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Allelic variation of bile salt hydrolase genes contributes to, but is not the sole determinant of, bile resistance levels in *Lactobacillus salivarius*

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ABSTRACT

Commensal lactobacilli produce bile salt hydrolase (BSH) enzymes whose role in intestinal survival is unclear. Twenty-six strains of *Lactobacillus salivarius*, from different sources, all harboured a *bsh1* allele on their respective megaplasmid, related to the plasmid-borne *bsh1* gene of the probiotic strain UCC118. A second locus (*bsh2*) was found in the chromosome of two strains that had higher bile resistance levels. Four BSH1-encoding allele groups were identified, defined by truncation or deletions involving a conserved residue. *In vitro* analyses showed that this allelic variation correlated with widely varying bile-deconjugation phenotypes. Despite very low activity of the UCC118 BSH1 enzyme, a mutant lacking this protein had significantly lowered 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 effect 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.

INTRODUCTION

Lactobacilli are among the species most commonly used as probiotic agents, due to the wide range of consumer benefits associated with their consumption (34). 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 (50). Bile is a detergent solution of organic and inorganic compounds, which varies in composition in different animals (44). The major constituents include bile acids, cholesterol and phospholipids (2). Human bile acids are synthesized in the liver and then circulated in the gastrointestinal tract, with high concentrations in the duodenum, jejunum and proximal ileum (48). 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 enzymes is a common bile resistance mechanism in bacteria. Bile salt hydrolases (BSH), or conjugated bile acid hydrolases (CBAH; EC 3.5.1.24) belong to the choloylglycine hydrolase family which form part of the N-terminal nucleophilic (Ntn) 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 (30). BSH enzymes act upon a wide range of bile acids conjugates and salts including six major human conjugated bile acids (taurocholic acid, TCA; taurodeoxycholic acid, TDCA; taurochenodeoxycholic acid, TCDCA; glycocholic acid, GCA; glycodeoxycholic acid, GDCA; glycochenodeoxycholic acid, GCDCA). Homologues of the *bsh* gene have been detected in many intestinal bacteria (28). 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 (16).

The presence and genetic organization of *bsh* genes in lactobacilli is very variable. In addition to presence in 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* ATCC33323 (two genes) (32). 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-conjugated bile acids (CBA) (36, 42). Resistance of *bsh* mutants of *Lactobacillus amylovorus* and *Lactobacillus plantarum* to bile acids/salts was reduced compared to the respective wild type strains (13, 14, 25). 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 persistence compared to the parental strain (17). 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 (24, 49, 53). 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 (53). BSH enzymes recognize bile acids on both the cholate steroid nucleus and the amino acid moiety. The crystal structure of *C. perfringens* BSH revealed that activity is conferred by a hydrophobic pocket that recognizes the cholyl moiety of the substrate (49). The crystal structures and biochemical properties of BSH from *B. longum* (30) and *C. perfringens* (49) have been well characterized.

Within the phylogenetically diverse genus *Lactobacillus*, BSHs have only been biochemically characterized from *L. acidophilus* PF01 (42, 46) and *L. johnsonii* 100-100 (20). Inactivation of *bshB* in *L. acidophilus* NCFM revealed that the strain lost hydrolytic activity for tauro-conjugated bile salts (42). Given that lactobacilli are the main contributors to BSH activity in the murine and chicken intestinal tracts (27, 57), 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 FBAs, by one of these major strategies: precipitation or 7-dehydroxylation and precipitation at moderately acidic pH; catabolism by CoA-ligase; transport (efflux) outside the bacterial cell. In *Bacteroides fragilis*, the presence or absence of BSH activity correlates with production of 7- α -hydroxysteroid dehydrogenase (54). How BSH-producing *Lactobacillus* species like *L. salivarius*, that are non-producers of 7- α -hydroxysteroid dehydrogenase, resist FBAs is not clear, and 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 (28). However, there is also evidence that the production levels and the enzymatic activity of BSH are not directly related to overall bile resistance levels (25, 43). In addition to *bsh*, other genes (*pva*, *btlB*) and the sigma factor σ^B were shown to contribute to bile resistance in *L. monocytogenes* EGDe (3). Furthermore, microarray analysis of the bile-induced transcriptome identified genes including MDR transporters, chaperone, esterase, and a histidine protein kinase that were implicated in bile resistance in *L. acidophilus* NCFM and

109 *Lactobacillus reuteri* ATCC55730 (47, 62). Genes involved in DNA repair, oxidative response,
110 transcriptional regulation, dGTP hydrolysis, membrane composition, and cell wall synthesis were
111 differentially expressed upon exposure of *Enterococcus faecalis* or *L. plantarum* WCFS1 cells to
112 bile (6, 7).

113 *L. salivarius* UCC118 is a well characterized strain (11) with probiotic properties (18). This
114 strain harbours a 242 kb megaplasmid pMP118, that interdigitates with chromosomally-encoded
115 functions to confer metabolic flexibility (37, 45). *L. salivarius* is common in the gastrointestinal
116 tract of many animals including humans (1), and chickens (27), but its survival mechanisms *in vivo*
117 are poorly understood. In this study, we therefore examined the contribution of allelic variants of
118 *bsh* to bile resistance of *L. salivarius*, as well as other bile resistance mechanisms.

MATERIALS AND METHODS

Bacterial strains, plasmids and growth conditions. Bacterial strains and plasmids used in this study are listed in Table 1. *L. salivarius* was grown under microaerobic conditions (5% CO₂) in de Man-Rogosa-Sharpe (MRS) medium (Oxoid Ltd., United Kingdom) at 37°C. *E. coli* was grown in Luria-Bertani (LB) broth (51) 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 (Em) and chloramphenicol (Cm) were used at 5 µg/ml for *L. salivarius* and *L. lactis*. Tetracycline (Tet) was added at 5µg/ml for *L. salivarius* and 10µg/ml for *L. lactis*. Ampicillin (Amp) and chloramphenicol were supplemented at 50 µg/ml and 34 µg/ml for *E. coli*, respectively.

DNA manipulation. Primers used for PCR were purchased from MWG Biotech (Ebersberg, Germany) and are listed in Table S1 of the Supporting 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 their instructions. For making constructs (pEB118 and pEB1046) for overexpression of *bsh1* (LSL_1801) and *1046bsh1*, KOD HiFi polymerase (Novagen, Darmstadt, Germany) and In-Fusion™ Dry-Down PCR cloning kit (Clontech, U. S. A.) were used for PCR amplification and cloning according to manufacturers' instructions. Plasmid DNA electrotransformation, *L. salivarius* genomic DNA isolation, pulsed-field gel electrophoresis (PFGE) (plug preparation, S1 nuclease treatment and electrophoresis) were performed as described previously (21). Southern blot analysis followed a standard protocol (51).

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. 500 ng of RNA 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 reaction consisted 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 qRT-PCR amplifications were performed on an ABI Prism 7000 using SYBR green I.

Type I microarray procedures. The *L. salivarius* array contains 1500 Agilent quality control spots and 60 nt oligonucleotides corresponding to 2184 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 have fewer probes) by eArray (<https://earray.chem.agilent.com/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. The array design and microarray data can be found at EMBL-EBI ArrayExpress under accession no. XXX1 and XXX2, respectively.

Overnight cultures of *L. salivarius* UCC118, LS201 and LS201Δ*bsh1* were diluted 50 fold in MRS without antibiotics and grown at 37°C to an OD₆₀₀ of 0.3. The cultures were divided in two and were either untreated or treated with 0.1% porcine bile or 1mM cholate (sodium cholate hydrate, Sigma C6445). After 15 min incubation, 12 ml samples were harvested by centrifugation (13,000 × g for 15 sec) at room temperature. Cell pellets were washed once with RNAprotect Bacteria Reagent (Qiagen) and immediately frozen at -80°C. Cells were disrupted by a bead-beater homogenizer (three times 1 min treatment 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. RNA quality was checked by Agilent Bioanalyzer 2100 using the RNA 6000 Nano assay kit (Agilent). 4 µg RNA derived from cells treated or untreated was used for complementary DNA 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, 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 hrs. Slides were scanned using Agilent Microarray Scanner System (G2505B) with Agilent scan control software version 7.0 for the 44k microarray at resolution of 5µm 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 (26). *P* values were calculated according to the Cyber-T test (38).

Phylogenetic analysis. BSH sequences were aligned by ClustalW provided by Molecular Evolutionary Genetics Analysis (MEGA) software version 4 (55). The neighbor-joining tree of BSH sequences was built by running MEGA4 using the *p*-distances amino acid model with 500 bootstrap replications. Penicillin V acylase (PVA) (P12256) from *Bacillus sphaericus* that 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* (1046*bsh1* and LSL_1801) and *lacZ* (LSL_0376), respectively. The corresponding PCR products were restricted with *Bam*HI and *Eco*RI 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 *1046bsh1* 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 minimum inhibition concentration (MIC) assay and bile challenge experiment procedures. *L. salivarius* strains were tested for hydrolase activity against tauro- or glyco-conjugated bile acids (CBA) by using a plate assay method (12). Overnight MRS broth cultures were streaked on MRS agar supplemented with 0.5 % (wt/vol) sodium taurodeoxycholate hydrate (TDCA, Sigma T0875) or 2 mM sodium glycodeoxycholate (GDCA, Sigma G3258). The plate was then incubated anaerobically for 48 hrs 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 l, agar 15 g, tryptone 10 g, yeast extract 5 g, NaCl 5 g, CaCl₂ 2H₂O 0.35 g, glucose 10 g, IPTG 1 mM, pH6.5) containing 5 g/l TDCA or 2mM GDCA (10) was used.

To measure minimum inhibition concentrations (MIC), 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. Cultures were then incubated at 37°C for 24 hrs 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. The cells were harvested by centrifugation. The cells

211 were washed once with MRS broth followed by resuspension in MRS broth containing a sub-lethal
212 concentration of porcine bile (0.2% for LS201 and its derivatives, 0.1% for JCM1046 and its
213 derivative) and incubated at 37°C (5 % CO₂) for 5 hrs. Samples were removed from the culture at
214 different time intervals, were diluted, and plated on MRS, MRS Em 5 or MRS Tet 5 plates for
215 viable cell counting.

216 **Expression and purification of BSH.** The genes for 118BSH1 (LSL_1801) or its homologs
217 from strain JCM1046 were amplified by PCR using primers EBF-EB118R or EBF-EB1046R.
218 Purified PCR products were cloned into the linearised T7 promoter-based pOPINE expression
219 vector (OPPF) by In-Fusion™ reactions. The resulting plasmids pEB118 and pEB1046 were
220 transformed into strain *E. coli* BL21(DE3)pLysS for overexpressing C-6 × His-tagged *bsh*. For
221 production of BSH, 40 ml of *E. coli* BL21(DE3)pLysS (pEB118 or pEB1046) overnight culture for
222 expression of corresponding *bsh* was inoculated into a biofermentor (Biolab, B. Braun Biotech Ltd.,
223 Germany) charged with 2 l of LB medium supplemented with 34 µg/ml Cm and 50 µg/ml Amp.
224 The culture was grown at 37°C with oxygen supplementation and agitation of 200 rpm to an OD₆₀₀
225 value of 0.6. The culture was then induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG)
226 at 37°C for 5 hrs. For expression of *118bsh1*, the culture was immediately cooled to 20°C followed
227 by induction with 0.1mM IPTG for 20 hrs. The cells were harvested by centrifugation and the cell
228 pellet was resuspended in 80 ml 50 mM Tris-HCl pH7.5, 500 mM NaCl, 20 mM imidazole. Cells
229 were disrupted by sonication. Cell debris was removed by centrifugation at 45,000 ×g for 30 min
230 at 4°C. BSH was purified by immobilized metal ion affinity chromatography (IMAC) and gel
231 filtration with the ÄKTAprime™ plus FPLC (GE Healthcare Life Sciences). BSH was eluted with
232 buffer (50 mM Tris-HCl pH7.5, 500 mM NaCl, 500 mM imidazole) from a HisTrap HP 1ml
233 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 DTT. 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 (30, 56). The reaction was set up in PCR strip tubes. In a 20 μ l reaction, a mixture of 0.1 M sodium phosphate buffer pH 5.5, 10 mM DTT, 10 mM T/G-CBA and BSH (100 nM 1046BSH1 or 400 nM 118BSH1) was incubated at 37°C (30 min for 1046BSH1 or 3 hrs for 118BSH1). Immediately, the reaction was stopped by adding 20 μ l 15% (wt/vol) trichloroacetic acid. The samples were then centrifuged at 10,000 $\times$ g for 1 min. 5 μ l of the supernatants or their appropriate dilutions was mixed with 95 μ l 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. Reactions were cooled and transferred to a 96-well plate and absorbance at 570 nm read. The absorbance at 570 nm was converted into the amount of amino acid, by reference to a glycine standard curve. One unit of BSH activity was defined as the amount of enzyme that released one μ mol of taurine/glycine from substrate per min. The same reaction conditions were used to determine the V_{max} and K_m for the 1046BSH1 enzyme with the enzyme concentration fixed at 140 nM the substrate concentration varied from 0.5 mM to 8 mM. To determine the optimum pH for BSH activity, 10 mM GDCA was used for 118BSH1, 10 mM TDCA was used for 1046BSH1, as these 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); 0.1 M sodium phosphate buffer (for pH 5.5 to pH 8).

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

Murine intestinal tract survival. Spontaneous rifampicin-resistant (rif^R) mutant of *L. salivarius* strain LS201Δ*bsh1* and streptomycin-resistant (strep^R) mutant of strain LS201Δ*lacZ* were isolated as follows. A 20 ml of overnight cultures of respective LS201 derivatives was centrifuged. Cell pellets were resuspended in 200 µl of PBS, and plated onto MRS/Tet5 supplemented with 50 µg/ml rifampicin or 1 mg/ml streptomycin. Murine inoculation experiments were approved by the institutional ethics committee and complied with all relevant legislation. For each group, five 9-week old Balb/C male mice were orally administered with either 100 µl PBS (control group), or a mixture of *L. salivarius* LS201Δ*bsh1* and LS201Δ*lacZ* cells at a dose of 10⁹ CFU each strain, in 100 µl (competitive experiment group) by oral gavage. Mice were given access to water and food after administering *Lactobacillus* strains or PBS. Faeces was collected individually at different time intervals and resuspended in 1 ml PBS by vortexing to homogenize. The faeces suspensions were centrifuged at 100 × g for 2 min. Supernatants were taken for dilution and viable cell counting on Tet-Rif or Tet-Strep 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 was analyzed by two-way analysis of variance (ANOVA).

RESULTS

Distribution of bsh alleles in *L. salivarius* strains. The genome of *L. salivarius* UCC118 contains two genes that were originally annotated as choloylglycine hydrolases: the chromosomally located LSL_0518, and the megaplasmid located LSL_1801 (11). The amino acid sequence of the LSL_0518 gene product shows slightly higher identity (31%) to characterized penicillin V acylases from *Bacillus subtilis* (accession CAJ77223) and *L. plantarum* WCFS1 (CAD65471) (31) than that (29%) to the conjugated bile acid hydrolase (2RF8_A) from *Clostridium perfringens*. The sequence of the LSL_1801 product is 53% identical to functionally characterized conjugated bile acid hydrolase (CAD00145) from *L. monocytogenes* EGDe (3), while it shows lower residue identity (34%) to penicillin V acylase (ZP_00394048.1) from *Bacillus anthracis* str. A2012.

LSL_1801 is located on the megaplasmid pMP118 in strain UC118, and megaplasms with a related replication origin were previously detected in all 33 *L. salivarius* strains examined (37). Among 28 *L. salivarius* strains investigated by Southern hybridization (Fig. 1), 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 (Raftis and O'Toole, unpublished data). This BSH, which will be the subject of a separate study, is 45 % identical to LSL_1801 at protein level, and its chromosomal gene did not hybridize with the LSL_1801 probe. A PCR survey failed to amplify this second *bsh* gene from 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 as 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 4 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 (165-171: 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 in group A. In Group C (JCM1046 group) BSH1 sequences are complete, relative to all the other sequences aligned. CCUG43299BSH1 is identical to 01M14315BSH1; other group C proteins are 94-99% 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)] (48) as indicated in Fig. 2. Group A and B BSH1 molecules lack the conserved Asn 171 residue. The sequence of *bsh2* from strain LMG14476 is identical to that from strain JCM1046. Pair-wise sequence alignment indicates that BSH2 shows highest sequence identity to BSH (ZP_03073770) from *L. reuteri* 100-23 (68.9%), compared to 47.2% identity with JCM1046 BSH1. Furthermore, the *L. salivarius* BSH2 sequence contains all 6 conserved BSH active site residues.

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 out-group. BSH sequences from Gram-positive bacteria could thus be divided into a clostridial clade and a non-clostridial clade (Fig. S1). All *Lactobacillus* BSH sequences were in the non-clostridial 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* BSHs 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 (28).

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) exemplified in Fig. 3, and summarized in full in Table 2. Strains harbouring the group B *bsh1* allele failed to demonstrate convincing activity in the plate assay. Apart from strains JCM1046 and LMG14476 that have two *bsh* genes in their genomes, BSH activity in group C strains was only detected against sodium taurodeoxycholate hydrate (TDCA). Strains JCM1046 and LMG14476 showed activity against both TDCA and sodium glycodeoxycholate (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 presence of an unrelated *bsh* gene. The 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 resistance levels to bile and bile components, as shown by the minimum inhibition concentration (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 conjugated bile acids (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 MIC values 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 have BSH1 only. This strengthens the linkage of the *bsh2* allele with GDCA deconjugation.

Comparison of *bsh1* transcription levels in *L. salivarius* strains. The preceding analysis identified inconsistencies in *bsh1* allele groupings and bile MIC values. Among the potential reasons for this was varying *bsh1* transcription levels. Nucleotide comparison of amplified flanking sequences upstream of *bsh1* revealed that the presumptive promoter and ribosome binding site of 24 *L. salivarius* *bsh1* genes appeared to be very conserved, and could be described by the following consensus sequence:

ATTATTAG-TTKAWW-N₆₋₈-TTGATAC-TYTWAT-A-GGAAG-N₈-ATG. (-35, -10 boxes and ribosome binding site were underlined; where 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 level of *bsh1* in three representative *L. salivarius* strains (UCC118, CCUG47825 and JCM1046, allele groups A through C) was 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. As shown in Fig. 4, the transcription level of *bsh1* in CCUG47825 was

modestly but significantly ($p < 0.01$) higher than that of strain UCC118. The increase was only 1.36-fold and 1.31-fold for exponential and stationary phases, respectively. Notwithstanding 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 grouping 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 interrupted in strain LS201, and strain JCM1046, by plasmid integration. LS201 is a derivative of UCC118 generated by curing of 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 LS201 Δ *bsh* was confirmed by Southern hybridization (Fig. 5A). A control integrant strain of LS201 (LS201 Δ *lacZ*) was constructed by disruption of the *lacZ* gene with plasmid pLS217. JCM1046 Δ *bsh1* was generated by integration of pLS218 into the megaplasmid pMP1046. Disruption of *bsh1* in strain LS201 led to a significant reduction in resistance to porcine bile (Fig. 6A). The relative survival rates of LS201, LS201 Δ *lacZ* and LS201 Δ *bsh* were 93%, 29% and 0.2% after 2 hrs of bile challenge. The cell numbers of LS201 Δ *bsh* were reduced by four logs after 5 hrs 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 LS201 Δ *bsh* to the resistance level of the LS201 Δ *lacZ* integrant. Transformation by the empty vector pLS209 had no effect (Fig. 6A). The JCM1046 Δ *bsh1* mutant also appeared more sensitive to bile than the wild type strain (Fig. 6B), but the relative reduction in bile resistance was smaller than that caused by *bsh1* disruption in

389 LS201. Some 20% of JCM1046 $\Delta bsh1$ cells survived 2 hrs of bile challenge. BSH plate assay
390 showed that the JCM1046 $\Delta bsh1$ mutant had completely lost deconjugation activity for TDCA (not
391 shown) but it still deconjugated GDCA, increasing the likelihood that *bsh2* as responsible for
392 activity against GDCA. Paradoxically however, the GDCA MIC values for LS201 Δbsh and
393 JCM1046 $\Delta bsh1$ were also decreased compared to their parental strains (Table 2), indicating some
394 degree of activity of *bsh1* against both TDCA and GDCA (see also below).

395 Porcine bile was used for the bile challenge experiment, because it is very similar in
396 composition to human bile (44). A previous study showed that *L. salivarius* UCC118 can survive
397 transit through the murine GI tract, (18) but the importance of bile resistance for this transit was
398 unknown. Spontaneous rifampicin or streptomycin-resistant derivatives of the *bsh1* mutant
399 (LS201 Δbsh) and the control strain (LS201 $\Delta lacZ$) were tested for competitive survival in a murine
400 GI tract transit model. In the control group of mice inoculated with phosphate-buffered saline
401 (PBS), no antibiotic resistant bacteria were detected in faeces at any time points. Both LS201 Δbsh
402 and LS201 $\Delta lacZ$ strains were detected 2 hrs after administration (Fig. 6C), but there were
403 significantly more cells recovered of LS201 $\Delta lacZ$ than that of LS201 Δbsh at times 2, 4, and 6
404 hours after administration. Cells of the LS201 Δbsh mutant could not be cultured 24 hours after
405 administration, whereas the LS201 $\Delta lacZ$ strain was still detectable in faeces 3 days after the oral
406 administration. Survival of strain LS201 Δbsh after transit through the murine GI tract was
407 significantly lower than that of strain LS201 $\Delta lacZ$ ($p < 0.01$).

408
409 **Biochemical characterization of recombinant BSH1 proteins.** The *118bsh1* and *1046bsh1*
410 genes, representing BSH1 groups A and C, were amplified and cloned into the *E. coli* expression
411 vector pOPINE and expressed as C-terminally His-tagged proteins. When tested by the BSH plate

412 assay, only *E. coli* strains that harboured the construct for expressing 1046BSH1 showed
413 deconjugation activity on both TDCA and GDCA (data not shown); *E. coli* harbouring the *118bsh1*
414 construct had no detectable BSH activity. 118BSH1 and 1046BSH1 were over-expressed in *E. coli*
415 Rosetta BL21 (DE3) and purified (Fig. 7). The predicted molecular weights of 118BSH and
416 1046BSH1 are 35,714 Da and 36,494 Da, respectively. Based on their elution profiles on calibrated
417 size exclusion chromatography columns, both 118BSH1 and 1046BSH1 were a mixture of dimer
418 and monomer forms (data not shown), although the dimer to monomer ratio for 1046BSH1 (2:1)
419 was twice that for 118BSH1 (1:1). Repeated attempts to express and purify the group B protein
420 (internal deletion and carboxy-terminal truncation) were unsuccessful, because the protein
421 (47825BSH1) was insoluble, and could not be refolded from inclusion bodies.

422 A comparison of the specific activities of the two enzymes (118BSH1 and 1046BSH1) on a
423 range of tauro- and glyco- CBAs was undertaken (Fig 8A). This indicated that the enzymes had
424 different substrate preferences. The 118BSH1 had greater activity against the glyco-CBAs than
425 against the tauro-CBAs. The limited activity against tauro-CBAs varied with very low
426 ($<1\mu\text{mol/mg}$) activity against the TCA compared with 8.8 and 9.8 $\mu\text{mol/mg}$ for TDCA and TCDCA
427 respectively. Activity against the better substrates, glyco-CBAs, also indicated that the CA
428 conjugate was the poorest of the three tested (68, 45, 58 $\mu\text{mol/mg}$ for GDCA, GCA and GCDCA
429 respectively), and showed a clear preference for glyco-conjugated bile acids. Of significance was
430 the switch in substrate preference for the 1046BSH1 enzyme, with clearly higher catalytic
431 capabilities against the tauro-conjugated substrates. The activity of this enzyme against
432 glyco-conjugated substrates was higher (ranging from 104-441 $\mu\text{mol/mg}$) than the 118 enzyme
433 activity for all substrates tested. More importantly, there was a very large increase in the activity
434 against the tauro-CBAs, with a specific activity of $> 1300\mu\text{mol/mg}$ against the best substrate

TCDCA, compared with $< 10 \mu\text{mol/mg}$ for the best tauro-conjugated substrate tested with 118BSH1. This would suggest that the 8 amino acid deletion in the 118BSH1 enzyme has a dramatic impact on the rate of hydrolysis and substrate selection.

It is evident from data presented Fig. 8 that both variants of BSH1 have activity over a broad range of pH but with a slight shift in pH optima. 1046BSH1 had maximal activity at pH 5.5 and 118BSH1 an optimum of pH 6.5. The more active 1046BSH1 enzyme was chosen for kinetic analysis. Using TCA as a substrate (0.5 – 8 mM), the K_m and V_{max} were calculated using a standard Michaelis-Menten kinetic analysis and were determined to be 1.979 mM and $3.169 \text{ nmol sec}^{-1}$ respectively (Fig 8B). The turnover number k_{cat} , defined as $V_{max}/[E]$, was 22.636 sec^{-1} .

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, and are summarized diagrammatically in Fig. 9. The complete datasets are available at ArrayExpress under accession no. XXX (Note: for review purposes, please see temporary Supporting Information Tables S2 and S3). 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 to bile, but each treatment led to differential expression of distinct gene clusters. As is evident from Fig. 9, cholate exposure caused differential expression of genes uniformly distributed around the chromosome and pMP118. Bile treatment affected expression of genes uniformly distributed round pMP118, but preferentially in the “top half” or *ori*-side of the chromosome. This is indicative that genes that are differentially regulated during bile stress also benefit from an increased gene dosage effect (52) by virtue of being close to the replication origin.

Challenge with 0.1% porcine bile resulted in a total of 123 and 68 genes being differentially expressed in LS201 and the LS201 $\Delta bsh1$ mutant, respectively, using as cut-off a p value < 0.05 and a ≥ 2 -fold expression change. Inability to produce BSH1 did not result in significantly different genes being expressed in response to bile, nor different levels of expression. The *bsh1* gene itself was not induced by bile exposure. In both the wild-type and *bsh1* mutant (Table S2), a conserved set of genes was up-regulated, 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 PTS system (LSL_1713-6) was highly induced (8-10 fold) by bile. Genes involved in 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; Fig. S2A), was up-regulated in both wild-type and *bsh1* mutant. This operon was also induced by cholate exposure (see below). Down regulated genes in both strains included those for a putative EPS biosynthesis cluster, prophage Sal2 (60), arginine and proline metabolism, amino acid transporters and a manganese transport protein.

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

The most significant changes were class I heat-shock genes (*groELS*, *grpE*); a gene (*hrcA*) encoding their repressor. Genes encoding other chaperone proteins (LSL_0578-9, LSL_0863) and

481 the ATP-dependent ClpP protease (LSL_1168) were also up-regulated by cholate, as was
482 expression of the *clpP* expression regulon *ctsR*.

483 A diverse collection of genes encoding transporters, efflux pumps, Na^+/H^+ antiporter, oxidase
484 proteins, reductase proteins, membrane proteins and diverse hydrolases were also up-regulated by
485 cholate exposure, indicating that the products of bile deconjugation put osmotic, oxidative, and pH
486 homeostasis burdens on *L. salivarius*.

DISCUSSION

Members of the species *L. salivarius* have a broad ecological distribution, reflected in the strains chosen for this study (Table 2). That all strains examined have a *bsh1* gene resident on their respective circular megaplasmid testifies to the biological selection on 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 draft genome sequence was available for one of these strains. When assessing the overall bile resistance phenotypes of the strains herein, 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, this strain showed deconjugation activity for TDCA. Phylogenetic analysis showed that the *L. salivarius* BSH1 and BSH2 protein are in two different branches of the BSH tree. 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 (41).

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 herein to have relatively low activity according to a traditional plate test, the MIC values of the strain for GDCA, porcine bile and bovine bile were as high, or higher, than almost all strains tested. More consistent with the plate assay, the recombinant 118BSH1 protein was less active against all substrates tested than the corresponding BSH1 protein from strain JCM1046, in particular against tauro-conjugated bile acids. The biochemical characterization also revealed a striking difference in preference for glyco-conjugated substrates shown by the 118BSH1 enzyme, compared with the tauro-conjugate preference shown by the 1046BSH1 enzyme. These differences

510 may be due in part to the internal deletion of eight residues in the group A BSH1 enzyme, including
511 Asn171, considered to be part of the conserved active site. However, there are 12 other single
512 amino acid residue differences (of which seven are conservative substitutions) between 1046BSH1
513 and 118BSH1, that may also be important. A structural comparison between these two enzymes
514 will give an interesting insight into how these changes impact on folding, substrate recognition and
515 activity. Notably however, disruption of the *bsh1* gene (LSL_1801) led to a dramatic reduction in
516 bile tolerance *in vitro*, and significant reduction in murine transit survival. It is significant therefore
517 that the relatively low activity of the 118BSH1 protein does not detract from its likely biological
518 importance. We were unable to purify soluble 47825BSH1 protein, corresponding to group B
519 proteins that harbor the internal deletion spanning Asn171 as well as the carboxy-terminal
520 truncation. Three of the five strains encoding group B BSH proteins are extra-intestinal in origin,
521 where production of active BSH would be less critical. However, *L. salivarius* strains of
522 extra-intestinal origin were not consistently more bile-sensitive than intestinal isolates. This
523 probably reflects the unreliability of assigning definitive origins/sources to strains of a species that
524 can survive in many niches.

525 The enzymatic properties of the more active BSH1 protein of JCM1046 are presented for
526 comparative purposes with other bacterial BSH enzymes in Table 4. The K_m of *L. salivarius*
527 1046BSH1 for TCA is higher than those from *L. johnsonii* and *B. longum*, suggesting that TCA is a
528 better substrate for these enzymes. However, the C-terminal His-tag present on the 1046BSH1
529 enzyme may affect the affinity for the substrate. Unlike BSH from *B. longum*, 10461BSH1 from *L.*
530 *salivarius* JCM1046 showed higher activity against tauro-conjugated bile acids than
531 glyco-conjugated ones. This may be related to different locations of lactobacilli and bifidobacteria
532 in the GI tract, or differences in the other bile detoxifying mechanisms in the respective species.

1046bsh2 is most similar to Lreu23DRAFT_0782 (JGI gene ID 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. Plate assay showed that JCM1046Δ*bsh1* had lost activity against TDCA and its activity against GDCA was retained, albeit at 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 118BSH1, 1046BSH1 and 1046BSH2 will provide valuable insights into related BSH molecules of a single species that have dramatically different activities and substrate profiles.

Exposure of *L. salivarius* early log-phase cells to bile or cholate did not induce the expression of *bsh1*. Genes for BSH were also not induced by bile in *L. acidophilus* (47) 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 MIC values 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* (47) 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 (47), 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.*

556 *salivarius*, although one gene product, LBA1425, shows 44% amino acid identity to LSL_1464,
557 which was significantly up-regulated by bile. LSL_1464 is a putative alpha-beta hydrolase of
558 unknown function that is conserved in other lactobacilli, *Listeria*, and some other Firmicutes, and
559 that merits functional characterization. A presumptive ABC transporter locus (Fig. S2A) that might
560 act as an efflux pump for bile was significantly up-regulated in *L. salivarius*, and this ABC
561 transporter locus is conserved in many bacteria (not shown). A second ABC transporter locus
562 induced by cholate (LSL_0031-0033) was 29% identical at protein level to Lr1265, which was
563 induced by bile stress in *L. reuteri* (62). Although altered carbon metabolism might be required to
564 maintain cellular ATP levels to energize bile export processes, it is more likely that induction of e.g
565 the mannose and sorbitol PTS systems is due to denaturation of their presumptive regulators.

566 The LSL_1335 gene was up-regulated 2.6 fold in response to bile. This gene encodes a
567 putative mucin-binding protein and candidate adhesion LspC, and was previously shown by us not
568 to be expressed *in vitro* (59). Bile may thus be used as a signaling molecule by commensal
569 lactobacilli like *L. salivarius* to modulate host-interaction genes. Consistent with this notion, genes
570 for three surface proteins of *L. acidophilus*, including two putative mucin-binding proteins, were
571 up-regulated upon bile exposure (47).

572 The toxicity of free bile acids produced by BSH activity can be avoided either through
573 catabolism or by export. According to the annotated genome, *L. salivarius* UCC118 has neither
574 7 α -dehydroxylate nor 7 α -dehydrogenate activity for unconjugated bile acid, nor does it contain
575 genes encoding cholate-CoA ligase (EC 6.2.1.7) which can further break down free bile acids
576 (cholate, deoxycholate and chenodeoxycholate). Thus the cholate-induced efflux pumps and
577 transporters likely play a role in removal of unconjugated bile acids in *L. salivarius* strains. Among
578 those cholate responsive transporters, the *L. salivarius* MDR transporter (LSL_0078, MDR protein

579 B) showed highly identity (71% and 59%) to the characterized MDR transporters from *L.*
580 *acidophilus* NCFM (LBA1429) (47) and *L. reuteri* ATCC55730 (lr1584) (62) which contribute to
581 bile resistance. Another cholate induced *L. salivarius* MDR transporter (Fig. S2B, LSL_0032-3)
582 are 52% and 53% identical to the *L. lactis* LmrCD cholate transporter, which also confer bile
583 resistance on these bacteria (64). The Opp system (LSL_2026-7) located on the 44-kb plasmid
584 pSF118-44 which is responsible for glycine-betaine uptake was also induced by cholate. These
585 gene products show high homology to the *L. monocytogenes* Bile system (57% and 45% identity
586 to BilB and BilA) which has been shown to enhance bile resistance when introduced into
587 *Bifidobacterium* and *Lactococcus* (61). Thus the products of BSH activity can induce the
588 expression of genes and gene products that potentiate the bile resistance phenotype of the organism,
589 potentially amplifying the phenotypic significance of the BSH kinetics of a particular enzyme
590 complement in a given strain.

591 BSH enzymes are clearly key contributors to bile resistance levels, and might conceivably be
592 the most important determinant under growth phase and nutritional conditions that cannot be
593 reproduced easily outside the gut. However, the *Lactobacillus* cell is equipped with a repertoire of
594 other mechanisms involved in protecting the cell against the inimical properties of bile, and which
595 result in a bile resistance level that cannot be predicted simply from consideration of the BSH
596 enzyme complement alone. Independent of its function in dietary fat emulsification, bile is a key
597 signaling molecule regulating its own biosynthesis, lipid absorption, cholesterol homeostasis, and
598 local mucosal defenses in the intestine (29). Weight gain in chickens is inversely correlated with
599 intestinal BSH activity, much of which comes from the dominant lactobacillus species in poultry, *L.*
600 *salivarius* (27). Excessive deconjugation of bile in the gut may be linked with “contaminated small
601 bowel syndrome” (23, 58). Thus it may be significant that *L. salivarius* strain UCC118, selected as

602 a human probiotic, has low BSH activity, but high overall bile resistance. This study shows the
603 complexity of bile-resistance level determination in commensal *L. salivarius* strains, the
604 integration of redundant mechanisms, and the potential for bile to act as an environmental cue in
605 probiotic lactobacilli.

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Figure legends

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

Fig. 2. Sequence alignment of *L. salivarius* LSL_1801 homologues encoding BSH1 enzymes. Sequences were aligned with ClustalW (<http://www.ebi.ac.uk/Tools/clustalw/>). Identical amino acids are marked by an asterisk, conserved and semi-conserved substitutions are marked by two dots and a single dot, respectively. Shaded residues are conserved amino acids implicated in active site. *L. salivarius* BSH1 gene amplicons were sequenced with primers FF029 and FF30 and were deposited in Genbank (Accession No. FJ591067-1083, FJ591085-92, and FJ607064).

Fig. 3. Detection of *L. salivarius* BSH activity by plate assay.
-, no BSH activity; +, w, positive bile salt hydrolase activity, production of opaque white colonies; +, p, positive, formation of precipitation; -/+, w, weak BSH activity, formation of opaque white colonies

Fig. 4. Relative expression of *bsh1* genes in 3 representative *L. salivarius* strains. Expression values graphed are ratios of *bsh1*: *groEL* gene expression in respective strains, normalized against the corresponding ratio in *L. salivarius* UCC118.

Fig. 5. 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 LS201 Δ *bsh1* genomic DNA were digested with *SpeI* (Lane 1-2) and hybridized with a labeled 582-bp amplicon of LSL_1801 (primers FF027 and FF028) as a probe. M, labeled DNA marker; 1, *L. salivarius* LS201 (derivative of *L. salivarius* UCC118 cured of pSF118-20; 2, *L. salivarius* LS201 Δ *bsh1*. DNA sizes are indicated by arrows.

(B) Schematic representation of the relevant regions of the LS201 and LS201 Δ *bsh1* genomes. Heavy lines represent megaplasmid DNA; thin lines represent plasmid DNA. *bsh1* (LSL_1801) is presented by the arrow, and the grey box is the *bsh1* internal fragment corresponding to the hybridization probe. *SpeI* sites are indicated

Fig. 6. 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. Big squares, *L. salivarius* LS201; triangles, *L. salivarius* LS201 Δ *bsh1*; cycles, *L. salivarius* LS201 Δ *lacZ*; diamonds, *L. salivarius* LS201 Δ *bsh1*(pLS219 [pLS209+118*bsh1*]); small squares, *L. salivarius* LS201 Δ *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* JCM1046 Δ *bsh1*

(C) Disruption of the *bsh1* gene of LS201 reduces survival during murine intestinal tract transit. Grey bars, *L. salivarius* LS201 Δ *bsh1*; white bars, *L. salivarius* LS201 Δ *lacZ*.

Fig. 7. Purification of recombinant *L. salivarius* BSH1 proteins.

Purification of *L. salivarius* 118BSH1 and 1046BSH1. M, broad range protein marker; E, *E. coli*

858 Rosetta DE3 cell lysate showing expression of His-tagged BSH1; H, IMAC-purified BSH1; G,
859 gel filtration-purified BSH1. Protein marker sizes are indicated.

860

861 Fig. 8. (A) pH and substrate dependence of *L. salivarius* BSH1 enzymes.

862 White bars, tauro-CBAs; Grey bars, glyco-CBAs; T/GDCA, tauro/glycodeoxycholate; T/GCA,
863 tauro/glycocholate; T/GCDCA, tauro/glycochenodeoxycholate

864 (B) Measurement of 1046BSH1 K_m and V_{max} for TCA

865

866 Fig. 9. Genome atlas of global bile-responsive and cholate-responsive transcriptomes in *L.*

867 *salivarius* strains.

868 Genome wheels with microarray results were generated by Microbial Genome Viewer

869 (<http://www.cmbi.ru.nl/genome/>). The scale represents the $\log_2$ transformation of the changes in

870 gene expression (treated/untreated) projected on a linear color gradient.

871 Table 1. Bacterial strains and plasmids

Strain or plasmid	Relevant properties ^a	Source or reference
Strains		
<i>L. salivarius</i>		
UCC118	Ileocaecal isolate from a human adult	(11)
LS201	pSF118-20 free derivative of strain UCC118	(21)
LS201Δ <i>bsh1</i>	LS201 integrant LSL_1801 (<i>bsh1</i>):pLS216	This work
LS201Δ <i>lacZ</i>	LS201 integrant LSL_0376 (<i>lacZ</i>):pLS217	This work
JCM1046Δ <i>bsh1</i>	JCM1046 integrant <i>bsh1</i> ::pLS218	This work
<i>E. coli</i>		
Top10	F ⁻ <i>mcrA</i> Δ(<i>mrr</i> - <i>hsdRMS</i> - <i>mcrBC</i>) φ80 <i>lacZ</i> ΔM15 Δ <i>lacX74</i> <i>recA1</i> <i>ara</i> Δ139 Δ(<i>ara-leu</i>)7697 <i>galU galK rpsL</i> (Str ^R) <i>endA1 nupG</i>	Invitrogen
Rosetta BL21(DE3)pLysS	F ⁻ <i>ompT hsdS_B</i> (r _B ⁻ m _B ⁻) <i>gal dcm</i> (DE3) pLysS (Cm ^R)	Invitrogen
<i>Lactococcus lactis</i>		
LL108	Strain with <i>repA</i> gene integrated in chromosome	(35)
Plasmids		
pORI19	Em ^r Ori ⁺ RepA ⁻ <i>lacZ</i> ⁻ derivative of pROI28	(33)
pPTPL	Tet ^r , promoter probe vector	(8)
pVE6007	Cm ^r , temperature sensitive, derivative of pWV01, lactococcal cloning vector	(40)
pLS209	Em ^r , <i>Lactobacillus</i> gene cloning vector, a derivative of pLS203 produced by PCR	(21)
pLS215	Tet ^r , derivative of pORI19, <i>erm</i> is replaced with <i>tet</i> from pPTPL	Unpublished results ^b
pLS216	Tet ^r , derivative of pLS215 containing a 558-bp internal gene fragment of <i>bsh1</i> (UCC118)	This work
pLS217	Tet ^r , derivative of pLS215 containing a 1002-bp internal gene fragment of <i>lacZ</i> (LSL_0376)	This work
pLS218	Em ^r , derivative of pORI19 containing a 582-bp internal gene fragment of <i>bsh1</i> (JCM1046)	This work
pLS219	Em ^r , derivative of pLS209 containing <i>bsh1</i> (LSL_1801) gene and its promoter region	This work
pOPINE	Amp ^r , derivative of pTriEx2 with a C-6 × His-tag fusion	OPPF ^c
pEB118	Amp ^r , derivative of pOPINE for expression of C-His-tagged <i>118bsh1</i>	This work
pEB1046	Amp ^r , derivative of pOPINE for expression of C-His-tagged <i>1046bsh1</i>	This work

872 ^a, Cm^r, chloramphenicol resistant; Em^r, erythromycin resistant; Tet^r, tetracycline resistant; Amp^r,

873 ampicillin resistant

874 ^b, contributed by Jan-Peter van Pijkeren

875 ^c, In-fusion cloning vector contributed by Oxford Protein Production Facility (OPPF)

876 Table 2. BSH activity and bile resistance of *L. salivarius* strains

BSH1 group	Strain	Origin	Plate assay activity		MIC		
			TDCA	GDCA	GDCA (mM)	Bovine bile (%)	Porcine bile (%)
A	UCC118	Human ileal-caecal region	-/+ (w)	-	6	>20	>5.0
A	NCIMB 8818	St. Ivel cheese	-/+ (w)	-	5	10	1.0
A	CCUG 27530B	Human abdomen	-/+ (w)	-	5	7.5	0.3
A	JCM 1047	Swine intestine	-	-	4	6.0	0.2
B	CCUG 47825	Human blood	-	-	4	>20	>5.0
B	CCUG 45735	Human blood	-	-	6	>20	>5.0
B	CCUG 38008	Human gall	-	-	6	15	>5.0
B	CCUG 47826	Human blood	-	-	6	15	>5.0
B	L21	Human feces	-	-	4	15	1.0
B	AH 4231	Human ileum-caecal	-	-	6	12	0.5
C	JCM 1046	Swine intestine	+ (p)	+ (p)	>15	>20	>5.0
C	LMG 14476	Cat with myocarditis	+ (p)	+ (p)	>15	>20	>5.0
C	DSM 20492	Human saliva	+ (w)	-	10	>20	>5.0
C	01M14315	Human gallbladder pus	+ (w)	-	6	>20	>5.0
C	JCM 1040	Human intestine	+ (w)	-	6	15	>5.0
C	CCUG 44481	Bird	+ (p)	-	4	>20	>5.0
C	DSM 20555	Human saliva	+ (w)	-	4	12	>5.0
C	JCM 1045	Human intestine	+ (p)	-	5	>20	1.5
C	CCUG 47171	Human tooth plaque	+ (p)	-	5	12	1.0
C	CCUG 43299	Human blood	+ (w)	-	6	>20	0.4
C	NCIMB702343	unknown	+ (p)	-	5	>20	0.4
C	DSM 20554	Human saliva	+ (p)	-	5	10	0.4
C	NCIMB 8817	Turkey feces	+ (p)	-	6	10	0.8
C	UCC119	Chicken intestine	+ (w)	-	4	15	0.2
D	NCIMB 8816	Human saliva	+ (p)	-	6	12	>5.0
D	JCM 1042	Human intestine	-	-	4	10	1.0
	JCM 1230	Chicken intestine	-	-	6	>20	>5.0
	LS201	UCC118 derivative	-	-	>15	>20	>5.0
	LS201Δ <i>bsh1</i>	This study	-	-	3	4.0	0.1
	JCM1046Δ <i>bsh1</i>	This study	-	+ (p)	6	>20	>5.0

877 TDCA, sodium taurodeoxycholate hydrate; GDCA, sodium glycodeoxycholate; MIC, minimum
878 inhibitory concentration; -, no BSH activity; + (w), positive bile salt hydrolase activity, production
879 of opaque white colonies; + (p), positive, formation of precipitation; -/+ (w), weak BSH activity,
880 formation of opaque white colonies; >, strain is resistant to the highest concentration of bile tested.

Table 3. Comparison of number of genes ordered in COG categories significantly affected by bile extract porcine with that by cholate

Bile/Cholate induced gene regulation		Up-regulated				Down-regulated			
Functional categories		LS201		LS201 Δ bsh		LS201		LS201 Δ bsh	
		bile	cholate	bile	cholate	bile	cholate	bile	cholate
Information storage and processing		3	17	2	26	6	37	5	65
J	Translation, ribosomal structure and biogenesis	1	0	1	2	0	21	0	42
A	RNA processing and modification	0	0	0	0	0	0	0	0
K	Transcription	2	8	1	11	1	6	3	12
L	Replication, recombination and repair	0	9	0	13	5	10	2	11
B	Chromatin structure and dynamics	0	0	0	0	0	0	0	0
Cellular processes and signaling		8	49	7	68	8	25	9	43
D	Cell cycle control, cell division, chromosome partitioning	0	0	0	2	1	4	1	4
V	Defense mechanisms	2	13	2	18	2	5	1	13
T	Signal transduction mechanisms	0	2	0	4	2	4	2	5
M	Cell wall/membrane/envelope biogenesis	4	7	4	9	2	9	5	15
N	Cell motility	0	0	0	1	0	1	0	1
Z	Cytoskeleton	0	0	0	0	0	0	0	0
W	Extracellular structures	0	0	0	0	0	0	0	0
U	Intracellular trafficking, secretion, and vesicular transport	0	1	0	1	1	2	0	2
O	Posttranslational modification, protein turnover,	2	26	1	33	0	0	0	3
Metabolism		37	134	17	165	28	93	24	150
C	Energy production and conversion	5	13	3	20	1	3	1	7
G	Carbohydrate transport and metabolism	23	18	8	22	3	25	6	37
E	Amino acid transport and metabolism	3	58	3	64	19	32	13	59
F	Nucleotide transport and metabolism	0	1	0	2	2	3	1	6
H	Coenzyme transport and metabolism	0	12	0	12	0	0	0	2
I	Lipid transport and metabolism	1	5	1	10	0	12	0	14
P	Inorganic ion transport and metabolism	5	23	2	29	3	12	3	18
Q	Secondary metabolites biosynthesis, transport and	0	4	0	6	0	6	0	7
Poorly characterized		20	40	4	55	5	21	2	32
R	General function prediction only	6	32	4	44	2	14	1	22
S	Function unknown	3	8	0	11	3	7	1	10
Unknown COG functions		11	0	0	2	0	0	0	0
Total number of gene expressed differentially		64	189	34	248	59	151	34	225

Table 4. Comparison of BSH enzymatic properties.

Enzyme	Sub unit MW/kDa	pH	Substrate preference	K_m (mM TCA)	K_m (mM GCA)	Reference
LS1046BSH1	36.5	3.5-7.5	tauro-CBA	1.976	ND	This study
LJBSHA/B	42	3.8-4.5	equal	0.76/0.95	NT	(39)
LABSH	34	5-7	tauro-CBA	NT	NT	(46)
BLBSH	35	5-7	glyco-CBA	1.12	0.16	(56)
BLBSH	-	6.5	equal	0.032	0.022	(30)
BFBSH	32.5	4.2	equal	0.45	0.35	(54)
BRBSH	28	3-11	glyco-CBA	NT	3.08 μ M (GDCA)	(53)
CPBSH	56	5.8-6.4	glyco-CBA	NT	0.5	(22)
XMCGH	52	7.9-8.5	equal	NT	1.1	(15)

LS, *L. salivarius*; LJ, *L. johnsonii*; LA, *L. L. acidophilus*; BL, *Bifidobacterium longum*; BF, *Bacteroides fragilis*; BR, *Brevibacillus* sp; CP, *Clostridium perfringens*; XM, *Xanthomonas maltophilia*; CGH, cholylglycine hydrolase; equal, BSH does not differ in substrate specificity; ND, not detectable; NT, not tested

Supplemental material

Fig. S1. Phylogenetic analysis of BSH from *Lactobacillus* and other bacteria

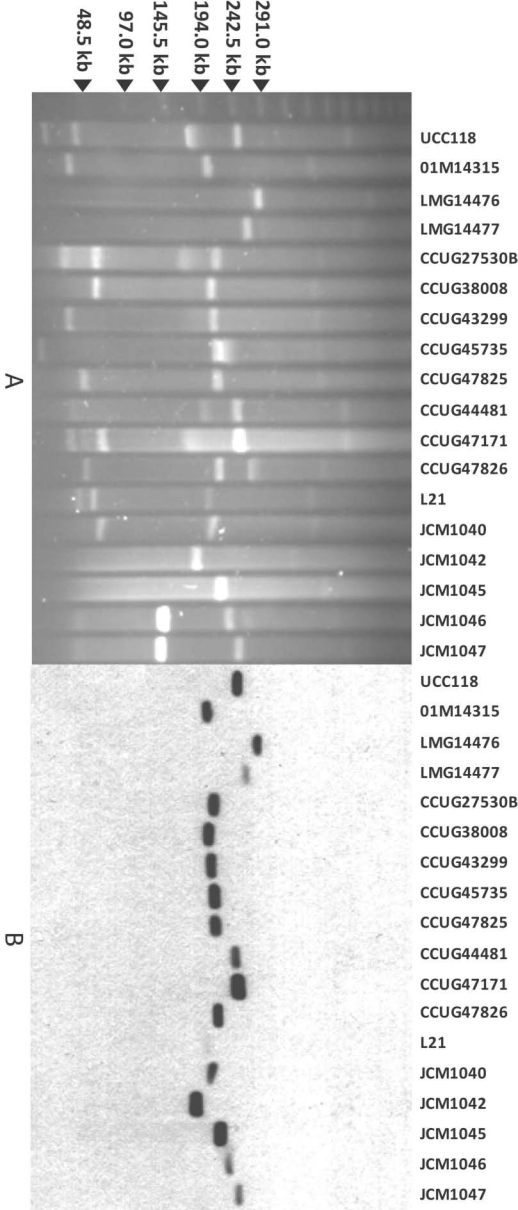
Neighbor-joining tree of BSH from different bacteria. Sequences were aligned and analyzed by MEGA4 using the *p*-distances amino acid model with 500 bootstrap replications. P12256 (penicillin V acylase) from *Bacillus sphaericus* was used as an outgroup. Accession numbers are to right of strain name abbreviations. BL, *Bifidobacterium longum*; BS, *Bacillus sphaericus*; CP, *Clostridium perfringens*; EF, *Enterococcus faecium*; LA, *L. acidophilus*; LG, *L. gasseri*; LJ, *L. johnsonii*; LM, *Li. monocytogenes*; LP, *L. plantarum*; LR, *L. reuteri*; LS, *L. salivarius*; MS, *Methanobrevibacter smithii*

Fig. S2. Bile and cholate induced ABC transporter operons in *L. salivarius*

Table S1. Primers used in this study

Table S2. Genes significantly up/down-regulated by porcine bile extract (expression ratio ≥ 2 -fold, $p < 0.05$)

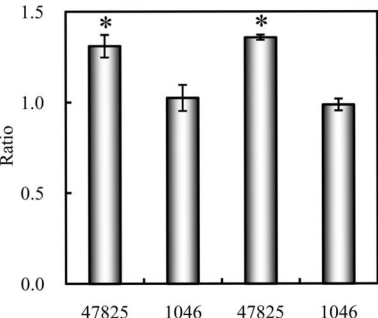
Table S3. Genes significantly induced by cholate (expression ratio ≥ 2 -fold, $p < 0.05$, ArrayExpress accession no. XXX)

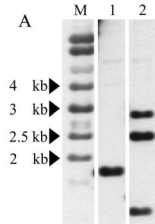


Strain	BSH1 Group	MRS	TDCA	GDCA	BSH activity	
					TDCA	GDCA
UCC118	A				-/+, w	-
CCUG47825	B				-	-
JCM1046	C				+, p	+, p
NCIMB8816	D				+, w	-

stationary phase

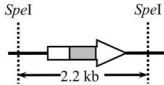
log phase



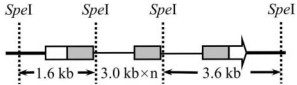


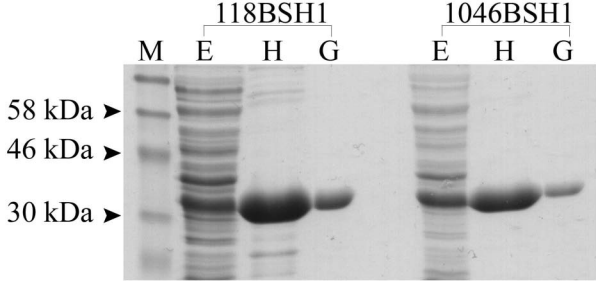
B

LS201

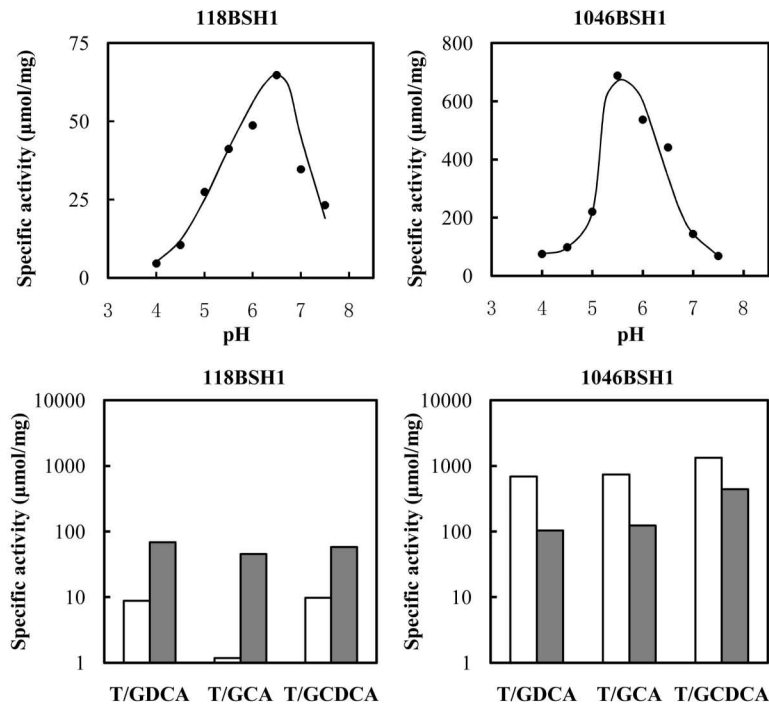


LS201 $\Delta bsh1$

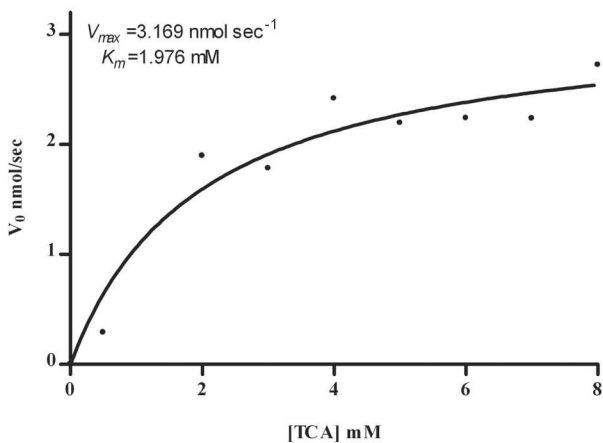


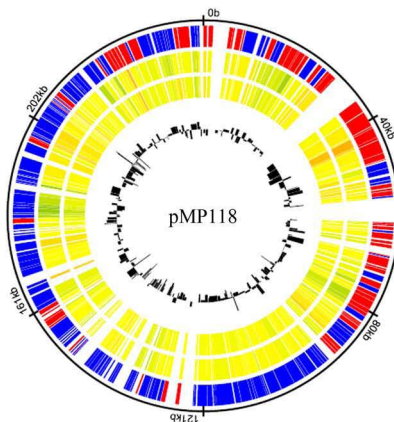
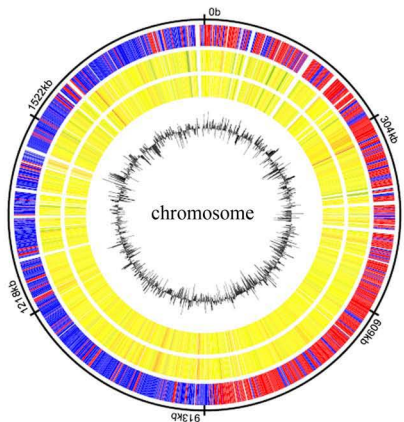


A



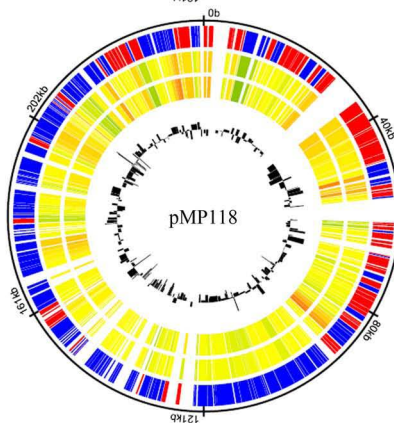
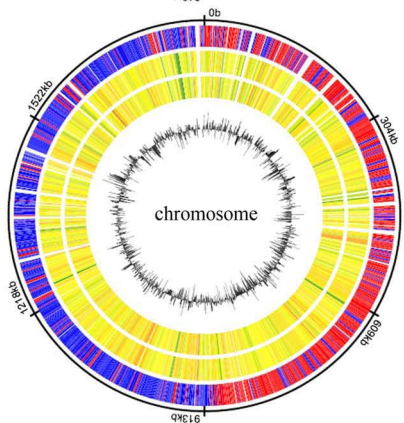
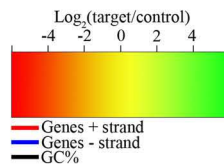
B





bile transcriptome

Transcriptome
Inner ring 1: LS201Δ*bsh1*
ring 2: LS201



cholate transcriptome