ end of the 16S rRNA of bifidobacteria with the regions upstream of the ATG start codon of all known bifidobacterial genes revealed that only the AGG of the canonical GGAGG sequence (54) is conserved (Table 6). Also, *bsh* is preceded by two possible SD sequences with central AGG motives. This could imply that the translation start in bifidobacteria has slightly different requirements than the translation start in, for instance, *Bacillus subtilis* (51, 54). The gene following *bsh*, *glnE*, does not have this AGG motive and has only a very short putative SD sequence (GGA) that has a free energy of −7.2 kcal, which is very low for the gram-positive translation machinery. However, the stem-loop structure in front of *glnE* might facilitate a translational restart by

TABLE 7. Overview of *bsh* mutants

Mutant	Mutation	Activity in *B. longum*	Activity in *E. coli*	Protein in *B. longum*[a]	Protein in *E. coli*[a]
A3	Spontaneous mutant, G-A transition creating a stop codon in place of amino acid 200 (Trp), deletion of 117 amino acids	No activity detected	Activity	No protein detected	Protein of original size
2503	UV-induced T-C transition causing Leu80Ser exchange	No activity detected	No activity detected	Protein present	Protein present
2001	Insertion of G after GG of Gly-267, causing a frameshift and loss of the last 50 amino acids	No activity	No activity	No protein	No protein
C1A[b]	Site-directed mutagenesis of TG of Cys-1 to GC, resulting in Cys1Ala exchange		No activity		Protein present

[a] The presence of BSH was determined by Western blotting with polyclonal antibodies.
[b] Mutation present only in *bsh* cloned in *E. coli*.

bringing the stop codon of *bsh* close to the start codon of *glnE* (54).

B. longum BSH exhibits high levels of homology with BSH of various lactobacilli and *C. perfringens*, with a hypothetical protein of *Bacillus subtilis*, and with PVA of *Bacillus sphaericus* (42). Recently, the crystal structure of PVA has been determined. A homology analysis of BSH and PVA revealed that four of the five amino acids at the active site of PVA are conserved in BSH, while Tyr-82 is replaced by Asn-81 in BSH. The catalytically important part of Tyr-82 in PVA is the NH group of the peptide bond. Therefore, probably, Tyr-82 could be replaced easily by another amino acid if the NH group is kept in the right position (for BSH, Asn-81). The fact that Asn-81 is conserved in the different BSHs could have to do with different steric requirements for binding of bile salts compared to binding of penicillin V. The striking similarities in the primary structure and in the active-site amino acids indicate that *B. longum* BSH and all other known BSHs belong to the same group of N-terminal nucleophile hydrolases. Therefore, the secondary and tertiary structures are probably also very similar. It is not clear, however, how much the tertiary structure is conserved in *L. johnsonii* 100/100 BSH, for which a heterotrimeric structure has been proposed (26).

Downstream of *bsh* a gene with a very high level of homology to glutamine synthetase adenylyltransferase (*glnE*) was found. This enzyme transfers adenylyl residues from ATP to glutamine synthetase and is part of the nitrogen regulation cascade (27, 53) in *E. coli* and, by analogy, probably also in *Haemophilus influenzae*, various mycobacteria, and pseudomonads. The intergenic region between *bsh* and *glnE* is rather short and lacks a strong termination signal. RT-PCR experiments have shown that the two genes are transcriptionally coupled, indicating that in *B. longum bsh* is part of an operon. The extent of this operon is not known, since only the 5′ part of *glnE* has been cloned and the transcript extends upstream of *bsh* (see above). Homology searches for the region 250 bp upstream of *bsh* produced no results. This genetic organization is different from that found in two *Lactobacillus* strains, while no adjacent sequences have been found for *C. perfringens bsh* (7). In *L. plantarum bsh* is monocistronic (6), and in *L. johnsonii bsh* is preceded by two genes that encode bile salt transport systems, but the exact extent of this operon is not known (11).

The combination of *bsh* and *glnE* in one operon is rather surprising, since there is no obvious functional relationship between the two enzymes. However, the coupling of *bsh* to a gene of the nitrogen regulation cascade reminds us of the hypothesis that hydrolysis of bile salts makes the amino acid nitrogen atoms of the released amino acids available for cells (18, 52).

Interestingly, the A3 *bsh* mutation, which did not produce a protein in *B. longum*, did produce activity and a protein in *E. coli* (Table 7). Apparently, in *E. coli* the stop codon created was overread in a translational readthrough process (12), which resulted in an intact molecule. One UV-induced mutation, the mutant 2503 *bsh* mutation, resulted in a leucine-to-serine change at the position next to active site residue Asn-81 which probably resulted in a substantial steric change which led to inactivation of the protein. With the other UV-induced mutation, the mutant 2001 *bsh* mutation, 50 C-terminal amino acids were removed, activity was lost, and there was rapid turnover of BSH, as the protein could not be detected in *B. longum* or in *E. coli*.

The N-terminal amino acid sequence determined for purified BSH did not include a Met residue. This means that formylmethionyl is processed and that Cys is the first amino acid of the mature protein. These findings are identical to findings obtained with PVA, in which Cys-1 plays a central role at the active site (42). The important role of Cys-1 was confirmed by the finding that a change of Cys-1 to Ala led to complete inactivation of BSH.

The biochemical and genetic data reported in this paper provide a sound foundation for investigating the role of bile salt hydrolysis in both the bacterial producers and their mammalian hosts. Furthermore, we created BSH-negative mutants which can be used in animal experiments and human studies to investigate the effects of enhanced bile salt deconjugation in the small intestine and to evaluate in a mechanistic way the hypothesis that BSH lowers serum cholesterol.

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