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Allelic Variation of Bile Salt Hydrolase Genes in *Lactobacillus salivarius* Does Not Determine Bile Resistance Levels▽†

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Commensal lactobacilli frequently produce bile salt hydrolase (Bsh) enzymes whose roles in intestinal survival are unclear. Twenty-six *Lactobacillus salivarius* strains from different sources all harbored a *bsh1* allele on their respective megaplasmids. This allele was related to the plasmid-borne *bsh1* gene of the probiotic strain UCC118. A second locus (*bsh2*) was found in the chromosomes of two strains that had higher bile resistance levels. Four Bsh1-encoding allele groups were identified, defined by truncations or deletions involving a conserved residue. In vitro analyses showed that this allelic variation was correlated with widely varying bile deconjugation phenotypes. Despite very low activity of the UCC118 Bsh1 enzyme, a mutant lacking this protein had significantly lower bile resistance, both in vitro and during intestinal transit in mice. However, the overall bile resistance phenotype of this and other strains was independent of the *bsh1* allele type. Analysis of the *L. salivarius* transcriptome upon exposure to bile and cholate identified a multiplicity of stress response proteins and putative efflux proteins that appear to broadly compensate for, or mask, the effects of allelic variation of *bsh* genes. Bsh enzymes with different bile-degrading kinetics, though apparently not the primary determinants of bile resistance in *L. salivarius*, may have additional biological importance because of varying effects upon bile as a signaling molecule in the host.

Lactobacilli are among the species most commonly used as probiotic agents, due to the wide range of consumer benefits associated with their consumption (32). During intestinal transit, the host suppresses bacterial survival and persistence by using a variety of mechanisms, including low pH, rapid transit time, and production of bile, digestive enzymes, and antimicrobial peptides. Bile resistance is one of the main criteria used for selecting bacterial strains for probiotic applications (49). Bile is a detergent solution of organic and inorganic compounds that varies in composition in different animals (42). The major constituents include bile acids, cholesterol, and phospholipids (2). Human bile acids are synthesized in the liver and then circulated in the gastrointestinal (GI) tract, with high concentrations in the duodenum, jejunum, and proximal ileum (46). Bile is toxic to bacterial cells, causing membrane damage, secondary-structure formation in RNA, DNA damage, and oxidative and osmotic stresses (2).

Production of bile salt hydrolase (Bsh) enzymes is a common bile resistance mechanism in bacteria. Bsh proteins, or conjugated bile acid hydrolase proteins (CBAH) (EC 3.5.1.24), belong to the choloylglycine hydrolase family, which forms part of the N-terminal nucleophilic hydrolase superfamily of enzymes (2). The choloylglycine hydrolase family also includes penicillin V amidase (PVA) (EC 3.5.1.11), whose evolutionary relationship with Bsh has been elucidated for the *Bifidobacterium longum* proteins (29). Bsh enzymes act upon a wide range of bile acid conjugates and salts, including six major human conjugated bile acids (CBA) (taurocholic acid [TCA], taurodeoxycholic acid [TDCA]; taurochenodeoxycholic acid [TCDCA]; glycocholic acid [GCA]; glycodeoxycholic acid [GDCA]; and glycochenodeoxycholic acid [GCDCA]). Homologues of the *bsh* gene have been detected in many intestinal bacteria (27). In some pathogens, including *Listeria monocytogenes*, *bsh* has been identified as a virulence factor (19). *bsh* was also demonstrated to be required for the persistence of *L. monocytogenes* in the murine intestine (3) and for the ability of *Brucella* to infect mice (14).

The presence and genetic organization of *bsh* genes in lactobacilli is very variable. In addition to presence in a single copy in some species, multiple copies of *bsh* were annotated in *Lactobacillus acidophilus* NCFM (*bshA* and *bshB*), *Lactobacillus johnsonii* NCC533 (three genes), and *Lactobacillus gasseri* ATCC 33323 (two genes) (30). In some *Lactobacillus* strains, *bsh* was part of an operon (20). Disruption and deletion of *bsh* in lactobacilli caused loss of corresponding activity against tauro/glyco-CBA (34, 40). The resistance of *bsh* mutants of *Lactobacillus amylovorus* and *Lactobacillus plantarum* to bile acids/salts was reduced compared to the respective wild-type strains (13, 16, 24). However, no convincing in vivo experiments have so far demonstrated that *bsh* contributes to bile resistance in these or other probiotic bacteria. A triple *bsh* mutant of *L. johnsonii* NCC533 (i.e., lacking all three Bsh proteins) did not exhibit significantly reduced murine gut per-

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sistence compared to the parental strain (15). The role of *bsh* in intestinal tract survival of probiotic lactobacilli is generally unclear.

Bsh enzymes from a variety of sources differ in structure, substrate specificity, and optimal temperature and pH range for enzyme function (23, 48, 52). Bsh subunit sizes range from 28 kDa to 56 kDa, and the enzymes are generally more active at an acidic pH range (4–7). The most thermostable Bsh was detected in *Brevibacillus* sp., whose optimal temperature is 60°C (52). Bsh enzymes recognize bile acids on both the cholate steroid nucleus and the amino acid moiety. The crystal structure of *Clostridium perfringens* Bsh revealed that activity is conferred by a hydrophobic pocket that recognizes the cholyl moiety of the substrate (48). The crystal structures and biochemical properties of Bsh from *B. longum* (29) and *C. perfringens* (48) have been well characterized.

Within the phylogenetically diverse genus *Lactobacillus*, Bsh proteins have been biochemically characterized only from *L. acidophilus* PF01 (40, 43) and *L. johnsonii* 100-100 (20). Inactivation of *bshB* in *L. acidophilus* NCFM revealed that the strain lost hydrolytic activity for tauroconjugated bile salts (40). Given that lactobacilli are the main contributors to Bsh activity in the murine and chicken intestinal tracts (26, 56) and could be physiologically important when produced by lactobacilli in the human gut, biochemical characterization of the corresponding Bsh enzymes is desirable.

The unconjugated bile acids, or free bile acids (FBA), generated by Bsh enzymes are more toxic than the conjugated substrate forms, and they strongly inhibit the growth of intestinal bacteria (4). Bacteria that hydrolyze bile must therefore detoxify or remove FBA by one of the following major strategies: precipitation or 7-dehydroxylation and precipitation at moderately acidic pH, catabolism by coenzyme A ligase, or transport (efflux) outside the bacterial cell. In *Bacteroides fragilis*, the presence or absence of Bsh activity is correlated with production of 7-α-hydroxysteroid dehydrogenase (53). How Bsh-producing *Lactobacillus* species, like *L. salivarius*, that are nonproducers of 7-α-hydroxysteroid dehydrogenase resist FBA is not clear, and this motivated our transcriptome analysis of cholate response in this study.

Bile exposure appears to have driven the dissemination and evolution of *bsh* genes in the human intestinal microbial metagenome (27). However, there is also evidence that the production levels and enzymatic activity of Bsh are not directly related to overall bile resistance levels (24, 41). In addition to *bsh*, other genes (*pva* and *btlB*) and the sigma factor σ^B have been shown to contribute to bile resistance in *L. monocytogenes* EGDe (3). Furthermore, microarray analysis of the bile-induced transcriptome identified genes, including those encoding multidrug resistance (MDR) transporters, chaperone, esterase, and a histidine protein kinase, that were implicated in bile resistance in *L. acidophilus* NCFM and *Lactobacillus reuteri* ATCC 55730 (45, 62). Genes involved in DNA repair, oxidative response, transcriptional regulation, dGTP hydrolysis, membrane composition, and cell wall synthesis were differentially expressed upon exposure of *Enterococcus faecalis* or *L. plantarum* WCFS1 cells to bile (6, 7).

L. salivarius UCC118 is a well-characterized strain (11) with probiotic properties (18). This strain harbors a 242-kb megaplasmid, pMP118, that interdigitates with chromosomally encoded functions to confer metabolic flexibility (35, 44). *L. salivarius* is common in the GI tracts of many animals, including humans (1) and chickens (26), but its survival mechanisms in vivo are poorly understood. In this study, therefore, we examined the contributions of allelic variants of *bsh* to the bile resistance of *L. salivarius*, as well as other bile resistance mechanisms.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *L. salivarius* was grown under microaerobic conditions (5% CO_2) in de Man-Rogosa-Sharpe (MRS) medium (Oxoid Ltd., United Kingdom) at 37°C. *Escherichia coli* was grown in Luria-Bertani (LB) broth (50) with aeration at 37°C. *Lactococcus lactis* was grown at 30°C in M17 broth (Oxoid Ltd., United Kingdom) supplemented with 0.5% (wt/vol) glucose. Erythromycin and chloramphenicol were used at 5 μg/ml for *L. salivarius* and *L. lactis*. Tetracycline (Tet) was added at 5 μg/ml for *L. salivarius* and 10 μg/ml for *L. lactis*. Ampicillin and chloramphenicol were supplemented at 50 μg/ml and 34 μg/ml, respectively, for *E. coli*.

DNA manipulation. The primers used for PCR were purchased from MWG Biotech (Ebersberg, Germany) and are listed in Table S1 in the supplemental material. *Pwo* polymerase (Roche, Mannheim, Germany) was used for PCR amplifications. Restriction enzymes, T4 DNA ligase, and PCR purification kits were purchased from Roche (Mannheim, Germany) and used according to the manufacturer's instructions. For making constructs (pEB118 and pEB1046) for overexpression of *bsh1* (LSL_1801) and $bsh1_{JCM1046}$, KOD HiFi polymerase (Novagen, Darmstadt, Germany) and an In-Fusion Dry-Down PCR cloning kit (Clontech) were used for PCR amplification and cloning according to the manufacturers' instructions. Plasmid DNA electrotransformation, *L. salivarius* genomic DNA isolation, and pulsed-field gel electrophoresis (plug preparation, S1 nuclease treatment, and electrophoresis) were performed as described previously (21). Southern blot analysis followed a standard protocol (50).

Analysis of *bsh* expression by qRT-PCR. *bsh1* transcription levels in *L. salivarius* strains were determined relative to that of the *groEL* gene. RNA was isolated from both exponential- and stationary-growth-phase cells of *L. salivarius* strains (three biological replicates) using an RNA-easy kit (Ambion, Cambridgeshire, United Kingdom). Random primers were purchased from MWG Biotech, Germany. RNA (500 ng) was reverse transcribed using Improm-II reverse transcriptase (Promega). PCR amplification was performed according to the manufacturer's instructions. Briefly, a 12.5-μl PCR mixture consisted of 6.25 μl 2× master mix (Biogene, United Kingdom), 50 nM of each primer, 1/60,000 SYBR green I (Biogene, United Kingdom), and 1 μl cDNA. The quantitative reverse transcription (qRT)-PCR amplifications were performed on an ABI Prism 7000 using SYBR green I.

Type I microarray procedures. The *L. salivarius* array contained 1,500 Agilent quality control spots and 60-nucleotide oligonucleotides corresponding to 2,184 genes (including annotated pseudogenes) in the genome of *L. salivarius* UCC118. A maximum of four probes (21 replicates) for each gene were designed from each open reading frame (smaller genes had fewer probes) by eArray (Agilent Technologies). These probes were spaced throughout the coding regions and designed to have melting temperatures between 58°C and 60°C. The probes were printed in spots, were randomly distributed across the array, and were printed by Agilent Technologies.

Overnight cultures of *L. salivarius* LS201 and LS201Δ*bsh1* were diluted 50-fold in MRS medium without antibiotics and grown at 37°C to an optical density at 600 nm of 0.3. The cultures were divided in two and were either untreated or treated with 0.1% porcine bile (Sigma; B8631) or 1 mM cholate (sodium cholate hydrate; Sigma C6445). After 15 min of incubation, 12-ml samples were harvested by centrifugation (13,000 × *g* for 15 s) at room temperature. Cell pellets were washed once with RNAprotect Bacteria Reagent (Qiagen) and immediately frozen at −80°C. Cells were disrupted with a bead beater homogenizer (a 1-min treatment three times with 1-min intervals on ice). Total RNA was isolated using the SV Total RNA Isolation System (Promega) with an additional 30-min Turbo DNase treatment. The RNA quality was checked with an Agilent Bioanalyzer 2100 using the RNA 6000 Nano assay kit (Agilent). RNA (4 μg) derived from treated or untreated cells was used for cDNA synthesis and labeled with Cy3/5-dCTP (GE Healthcare Life Sciences) with a SuperScript II reverse transcriptase kit (Invitrogen) at 42°C for 90 min. Cy3- and Cy5-labeled cDNAs were purified using the MinElute PCR purification kit (Qiagen) and quantified using the NanoDrop ND-1000 UV-Vis spectrophotometer (NanoDrop Technologies,

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant properties[a]	Source or reference
Strains		
L. salivarius		
UCC118	Ileocecal isolate from a human adult	11
LS201	pSF118-20-free derivative of strain UCC118	21
LS201Δ*bsh1*	LS201 integrant LSL_1801 (*bsh1*)::pLS216	This work
LS201Δ*lacZ*	LS201 integrant LSL_0376 (*lacZ*)::pLS217	This work
JCM1046Δ*bsh1*	JCM1046 integrant *bsh1*::pLS218	This work
E. coli		
Top10	F^- *mcr*A Δ(*mrr-hsdRMS-mcrBC*) φ80*lacZ*ΔM15 Δ*lac*X74 *recA1 ara*Δ*139* Δ(*ara-leu*)*7697 galU galK rpsL*(Str^r) *endA1 nupG*	Invitrogen
Rosetta BL21(DE3)pLysS	F^- *ompT hsdS*$_B$ ($r_B^-m_B^-$) *gal dcm* (DE3) pLysS (Cm^r)	Invitrogen
L. lactis		
LL108	Strain with *repA* gene integrated into the chromosome	33
Plasmids		
pORI19	Em^r Ori^+ $RepA^-$ lacZ′ derivative of pROI28	31
pPTPL	Tet^r; promoter/probe vector	8
pVE6007	Cm^r; temperature sensitive; derivative of pWV01; lactococcal cloning vector	38
pLS209	Em^r; *Lactobacillus* gene-cloning vector; derivative of pLS203 produced by PCR	21
pLS215	Tet^r; derivative of pORI19; *erm* is replaced with *tet* from pPTPL	Unpublished results[b]
pLS216	Tet^r, derivative of pLS215 containing a 558-bp internal gene fragment of *bsh1* (UCC118)	This work
pLS217	Tet^r; derivative of pLS215 containing a 1,002-bp internal gene fragment of *lacZ* (LSL_0376)	This work
pLS218	Em^r; derivative of pORI19 containing a 582-bp internal gene fragment of *bsh1* (JCM1046)	This work
pLS219	Em^r; derivative of pLS209 containing *bsh1* (LSL_1801) gene and its promoter region	This work
pOPINE	Amp^r; derivative of pTriEx2 with a C-terminal six-His-tag fusion	OPPF[c]
pEB118	Amp^r; derivative of pOPINE for expression of C-terminally His-tagged $bsh1_{UCC118}$	This work
pEB1046	Amp^r; derivative of pOPINE for expression of C-terminally His-tagged $bsh1_{JCM1046}$	This work

[a] Cm^r, chloramphenicol resistant; Em^r, erythromycin resistant; Tet^r, tetracycline resistant; Amp^r, ampicillin resistant.
[b] Contributed by Jan-Peter van Pijkeren.
[c] In-fusion cloning vector contributed by Oxford Protein Production Facility (OPPF).

Rockland, DE). An Agilent Oligo aCGH/Chip-on chip hybridization kit was used for hybridization. Hybridizations were performed in an Agilent hybridization oven (G2545A) at 65°C for 24 h. Slides were scanned using the Agilent Microarray Scanner System (G2505B) with Agilent scan control software version 7.0 for the 44k microarray at a resolution of 5 μm and Red and Green PMT at 10. Agilent Feature Extraction software version 9.1 was used for feature extraction. Microarray data outliers were removed with the Grubbs test (25). *P* values were calculated according to the Cyber-*t* test (36).

Phylogenetic analysis. Bsh sequences were aligned by ClustalW provided by Molecular Evolutionary Genetics Analysis software version 4 (MEGA4) (54). The neighbor-joining tree of Bsh sequences was built by running MEGA4 using the *p* distances amino acid model with 500 bootstrap replications. PVA (P12256) from *Bacillus sphaericus*, which belongs to the same choloylglycine hydrolase family (CBAH) (PF02275 [http://pfam.sanger.ac.uk]) as Bsh, was used as an outgroup.

Construction of *L. salivarius bsh1* and *lacZ* mutants. *L. salivarius bsh1* and *lacZ* integrants were obtained by plasmid integration as described previously (59). Primer pairs FF025-FF026, FF027-FF028, and JP076-JP081 were used to PCR amplify internal fragments of *bsh* ($bsh1_{JCM1046}$ and LSL_1801) and *lacZ* (LSL_0376), respectively. The corresponding PCR products were restricted with BamHI and EcoRI and ligated to similarly digested pORI19 or pLS215. *L. lactis* LL108 was used as the cloning host for these constructs. The resulting plasmids, pLS216 and pLS217, were transformed into *L. salivarius* LS201 for construction of the *bsh1* (LSL_1801) integrant (LS201Δ*bsh1*) and the *lacZ* (LSL_0376) integrant (LS201Δ*lacZ*). pLS218 was transformed into *L. salivarius* JCM1046 to generate the $bsh1_{JCM1046}$ integrant JCM1046Δ*bsh1*. Integrants of pORI constructs were selected through curing of pVE6007 by growth at elevated temperature, as described previously (59).

Bsh plate assay, bile MIC assay, and bile challenge experiment procedures. *L. salivarius* strains were tested for hydrolase activity against tauro- or glyco-CBA by using a plate assay method (12). Overnight MRS broth cultures were streaked on MRS agar supplemented with 9.6 mM (0.5% [wt/vol]) TDCA (Sigma; T0875) or 2 mM GDCA (Sigma; G3258). The plate was then incubated anaerobically for 48 h at 37°C. Bsh activity was detectable when deoxycholic acid precipitated in the agar medium below and around a colony. For detecting Bsh activity of *E. coli* expressing various constructs, an optimized LB bile acids medium (for 1 liter, 15 g agar, 10 g tryptone, 5 g yeast extract, 5 g NaCl, 0.35 g $CaCl_2 \cdot 2H_2O$, 10 g glucose, 1 mM isopropyl-β-D-thiogalactopyranoside [IPTG], pH 6.5) containing 5 g/liter TDCA or 2 mM GDCA (10) was used.

To measure MICs, overnight cultures of *L. salivarius* strains were inoculated at 1% into MRS medium containing different concentrations of porcine or bovine bile (Sigma; B8631 and B8381) or GDCA. The cultures were then incubated at 37°C for 24 h, and 10 μl was spotted on MRS agar plates. Growth on the plate was indicative of resistance to the corresponding bile/bile salt concentration of the strain.

For survival experiments, *L. salivarius* LS201, JCM1046, and the corresponding *bsh1* or *lacZ* integrants were grown to stationary phase, and the cells were harvested by centrifugation. The cells were washed once with MRS broth, followed by resuspension in MRS broth containing a sublethal concentration of porcine bile (0.2% for LS201 and its derivatives; 0.1% for JCM1046 and its derivative) and incubation at 37°C (5% CO_2) for 5 h. Samples were removed from the culture at different time intervals, diluted, and plated on MRS, MRS erythromycin 5 (μg/ml), or MRS Tet 5 (μg/ml) plates for viable-cell counting.

Expression and purification of Bsh. The genes for $Bsh1_{UCC118}$ (LSL_1801) or its homologues from strain JCM1046 were amplified by PCR using primers EBF-EB118R or EBF-EB1046R. Purified PCR products were cloned into the linearized T7 promoter-based pOPINE expression vector (OPPF) by In-Fusion reactions. The resulting plasmids, pEB118 and pEB1046, were transformed into *E. coli* strain BL21(DE3)pLysS for overexpressing C-terminally six-His-tagged *bsh*. For production of Bsh, 40 ml of *E. coli* BL21(DE3)pLysS (pEB118 or pEB1046) overnight culture for expression of the corresponding *bsh* was inoculated into a biofermentor (Biolab; B. Braun Biotech Ltd., Germany) charged with 2 liters of LB medium supplemented with 34 μg/ml chloramphenicol and 50 μg/ml ampicillin. The culture was grown at 37°C with oxygen supplementation and agitation at 200 rpm to an optical density at 600 nm of 0.6. The culture was then induced with 1 mM IPTG at 37°C for 5 h. For expression of $bsh1_{UCC118}$, the culture was immediately cooled to 20°C, followed by induction with 0.1 mM IPTG for 20 h. The cells were harvested by centrifugation, and the cell pellet was resuspended in 80 ml 50 mM Tris-HCl, pH 7.5, 500 mM NaCl, 20 mM imidazole. The cells were disrupted by sonication, and the cell debris was removed by centrifugation at 45,000 × *g* for 30 min at 4°C. Bsh was purified by immobilized metal ion affinity chromatography (IMAC) and gel filtration with ÄKTAprime

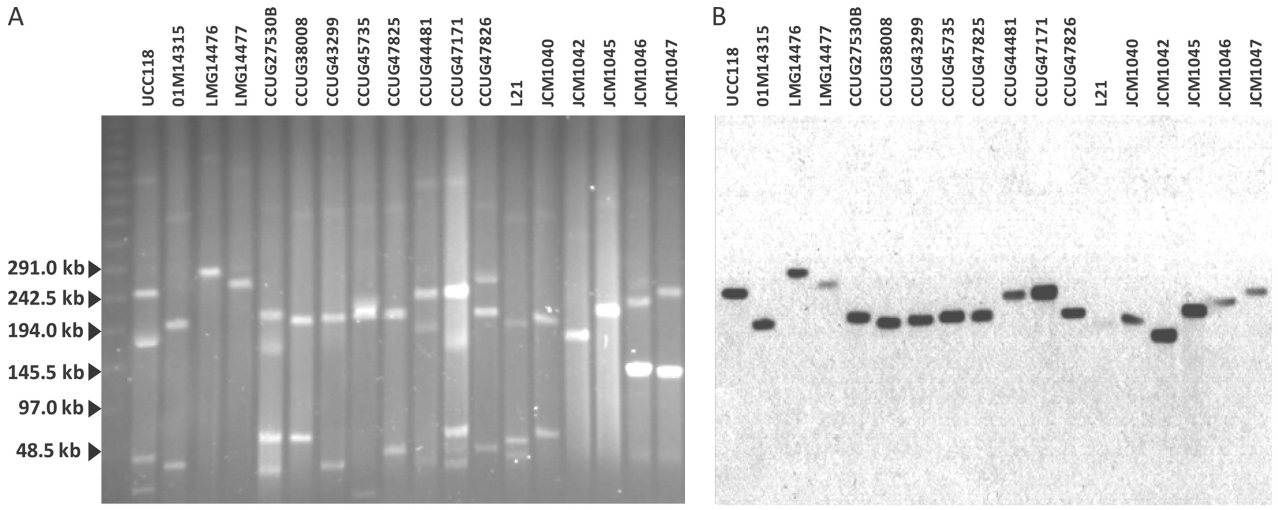

FIG. 1. Southern hybridization analysis of the presence of *bsh1* (LSL_1801) homologues in *L. salivarius* strains. (A) separation of S1 nuclease-treated genomic DNA of *L. salivarius* strains by pulsed-field gel electrophoresis. (B) Corresponding Southern hybridization using the *L. salivarius* UCC118 *bsh1* (LSL_1801) probe.

plus fast protein liquid chromatography (GE Healthcare Life Sciences). Bsh was eluted with buffer (50 mM Tris-HCl, pH 7.5, 500 mM NaCl, 500 mM imidazole) from a HisTrap HP 1-ml column. IMAC-purified Bsh was buffer exchanged, concentrated in a Centriprep (Amicon) concentrator, and then applied to a Superdex 200 gel filtration column. Bsh was eluted with a buffer containing 20 mM Tris-HCl, pH 7.5, 200 mM NaCl, 10 mM dithiothreitol. Fractions containing Bsh were pooled and concentrated.

***L. salivarius* Bsh protein and activity assay.** Bsh specific activity was determined by measuring amino acid release from conjugated bile salts (29, 55). The reaction was set up in PCR strip tubes. In a 20-μl reaction mixture, a mixture of 0.1 M sodium phosphate buffer, pH 5.5, 10 mM dithiothreitol, 10 mM T/G-CBA, and Bsh (100 nM $Bsh1_{JCM1046}$ or 400 nM $Bsh1_{UCC118}$) was incubated at 37°C (30 min for $Bsh1_{JCM1046}$ or 3 h for $Bsh1_{UCC118}$). Immediately, the reaction was stopped by adding 20 μl 15% (wt/vol) trichloroacetic acid. The samples were then centrifuged at 10,000 × *g* for 1 min. Five microliters of the supernatants or their appropriate dilutions was mixed with 95 μl ninhydrin reagent (19 ml ninhydrin solution contains 5 ml 1% ninhydrin in 0.5 M citrate buffer, pH 5.5, 12 ml glycerol, 2 ml 0.5 M citrate buffer, pH 5.5) and incubated at 100°C for 15 min. The reaction mixtures were cooled and transferred to a 96-well plate, and the absorbance at 570 nm was read. The absorbance at 570 nm was converted into the amount of amino acid by reference to a glycine standard curve. Control reactions were set up to check spontaneous hydrolysis of bile acids under the enzymatic reaction conditions in the absence of Bsh. No products were detected for any of the control reactions. One unit of Bsh activity was defined as the amount of enzyme that released 1 μmol of taurine/glycine from the substrate per min. The same reaction conditions were used to determine the V_{max} and K_m for the $Bsh1_{JCM1046}$ enzyme. With the enzyme concentration fixed at 140 nM, the substrate concentrations varied from 0.5 mM to 8 mM. To determine the optimum pH for Bsh activity, 10 mM GDCA was used for $Bsh1_{UCC118}$ and 10 mM TDCA was used for $Bsh1_{JCM1046}$, as they were shown to be good substrates for the respective enzymes. The following buffering systems were used in this study: 0.1 M citrate phosphate buffer (for pH 3 to pH 5) and 0.1 M sodium phosphate buffer (for pH 5.5 to pH 8).

The protein concentration was determined by the Bradford method (5) using Bio-Rad Protein Assay reagents. Bovine serum albumin was used as the standard.

Murine intestinal tract survival. A spontaneous rifampin (rifampicin)-resistant mutant of *L. salivarius* strain LS201Δ*bsh1* and a streptomycin-resistant mutant of strain LS201Δ*lacZ* were isolated as follows. Twenty milliliters of overnight culture of each LS201 derivative was centrifuged. The cell pellets were resuspended in 200 μl of phosphate-buffered saline (PBS) and plated onto MRS Tet 5 supplemented with 50 μg/ml rifampin or 1 mg/ml streptomycin. Mutants were purified by serial streaking on selective agar. Both mutations were 100% stable for up to 30 generations (72 h) in nonselective MRS medium culture, as evidenced by lack of reversion to antibiotic sensitivity (data not shown). Murine inoculation experiments were approved by the institutional ethics committee and complied with all relevant legislation. Bacterial cells were prepared by harvesting overnight MRS medium cultures by centrifugation, washing them once in PBS, and resuspending them to the required cell density. For each group, five 9-week-old male BALB/c mice were orally administered either 100 μl PBS (control group) or a mixture of *L. salivarius* LS201Δ*bsh1* and LS201Δ*lacZ* cells at a dose of 10^9 CFU for each strain in 100 μl (competitive-experiment group) by oral gavage. The mice were given access to water and food after the *Lactobacillus* strains or PBS was administered. Feces were collected individually at different time intervals and resuspended in 1 ml PBS by vortexing to homogenize them. The feces suspensions were centrifuged at 100 × *g* for 2 min. The supernatants were taken for dilution and viable-cell counting on tetracycline-rifampin or tetracycline-streptomycin for LS201Δ*bsh1* and LS201Δ*lacZ*, respectively. The study was powered to determine differences between groups at a significant level. Data pertaining to the comparative survival of strains over time were analyzed by two-way analysis of variance.

Accession numbers. The array design and microarray data can be found at EMBL-EBI ArrayExpress under accession no. E-MEXP-2224, -2225, -2226, and -2228. *L. salivarius* Bsh1 gene amplicons were deposited in GenBank (accession no. FJ591067-81, FJ591083, FJ591085-86, FJ591088-92, and FJ607064), and Bsh2 gene amplicons were deposited with accession no. FJ591082 and FJ591087.

RESULTS

Distribution of *bsh* alleles in *L. salivarius* strains. The genome of *L. salivarius* UCC118 contains two genes that were originally annotated as encoding choloylglycine hydrolases: the chromosomally located LSL_0518 and the megaplasmid-located LSL_1801 (11). Based upon sequence alignment, phylogenetic clustering, and protein homology modeling, the product of LSL_0518 has recently been identified unequivocally as a PVA (30), along with many other misannotated genes for presumptive Bsh enzymes. The sequence of the LSL_1801 product, whose annotation as a Bsh is supported, is 53% identical to that of functionally characterized CBA hydrolase (CAD00145) from *L. monocytogenes* EGDe (3).

LSL_1801 is located on the megaplasmid pMP118 in strain UC118, and megaplasmids with a related replication origin were previously detected in all 33 *L. salivarius* strains examined (35). Among 28 *L. salivarius* strains investigated by Southern hybridization (Fig. 1; see Fig. S1 in the supplemental material),

a single *bsh* allele located on the circular megaplasmid was detected in all strains except JCM1230 (not shown). A second *bsh* locus was detected in strain JCM1046 by annotation of a draft genome sequence (E. Raftis and P. W. O'Toole, unpublished data). This Bsh, which will be the subject of a separate study, is not present in any other *L. salivarius* strain except LMG14476. For clarity, we refer to LSL_1801-related proteins as Bsh1 (preceded where appropriate by the strain number), and we designated the additional enzyme present in JCM1046 and LMG14476 Bsh2. The apparently universal presence of *bsh1* homologues in *L. salivarius*, despite their location on an extrachromosomal element, suggested selection and biological significance that we proceeded to investigate.

Allelic variation of *bsh1* in *L. salivarius*. *bsh1* (LSL_1801) homologues from 26 *L. salivarius* strains were amplified and sequenced. The predicted Bsh1 proteins from these *L. salivarius* strains were greater than 93% identical to each other (Fig. 2). Based on the sequence alignment, the Bsh1 proteins could be divided into four major groups (Table 2 and Fig. 3). Group A (UCC118 group) Bsh1 sequences are identical to each other. Relative to other Bsh proteins (Fig. 2), group A proteins contain an internal deletion of 8 amino acids (165 to 171; NPI/VGVLTN) in the middle of the sequence. Group B (CCUG47825 group) Bsh1 sequences are also identical to each other. The sequence has a C-terminal truncation, and it has the same internal deletion as group A. In group C (JCM1046 group), Bsh1 sequences are complete, relative to all the other sequences aligned. CCUG43299Bsh1 is identical to 01M1431 5Bsh1; other group C proteins are 94 to 99% identical. Group D Bsh1 proteins (NCIMB8816 and JCM1042) represent a pseudogene group (data not shown); these sequences are interrupted by a stop codon at amino acid 74. Bsh1 proteins in group C contain all reported conserved active-site amino acids in Bsh enzymes (cysteine 2 [Cys 2], arginine 16 [Arg 16], aspartic acid 19 [Asp 19], asparagine 79 and 171 [Asn 79 and 171], and arginine 224 [Arg 224]) (46), as indicated in Fig. 2. Group A and B Bsh1 molecules lack the conserved Asn 171 residue.

Bsh phylogenetic analysis. The phylogeny of Bsh1 and Bsh2 from *L. salivarius* strain JCM1046 was investigated by tree construction with representative gram-positive bacterial Bsh sequences, employing PVA from *B. sphaericus* as the outgroup. Bsh sequences from gram-positive bacteria could thus be divided into a clostridial clade and a nonclostridial clade (see Fig. S2 in the supplemental material). All lactobacillus Bsh sequences were in the nonclostridial clade, and most of them were in a large group represented by the *L. salivarius* Bsh1 branch and the *L. salivarius* Bsh2 branch. A few *Lactobacillus* Bsh proteins separated into the *Bifidobacterium* Bsh group. Lack of complete *bsh* gene concordance with 16S gene phylogeny supports dissemination of the corresponding *bsh* genes by selection and lateral gene transfer (27).

Bsh activity and bile resistance of *L. salivarius* strains. Bsh activity in *Lactobacillus* cells was detected by a plate method (12). Bsh activity is indicated by either white colonies with surrounding precipitation zones, in the case of high activity, or opaque white colonies without precipitation haloes, as shown for representative strains in Fig. 3. *L. salivarius* strains with group A Bsh1 enzymes exhibited weak Bsh activity against TDCA in this assay (formation of opaque white colonies), as exemplified in Fig. 3 and summarized in full in Table 2. Strains harboring the group B *bsh1* allele failed to demonstrate convincing activity in the plate assay. Apart from strains JCM1046 and LMG14476, which have two *bsh* genes in their genomes, Bsh activity in group C strains was detected only against TDCA. Strains JCM1046 and LMG14476 showed activity against both TDCA and GDCA, suggesting the latter activity was due to the presence of the additional *bsh2* allele. Among the group D strains (pseudogene group), white-colony formation was recorded for strain NCIMB8816 (Fig. 3), suggesting the presence of an unrelated *bsh* gene. Strain JCM1230, lacking a *bsh* allele detectable by hybridization or PCR, also lacked detectable Bsh activity in this assay (not shown).

L. salivarius strains exhibited widely variant levels of resistance to bile and bile components, as shown by the MIC values in Table 2. Strains whose genomes encoded Bsh1 enzymes from the same group did not necessarily have the same MIC for either bile or CBA. The non-Bsh-producing strain JCM1230 had a higher MIC for GDCA than some Bsh-producing strains. All the *L. salivarius* strains were resistant to the highest concentration (100 mM) of TDCA tested (data not shown). The MICs for GDCA for all *L. salivarius* strains were very similar, except for those of strains JCM1046 and LMG14476, which could resist much higher concentrations (>15 mM) of GDCA than the other *L. salivarius* strains that had Bsh1 only. This strengthens the linkage of the *bsh2* allele with GDCA deconjugation. However the lack of correlation between the bile MICs of strains and the *bsh1* allele types and plate assay phenotypes indicated that factors other than bile hydrolysis were important for determining the overall bile resistance level of the strain.

Comparison of *bsh1* transcription levels in *L. salivarius* strains. The preceding analysis identified inconsistencies in *bsh1* allele groupings and bile MICs. Among the potential reasons for this was varying *bsh1* transcription levels. Nucleotide comparison of amplified flanking sequences upstream of *bsh1* revealed that the presumptive promoters and ribosome binding sites of 24 *L. salivarius bsh1* genes spanning allele groups A, B, and C appeared to be very conserved and could be described by the following consensus sequence: ATTATT AG-TTKAWW-N_{6-8}-TTGATAC-TYTWAT-A-GGAAG-N_8-ATG (the −35 and −10 boxes and ribosome binding site are underlined; K = T or G; W = A or T; Y = C or T; R = A or G; D = A, G, or T; N = A, T, G, or C). The transcription levels of *bsh1* in three representative *L. salivarius* strains (UCC118, CCUG47825, and JCM1046; allele groups A through C) were analyzed by qRT-PCR at two growth phases, using *groEL* as a reference gene and relating expression levels to those of *bsh1* in *L. salivarius* UCC118. The transcription level of *bsh1* in CCUG47825 was modestly but significantly ($P < 0.01$) higher than that of strain UCC118 and was increased only 1.36-fold and 1.31-fold for exponential and stationary phases, respectively. Despite minor sequence differences, the consensus promoter region of *bsh1* and the qRT-PCR data collectively indicate that *bsh1* is transcribed at broadly similar levels in the *L. salivarius* strains examined. Thus, the lack of correlation of *L. salivarius* bile resistance levels and their Bsh1 groupings is probably not due to the transcription level of *bsh1*.

Biological characterization of Bsh1 enzymes in *L. salivarius*. To further characterize the function of *bsh1*, the gene was

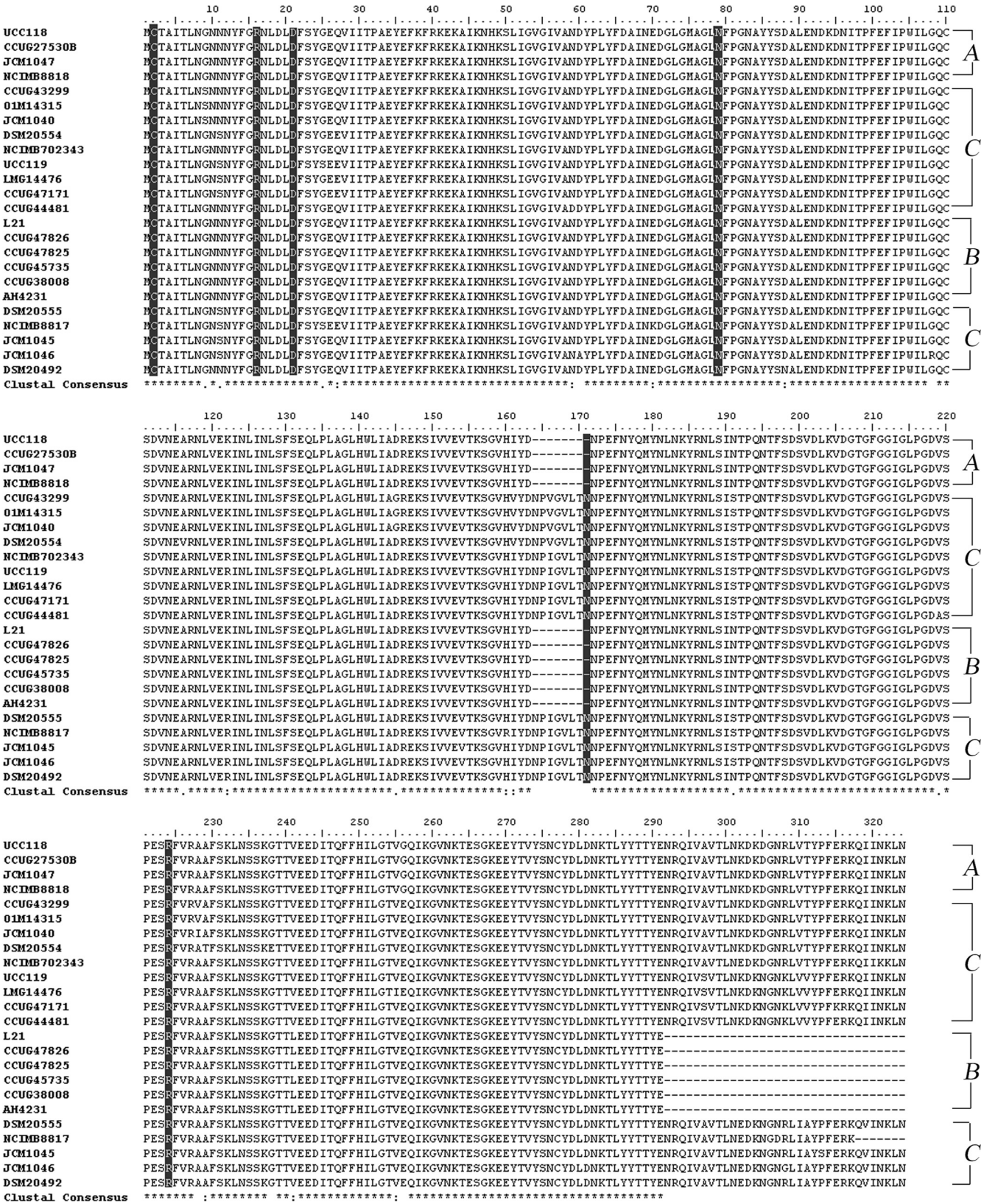

FIG. 2. Sequence alignment of *L. salivarius* LSL_1801 homologues encoding Bsh1 enzymes. The sequences were aligned with ClustalW (http://www.ebi.ac.uk/Tools/clustalw/). Identical amino acids are marked by asterisks, and conserved and semiconserved substitutions are marked by two dots and a single dot, respectively. The shaded residues are conserved amino acids implicated in the active site. *L. salivarius* Bsh1 gene amplicons were sequenced with primers FF029 and FF30 and were deposited in GenBank.

TABLE 2. Bsh activities and bile resistance of *L. salivarius* strains

Bsh1 group	Strain	Origin	Plate assay activity[a]		MIC[b]		
			TDCA	GDCA	GDCA (mM)	Bovine bile (%)	Porcine bile (%)
A	UCC118	Human ileal-cecal region	−/+ (w)	−	6	>20	>5.0
A	NCIMB 8818	St. Ivel cheese	−/+ (w)	−	5	10	1.0
A	CCUG27530B	Human abdomen	−/+ (w)	−	5	7.5	0.3
A	JCM1047	Swine intestine	−	−	4	6.0	0.2
B	CCUG47825	Human blood	−	−	4	>20	>5.0
B	CCUG45735	Human blood	−	−	6	>20	>5.0
B	CCUG38008	Human gall	−	−	6	15	>5.0
B	CCUG47826	Human blood	−	−	6	15	>5.0
B	L21	Human feces	−	−	4	15	1.0
B	AH4231	Human ileum-cecum	−	−	6	12	0.5
C	JCM1046	Swine intestine	+ (p)	+ (p)	>15	>20	>5.0
C	LMG14476	Cat with myocarditis	+ (p)	+ (p)	>15	>20	>5.0
C	DSM20492	Human saliva	+ (w)	−	10	>20	>5.0
C	01M14315	Human gallbladder pus	+ (w)	−	6	>20	>5.0
C	JCM1040	Human intestine	+ (w)	−	6	15	>5.0
C	CCUG44481	Bird	+ (p)	−	4	>20	>5.0
C	DSM20555	Human saliva	+ (w)	−	4	12	>5.0
C	JCM045	Human intestine	+ (p)	−	5	>20	1.5
C	CCUG47171	Human tooth plaque	+ (p)	−	5	12	1.0
C	CCUG43299	Human blood	+ (w)	−	6	>20	0.4
C	NCIMB702343	Unknown	+ (p)	−	5	>20	0.4
C	DSM20554	Human saliva	+ (p)	−	5	10	0.4
C	NCIMB8817	Turkey feces	+ (p)	−	6	10	0.8
C	UCC119	Chicken intestine	+ (w)	−	4	15	0.2
D	NCIMB8816	Human saliva	+ (p)	−	6	12	>5.0
D	JCM1042	Human intestine	−	−	4	10	1.0
NA[c]	JCM1230	Chicken intestine	−	−	6	>20	>5.0
NA	LS201	UCC118 derivative	−	−	>15	>20	>5.0
NA	LS201Δ*bsh1*	This study	−	−	3	4.0	0.1
NA	JCM1046Δ*bsh1*	This study	−	+ (p)	6	>20	>5.0

[a] -, no Bsh activity; + (w), positive bile salt hydrolase activity and production of opaque white colonies; + (p), positive with formation of precipitation; -/+ (w), weak Bsh activity and formation of opaque white colonies.
[b] >, strain is resistant to the highest concentration of bile tested.
[c] NA, not applicable.

interrupted in strain LS201 and strain JCM1046 by plasmid integration. LS201 is a derivative of UCC118 generated by curing of the resident plasmid pSF118-20; this strain was used to allow complementation of the mutated *bsh1* allele with a copy cloned into a low-copy-number vector that we derived from pSF118-20 (21). The integration of plasmid pLS216 into pMP118 in strain LS201Δ*bsh1* was confirmed by Southern hybridization (Fig. 4A). A control integrant strain of LS201 (LS201Δ*lacZ*) was constructed by disruption of the *lacZ* gene

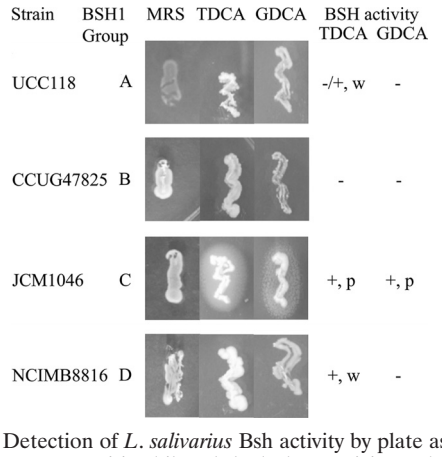

FIG. 3. Detection of *L. salivarius* Bsh activity by plate assay. −, no Bsh activity; +, w, positive bile salt hydrolase activity and production of opaque white colonies; +, p, positive with formation of precipitation; −/+, w, weak Bsh activity and formation of opaque white colonies.

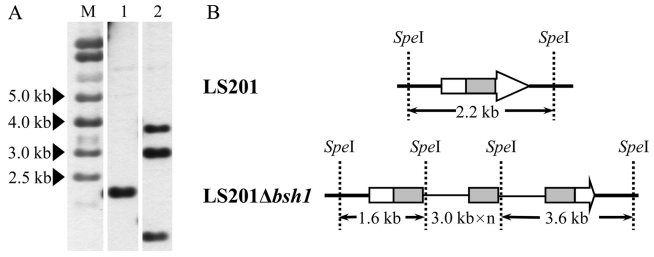

FIG. 4. Disruption of *bsh1* (LSL_1801) in *L. salivarius* LS201. (A) Southern hybridization analysis of insertional inactivation of the *bsh1* gene in *L. salivarius* LS201. LS201 and LS201Δ*bsh1* genomic DNAs were digested with SpeI (lanes 1 and 2) and hybridized with a labeled 582-bp amplicon of LSL_1801 (primers FF027 and FF028) as a probe. Lanes: M, labeled DNA marker; 1, *L. salivarius* LS201 (derivative of *L. salivarius* UCC118 cured of pSF118-20; 2, *L. salivarius* LS201Δ*bsh1*). DNA sizes are indicated by arrowheads. (B) Schematic representation of the relevant regions of the LS201 and LS201Δ*bsh1* genomes. The heavy lines represent megaplasmid DNA; the thin lines represent plasmid DNA. *bsh1* (LSL_1801) is represented by the arrows, and the gray boxes are the *bsh1* internal fragment corresponding to the hybridization probe. SpeI sites are indicated.

with plasmid pLS217. JCM1046Δ*bsh1* was generated by integration of pLS218 into the megaplasmid pMP1046. Porcine bile was used for a bile challenge experiment because it is very similar in composition to human bile (42). Disruption of *bsh1* in strain LS201 led to a significant reduction in resistance to porcine bile (Fig. 5A). The relative survival rates of LS201, LS201Δ*lacZ*, and LS201Δ*bsh1* were 93%, 29%, and 0.2% after 2 h of bile challenge. The cell numbers of LS201Δ*bsh1* were reduced by 4 log units after 5 h of bile challenge. Expression of *bsh1* (LSL_1801) in *trans* from its native promoter (i.e., when cloned in plasmid pLS219) restored the bile resistance of LS201Δ*bsh1* to the resistance level of the LS201Δ*lacZ* integrant. Transformation by the empty vector pLS209 had no effect (Fig. 5A). The JCM1046Δ*bsh1* mutant also appeared more sensitive to bile than the wild-type strain (Fig. 5B), but the relative reduction in bile resistance was smaller than that caused by *bsh1* disruption in LS201. Some 20% of JCM1046Δ*bsh1* cells survived 2 h of bile challenge. A Bsh plate assay showed that the JCM1046Δ*bsh1* mutant had completely lost deconjugation activity for TDCA (not shown), but it still deconjugated GDCA, increasing the likelihood that *bsh2* was responsible for activity against GDCA. Paradoxically, however, the GDCA MICs for LS201Δ*bsh1* and JCM1046Δ*bsh1* were also decreased compared to those of their parental strains (Table 2), indicating some degree of activity of *bsh1* against both TDCA and GDCA (see below).

A previous study showed that *L. salivarius* UCC118 can survive transit through the murine GI tract (18), but the importance of bile resistance for this transit was unknown. Spontaneous rifampin- or streptomycin-resistant derivatives of the *bsh1* mutant (LS201Δ*bsh1*) and the control strain (LS201Δ*lacZ*) were tested for competitive survival in a murine GI tract transit model. In the control group of mice inoculated with PBS, no antibiotic-resistant bacteria were detected in feces at any time points. Both the LS201Δ*bsh1* and LS201Δ*lacZ* strains were detected 2 h after administration (Fig. 5C), but there were significantly more cells of LS201Δ*lacZ* than of LS201Δ*bsh1* recovered at 2, 4, and 6 h after administration. Cells of the LS201Δ*bsh1* mutant could not be cultured 24 h after administration, whereas the LS201Δ*lacZ* strain was still detectable in feces 3 days after oral administration, similar to our unpublished studies of wild-type *L. salivarius* UCC118 (data not shown). Survival of strain LS201Δ*bsh1* after transit through the murine GI tract was significantly lower than that of strain LS201Δ*lacZ* ($P < 0.01$).

Biochemical characterization of recombinant Bsh1 proteins. The $bsh1_{UCC118}$ and $bsh1_{JCM1046}$ genes, representing Bsh1 groups A and C, were amplified and cloned into the *E. coli* expression vector pOPINE and expressed as C-terminally His-tagged proteins. When tested by the Bsh plate assay, only *E. coli* strains that harbored the construct for expressing $Bsh1_{JCM1046}$ showed deconjugation activity on both TDCA and GDCA (data not shown); *E. coli* harboring the $bsh1_{UCC118}$ construct had no detectable Bsh activity. $Bsh1_{UCC118}$ and $Bsh1_{JCM1046}$ were overexpressed in *E. coli* Rosetta BL21(DE3) and purified (Fig. 6). The predicted molecular masses of 118Bsh and $Bsh1_{JCM1046}$ are 35,714 Da and 36,494 Da, respectively. Based on their elution profiles on calibrated size exclusion chromatography columns, both $Bsh1_{UCC118}$ and $Bsh1_{JCM1046}$ were mixtures of dimer and monomer forms (data

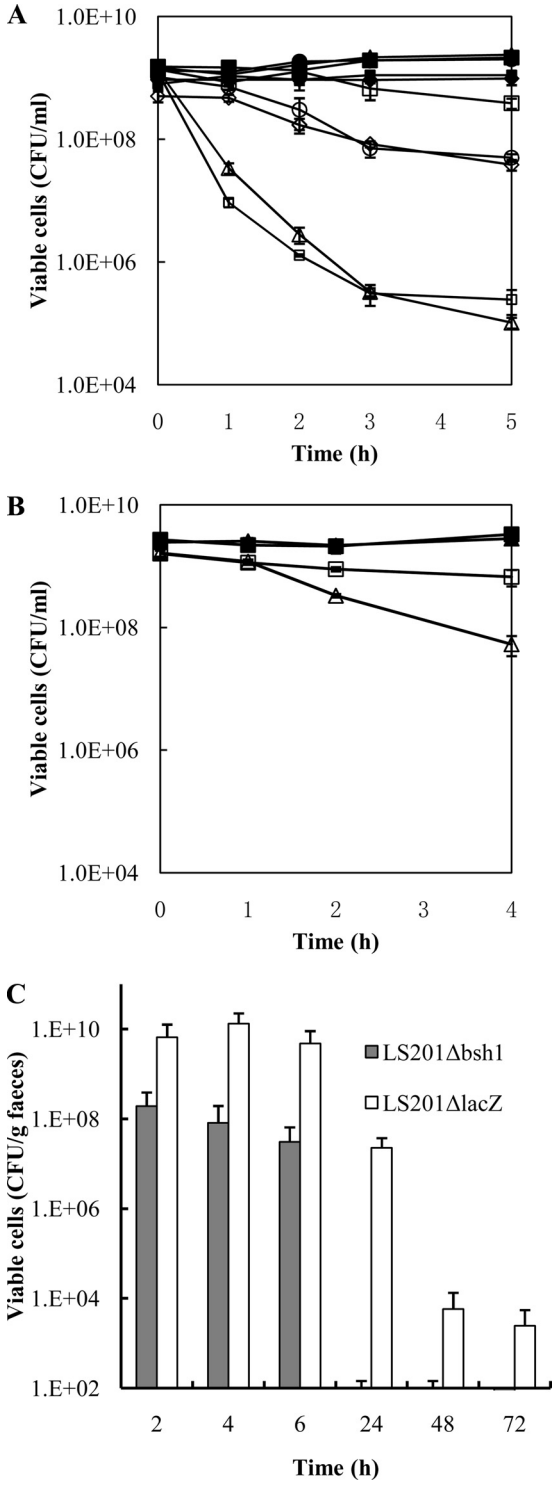

FIG. 5. Bsh1 and Bsh2 contribute to bile resistance in *L. salivarius*. (A) Survival of *L. salivarius* LS201 in the presence (open symbols) or absence (closed symbols) of 0.2% porcine bile. Large squares, *L. salivarius* LS201; triangles, *L. salivarius* LS201Δ*bsh1*; circles, *L. salivarius* LS201Δ*lacZ*; diamonds, *L. salivarius* LS201Δ*bsh1*(pLS219); small squares, *L. salivarius* LS201Δ*bsh1*(pLS209). (B) Survival of *L. salivarius* JCM1046 in the presence (open symbols) or absence (closed symbols) of 0.1% porcine bile. Squares, wild type *L. salivarius* JCM1046; triangles, *L. salivarius* JCM1046Δ*bsh1*. (C) Disruption of the *bsh1*gene of LS201 reduces survival during murine intestinal tract transit. The error bars indicate standard deviations.

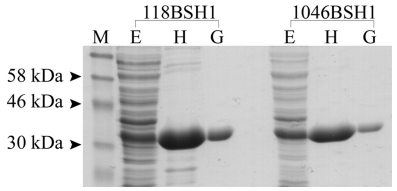

FIG. 6. Purification of recombinant *L. salivarius* Bsh1 proteins ($Bsh1_{UCC118}$ and $Bsh1_{JCM1046}$). Lanes: M, broad-range protein marker; E, *E. coli* Rosetta DE3 cell lysate showing expression of His-tagged Bsh1; H, IMAC-purified Bsh1; G, gel filtration-purified Bsh1. Protein marker masses are indicated.

not shown), although the dimer-to-monomer ratio for $Bsh1_{JCM1046}$ (2:1) was twice that for $Bsh1_{UCC118}$ (1:1). Repeated attempts to express and purify the group B protein (internal deletion and carboxy-terminal truncation) were unsuccessful, because the protein (47825Bsh1) was insoluble and could not be refolded from inclusion bodies.

A comparison of the specific activities of the two enzymes ($Bsh1_{UCC118}$ and $Bsh1_{JCM1046}$) on a range of tauro- and glyco-CBA was undertaken (Fig. 7A). This indicated that the enzymes had different substrate preferences. $Bsh1_{UCC118}$ had greater activity against the glyco-CBA than against the tauro-CBA. The limited activity against tauro-CBA varied, with very low (<0.01 μmol min^{-1} mg^{-1}) activity against the TCA compared with 0.049 and 0.055 μmol min^{-1} mg^{-1} for TDCA and TCDCA, respectively. Activity against the better substrates, glyco-CBA, also indicated that the CA conjugate was the poorest of the three tested (0.38, 0.25, and 0.32 μmol min^{-1} mg^{-1} for GDCA, GCA, and GCDCA, respectively) and showed a clear preference for glyco-CBA. Of significance was the switch in substrate preference for the $Bsh1_{JCM1046}$ enzyme, with clearly higher catalytic capabilities against the tauroconjugated substrates. The activity of this enzyme against glycoconjugated substrates was higher (ranging from 3.5 to 14.7 μmol min^{-1} mg^{-1}) than the $Bsh1_{UCC118}$ enzyme activity for all substrates tested. More importantly, there was a very large increase in the activity against the tauro-CBA, with a specific activity of >40 μmol min^{-1} mg^{-1} against the best substrate, TCDCA, compared with <0.06 μmol/mg for the best tauroconjugated substrate tested with $Bsh1_{UCC118}$. This suggests that the 8-amino-acid deletion in the $Bsh1_{UCC118}$ enzyme has a dramatic impact on the rate of hydrolysis and substrate selection.

It is evident from the data presented Fig. 7 that both variants of Bsh1 have activity over a broad range of pH but with a slight shift in pH optima. $Bsh1_{JCM1046}$ had maximal activity at pH 5.5 and $Bsh1_{UCC118}$ had an optimum of pH 6.5. The more active $Bsh1_{JCM1046}$ enzyme was chosen for kinetic analysis. Using TCA as a substrate (0.5 to 8 mM), the K_m and V_{max} were calculated using a standard Michaelis-Menten kinetic analysis and were determined to be 2.02 mM and 11.2 μmol min^{-1} mg^{-1}, respectively (Fig. 7B). The turnover number k_{cat}, defined as V_{max}/[E], was 22.636 s^{-1}, where [E] is the molar enzyme concentration.

The bile- and cholate-induced transcriptomes of *L. salivarius*. To identify mechanisms for resisting bile other than Bsh and ways in which the deconjugation products of Bsh might be dealt with, the bile- and cholate-induced transcriptomes of *L. salivarius* were investigated. Exposure was performed with cells in exponential phase, since stationary-phase lactobacillus cells are generally more resistant and less responsive to bile (57, 62). The differential gene expression data are summarized diagrammatically in Fig. 8. The complete data sets are available at ArrayExpress. Responsive genes were located on both the chromosome and the megaplasmid pMP118, with some discrete clusters evident. Many more genes were differentially expressed upon exposure to cholate than on exposure to bile, but each treatment led to differential expression of distinct gene clusters. As is evident from Fig. 8, cholate exposure caused differential expression of genes uniformly distributed around the chromosome and pMP118. Bile treatment affected expression of genes uniformly distributed around pMP118, but preferentially in the "top half" or *ori* side of the chromosome. This may indicate that genes that are differentially regulated during bile stress also benefit from an increased gene dosage effect (51) by virtue of being close to the replication origin (17, 47).

Challenge with 0.1% porcine bile resulted in a total of 123 and 68 genes being differentially expressed in LS201 and the LS201Δ *bsh1* mutant, respectively, using as the cutoff a P value of <0.05 and a $\geq$2-fold expression change. Inability to produce Bsh1 did not result in significantly different genes being expressed in response to bile or different levels of expression. The *bsh1* gene itself was not induced by bile exposure. In both the wild-type and the *bsh1* mutant (see Table S2 in the supplemental material), a conserved set of genes was upregulated, including those involved in carbohydrate transport and metabolism, energy production and conversion, cell wall/membrane/envelope biogenesis, amino acid transport and metabolism, and inorganic ion transport and metabolism. A mannose-specific phosphotransferase system (LSL_1713-6) was highly induced (8- to 10-fold) by bile. Genes involved in the transport and metabolism of other carbohydrates, such as glycerol, galactose, rhamnose, and sorbitol, were also induced by bile. A putative ABC transporter operon (LSL_0220-0222) (see Fig. S3A in the supplemental material) was upregulated in both the wild type and the *bsh1* mutant. This operon was also induced by cholate exposure (see below). Downregulated genes in both strains included those for a putative exopolysaccharide biosynthesis cluster, prophage Sal2 (60), arginine and proline metabolism, amino acid transporters, and a manganese transport protein.

A much larger gene set (813 for the wild type and mutant combined) was differentially expressed upon exposure to cholate, and the range of expression change values was considerably greater (Table 3). Prominent among these genes were those for classical stress response proteins (GroEL, GroES, chaperones, and Clp proteases), as well as diverse transporters (Opp system, ABC transporters, and MDR transporters). There was generally excellent concordance between the wild type and the *bsh1* mutant, both in the identities and changes of the genes.

The most significant changes were in class I heat shock genes (*groELS* and *grpE*) and a gene (*hrcA*) encoding their repressor. Genes encoding other chaperone proteins (LSL_0578-9 and LSL_0863) and the ATP-dependent ClpP protease (LSL_1168) were also upregulated by cholate, as was expression of the *clpP* expression regulon *ctsR*. A diverse collection of genes encoding transporters, efflux pumps, Na^+/H^+ antiporters, oxidase proteins, reductase proteins, membrane proteins, and diverse hydrolases were also upregulated by cholate exposure, indicating that the products of bile deconjugation put osmotic, oxidative, and pH

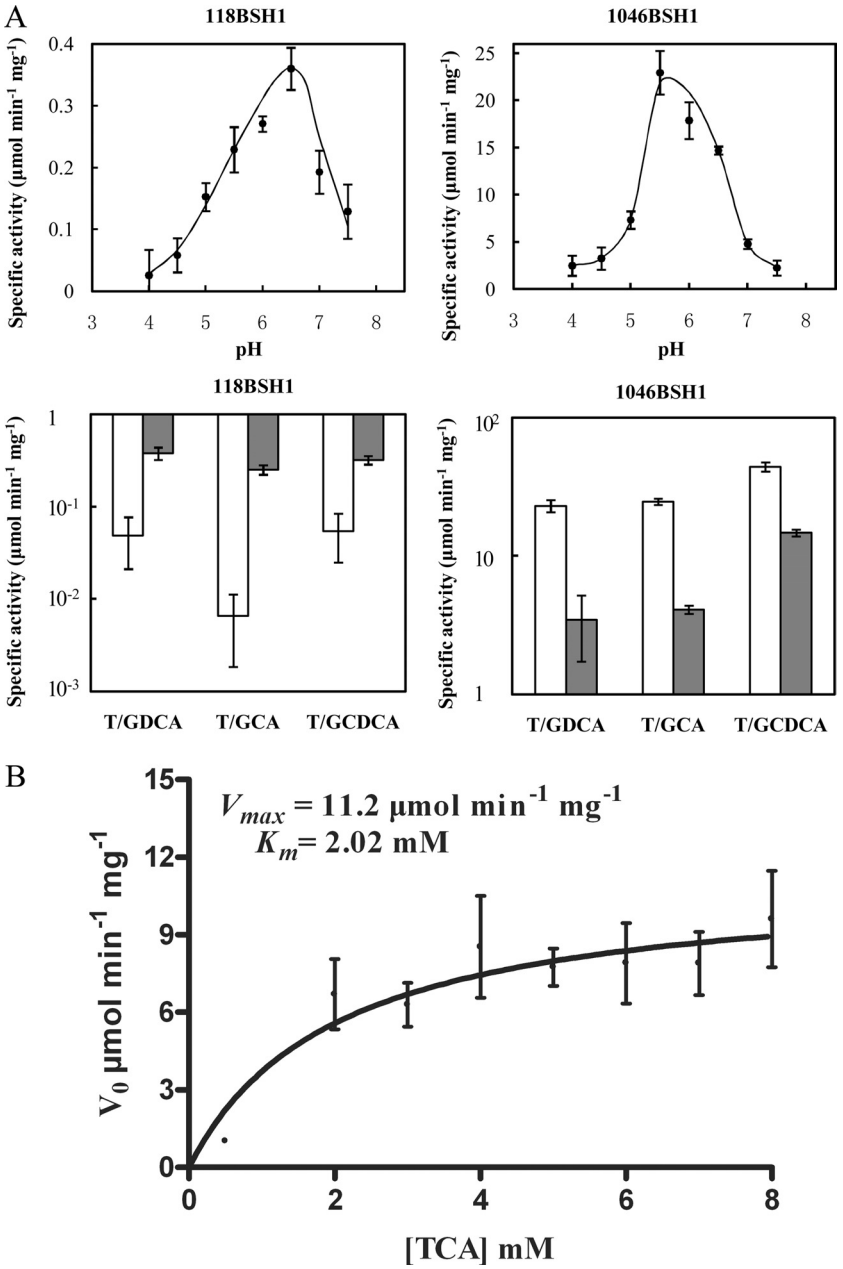

FIG. 7. (A) pH and substrate dependence of *L. salivarius* Bsh1 enzymes. White bars, tauro-CBA; gray bars, glyco-CBA. (B) Measurement of $Bsh1_{JCM1046}$ K_m and V_{max} for TCA. The error bars indicate standard deviations.

homeostasis burdens on *L. salivarius*. The transcriptome analysis highlights the multiplicity of mechanisms by which *L. salivarius* reacts to bile and unconjugated bile acids, which likely contributes to overall bile resistance levels.

DISCUSSION

Members of the species *L. salivarius* have a broad ecological distribution, reflected in the strains chosen for this study (Table 2). It is clear that, while an intestinal niche may not be permanent, many strains pass through the small intestine, perhaps by being shed from an upper GI tract colonization site or in food. The ability to deconjugate bile is thus a logical survival trait for the diversity of *L. salivarius* strains examined here. The fact that all strains examined have a *bsh1* gene resident on their respective circular megaplasmids testifies to the biological selection for this gene and the stability of the megaplasmid as its physical location. Two strains were shown to have a second, *bsh2*, gene, but only because a draft genome sequence was available for one of the strains. When assessing the overall bile resistance phenotypes of the strains here, it must be noted that other additional *bsh* genes may be present in a given strain. Thus, although the group D Bsh1 enzyme present in strain NCIMB8816 is expected to be inactive, the strain showed deconjugation activity for TDCA. Phylogenetic analysis showed

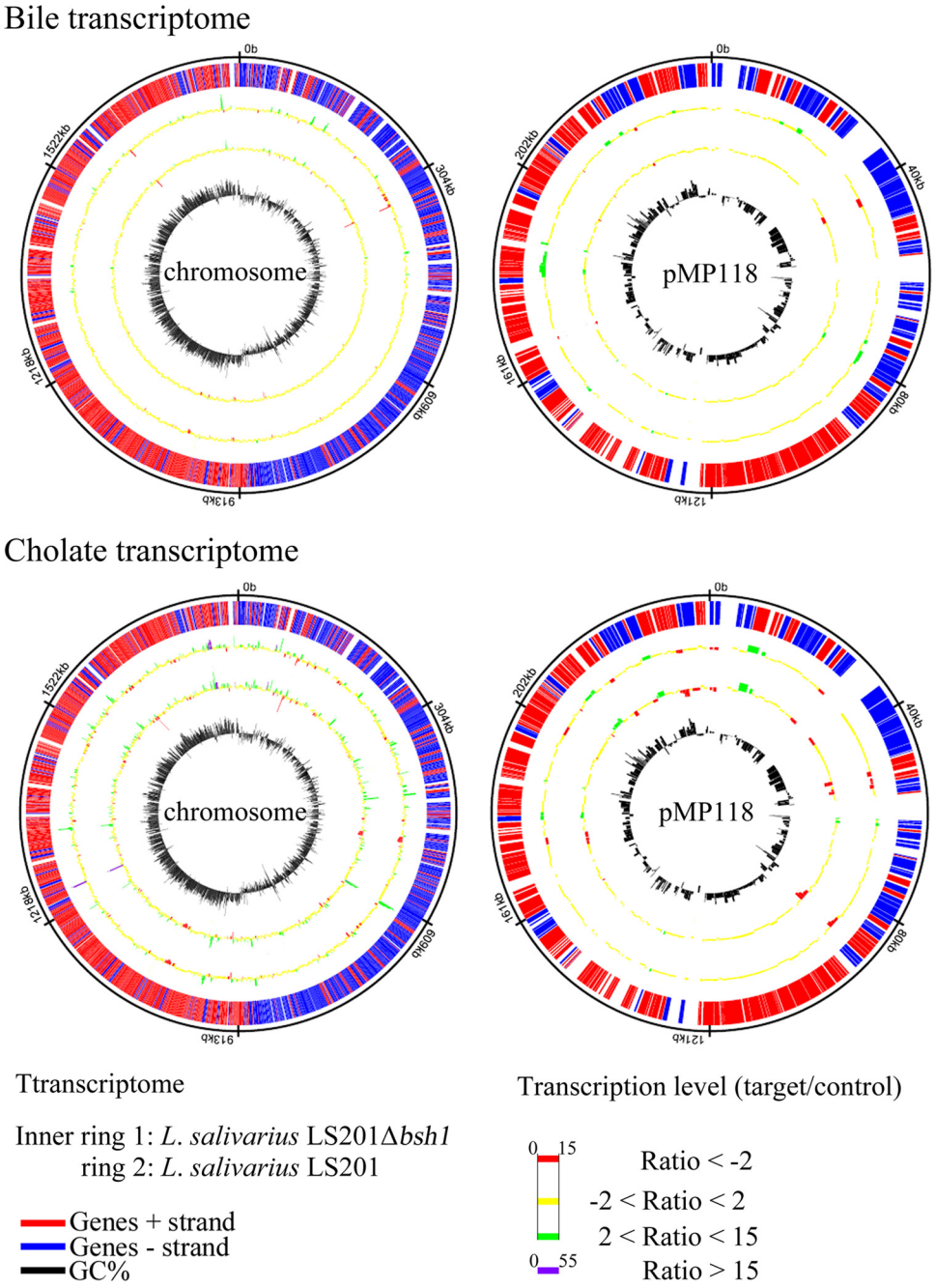

FIG. 8. Genome atlas of global bile-responsive and cholate-responsive transcriptomes in *L. salivarius* strains. The genome wheels with microarray results were generated by Microbial Genome Viewer (http://www.cmbi.ru.nl/genome/). The scale represents the changes in gene expression (treated/untreated) projected on color differences and bar lengths.

that the *L. salivarius* Bsh1 and Bsh2 proteins are in two different branches of the Bsh tree (see Fig. S2 in the supplemental material). This is unsurprising, given that a distinguishing feature of the genus *Lactobacillus* is its extraordinary phenotypic and genomic diversity (9) and that lateral gene transfer is an important element in generating this diversity (39).

The strain *L. salivarius* UCC118 was selected for human probiotic applications based initially upon a number of criteria, including bile resistance (18). Thus, although the Bsh of this strain was shown here to have relatively low activity according to a traditional plate test, the MICs of the strain for GDCA, porcine bile, and bovine bile were as high as, or higher than, those of almost all strains tested. More consistent with the plate assay, the recombinant $Bsh1_{UCC118}$ protein was less active against all substrates tested than the corresponding Bsh1 protein from strain JCM1046, in particular against tauro-CBA. The biochemical characterization also revealed a striking difference in preference for glycoconjugated substrates shown by

TABLE 3. Comparison of numbers of genes ordered in COG[a] categories significantly affected by bile extract porcine with those significantly affected by cholate

Function	Bile/cholate-induced gene regulation functional category	No. upregulated				No. downregulated			
		LS201		LS201Δ*bsh1*		LS201		LS201Δ*bsh1*	
		Bile	Cholate	Bile	Cholate	Bile	Cholate	Bile	Cholate
Information storage and processing (total no.)		3	17	2	26	6	37	5	65
Translation, ribosomal structure, and biogenesis	J	1	0	1	2	0	21	0	42
RNA processing and modification	A	0	0	0	0	0	0	0	0
Transcription	K	2	8	1	11	1	6	3	12
Replication, recombination, and repair	L	0	9	0	13	5	10	2	11
Chromatin structure and dynamics	B	0	0	0	0	0	0	0	0
Cellular processes and signaling (total no.)		8	49	7	68	8	25	9	43
Cell cycle control, cell division, chromosome partitioning	D	0	0	0	2	1	4	1	4
Defense mechanisms	V	2	13	2	18	2	5	1	13
Signal transduction mechanisms	T	0	2	0	4	2	4	2	5
Cell wall/membrane/envelope biogenesis	M	4	7	4	9	2	9	5	15
Cell motility	N	0	0	0	1	0	1	0	1
Cytoskeleton	Z	0	0	0	0	0	0	0	0
Extracellular structures	W	0	0	0	0	0	0	0	0
Intracellular trafficking, secretion, and vesicular transport	U	0	1	0	1	1	2	0	2
Posttranslational modification, protein turnover, chaperones	O	2	26	1	33	0	0	0	3
Metabolism (total no.)		37	134	17	165	28	93	24	150
Energy production and conversion	C	5	13	3	20	1	3	1	7
Carbohydrate transport and metabolism	G	23	18	8	22	3	25	6	37
Amino acid transport and metabolism	E	3	58	3	64	19	32	13	59
Nucleotide transport and metabolism	F	0	1	0	2	2	3	1	6
Coenzyme transport and metabolism	H	0	12	0	12	0	0	0	2
Lipid transport and metabolism	I	1	5	1	10	0	12	0	14
Inorganic-ion transport and metabolism	P	5	23	2	29	3	12	3	18
Secondary-metabolites biosynthesis, transport, and catabolism	Q	0	4	0	6	0	6	0	7
Poorly characterized (total no.)		20	40	4	55	5	21	2	32
General function prediction only	R	6	32	4	44	2	14	1	22
Function unknown	S	3	8	0	11	3	7	1	10
Unknown COG functions		11	0	0	2	0	0	0	0
Total no. of gene expressed differentially		64	189	34	248	59	151	34	225

[a] COG, clusters of orthologous groups.

the $Bsh1_{UCC118}$ enzyme compared with the tauroconjugate preference shown by the $Bsh1_{JCM1046}$ enzyme. These differences may be due in part to the internal deletion of 8 residues in the group A Bsh1 enzyme, including Asn 171. This residue is considered, based on sequence alignment, to be part of the conserved active site (2), even though there are two other Asn residues nearby in the sequence. Importantly, however, the deletion in this region of the group A Bsh enzyme also spans Thr 174 (*Bifidobacterium* and *Clostridium* enzyme coordinates), which is part of a loop forming part of the active site (48). However, there are also 12 other single-amino-acid-residue differences (7 of which are conservative substitutions) between $Bsh1_{JCM1046}$ and $Bsh1_{UCC118}$ that may also be functionally important. A structural comparison between these two enzymes will give interesting insight into how these changes impact folding, substrate recognition, and activity. Notably, however, disruption of the *bsh1* gene (LSL_1801) led to a dramatic reduction in bile tolerance in vitro and significant reduction in murine transit survival. It is significant, therefore, that the relatively low activity of the $Bsh1_{UCC118}$ protein does not detract from its likely biological importance. We were unable to purify soluble 47825Bsh1 protein, corresponding to group B proteins that harbor the internal deletion spanning Asn 171, as well as the carboxy-terminal truncation. Three of the five strains encoding group B Bsh proteins are extraintestinal in origin, where production of active Bsh would be less critical. However, *L. salivarius* strains of extraintestinal origin were not consistently more bile sensitive than intestinal isolates. This probably reflects the unreliability of assigning definitive origins/sources to strains of a species that can survive in many niches.

The enzymatic properties of the more active Bsh1 protein of JCM1046 were determined for comparison with those of other bacterial Bsh enzymes. The K_m of *L. salivarius* $Bsh1_{JCM1046}$ for TCA (2.02 mM) is higher than those from *L. johnsonii* (0.76 mM [37]) and *B. longum* (1.12 mM [29]), suggesting that TCA is a better substrate for these enzymes. However, the C-terminal His tag present on the $Bsh1_{JCM1046}$ enzyme may affect the affinity for the substrate. Unlike Bsh from *B. longum*, 10461Bsh1 from *L. salivarius* JCM1046 showed higher activity against tauro-CBA than glyco-CBA. This may be related to different locations of lactobacilli and bifidobacteria in the GI tract or differences in the other bile-detoxifying mechanisms in the respective species.

$bsh2_{JCM1046}$ is most similar to Lreu23DRAFT_0782 (Joint Genome Institute gene identifier, 639134569), which is not biologically characterized. The *bsh2* gene is the only additional enzyme related to bile degradation that we annotated in the JCM1046 draft genome sequence. A plate assay showed that JCM1046Δ*bsh1* had lost activity against TDCA and that its

activity against GDCA was retained, albeit at a reduced level compared to the wild-type strain, JCM1046. This suggested that *bsh2* was responsible for enhanced GDCA resistance in strains JCM1046 and LMG14476. Detailed structural comparison of $Bsh1_{UCC118}$, $Bsh1_{JCM1046}$, and $Bsh2_{JCM1046}$ will provide valuable insights into related Bsh molecules of a single species that have dramatically different activities and substrate profiles.

From the literature, bile and cholate exposure is likely most stressful to lactobacillus cells in exponential phase, since stationary-phase cells are very resistant to bile stress (57, 62). Thus, the array experiments were performed with early-log-phase cells. Exposure of *L. salivarius* cells to bile or cholate did not induce the expression of *bsh1*. Genes for Bsh were also not induced by bile in *L. acidophilus* (45), contrasting with induction of *bsh* expression in *L. plantarum* WCFS1 by bile (7) and in *B. longum* NCC2705 by simulated intestinal stress conditions (63). It remains possible that *L. salivarius bsh1* expression is inducible in vivo, modulated by factors other than bile. Bile exposure caused differential expression of a large set of genes whose products are implicated in the Bsh-independent bile MICs of the strains tested. The altered cell activities are primarily in the categories of carbohydrate metabolism, cell surface remodeling, stress response, and transport/efflux, many of which are readily rationalized as contributing to resistance to a detergent-like molecule. Broadly similar categorizations of bile response were demonstrated for *L. acidophilus* (45) and *L. reuteri* (62), with important genes distinctive to each species. For example, differential expression of a 7-kb eight-gene operon encoding a two-component regulatory system was central to *L. acidophilus* bile response (45), whereas none of the *L. salivarius* two-component regulator systems were differentially expressed. The bile-inducible operon identified in *L. acidophilus* is not present in *L. salivarius*, although one gene product, LBA1425, shows 44% amino acid identity to LSL_1464, which was significantly upregulated by bile. LSL_1464 is a putative alpha-beta hydrolase of unknown function that is conserved in other lactobacilli, *Listeria*, and some other *Firmicutes* and that merits functional characterization. A presumptive ABC transporter locus (see Fig. S3A in the supplemental material) that might act as an efflux pump for bile was significantly upregulated in *L. salivarius*, and this ABC transporter locus is conserved in many bacteria (not shown). A second ABC transporter locus induced by cholate (LSL_0031-0033) was 29% identical at the protein level to Lr1265, which was induced by bile stress in *L. reuteri* (62). Although altered carbon metabolism might be required to maintain cellular ATP levels to energize bile export processes, it is more likely that induction of, e.g., the mannose and sorbitol phosphotransferase systems is due to denaturation of their presumptive regulators.

The LSL_1335 gene was upregulated 2.6-fold in response to bile. This gene encodes a putative mucin-binding protein and candidate adhesin molecule LspC and was previously shown by us not to be expressed in vitro (59). Bile may thus be used as a signaling molecule by commensal lactobacilli like *L. salivarius* to modulate host interaction genes. Consistent with this notion, genes for three surface proteins of *L. acidophilus*, including two putative mucin-binding proteins, were upregulated upon bile exposure (45).

The toxicity of FBA produced by Bsh activity can be avoided either through catabolism or by export. According to the annotated genome, *L. salivarius* UCC118 has neither 7-α-dehydroxylate nor 7-α-dehydrogenate activity for unconjugated bile acid, nor does it contain genes encoding cholate-coenzyme A ligase (EC 6.2.1.7), which can further break down FBA (into cholate, deoxycholate, and chenodeoxycholate). Thus, the cholate-induced efflux pumps and transporters likely play a role in removal of unconjugated bile acids in *L. salivarius* strains. Among those cholate-responsive transporters, the *L. salivarius* MDR transporter (LSL_0078; MDR protein B) showed high identity (71% and 59%) to the characterized MDR transporters from *L. acidophilus* NCFM (LBA1429) (45) and *L. reuteri* ATCC 55730 (lr1584) (62), which contribute to bile resistance. Another cholate-induced *L. salivarius* MDR transporter (see Fig. S3B, LSL_0032-3, in the supplemental material) is 52% and 53% identical to the *L. lactis* LmrCD cholate transporter, which also confers bile resistance on these bacteria (64). The Opp system (LSL_2026-7), located on the 44-kb plasmid pSF118-44, which is responsible for glycine-betaine uptake, was also induced by cholate. These gene products show high homology to the *L. monocytogenes* BilE system (57% and 45% identity to BilB and BilA), which has been shown to enhance bile resistance when introduced into *Bifidobacterium* and *Lactococcus* (61). Thus, the products of Bsh activity can induce the expression of genes and gene products that potentiate the bile resistance phenotype of the organism, potentially amplifying the phenotypic significance of the Bsh kinetics of a particular enzyme complement in a given strain.

Bsh enzymes are clearly key contributors to bile resistance levels and might conceivably be the most important determinant under growth phase and nutritional conditions that cannot be reproduced easily outside the gut. Thus, the apparent discrepancy between the *bsh* allele type and the bile MIC reflected by the in vitro assay may be less significant in the gut. Unconjugated bile acids produced by the actions of Bsh enzymes might be removed or sequestered in the gut lumen by the host or other microbes. Furthermore, the study clearly shows that the *L. salivarius* cell is equipped with a repertoire of other mechanisms involved in protecting the cell against the inimical properties of bile and which result in a bile resistance level that cannot be predicted simply from consideration of the Bsh enzyme complement alone. Independent of its function in dietary fat emulsification, bile is a key signaling molecule regulating its own biosynthesis, lipid absorption, cholesterol homeostasis, and local mucosal defenses in the intestine (28). Weight gain in chickens in inversely correlated with intestinal Bsh activity, much of which comes from the dominant lactobacillus species in poultry, *L. salivarius* (26). Excessive deconjugation of bile in the gut may be linked with "contaminated small bowel syndrome" (22, 58). Thus, it may be significant that *L. salivarius* strain UCC118, selected as a human probiotic, has low Bsh activity but high overall bile resistance. This study shows the complexity of bile resistance level determination in commensal *L. salivarius* strains, the integration of redundant mechanisms, and the potential for bile to act as an environmental cue in probiotic lactobacilli.

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