Genes encoding bile salt hydrolases and conjugated bile salt transporters in *Lactobacillus johnsonii* 100-100 and other *Lactobacillus* species

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***Lactobacillus johnsonii* strain 100-100 expresses two antigenically distinct conjugated bile salt hydrolases (BSH), α and β , that combine to form native homo- and heterotrimers. This paper reports characterization of loci within the genome that encode this capacity. A locus that encodes BSH β (*cbsH β*), a partial (*cbsT1*) and a complete conjugated bile salt transporter (*cbsT2*) was identified previously. DNA sequence analysis at this locus was extended and revealed a complete ORF for *cbsT1* and no other ORFs in tandem. The three genes, *cbsT1*, *cbsT2* and *cbsH β* , probably constitute an operon; a putative promoter was identified upstream of *cbsT1*. A second locus that expresses BSH activity in strain 100-100 was identified. Sequence analysis of the clone predicted a 978 nt ORF that did not share tandem organization with other ORFs, was similar in sequence to other BSH genes, and matched, in predicted protein sequence, the first 25 amino acids of BSH α . A phenotypic screen for BSH activity and a genetic screen for the *cbsH β* locus were performed on 50 *Lactobacillus* isolates from humans or dairy products. Nearly all of the isolates that were positive for *cbsH β* were from human sources. Variability in the BSH phenotype and *cbsH β* genotype was identified in isolates of the same species. DNA sequence was obtained and analysed from the *cbsH β* locus of one human isolate, *L. acidophilus* strain KS-13. This organism has *cbsT1*, *cbsT2* and *cbs β* genes that are 84, 87 and 85% identical in DNA sequence to those of strain 100-100. DNA sequence identity to strain 100-100 ends in regions flanking this locus. The findings of this study suggest that BSH genes have been acquired horizontally and that BSH activity is important at some level for lactobacilli to colonize the lower gastrointestinal tract.**

Keywords: intestinal microflora, probiotics, major facilitator superfamily, horizontal gene transfer, lactic acid bacteria

INTRODUCTION

The autochthonous microbiota inhabiting the lower gastrointestinal tract of animals (Savage, 1977) have evolved the ability to alter bile acids biochemically

Abbreviations: BSH, bile salt hydrolase; EF, extracellular factor; LDGW, long-distance genome walking; LPEA, long primer extension and amplification; MFS, major facilitator superfamily.

The GenBank accession numbers for the sequences determined in this work are AF054971, AF091248 (sequences of the complete BSH operons in *L. johnsonii* strain 100-100 and *L. acidophilus* strain KS-13, respectively) and AF297873 (sequence of *cbsH α*).

(Baron & Hylemon, 1997). Prokaryotic organisms from several genera express proteins that catalyse hydrolysis of an amide bond between the C-24 position of the steroid moiety and an amino acid, either glycine or taurine (Savage *et al.*, 1995; Baron & Hylemon, 1997; Savage, 2000). This activity may affect animal health, including that of humans. For example, it is associated with growth depression in monogastric animals grown for food (Eyssen & De Somer, 1963; Cole & Fuller, 1974). In humans, it may contribute to colon carcinogenesis (Kay, 1981; Cheah, 1990; Kandell & Bernstein, 1991) but may lower blood cholesterol levels (De Smet *et al.*, 1994). Some bacterial species express enzymes

that catalyse side-chain (sterol) transformations such as 7α - and 7β -dehydroxylation, 6β -dehydrogenation and desulfation (Baron & Hylemon, 1997; Hylemon & Harder, 1999). Bile salt hydrolysis, however, is the principal microbial reaction in that it performs a 'gatekeeping' function: bile salts must be deconjugated before sterol transformations can occur (Batta *et al.*, 1990).

The class of microbial enzymes that catalyse deconjugation have been collectively named conjugated bile salt hydrolases (BSH, EC 3.5.1.24). These enzymes can have demonstrable specificity for tauro- versus glycoconjugates (Aries & Hill, 1970; Gilliland & Speck, 1977), and with the exception of one expressed by *Clostridium perfringens* (Kishinaka *et al.*, 1994), are soluble cytosolic proteins (Stellwag & Hylemon, 1976; Gopal-Srivastava & Hylemon, 1988; Kawamoto *et al.*, 1989; Lundeen & Savage, 1990; Grill *et al.*, 1995; Tanaka *et al.*, 2000). The molecular fate of the amino acid released by deconjugation in the bacterial cell and the benefit that the organisms may derive from such activity is unclear (De Smet *et al.*, 1995). The deconjugated product, or primary bile acid, is a toxic hydrophobic molecule that can disaggregate the cytoplasmic membrane (Thanassi *et al.*, 1997). These molecules are not metabolized and leave the cell passively or through some unidentified bile acid export mechanism. At the physiological pH of the intestinal lumen, deconjugates can be actively transported through the epithelium (Wong *et al.*, 1994; Hylemon & Harder, 1999) and into the bloodstream of the host, or are precipitated in the faeces and excreted (Baron & Hylemon, 1997).

BSH enzymes have been purified and characterized from *Bacteroides vulgatus* VI-31 (Kawamoto *et al.*, 1989), *Bacteroides fragilis* subsp. *fragilis* (Stellwag & Hylemon, 1976), *Bifidobacterium longum* BB536 (Grill *et al.*, 1995) and SBT2928 (Tanaka *et al.*, 2000), *C. perfringens* MCV 815 (Gopal-Srivastava & Hylemon, 1988) and *Lactobacillus johnsonii* strain 100-100 (Lundeen & Savage, 1990, 1992a). Genes encoding BSH activity have been cloned from *Lactobacillus plantarum* 80 (Christiaens *et al.*, 1992), *C. perfringens* 13 (Coleman & Hudson, 1995), *L. johnsonii* strain 100-100 (Elkins & Savage, 1998) and *Bif. longum* SBT2928 (Tanaka *et al.*, 2000). Genetic characterization of these loci reveals different architectures: the *L. plantarum* 80 BSH transcript is monocistronic (Christiaens *et al.*, 1992); the *L. johnsonii* strain 100-100 BSH gene is arranged in tandem with at least two other functionally related genes (Elkins & Savage, 1998); and the *Bif. longum* SBT2928 BSH gene is coordinately regulated with at least one other gene that shares a high level of amino acid homology with glutamine synthetase adenylyltransferase (*glnE*; Tanaka *et al.*, 2000). The extent of the operon in *Bif. longum* SBT2928 and the putative operon in *L. johnsonii* strain 100-100 is not known (Elkins & Savage, 1998; Tanaka *et al.*, 2000); DNA sequence flanking the *C. perfringens* 13 BSH gene has not been characterized (Coleman & Hudson, 1995).

L. johnsonii strain 100-100 expresses high levels of BSH activity (Lundeen & Savage, 1990). In contrast to other bacteria studied, the activity in strain 100-100 involves four enzymes that exist as homo- and heterotrimers of two antigenically distinct proteins, designated α and β (Lundeen & Savage, 1992a). A 2977 bp *EcoRI* genomic clone, pIN-BSH2, that expresses hydrolase activity in *Escherichia coli* cells has been identified and shown to encode the β peptide (ORF3, hereafter referred to as *cbsH β* ; Elkins & Savage, 1998). A 651 nt partial ORF1 (hereafter referred to as *cbsT1*) and a 1353 nt complete ORF2 (hereafter referred to as *cbsT2*) were encoded on the fragment 5' of *cbsH β* . These ORFs share similarity with transport proteins of the major facilitator superfamily (MFS; Saier *et al.*, 1999) and approximately 80 % predicted amino acid sequence similarity to each other. They increase uptake of conjugated bile acids by as much as threefold over control levels when expressed in *E. coli* cells that were exposed to an extracellular factor (EF; Lundeen & Savage, 1990, 1992b) produced by strain 100-100 (Elkins & Savage, 1998).

We have characterized this locus and report here that *cbsT1*, *cbsT2* and *cbsH β* probably constitute a BSH operon. In addition, we report a second locus that encodes BSH α . We have also assayed a collection of 50 *Lactobacillus* spp. for BSH activity and the *cbsH β* locus. We speculate that BSH activity was acquired horizontally and implicate the activity as an important factor for colonization by lactobacilli of the gastrointestinal tract.

METHODS

Culture techniques. *L. johnsonii* strain 100-100 was maintained at -80°C in 15 % (v/v) glycerol for cryoprotection and grown anaerobically in MRS broth or agar medium (Becton Dickinson) as previously reported (Lundeen & Savage, 1990; Savage *et al.*, 1995). Strains of various *Lactobacillus* spp. obtained from several culture collections (see Table 2) were received as lyophilates, revived anaerobically in MRS medium, and subcultured. Glycerol stocks of each strain were prepared from isolated colonies and handled identically to strain 100-100.

Oligonucleotide primers. Primers used in this study (Table 1) were synthesized by Gibco-BRL Custom Primers Division under standard conditions.

Extension of genomic DNA sequence. Long-distance genome walking polymerase chain reaction (LDGW PCR; Min & Powell, 1998) was used to amplify and extend DNA sequence upstream and downstream of the strain 100-100 locus in pIN-BSH2 (Fig. 1). Long and accurate Advantage cDNA Polymerase Mix (ADP; Clontech), nested sequence-specific primers (SSPs), oligo-dG adaptor primer (AP), and thermocycling parameters established by Min & Powell (1998) were used. Genomic DNA was purified from strain 100-100 cells (Wizard Genomic DNA Purification Kit; Promega). LDGW was performed on genomic DNA with nested SSPs, UP-1 and UP-2, that were engineered to the upstream end of the 2977 bp *EcoRI* fragment. The LDGW protocol was modified to include a second nested amplification with UP-3 that eliminated contaminating DNA species and enriched for the genomic DNA sequence of interest. DNA from the second reaction was

Table 1. Oligonucleotide primers used in this study

Primer	Nucleotide sequence (5'–3')*	Strand†	Location	Use
AP	CGAGGAATTCGGGGGGGGGGGGGGG	NA	NA	LDGW, LPEA
UP-1	CCCTAAACCAATAATTAAGAGCCG	–	<i>cbsT1</i> (768–792)	LDGW
UP-2	GCCGATACTAAAAATCCAGAACCATTTC	–	<i>cbsT1</i> (744–771)	LDGW
UP-3, T1-1	CCATTTTCATGTGAAGAACTTGCTTGCCGCTCC	–	<i>cbsT1</i> (719–750)	LDGW, LPEA
DN-1	GCTACTCTTCTGGAAGCAAGACTTACTAC	+	<i>cbsHβ</i> (839–867)	LDGW
DN-2	CTACTGTAATTTTGAAGATGATTTTGAA	+	<i>cbsHβ</i> (864–891)	LDGW
DN-3	AAAGACTTATAAACTAGACGATCACAC	+	<i>cbsHβ</i> (894–920)	LDGW
Hβ-1	AATCCTGTCCAACATCTAGATTACG	–	<i>cbsHβ</i> (52–76)	LPEA
T1-2	ACACCTTGACCAGAATAAAAGTTGAACCAACC	–	<i>cbsT1</i> (360–392)	LPEA
T1strt	CGACAATATAAGTGgTAccGTCAAAGCTC‡	+	5' of <i>cbsT1</i> (64–93)	Cloning, pT1T2Hβ
T2strt	GGTTTTGCTATTGGTαCCCCACTATTTGGC‡	+	<i>cbsT1</i> (1174–1203)	Cloning, pT2Hβ
Hβstrt	GGCTATGCCATTGGTαCcCCACTATTTGG‡	+	<i>cbsT2</i> (1171–1199)	Cloning, pHβ
Hβend	CGACCAGGCTTGATgGATCCGC§	–	3' of <i>cbsHβ</i> (137–158)	Cloning, pT1T2Hβ, pT2Hβ and pHβ
Hβ675a	ATGTGTACTGGTTTAAGATTC	+	<i>cbsHβ</i> (1–21)	PCR screen for <i>cbsHβ</i>
Hβ675b	GGCTGGAATGCTGTCACCAGGCAGACC	–	<i>cbsHβ</i> (649–675)	PCR screen for <i>cbsHβ</i>
La-Hβa	GCTATTGGTαCcCCATTATTCGG‡	+	<i>cbsT2</i> (1177–1199)	Cloning, pLaHβ
La-Hβb	GTCATTTGGGAgGTαCTGTTGAATCAGG‡	–	3' of <i>cbsHβ</i> (163–191)	Cloning, pLaHβ

* Lower-case nucleotides indicate base substitutions in the sequence used to create restriction enzyme sites (underlined).

† Primers were engineered to the coding strand (+) or the noncoding strand (–) of the *cbsT1*, *cbsT2* and *cbsHβ* locus in *L. johnsonii* strain 100-100. NA, Not applicable; AP primer duplicated from procedure outlined by Min & Powell (1998).

‡ *KpnI* site underlined.

§ *BamHI* site underlined.

|| Primers were engineered to the *cbsHβ* locus in *L. acidophilus* strain KS-13.

purified (Wizard PCR Preps DNA Purification System) and sequenced with a fourth nested SSP and walking primers. The sequence was reconfirmed by directly amplifying genomic DNA with UP-1 and a primer engineered to the downstream end of the new sequence. The same strategy was applied to DNA sequence downstream of the cloned locus with primers, DN-1, DN-2 and DN-3. DNA sequence upstream and downstream of a 675 bp PCR amplified locus in *L. acidophilus* strain KS-13 was also obtained with LDGW (primers not shown).

RNA transcript analysis. Strain 100-100 is known to express BSH activity upon entry into stationary phase (Lundeen & Savage, 1990). Whole-cell RNA was harvested from stationary-phase cells (TRI Reagent; Molecular Research Center). Approximately 2 ml of cells were incubated with a 120 µl solution of 10 mg ml^{–1} lysozyme (Sigma)/10 mg ml^{–1} lysostaphin (Sigma) for 1 h at 37 °C prior to RNA isolation. Analysis of the mRNA that encodes *cbsHβ* was performed with long primer extension and amplification (LPEA) PCR developed in our laboratory as a hybrid protocol of rapid amplification of cDNA Ends (RACE; Edwards *et al.*, 1995) and LDGW (Min & Powell, 1998). The method involves use of Moloney Murine Leukaemia Virus-reverse transcriptase (M-MLV RT) and ADP to extend over long distances (>7 kb) on template nucleic acids. A microcentrifuge tube containing approximately 2 µg strain 100-100 RNA and 50 pmol primer Hβ-1 was adjusted to a final volume of 15 µl with distilled H₂O, heated to 70 °C for 5 min, and immediately cooled on ice. Two hundred units of M-MLV RT (Promega), 5 µl of 5 × M-MLV RT reaction buffer, 10 pmol of each dNTP (UltraPure; Clontech) and 25 units of rRNasin Ribonuclease inhibitor (Promega) were added to a final volume of 25 µl. The

tube was incubated at a stringent temperature for the M-MLV RT of 42 °C for 60 min. DNA was purified (Wizard PCR Preps DNA Purification System) from two such RT reactions and concentrated to approximately 15 µl. A dCTP nucleotide tail was added to the 3' end of the cDNA. Two successive, nested PCR reactions were accomplished as described by Min & Powell (1998) with tailed cDNA, AP and T1-1 (see Fig. 1) and then template DNA produced in the first reaction, T1-2 and AP. Total DNA from the second PCR reaction was purified and sequenced with T1-2. The DNA complement of the nucleotide adjacent to the oligo-dC tail was identified as the putative transcriptional start site.

Recombinant DNA methods. Plasmid vector pSportI (Gibco-BRL) was used in all recombinant DNA procedures. Three constructs that harbour strain 100-100 genomic loci encoding *cbsT1-cbsT2-cbsHβ*, *cbsT2-cbsHβ* or *cbsHβ* were cloned under control of the pSportI *lac*-inducible promoter. Primers containing engineered *KpnI* sites, T1strt, T2strt and Hβstrt, were constructed 5' of *cbsT1*, *cbsT2* and *cbsHβ* respectively and used with a primer containing an engineered *BamHI* site 3' of *cbsHβ*, Hβend. Genomic DNA was amplified by PCR, digested with *KpnI* and *BamHI* (Promega), and cloned into identically digested vector DNA with T4 DNA ligase (Promega). The chimeras were designated pT1T2Hβ, pT2Hβ and pHβ, respectively, and transformed into *E. coli* DH5α cells. Constructs were identified by blue/white colony screening on Luria–Bertani (LB) medium containing 100 µg ampicillin (Amp) ml^{–1}, 0.5 mM IPTG and 40 µg X-Gal ml^{–1} (LB-Amp-IPTG-X-Gal). Plasmid DNA for each construct was purified (Wizard Plus Minipreps DNA Purification Kit) and

Fig. 1. LDGW PCR (Min & Powell, 1998) was used to amplify strain 100-100 genomic DNA 5' and 3' of an *EcoRI* cloned locus, pIN-BSH2 (Elkins & Savage, 1998), and revealed a complete ORF for *cbsT1* and three additional ORFs: *arbX*, *arbZ* and *mat*. BLAST analysis identified high similarity of the three ORFs to glycosyltransferases, phospho- β -glycosidases and group II intron encoding maturases, respectively. Locations of primers used in this study (Table 1) are shown. A scale in bp is shown at the bottom.

sequenced to verify content and orientation. To clone and express the predicted BSH gene from *L. acidophilus* strain KS-13, chromosomal DNA was purified from strain KS-13 cells and PCR-amplified with primers La-H β a and La-H β b. The fragment was cloned at the *KpnI* site of pSportI. Transformants were screened for BSH activity.

Generation of an *L. johnsonii* strain 100-100 genomic library.

A genomic library of strain 100-100 was created with pSportI and screened for BSH activity. Chromosomal DNA was purified from strain 100-100 cells and partially digested with *Sau3AI* (Promega). The fragments were cloned at the *BamHI* site of pSportI in *E. coli* DH5 α cells. The library was screened for clones expressing BSH activity.

DNA sequencing and analysis. DNA generated from LDGW and LPEA PCR, direct amplified DNA, and cloned DNA was sequenced by the ABI Prism automated fluorescent method (Molecular Biology Resource Facility, University of Tennessee, Knoxville). Direct amplified DNA and cloned DNA were sequenced bidirectionally using universal forward and reverse primers and/or synthesized oligonucleotide walking primers. DNA sequence was analysed for start and stop codons and translated into predicted amino acid sequence with MacVector. ORFs were putatively identified from similarity with published sequences using the advanced basic local alignment search tool (BLAST; NIH website).

Assay for BSH activity. Transformed *E. coli* DH5 α cells were screened for the ability to hydrolyse conjugated bile acids with an agar plate assay (Dashkevich & Feighner, 1989). Cultures were streaked or replica plated onto MRS agar containing 0.5% taurodeoxycholic acid (Sigma), a conjugated bile acid, 100 μ g Amp ml $^{-1}$ and 0.5 mM IPTG (MRS-TDCA-Amp-IPTG). Cultures were incubated anaerobically for 24–48 h. BSH activity was detected when a halo of deoxycholic acid precipitated around colonies in the medium.

Screen for *cbsH β* . Coleman & Hudson (1995) identified conserved amino acid motifs in cloned BSHs by aligning predicted protein sequences. A motif at the amino terminus and a motif centred around residue 225 were selected and used to construct primers H β 675a and H β 675b, respectively. The

primers were engineered to *cbsH β* sequence in strain 100-100 and encompass a region of approximately 675 bp. The primers were used in standard PCR reactions to amplify genomic DNA from other lactobacilli.

RESULTS

Genomic analysis of the *cbsH β* locus

Genomic DNA sequence upstream and downstream of the cloned locus in pIN-BSH2 was extended and analysed. DNA fragments of approximately 2000–2500 bp were amplified in both directions by LDGW (Fig. 1). Sequence upstream of the locus yielded a complete ORF of 1356 nt for *cbsT1* that is 75% identical and 86% similar in predicted amino acid sequence to *cbsT2*. A putative Shine–Dalgarno 16S rRNA sequence was identified that is centred 8–13 nt upstream of the ATG start site for *cbsT1*. Advanced BLAST analysis of CbsT1 and CbsT2 revealed similarities to several families of the MFS (Saier *et al.*, 1999). The highest scoring, with approximately 42% similarity to the first 209 and 223 amino acids of CbsT1 and CbsT2 (6 and 10% gaps), respectively, were oxalate:formate antiporters from *Archaeoglobus fulgidus* and *Oxalobacter formigenes* (Abe *et al.*, 1996). MacVector also predicted two additional clustered ORFs 305 nt upstream of *cbsT1* on the coding strand. These ORFs are highly similar in spatial arrangement and predicted amino acid sequence to two coordinately regulated genes, *arbX* and *arbZ*, in *L. delbrueckii* subsp. *lactis* that encode a putative glycosyltransferase and a phospho- β -glycosidase, respectively (Weber *et al.*, 1998). An ORF of 1245 nt was identified 119 nt downstream of *cbsH β* , in the opposite orientation. BLAST analysis putatively identified the ORF, designated *mat*, as a group II intron encoding maturase (Fig. 1; Edgell *et al.*, 2000).

Fig. 2. (a) Ethidium bromide-agarose gel electrophoresis of LPEA PCR products. Lane 1, 1 kb DNA ladder (Promega) with fragment sizes in bp; lane 2, DNA products from first, nested PCR reaction containing oligo-dC tailed cDNA, T1-1 and AP; lane 3, products from the second, nested PCR reaction containing DNA template from lane 2, T1-2 and AP. (b) The complement of the DNA sequence from second, nested LPEA PCR reaction (LPEA PCRc; panel a, lane 3) is identical to the genomic sequence from strain 100-100. A transcriptional start site (arrow) and putative promoter (underlined) were identified.

Fig. 3. A BSH-positive genomic clone from strain 100-100, pBSH2, with predicted ORFs. The predicted amino acid sequence of *cbsHx* matched the protein sequence of purified BSHx (Lundeen & Savage, 1992a). An inverted repeat (underlined) was identified downstream of the *cbsHx* stop codon.

BSH operon in strain 100-100

LPEA produced long cDNA that extended to the 5' terminus of the mRNA species complementary to primer H β -1. This cDNA was amplified with AP and nested primers, T1-1 and T1-2, in two successive nested LDGW PCR reactions. The first PCR reaction produced two visible bands of approximately 2 and 0.8 kbp when analysed by 1% agarose gel electrophoresis (Fig. 2a, lane 2). The second, nested reaction produced a single fragment of approximately 0.5 kbp (Fig. 2a, lane 3). Considering the respective placement of T1-1 and T1-2, a decrease of approximately 0.3 kbp from 0.8 to 0.5 kbp is proportional to and consistent with genomic DNA sequence from strain 100-100. We believe that the fragment of 2 kbp (Fig. 2a, lane 2) is an artifact. LDGW PCR is known to produce artifacts of unrelated sequence that can be eliminated, in our experience, in subsequent nested amplifications with SSPs. Alternatively, or in addition, the 2 kbp fragment could have been amplified from a similar primer-binding site for T1-1 that is found within *chsT2*. Purified DNA from the second reaction was sequenced with T1-2 and matched identically the

DNA sequence from the genome of strain 100-100 directly downstream of the T1-2 binding site (Fig. 2b). The complement of the DNA sequence extended 78 nt upstream of *cbtT1* and was tailed with a GS stretch. Sequences centred approximately -10 and -35 of the transcriptional start site, AATATAA and TTGATT respectively, were identified that resemble *E. coli* -10 and -35 consensus promoter sequences, TATAAT and TTGACA respectively, that are recognized by σ^{70} (Fig. 2b). Expression analysis of *cbtH β* was undertaken with three clones, pT1T2H β , pT2H β and pH β . All three clones expressed BSH activity when transformed into *E. coli* DH5 α cells incubated anaerobically on MRS-TDCA-Amp-IPTG plates (data not shown).

Identification of *cbsHα*

A genomic library of strain 100-100 was prepared in *E. coli* and colonies were screened anaerobically on MRS-TDCA-Amp-IPTG plates. Two colonies, designated pBSH1 and pBSH2, produced a halo of deoxycholic acid. DNA sequence analysis of pBSH1 revealed a fragment encoding the *cbsHβ* locus. The second BSH-

Table 2. BSH phenotype and *cbsH β* genotype of strains of *Lactobacillus* spp.

Species	Strain*	Source	BSH activity†	<i>cbsHβ</i> locus‡
<i>L. acidophilus</i>	ATCC 4356	Human	+	—
	ATCC 4357	Human	+	—
	ATCC 53544	Human infant, rectal swab	+	—
	BCCM 8151	Acidophilus milk	0	—
	JCM 1028	Human intestine	+	+
	JCM 1034	Human intestine	+	+
	JCM 1229	Human intestine	(+)	—
	NCSK 1070	Unknown	+	—
	(ATCC 700396)			
<i>L. brevis</i>	PI KS-13	Human	+	+
	ATCC 14869	Human faeces	—	(—)
	BCCM 7944	Human faeces	+	—
	BCCM 11998	Starter from dairy	+	—
	BCCM 18022	Zabady (yogurt)	+	—
<i>L. buchneri</i>	JCM 1068	Human intestine	—	—
	JCM 1069	Human intestine	+	—
<i>L. delbrueckii</i> subsp. <i>bulgaricus</i>	ATCC 11842	Bulgarian yogurt	0	+
	BCCM 12168	Homemade yogurt	0	—
	DSMZ 20080	Yogurt	0	(—)
<i>L. fermentum</i>	ATCC 11976	Intestine of infant	(+)	—
	ATCC 23271	Human intestine	—	—
	BCCM 6902	Fermented beets	—	—
	DSMZ 20050	Unknown	—	—
<i>L. gasseri</i>	ATCC 19992	Faeces	—	+
	BCCM 9203	Human	+	+
	JCM 1025	Human intestine	+	+
	NCSK 99	Unknown	(+)	+
<i>L. johnsonii</i>	JCM 1022	Human intestine	+	+
	JCM 8791	Human faeces	+	+
<i>L. plantarum</i>	BCCM 18021	Milk	(+)	—
	BCCM 18027	Laban rayeb	(+)	—
	BCCM 18035	Milk	—	—

* ATCC, American Type Culture Collection; JCM, Japanese Collection of Microorganisms; BCCM, Belgian Co-ordinated Collections of Microorganisms; DSMZ, Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (German Collection of Microorganisms and Cell Cultures); NCSK, North Carolina State University (Todd Klaenhammer); PI, Principal Investigator (our laboratory).

† From Moser & Savage (2001). *Lactobacillus* strains were streaked on MRS plates containing 0.5% taurodeoxycholic acid (MRS-TDCA). +, a halo of deoxycholic acid precipitate around bacterial growth (Dashkevich & Feighner, 1989); (+), weak halo; —, no halo present; 0, no growth on MRS-TDCA plates.

‡ The *cbsH β* locus was screened with oligonucleotide primers H β 675a and H β 675b using standard PCR. +, a single 675 bp PCR product; —, no amplification of genomic DNA; (—), results negative – a ladder of bands was obtained.

positive clone, pBSH2, contained a 1610 bp DNA fragment from the genome of strain 100-100 encoding a complete ORF of 978 nt and two putative partial ORFs of 123 and 315 nt. Two partial ORFs were located 229 nt upstream and 56 nt downstream of the complete ORF, respectively (Fig. 3). Advanced BLAST analysis of the complete ORF displayed sequence similarities (DNA and predicted amino acid) to all cloned BSH genes (Christiaens *et al.*, 1992; Coleman & Hudson, 1995;

Elkins & Savage, 1998; Tanaka *et al.*, 2000); the BSH gene from *L. plantarum* 80 (Christiaens *et al.*, 1992) scored the highest. The partial ORFs did not display strong sequence similarity to any known genes. Microsequencing of the first 25 amino acids of the α hydrolase (BSH α) was done previously on purified protein blotted onto PVDF membranes (Lundeen & Savage, 1992a). The predicted amino acid sequence from the amino terminus of the complete ORF, designated *cbsH α* , was

Fig. 4. The sequence of the *cbsHβ* locus in the genome of *L. acidophilus* strain KS-13 was generated by standard (675 bp) and LDGW PCR (Min & Powell, 1998). The DNA sequence identity to strain 100-100 (85%) ends 26 nt upstream of the putative promoter and immediately downstream of the *cbsHβ* stop codon.

virtually identical (23/25 amino acids) to the BSH α sequence (Fig. 3). DNA sequence centred approximately 8 to 13 nt 5' of *cbsH α* , TAAGGAGGAAT, closely matched the Shine–Dalgarno 16S rRNA sequence of TAAGGAGGTGA. An inverted repeat capable of forming a stem–loop structure was identified 12 nt 3' of the stop codon of this gene (Fig. 3).

Survey of 50 *Lactobacillus* isolates for the *cbsHβ* locus

Fifty strains of *Lactobacillus* species from humans or dairy products were obtained from culture banks and screened for BSH activity and the *cbsHβ* locus (Table 2). Genomic DNA from each was screened using standard PCR with primers Hβ675a and Hβ675b. Ten isolates screened positive for *cbsHβ*. Nine of these isolates were cultured from human sources and belong to the species *acidophilus*, *gasserii* and *johnsonii*. Only one dairy isolate from Bulgarian yogurt, *L. delbrueckii* subsp. *bulgaricus*, screened positive for the locus. Some isolates from the same species displayed a variable BSH phenotype as reported by Moser & Savage (2001), and *cbsHβ* genotype. Strains of species [number screened] *amylovorus* [2], *crispatus* [3], *curvatus* subsp. *curvatus* [1], *kefiri* [1], *maltaromicus* [1], *paracasei* subsp. *paracasei* [3], *reuteri* [2], *rhamnosus* [3], *ruminis* [1], undetermined [2] were screened for *cbsHβ*, were negative and were therefore not included in Table 2.

cbsHβ locus in *L. acidophilus* strain KS-13

Strain KS-13, a human isolate from our laboratory, screened positive for the *cbsHβ* locus (Table 2) and was chosen for an extended genetic study. Primers Hβ675a and Hβ675b amplified a single fragment of approximately 675 bp from the genome of strain KS-13 that was 85% similar in DNA sequence to that of *cbsHβ* of strain 100-100. DNA fragments flanking this region in strain KS-13 were obtained by LDGW and sequenced. The composite DNA sequence was analysed with MacVector and revealed an identical genomic architecture to the operon in strain 100-100 (Fig. 4). Three tandem ORFs of 1344, 1353 and 948 nt were predicted that displayed 84, 87 and 85% DNA sequence identity and 95, 95 and 97% predicted amino acid sequence similarity to *cbsT1*, *cbsT2* and *cbsHβ* of strain 100-100, respectively. Intergenic regions of 28 nt (*cbsT1*–*cbsT2*) and 21 nt (*cbsT2*–*cbsHβ*) in strain KS-13 compare with 27 nt and 19 nt in strain 100-100 (Elkins & Savage, 1998), respectively

(Fig. 4). Primers La-Hβa and La-Hβb, which contain engineered *KpnI* sites, were used to amplify and clone the predicted *cbsHβ* gene in strain KS-13. *E. coli* DH5 α cells were transformed with a pSport1 plasmid construct containing this ORF. Colonies from such cells precipitated deoxycholic acid in the surrounding medium when grown anaerobically on MRS-TDCA-Amp-IPTG plates.

DISCUSSION

We have demonstrated that BSH activity is encoded by two separate loci on the genome of *L. johnsonii* strain 100-100. One locus encodes *cbsHβ* and is part of an operon of three genes related in function. To our knowledge, this is the first report of a complete BSH operon. Unlike the partial operon in *Bif. longum* SBT2928 (Tanaka *et al.*, 2000), *cbsHβ* is encoded with functionally related genes. The second locus encodes another BSH, *cbsH α* , which is not arranged in tandem with other ORFs. This discovery is novel since other enteric bacteria are known to encode only one BSH (Stellwag & Hylemon, 1976; Gopal-Srivastava & Hylemon, 1988; Kawamoto *et al.*, 1989; Christiaens *et al.*, 1992; Kishinaka *et al.*, 1994; Coleman & Hudson, 1995; Grill *et al.*, 1995; Tanaka *et al.*, 2000).

The BSH operon of strain 100-100 contains two genes, *cbsT1* and *cbsT2*, that share a high level of similarity in their DNA and predicted amino acid sequence. This finding suggests that the genes are duplicates. Functional studies have shown that *cbsT2* and partial *cbsT1*, when expressed in *E. coli*, increase uptake of conjugated bile acids (Elkins & Savage, 1998). These genes, while demonstrating putative topologies characteristic of MFS transporters (Saier *et al.*, 1999), do not display impressive sequence similarity, as identified by BLAST, to any known genes. Members of several subfamilies of the MFS provide the closest sequence similarity. In support of our observations, M. Saier and colleagues have phylogenetically classified *cbsT1* and *cbsT2* as members of a new subfamily of the MFS called the conjugated bile salt transporter family (BST) (Saier *et al.*, 1999; <http://www-biology.ucsd.edu/~msaier/transport/titlepage.html>).

We were able to produce and amplify cDNA from strain 100-100 using primers engineered to *cbsHβ* and *cbsT1*. LPEA produced one fragment of 0.5 kbp that was identical in sequence to genomic DNA from the upstream end of *cbsT1*. This sequence extended 78 nt

upstream of the start site of *cbsT1*. We conclude that *cbsT1*, *cbsT2* and *cbsH β* are probably encoded polycistronically. The expression analysis with *cbsH β* supported that conclusion. When expressed under *lac*-inducible promotion, clones containing *cbsT1-cbsT2-cbsH β* or *cbsT2-cbsH β* produced precipitates of deoxycholic acid in the medium. Therefore, *cbsT1* and *cbsT2* probably do not contain mRNA termination sequences, at least not ones that are recognized by *E. coli*. However, further expression evidence and DNA motif analysis are necessary to convincingly demonstrate the polycistronic expression of the three genes.

The genetic analysis of BSH activity supports protein characterization studies. Strain 100-100 produces two hydrolases, α and β , that combine to form homo- and heterotrimeric complexes. Purified α and β hydrolases have estimated molecular masses of 42 and 38 kDa, respectively (Lundeen & Savage, 1992a). The calculated molecular masses of the BSH from the *Sau3AI* genomic clone (complete ORF) and *cbsH β* are 36667 and 34916 Da, respectively. In addition to having the larger molecular mass, the BSH from the genomic clone had an amino-terminal sequence that matches, with only two differences, the first 25 amino acids of the α hydrolase (Lundeen & Savage, 1992a). The observed differences in amino acid sequence are probably due to problems in the peptide sequencing. To prepare the α peptide for sequencing, it was hydrolysed with 6 M HCl and derivatized with phenylisothiocyanate as previously reported (Lundeen & Savage, 1992a). Under such conditions, Cys cannot be resolved, and Glu and Gln cannot be distinguished during sequencing. Such problems undoubtedly explain the discrepancies between the chemical peptide sequence and the peptide sequence inferred from the nucleotide sequence. We conclude, therefore, that the complete 978 nt ORF from the *Sau3AI* genomic clone encodes BSH α and designate this ORF *cbsH α* . We also conclude that the known genetic elements responsible for BSH activity in strain 100-100 have been identified and cloned.

CbsH α shares the highest amino acid sequence similarity with the BSH from *L. plantarum* 80 (Christiaens *et al.*, 1992). Similar to the BSH in strain 80, *cbsH α* is not arranged in tandem with other ORFs and contains an inverted repeat 3' of the stop codon. The BSH protein(s), however, has not been isolated or characterized from strain 80 (Christiaens *et al.*, 1992). We suggest that the strain may also contain a second hydrolase locus encoding additional functionally related genes if the activity was acquired horizontally. We also tentatively conclude that *cbsH α* , similar to the BSH gene from 80, is encoded monocistronically.

Our phenotypic and genetic screens suggest that BSH activity was acquired horizontally in lactobacilli. Lactobacilli within the species *acidophilus*, *brevis*, *buchneri*, *fermentum*, *gasseri* and *plantarum* express a variable BSH phenotype. PCR screening within *L. acidophilus* and *L. delbrueckii* subsp. *bulgaricus* also produced variable results. Nearly all (9/10) of the *cbsH β* -positive

isolates were from human sources, which suggests that this locus is important for persistent colonization of the gastrointestinal tract. Taken in aggregate, these results suggest that the BSH phenotype and genotype is variable within a given species. It is not known whether BSH genes and related genes are encoded on mobile genetic elements. It is known, however, that the BSH gene from *Bif. longum* SBT2928 is flanked by inverted repeats (Tanaka *et al.*, 2000). It is interesting to note, furthermore, that strain 100-100 encodes a maturase, *mat*, downstream of *cbsH β* . Although primary literature on bacterial maturases is scarce, the enzymes have endonuclease and reverse transcriptase activity that facilitates movement and splicing of cDNA into the genome in a process known as retrohoming (Edgell *et al.*, 2000). Group II maturases are encoded by self-contained ORFs with independent promoters. Most group II maturases are inserted in or associated with mobile genetic elements (Edgell *et al.*, 2000). The putative maturase in strain 100-100 is located complementary to but in close proximity with DNA encoding the BSH operon.

A BSH operon identical in genomic architecture to *L. johnsonii* strain 100-100 is conserved in *L. acidophilus* strain KS-13. DNA sequence identity to strain 100-100 ends immediately upstream of the putative promoter and downstream of *cbsH β* . We suggest that this locus forms a genomic mobile element. *L. acidophilus* is one of only two organisms that respond to an EF of unknown composition produced by strain 100-100 (Lundeen & Savage, 1992b). Unlike other BSH systems, hydrolase activity in strain 100-100 is increased by as much as three- to fivefold within 20 min after conjugated bile acids are added to suspensions of stationary-phase cells. The increase is due to induction of a soluble extracellular molecule and not enzymic or regulatory proteins (Lundeen & Savage, 1992b). EF also enhances uptake of conjugated bile acids in *E. coli* cells expressing *cbsT2* and partial *cbsT1* from strain 100-100 (Elkins & Savage, 1998). Therefore, the transporters in strain KS-13 are related to the bacterium's ability to respond to EF.

The conserved DNA sequence upstream of *cbsT1* in strain KS-13 supported our data on the putative promoter for the *cbsH β* operon in strain 100-100. A region of approximately 135 nt upstream from *cbsT1* should be conserved in strain KS-13 if it is important for promotion and transcription of the genes. Lundeen & Savage (1990) demonstrated at a physiological level that induction of BSH expression corresponds with entry of strain 100-100 into stationary phase. LPEA analysis supported this conclusion as well. There are no conserved operator/enhancer elements since sequence identity between strain KS-13 and 100-100 ends upstream of the -35 promoter element. Thus, the RNA analysis supports simple sigma-dependent stationary-phase induction of BSH activity.

We have cloned the genetic determinants for BSH activity in *L. johnsonii* strain 100-100 and have extended the study into other *Lactobacillus* species. In strain 100-100, two genetic loci encode BSH activity. The *cbsH β*

locus is an operon of three genes encoding two bile-acid-related functions: hydrolysis and transport. This locus is conserved in other *Lactobacillus* species, specifically human isolates. Within some species, however, BSH activity and the *cbsH β* locus were not constant traits. *L. acidophilus* strain KS-13 encoded the *cbsH β* locus but DNA sequences flanking the three-gene operon were not conserved. Our data support a hypothesis that BSH activity is important at some level for colonization of the gastrointestinal tract and that BSH genes were acquired horizontally. Bile acid transport by contrast could be studied. Is there a bile acid exporter or is export activity catalysed concomitantly with import? What is the function of EF in bile acid transport? Experiments designed to identify the energy source and kinetics of transport may provide functional information that supports the phylogenetic assignment of *cbsT1* and *cbsT2* into a new family of the MFS. This information, once obtained, may lead to a working model for BSH activity in enteric bacteria.

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