Fang F., Li Y., Bumann M., Raftis E.J., Casey P.G., Cooney J.C., Walsh M.A., O'Toole P.W. 2009. Allelic variation of bile salt hydrolase genes in Lactobacillus salivarius does not determine bile resistance levels. Journal of Bacteriology 191(18):5743-57. doi: 10.1128/JB.00506-09

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

Fang Fang[1, 2], Yin Li[1, 2†], Mario Bumann[3], Emma J. Raftis[1], Patrick G. Casey[1, 2], Jakki C. Cooney[4], Martin A. Walsh[3], and Paul W. O'Toole[1, 2]*

Department of Microbiology [1], Alimentary Pharmabiotic Centre [2], University College Cork, Ireland.

MRC France, BM14, c/o ESRF, 6 rue Jules Horowitz, B.P. 220, 38043 Grenoble, France[3]

Department of Life Sciences, and Materials and Surface Science Institute, University of Limerick, Ireland[4]

* For correspondence. Email: pwotoole@ucc.ie; Tel. (+353) 21 490 3997; Fax: (+353) 21 4903101.

[†]Present address: Institute of Microbiology, Chinese Academy of Sciences, Beijing 100080, P. R. China

Running title: *Lactobacillus salivarius* bile salt hydrolase and bile resistance

Keywords: *Lactobacillus salivarius*, bile resistance, bile salt hydrolase, microarray, megaplasmid, transporter

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

64 required for the persistence of *L. monocytogenes* in the murine intestine (3) and for the ability of
65 *Brucella* to infect mice (16).

66 The presence and genetic organization of *bsh* genes in lactobacilli is very variable. In addition
67 to presence in single copy in some species, multiple copies of *bsh* were annotated in *Lactobacillus*
68 *acidophilus* NCFM (*bshA* and *bshB*), *Lactobacillus johnsonii* NCC533 (three genes) and
69 *Lactobacillus gasseri* ATCC33323 (two genes) (32). In some *Lactobacillus* strains, *bsh* was part of
70 an operon (20). Disruption and deletion of *bsh* in lactobacilli caused loss of corresponding activity
71 against tauro/glyco-conjugated bile acids (CBA) (36, 42). Resistance of *bsh* mutants of
72 *Lactobacillus amylovorus* and *Lactobacillus plantarum* to bile acids/salts was reduced compared to
73 the respective wild type strains (13, 14, 25). However, no convincing *in vivo* experiments have so
74 far demonstrated that *bsh* contributes to bile resistance in these or other probiotic bacteria. A triple
75 *bsh* mutant of *L. johnsonii* NCC533 (i.e. lacking all three BSH proteins) did not exhibit
76 significantly reduced murine gut persistence compared to the parental strain (17). The role of *bsh*
77 in intestinal tract survival of probiotic lactobacilli is generally unclear.

78 BSH enzymes from a variety of sources differ in structure, substrate specificity, and optimal
79 temperature and pH range for enzyme function (24, 49, 53). BSH subunit sizes range from 28 kDa
80 to 56 kDa, and the enzymes are generally more active at an acidic pH range (4-7). The most
81 thermostable BSH was detected in *Brevibacillus* sp whose optimal temperature is 60^{o}C (53). BSH
82 enzymes recognize bile acids on both the cholate steroid nucleus and the amino acid moiety. The
83 crystal structure of *C. perfringens* BSH revealed that activity is conferred by a hydrophobic pocket
84 that recognizes the cholyl moiety of the substrate (49). The crystal structures and biochemical
85 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

Lactobacillus reuteri ATCC55730 (47, 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 harbours a 242 kb megaplasmid pMP118, that interdigitates with chromosomally-encoded functions to confer metabolic flexibility (37, 45). *L. salivarius* is common in the gastrointestinal tract of many animals including humans (1), and chickens (27), but its survival mechanisms *in vivo* are poorly understood. In this study, we therefore examined the contribution of allelic variants of *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% CO_2) 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

142 RNA-easy kit (Ambion, Cambridgeshire, United Kingdom). Random primers were purchased
143 from MWG Biotech, Germany. 500 ng of RNA was reverse transcribed using Improm-II reverse
144 transcriptase (Promega). PCR amplification was performed according to the manufacturer's
145 instructions. Briefly, a 12.5 µl PCR reaction consisted 6.25 µl 2 × master mix (Biogene, United
146 Kingdom), 50 nM of each primer, 1/60,000 SYBR green I (Biogene, United Kingdom) and 1 µl
147 cDNA. The qRT-PCR amplifications were performed on an ABI Prism 7000 using SYBR green I.
148 **Type I microarray procedures.** The *L. salivarius* array contains 1500 Agilent quality control
149 spots and 60 nt oligonucleotides corresponding to 2184 genes (including annotated pseudogenes)
150 in the genome of *L. salivarius* UCC118. A maximum of four probes 21 replicates for each gene
151 were designed from each open reading frame (smaller genes have fewer probes) by eArray
152 (https://earray.chem.agilent.com/earray/, Agilent Technologies). These probes were spaced
153 throughout the coding regions and designed to have melting temperatures between 58 ^{o}C and 60^{o}C.
154 The probes were printed in spots, were randomly distributed across the array, and were printed by
155 Agilent Technologies. The array design and microarray data can be found at EMBL-EBI
156 ArrayExpress under accession no. XXX1 and XXX2, respectively.
157 Overnight cultures of *L. salivarius* UCC118, LS201 and LS201Δ*bsh1* were diluted 50 fold in
158 MRS without antibiotics and grown at 37^{o}C to an OD_{600} of 0.3. The cultures were divided in two
159 and were either untreated or treated with 0.1% porcine bile or 1mM cholate (sodium cholate
160 hydrate, Sigma C6445). After 15 min incubation, 12 ml samples were harvested by centrifugation
161 (13,000 × g for 15 sec) at room temperature. Cell pellets were washed once with RNAprotect
162 Bacteria Reagent (Qiagen) and immediately frozen at -80^{o}C. Cells were disrupted by a bead-beater
163 homogenizer (three times 1 min treatment with 1 min intervals on ice). Total RNA was isolated
164 using the SV Total RNA Isolation System (Promega) with an additional 30 min TURBO DNase

165 treatment. RNA quality was checked by Agilent Bioanalyzer 2100 using the RNA 6000 Nano assay
166 kit (Agilent). 4 μg RNA derived from cells treated or untreated was used for complementary DNA
167 synthesis and labeled with Cy3/5-dCTP (GE Healthcare Life Sciences) with a SuperScript™ II
168 reverse transcriptase kit (Invitrogen) at 42^{o}C for 90 min. Cy3-and Cy5- labeled cDNAs were
169 purified using the MinElute PCR purification kit (Qiagen) and quantified using the NanoDrop
170 ND-1000 UV-VIS spectrophotometer (NanoDrop Technologies, Rockland, DE). An Agilent Oligo
171 aCGH/CHIP-on chip hybridization kit was used for hybridization. Hybridizations were performed
172 in an Agilent hybridization oven (G2545A) at 65^{o}C for 24 hrs. Slides were scanned using Agilent
173 Microarray Scanner System (G2505B) with Agilent scan control software version 7.0 for the 44k
174 microarray at resolution of 5μm and Red and Green PMT at 10. Agilent Feature Extraction
175 software verion 9.1 was used for feature extraction. Microarray data outliers were removed with
176 the Grubbs test (26). *P* values were calculated according to the Cyber-T test (38).

177 **Phylogenetic analysis.** BSH sequences were aligned by ClustalW provided by Molecular
178 Evolutionary Genetics Analysis (MEGA) software version 4 (55). The neighbor-joining tree of
179 BSH sequences was built by running MEGA4 using the *p*-distances amino acid model with 500
180 bootstrap replications. Penicillin V acylase (PVA) (P12256) from *Bacillus sphaericus* that belongs
181 to the same choloylglycine hydrolase family (CBAH, PF02275 [http://pfam.sanger.ac.uk]) as BSH
182 was used as an outgroup.

183 **Construction of *L. salivarius bsh1* and *lacZ* mutants.** *L. salivarius bsh1* and *lacZ* integrants
184 were obtained by plasmid integration as described previously (59). Primer pairs FF025-FF026,
185 FF027-FF028 and JP076-JP081 were used to PCR amplify internal fragments of *bsh* (*1046bsh1*
186 and LSL_1801) and *lacZ* (LSL_0376), respectively. The corresponding PCR products were
187 restricted with *Bam*HI and *Eco*RI and ligated to similarly digested pORI19 or pLS215. *L. lactis*

188 LL108 was used as the cloning host for these constructs. The resulting plasmids pLS216 and
189 pLS217 were transformed into *L. salivarius* LS201 for construction of the *bsh1* (LSL_1801)
190 integrant (LS201Δ*bsh1*) and the *lacZ* (LSL_0376) integrant (LS201Δ*lacZ*). pLS218 was
191 transformed into *L. salivarius* JCM1046 to generate the *1046bsh1* integrant JCM1046Δ*bsh1*.
192 Integrants of pORI constructs were selected through curing of pVE6007 by growth at elevated
193 temperature, as described previously (59).

194 **BSH plate assay, bile minimum inhibition concentration (MIC) assay and bile challenge**
195 **experiment procedures.** *L. salivarius* strains were tested for hydrolase activity against tauro- or
196 glyco-conjugated bile acids (CBA) by using a plate assay method (12). Overnight MRS broth
197 cultures were streaked on MRS agar supplemented with 0.5 % (wt/vol) sodium taurodeoxycholate
198 hydrate (TDCA, Sigma T0875) or 2 mM sodium glycodeoxycholate (GDCA, Sigma G3258). The
199 plate was then incubated anaerobically for 48 hrs at 37^{o}C. BSH activity was detectable when
200 deoxycholic acid precipitated in the agar medium below and around a colony. For detecting BSH
201 activity of *E. coli* expressing various constructs, an optimized LB bile acids medium (for 1 l, agar
202 15 g, tryptone 10 g, yeast extract 5 g, NaCl 5 g, $CaCl_2$ $2H_2O$ 0.35 g, glucose 10 g, IPTG 1 mM,
203 pH6.5) containing 5 g/l TDCA or 2mM GDCA (10) was used.

204 To measure minimum inhibition concentrations (MIC), overnight cultures of *L. salivarius*
205 strains were inoculated at 1% into MRS medium containing different concentrations of porcine or
206 bovine bile (Sigma, B8631 and B8381) or GDCA. Cultures were then incubated at 37^{o}C for 24 hrs
207 and 10 µl was spotted on MRS agar plates. Growth on the plate was indicative of resistance to the
208 corresponding bile/bile salt concentration of the strain.

209 For survival experiments, *L. salivarius* LS201, JCM1046 and the corresponding *bsh1* or *lacZ*
210 integrants were grown to stationary phase. The cells were harvested by centrifugation. The cells

were washed once with MRS broth followed by resuspension in MRS broth containing a sub-lethal concentration of porcine bile (0.2% for LS201 and its derivatives, 0.1% for JCM1046 and its derivative) and incubated at 37^{o}C (5 % CO_2) for 5 hrs. Samples were removed from the culture at different time intervals, were diluted, and plated on MRS, MRS Em 5 or MRS Tet 5 plates for viable cell counting.

Expression and purification of BSH. The genes for 118BSH1 (LSL_1801) or its homologs from strain JCM1046 were amplified by PCR using primers EBF-EB118R or EBF-EB1046R. Purified PCR products were cloned into the linearised T7 promoter-based pOPINE expression vector (OPPF) by In-Fusion™ reactions. The resulting plasmids pEB118 and pEB1046 were transformed into strain *E. coli* BL21(DE3)pLysS for overexpressing C-6 × His-tagged *bsh*. For production of BSH, 40 ml of *E. coli* BL21(DE3)pLysS (pEB118 or pEB1046) overnight culture for expression of corresponding *bsh* was inoculated into a biofermentor (Biolab, B. Braun Biotech Ltd., Germany) charged with 2 l of LB medium supplemented with 34 μg/ml Cm and 50 μg/ml Amp. The culture was grown at 37^{o}C with oxygen supplementation and agitation of 200 rpm to an OD_{600} value of 0.6. The culture was then induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) at 37^{o}C for 5 hrs. For expression of *118bsh1*, the culture was immediately cooled to 20^{o}C followed by induction with 0.1mM IPTG for 20 hrs. The cells were harvested by centrifugation and the cell pellet was resuspended in 80 ml 50 mM Tris-HCl pH7.5, 500 mM NaCl, 20 mM imidazole. Cells were disrupted by sonication. Cell debris was removed by centrifugation at 45,000 ×g for 30 min at 4^{o}C. BSH was purified by immobilized metal ion affinity chromatography (IMAC) and gel filtration with the ÄKTAprime™ plus FPLC (GE Healthcare Life Sciences). BSH was eluted with buffer (50 mM Tris-HCl pH7.5, 500 mM NaCl, 500 mM imidazole) from a HisTrap HP 1ml 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 µl 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 37ºC (30 min for 1046BSH1 or 3 hrs for 118BSH1). 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. 5 µl of the supernatants or their appropriate dilutions was mixed with 95µl 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^{o}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 µmol 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 10^9 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 votexing 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 *Bacilllus 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 megaplasmids 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-N_{6-8}-TTGATAC-TYTWAT-A-GGAAG-N_8-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

366 modestly but significantly ($p < 0.01$) higher than that of strain UCC118. The increase was only
367 1.36-fold and 1.31-fold for exponential and stationary phases, respectively. Notwithstanding minor
368 sequence differences, the consensus promoter region of *bsh1* and the qRT-PCR data collectively
369 indicate that *bsh1* is transcribed at broadly similar levels in the *L. salivarius* strains examined. Thus,
370 the lack of correlation of *L. salivarius* bile resistance levels and their BSH1 grouping is probably
371 not due to the transcription level of *bsh1*.
372
373 **Biological characterization of BSH1 enzymes in *L. salivarius*.** To further characterize the
374 function of *bsh1*, the gene was interrupted in strain LS201, and strain JCM1046, by plasmid
375 integration. LS201 is a derivative of UCC118 generated by curing of resident plasmid pSF118-20;
376 this strain was used to allow complementation of the mutated *bsh1* allele with a copy cloned into a
377 low-copy number vector that we derived from pSF118-20 (21). The integration of plasmid pLS216
378 into pMP118 in strain LS201Δ*bsh* was confirmed by Southern hybridization (Fig. 5A). A control
379 integrant strain of LS201 (LS201Δ*lacZ*) was constructed by disruption of the *lacZ* gene with
380 plasmid pLS217. JCM1046Δ*bsh1* was generated by integration of pLS218 into the megaplasmid
381 pMP1046. Disruption of *bsh1* in strain LS201 led to a significant reduction in resistance to porcine
382 bile (Fig. 6A). The relative survival rates of LS201, LS201Δ*lacZ* and LS201Δ*bsh* were 93%, 29%
383 and 0.2% after 2 hrs of bile challenge. The cell numbers of LS201Δ*bsh* were reduced by four logs
384 after 5 hrs bile challenge. Expression of *bsh1* (LSL_1801) *in trans* from its native promoter
385 (i.e.when cloned in plasmid pLS219) restored the bile resistance of LS201Δ*bsh* to the resistance
386 level of the LS201Δ*lacZ* integrant. Transformation by the empty vector pLS209 had no effect (Fig.
387 6A). The JCM1046Δ*bsh1* mutant also appeared more sensitive to bile than the wild type strain (Fig.
388 6B), 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 hrs of bile challenge. 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* as responsible for activity against GDCA. Paradoxically however, the GDCA MIC values for LS201Δ*bsh* and JCM1046Δ*bsh1* were also decreased compared to their parental strains (Table 2), indicating some degree of activity of *bsh1* against both TDCA and GDCA (see also below).

Porcine bile was used for the bile challenge experiment, because it is very similar in composition to human bile (44). 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 rifampicin or streptomycin-resistant derivatives of the *bsh1* mutant (LS201Δ*bsh*) 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 phosphate-buffered saline (PBS), no antibiotic resistant bacteria were detected in faeces at any time points. Both LS201Δ*bsh* and LS201Δ*lacZ* strains were detected 2 hrs after administration (Fig. 6C), but there were significantly more cells recovered of LS201Δ*lacZ* than that of LS201Δ*bsh* at times 2, 4, and 6 hours after administration. Cells of the LS201Δ*bsh* mutant could not be cultured 24 hours after administration, whereas the LS201Δ*lacZ* strain was still detectable in faeces 3 days after the oral administration. Survival of strain LS201Δ*bsh* 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 *118bsh1* and *1046bsh1* 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 harboured the construct for expressing 1046BSH1 showed deconjugation activity on both TDCA and GDCA (data not shown); *E. coli* harbouring the *118bsh1* construct had no detectable BSH activity. 118BSH1 and 1046BSH1 were over-expressed in *E. coli* Rosetta BL21 (DE3) and purified (Fig. 7). The predicted molecular weights of 118BSH and 1046BSH1 are 35,714 Da and 36,494 Da, respectively. Based on their elution profiles on calibrated size exclusion chromatography columns, both 118BSH1 and 1046BSH1 were a mixture of dimer and monomer forms (data not shown), although the dimer to monomer ratio for 1046BSH1 (2:1) was twice that for 118BSH1 (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 (118BSH1 and 1046BSH1) on a range of tauro- and glyco- CBAs was undertaken (Fig 8A). This indicated that the enzymes had different substrate preferences. The 118BSH1 had greater activity against the glyco-CBAs than against the tauro-CBAs. The limited activity against tauro-CBAs varied with very low (<1μmol/mg) activity against the TCA compared with 8.8 and 9.8 μmol/mg for TDCA and TCDCA respectively. Activity against the better substrates, glyco-CBAs, also indicated that the CA conjugate was the poorest of the three tested (68, 45, 58 μmol/mg for GDCA, GCA and GCDCA respectively), and showed a clear preference for glyco-conjugated bile acids. Of significance was the switch in substrate preference for the 1046BSH1 enzyme, with clearly higher catalytic capabilities against the tauro-conjugated substrates. The activity of this enzyme against glyco-conjugated substrates was higher (ranging from 104-441 μmol/mg) than the 118 enzyme activity for all substrates tested. More importantly, there was a very large increase in the activity against the tauro-CBAs, with a specific activity of > 1300 μmol/mg against the best substrate

435 TCDCA, compared with < 10 μmol/mg for the best tauro-conjugated substrate tested with
436 118BSH1. This would suggest that the 8 amino acid deletion in the 118BSH1 enyzyme has a
437 dramatic impact on the rate of hydrolysis and substrate selection.

438 It is evident from data presented Fig. 8 that both variants of BSH1 have activity over a broad
439 range of pH but with a slight shift in pH optima. 1046BSH1 had maximal activity at pH 5.5 and
440 118BSH1 an optimum of pH 6.5. The more active 1046BSH1 enzyme was chosen for kinetic
441 analysis. Using TCA as a substrate (0.5 – 8 mM), the K_m and V_{max} were calculated using a standard
442 Michaelis-Menten kinetic analysis and were determined to be 1.979 mM and 3.169 nmol sec^{-1}
443 respectively (Fig 8B). The turnover number k_{cat}, defined as V_{max}/[E], was 22.636 sec^{-1}.

444

445 **The bile and cholate-induced transcriptomes of *L. salivarius*.** To identify mechanisms for
446 resisting bile other than BSH, and ways in which the deconjugation products of BSH might be
447 dealt with, the bile and cholate induced transcriptomes of *L. salivarius* were investigated, and are
448 summarized diagrammatically in Fig. 9. The complete datasets are available at ArrayExpress under
449 accession no. XXX (Note: for review purposes, please see temporary Supporting Information
450 Tables S2 and S3). Responsive genes were located on both the chromosome and the megaplasmid
451 pMP118, with some discrete clusters evident. Many more genes were differentially expressed upon
452 exposure to cholate than to bile, but each treatment led to differential expression of distinct gene
453 clusters. As is evident from Fig. 9, cholate exposure caused differential expression of genes
454 uniformly distributed around the chromosome and pMP118. Bile treatment affected expression of
455 genes uniformly distributed round pMP118, but preferentially in the "top half" or *ori*-side of the
456 chromosome. This is indicative that genes that are differentially regulated during bile stress also
457 benefit from an increased gene dosage effect (52) by virtue of being close to the replication origin.

458 Challenge with 0.1% porcine bile resulted in a total of 123 and 68 genes being differentially
459 expressed in LS201 and the LS201 Δ*bsh1* mutant, respectively, using as cut-off a p value < 0.05
460 and a ≥ 2-fold expression change. Inability to produce BSH1 did not result in significantly different
461 genes being expressed in response to bile, nor different levels of expression. The *bsh1* gene itself
462 was not induced by bile exposure. In both the wild-type and *bsh1* mutant (Table S2), a conserved
463 set of genes was up-regulated, including those involved in carbohydrate transport and metabolism,
464 energy production and conversion, cell wall/membrane/envelope biogenesis, amino acid transport
465 and metabolism, and inorganic ion transport and metabolism. A mannose specific PTS system
466 (LSL_1713-6) was highly induced (8-10 fold) by bile. Genes involved in transport and metabolism
467 of other carbohydrates such as glycerol, galactose, rhamnose, and sorbitol were also induced by
468 bile. A putative ABC transporter operon (LSL_0220-0222; Fig. S2A), was up-regulated in both
469 wild-type and *bsh1* mutant. This operon was also induced by cholate exposure (see below). Down
470 regulated genes in both strains included those for a putative EPS biosynthesis cluster, prophage
471 Sal2 (60), arginine and proline metabolism, amino acid transporters and a manganese transport
472 protein.

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

479 The most significant changes were class I heat-shock genes (*groELS*, *grpE*); a gene (*hrcA*)
480 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-congugated 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

may be due in part to the internal deletion of eight residues in the group A BSH1 enzyme, including Asn171, considered to be part of the conserved active site. However, there are 12 other single amino acid residue differences (of which seven are conservative substitutions) between 1046BSH1 and 118BSH1, that may also be important. A structural comparison between these two enzymes will give an interesting insight into how these changes impact on 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 118BSH1 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 Asn171 as well as the carboxy-terminal truncation. Three of the five strains encoding group B BSH proteins are extra-intestinal in origin, where production of active BSH would be less critical. However, *L. salivarius* strains of extra-intestinal 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 are presented for comparative purposes with other bacterial BSH enzymes in Table 4. The K_m of *L. salivarius* 1046BSH1 for TCA is higher than those from *L. johnsonii* and *B. longum*, suggesting that TCA is a better substrate for these enzymes. However, the C-terminal His-tag present on the 1046BSH1 enzyme may affect the affinity for the substrate. Unlike BSH from *B. longum*, 10461BSH1 from *L. salivarius* JCM1046 showed higher activity against tauro-conjugated bile acids than glyco-conjugated ones. 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.

533 *1046bsh2* is most similar to Lreu23DRAFT_0782 (JGI gene ID 639134569), which is not
534 biologically characterized. The *bsh2* gene is the only additional enzyme related to bile degradation
535 that we annotated in the JCM1046 draft genome sequence. Plate assay showed that
536 JCM1046Δ*bsh1* had lost activity against TDCA and its activity against GDCA was retained, albeit
537 at reduced level compared to the wild type strain JCM1046. This suggested that *bsh2* was
538 responsible for enhanced GDCA resistance in strains JCM1046 and LMG14476. Detailed
539 structural comparison of 118BSH1, 1046BSH1 and 1046BSH2 will provide valuable insights into
540 related BSH molecules of a single species that have dramatically different activities and substrate
541 profiles.

542 Exposure of *L. salivarius* early log-phase cells to bile or cholate did not induce the expression
543 of *bsh1*. Genes for BSH were also not induced by bile in *L. acidophilus* (47) contrasting with
544 induction of *bsh* expression in *L. plantarum* WCFS1 by bile (7) and in *B. longum* NCC2705 by
545 simulated intestinal stress conditions (63). It remains possible that *L. salivarius bsh1* expression is
546 inducible *in vivo*, modulated by factors other than bile. Bile exposure caused differential
547 expression of a large set of genes whose products are implicated in the BSH-independent bile MIC
548 values of the strains tested. The altered cell activities are primarily in the categories of
549 carbohydrate metabolism, cell surface remodeling, stress response, and transport/efflux, many of
550 which are readily rationalized as contributing to resistance to a detergent-like molecule. Broadly
551 similar categorizations of bile response were demonstrated for *L. acidophilus* (47) and *L. reuteri*
552 (62), with important genes distinctive to each species. For example, differential expression of a
553 7-kb eight-gene operon encoding a two-component regulatory system was central to *L. acidophilus*
554 bile response (47), whereas none of the *L. salivarius* two-component regulator systems were
555 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 7α-dehydroxylate nor 7α-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

B) showed highly identity (71% and 59%) to the characterized MDR transporters from *L. acidophilus* NCFM (LBA1429) (47) and *L. reuteri* ATCC55730 (lr1584) (62) which contribute to bile resistance. Another cholate induced *L. salivarius* MDR transporter (Fig. S2B, LSL_0032-3) are 52% and 53% identical to the *L. lactis* LmrCD cholate transporter, which also confer 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. However, the *Lactobacillus* 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 (29). Weight gain in chickens in inversely correlated with intestinal BSH activity, much of which comes from the dominant lactobacillus species in poultry, *L. salivarius* (27). Excessive deconjugation of bile in the gut may be linked with “contaminated small 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.

606 **ACKNOWLEDGEMENTS**

607 We thank R. Owen and N. Berrow (Oxford Protein Production Facility, Oxford University) for
608 help with expressing and purifying BSH proteins; M. W. Mangan for designing the microarrays;
609 A.L. Zomer, M.J. Claesson & M. Begley for valuable discussions and H. de Weerd for assistance
610 with array data analysis. This research was supported by Science Foundation Ireland through a
611 Centre for Science, Engineering and Technology award to the Alimentary Pharmabiotic Centre,
612 UCC. We are grateful to Alimentary Health Ltd., Ireland for providing *L. salivarius* strain AH4231
613 and UCC118.

614

615

REFERENCES

1. **Ahrné, S., S. Nobaek, B. Jeppsson, I. Adlerberth, A. E. Wold, and G. Molin.** 1998. The normal *Lactobacillus* flora of healthy human rectal and oral mucosa. J Appl Microbiol **85:**88-94.
2. **Begley, M., C. G. M. Gahan, and C. Hill.** 2005. The interaction between bacteria and bile. FEMS Microbiol Rev **29:**625-651.
3. **Begley, M., R. D. Sleator, C. G. M. Gahan, and C. Hill.** 2005. Contribution of three bile-associated loci, *bsh*, *pva*, and *btlB*, to gastrointestinal persistence and bile tolerance of *Listeria monocytogenes*. Infect Immun **73:**894-904.
4. **Binder, H. J., B. Filburn, and M. Floch.** 1975. Bile acid inhibition of intestinal anaerobic organisms. Am J Clin Nutr **28:**119-125.
5. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem **72:**248-254.
6. **Breton, Y. L., A. Maze, A. Hartke, S. Lemarinier, Y. Auffray, and A. Rince.** 2002. Isolation and characterization of bile salts-sensitive mutants of *Enterococcus faecalis*. Curr Microbiol **45:**434-439.
7. **Bron, P. A., D. Molenaar, W. M. de Vos, and M. Kleerebezem.** 2006. DNA micro-array-based identification of bile-responsive genes in *Lactobacillus plantarum*. J Appl Microbiol **100:**728-738.
8. **Burgess, C., M. O'Connell-Motherway, W. Sybesma, J. Hugenholtz, and D. van Sinderen.** 2004. Riboflavin production in *Lactococcus lactis*: potential for in situ production of vitamin-enriched foods. Appl Environ Microbiol **70:**5769-5777.
9. **Canchaya, C., M. J. Claesson, G. F. Fitzgerald, D. van Sinderen, and P. W. O'Toole.** 2006. Diversity of the genus *Lactobacillus* revealed by comparative genomics of five species. Microbiology **152:**3185-3196.
10. **Christiaens, H., R. J. Leer, P. H. Pouwels, and W. Verstraete.** 1992. Cloning and expression of a conjugated bile acid hydrolase gene from *Lactobacillus plantarum* by using a direct plate assay. Appl Environ Microbiol **58:**3792-3798.
11. **Claesson, M. J., Y. Li, S. Leahy, C. Canchaya, J. P. van Pijkeren, A. M. Cerdeno-Tarraga,**

J. Parkhill, S. Flynn, G. C. O'Sullivan, J. K. Collins, D. Higgins, F. Shanahan, G. F. Fitzgerald, D. van Sinderen, and P. W. O'Toole. 2006. Multireplicon genome architecture of *Lactobacillus salivarius*. Proc Natl Acad Sci U S A **103:**6718-6723.

12. **Dashkevicz, M. P., and S. D. Feighner.** 1989. Development of a differential medium for bile salt hydrolase-active *Lactobacillus* spp. Appl Environ Microbiol **55:**11-16.
13. **De Boever, P., and W. Verstraete.** 1999. Bile salt deconjugation by *Lactobacillus plantarum* 80 and its implication for bacterial toxicity. J Appl Microbiol **87:**345-352.
14. **De Smet, I., L. Van Hoorde, M. Vande Woestyne, H. Christiaens, and W. Verstraete.** 1995. Significance of bile salt hydrolytic activities of lactobacilli. J Appl Bacteriol **79:**292-301.
15. **Dean, M., C. Cervellati, E. Casanova, M. Squerzanti, V. Lanzara, A. Medici, P. Polverino de Laureto, and C. M. Bergamini.** 2002. Characterization of cholylglycine hydrolase from a bile-adapted strain of *Xanthomonas maltophilia* and its application for quantitative hydrolysis of conjugated bile salts. Appl Environ Microbiol **68:**3126-3128.
16. **Delpino, M. V., M. I. Marchesini, S. M. Estein, D. J. Comerci, J. Cassataro, C. A. Fossati, and P. C. Baldi.** 2007. A bile salt hydrolase of *Brucella abortus* contributes to the establishment of a successful infection through the oral route in mice. Infect Immun **75:**299-305.
17. **Denou, E., R. D. Pridmore, B. Berger, J. M. Panoff, F. Arigoni, and H. Brussow.** 2008. Identification of genes associated with the long-gut-persistence phenotype of the probiotic *Lactobacillus johnsonii* strain NCC533 using a combination of genomics and transcriptome analysis. J Bacteriol **190:**3161-3168.
18. **Dunne, C., L. O'Mahony, L. Murphy, G. Thornton, D. Morrissey, S. O'Halloran, M. Feeney, S. Flynn, G. Fitzgerald, C. Daly, B. Kiely, G. C. O'Sullivan, F. Shanahan, and J. K. Collins.** 2001. In vitro selection criteria for probiotic bacteria of human origin: correlation with in vivo findings. Am J Clin Nutr **73:**386S-392S.
19. **Dussurget, O., D. Cabanes, P. Dehoux, M. Lecuit, C. Buchrieser, P. Glaser, and P. Cossart.** 2002. *Listeria monocytogenes* bile salt hydrolase is a PrfA-regulated virulence factor involved in the intestinal and hepatic phases of listeriosis. Mol Microbiol **45:**1095-1106.
20. **Elkins, C. A., S. A. Moser, and D. C. Savage.** 2001. Genes encoding bile salt hydrolases and conjugated bile salt transporters in *Lactobacillus johnsonii* 100-100 and other *Lactobacillus* species. Microbiology **147:**3403-3412.
21. **Fang, F., S. Flynn, Y. Li, M. J. Claesson, J.-P. van Pijkeren, J. K. Collins, D. van**

677 **Sinderen, and P. W. O'Toole.** 2008. Characterization of endogenous plasmids from
678 *Lactobacillus salivarius* UCC118. Appl Environ Microbiol **74:**3216-3228.
679 22. **Gopal-Srivastava, R., and P. B. Hylemon.** 1988. Purification and characterization of bile salt
680 hydrolase from *Clostridium perfringens*. J Lipid Res **29:**1079-1085.
681 23. **Gracey, M.** 1983. The contaminated small bowel syndrome, p. 495-515. *In* D. J. Hentges (ed.),
682 Human intestinal microflora in health and disease. Acad Press New York.
683 24. **Grill, J., F. Schneider, J. Crociani, and J. Ballongue.** 1995. Purification and
684 characterization of conjugated bile salt hydrolase from *Bifidobacterium longum* BB536. Appl
685 Environ Microbiol **61:**2577-2582.
686 25. **Grill, J. P., C. Cayuela, J. M. Antoine, and F. Schneider.** 2000. Isolation and
687 characterization of a *Lactobacillus amylovorus* mutant depleted in conjugated bile salt
688 hydrolase activity: relation between activity and bile salt resistance. J Appl Microbiol
689 **89:**553-563.
690 26. **Grubbs, F. E.** 1969. Procedures for detecting outlying observations in samples.
691 Technometrics **11:**1-21.
692 27. **Guban, J., D. R. Korver, G. E. Allison, and G. W. Tannock.** 2006. Relationship of dietary
693 antimicrobial drug administration with broiler performance, decreased population levels of
694 *Lactobacillus salivarius*, and reduced bile salt deconjugation in the ileum of broiler chickens.
695 Poult Sci **85:**2186-2194.
696 28. **Jones, B. V., M. Begley, C. Hill, C. G. Gahan, and J. R. Marchesi.** 2008. Functional and
697 comparative metagenomic analysis of bile salt hydrolase activity in the human gut microbiome.
698 Proc Natl Acad Sci U S A **105:**13580-13585.
699 29. **Keitel, V., R. Kubitz, and D. Haussinger.** 2008. Endocrine and paracrine role of bile acids.
700 World J Gastroenterol **14:**5620-5629.
701 30. **Kumar, R. S., J. A. Brannigan, A. A. Prabhune, A. V. Pundle, G. G. Dodson, E. J. Dodson,**
702 **and C. G. Suresh.** 2006. Structural and functional analysis of a conjugated bile salt hydrolase
703 from *Bifidobacterium longum* reveals an evolutionary relationship with penicillin V acylase. J
704 Biol Chem **281:**32516-32525.
705 31. **Lambert, J. M., R. S. Bongers, W. M. de Vos, and M. Kleerebezem.** 2008. Functional
706 analysis of four bile salt hydrolase and penicillin acylase family members in *Lactobacillus*
707 *plantarum* WCFS1. Appl Environ Microbiol **74:**4719-4126.

32. **Lambert, J. M., R. J. Siezen, W. M. de Vos, and M. Kleerebezem.** 2008. Improved annotation of conjugated bile acid hydrolase superfamily members in Gram-positive bacteria. Microbiology **154:**2492-2500.

33. **Law, J., G. Buist, A. Haandrikman, J. Kok, G. Venema, and K. Leenhouts.** 1995. A system to generate chromosomal mutations in *Lactococcus lactis* which allows fast analysis of targeted genes. J Bacteriol **177:**7011-7018.

34. **Lebeer, S., J. Vanderleyden, and S. C. J. De Keersmaecker.** 2008. Genes and molecules of lactobacilli supporting probiotic action. Microbiol Mol Biol Rev **72:**728-764.

35. **Leenhouts, K., A. Bolhuis, G. Venema, and J. Kok.** 1998. Construction of a food-grade multiple-copy integration system for *Lactococcus lactis*. Appl Microbiol Biotechnol **49:**417-423.

36. **Leer, R. J., H. Christiaens, W. Verstraete, L. Peters, M. Posno, and P. H. Pouwels.** 1993. Gene disruption in *Lactobacillus plantarum* strain 80 by site-specific recombination: isolation of a mutant strain deficient in conjugated bile salt hydrolase activity. Mol Gen Genet **239:**269-272.

37. **Li, Y., C. Canchaya, F. Fang, E. Raftis, K. A. Ryan, J.-P. van Pijkeren, D. van Sinderen, and P. W. O'Toole.** 2007. Distribution of megaplasmids in *Lactobacillus salivarius* and other lactobacilli. J Bacteriol **189:**6128-6139.

38. **Long, A. D., H. J. Mangalam, B. Y. P. Chan, L. Tolleri, G. W. Hatfield, and P. Baldi.** 2001. Improved statistical inference from DNA microarray data using analysis of variance and a Bayesian statistical framework. Analysis of global gene expression in *Escherichia coli* K12. J Biol Chem **276:**19937-19944.

39. **Lundeen, S. G., and D. C. Savage.** 1990. Characterization and purification of bile salt hydrolase from *Lactobacillus* sp. strain 100-100. J Bacteriol **172:**4171-4177.

40. **Maguin, E., P. Duwat, T. Hege, D. Ehrlich, and A. Gruss.** 1992. New thermosensitive plasmid for gram-positive bacteria. J Bacteriol **174:**5633-5638.

41. **Makarova, K., A. Slesarev, Y. Wolf, A. Sorokin, B. Mirkin, E. Koonin, A. Pavlov, N. Pavlova, V. Karamychev, N. Polouchine, V. Shakhova, I. Grigoriev, Y. Lou, D. Rohksar, S. Lucas, K. Huang, D. M. Goodstein, T. Hawkins, V. Plengvidhya, D. Welker, J. Hughes, Y. Goh, A. Benson, K. Baldwin, J. H. Lee, I. Daíz-Muñiz, B. Dosti, V. Smeianov, W. Wechter, R. Barabote, G. Lorca, E. Altermann, R. Barrangou, B. Ganesan, Y. Xie, H. Rawsthorne,**

739 **D. Tamir, C. Parker, F. Breidt, J. Broadbent, R. Hutkins, D. O'Sullivan, J. Steele, G. Unlu,**
740 **M. Saier, T. Klaenhammer, P. Richardson, S. Kozyavkin, B. Weimer, and D. Mills.** 2006.
741 Comparative genomics of the lactic acid bacteria. Proc Natl Acad Sci U S A **103:**15611-15616.
742 42. **McAuliffe, O., R. J. Cano, and T. R. Klaenhammer.** 2005. Genetic analysis of two bile salt
743 hydrolase activities in *Lactobacillus acidophilus* NCFM. Appl Environ Microbiol
744 **71:**4925-4929.
745 43. **Moser, S. A., and D. C. Savage.** 2001. Bile salt hydrolase activity and resistance to toxicity of
746 conjugated bile salts are unrelated properties in lactobacilli. Appl Environ Microbiol
747 **67:**3476-3480.
748 44. **Nakayama, F.** 1969. Composition of gallstone and bile: species difference. J Lab Clin Med
749 **73:**623-630.
750 45. **O'Toole, P. W., and E. J. Raftis.** 2009. Megaplasmid pMP118 of *Lactobacillus salivarius*, p.
751 311-326. *In* E. Schwartz (ed.), Microbial megaplasmids, vol. 11. Springer London, Limited,
752 London.
753 46. **Oh, H. K., J. Y. Lee, S. J. Lim, M. J. Kim, G. B. Kim, J. H. Kim, S. K. Hong, and D. K.**
754 **Kang.** 2008. Molecular cloning and characterization of a bile salt hydrolase from *Lactobacillus*
755 *acidophilus* PF01. J Microbiol Biotechnol **18:**449-456.
756 47. **Pfeiler, E. A., M. A. Azcarate-Peril, and T. R. Klaenhammer.** 2007. Characterization of a
757 novel bile-inducible operon encoding a two-component regulatory system in *Lactobacillus*
758 *acidophilus*. J Bacteriol **189:**4624-4634.
759 48. **Ridlon, J. M., D.-J. Kang, and P. B. Hylemon.** 2006. Bile salt biotransformations by human
760 intestinal bacteria. J Lipid Res **47:**241-259.
761 49. **Rossocha, M., R. Schultz-Heienbrok, H. von Moeller, J. P. Coleman, and W. Saenger.**
762 2005. Conjugated bile acid hydrolase is a tetrameric N-terminal thiol hydrolase with specific
763 recognition of its cholyl but not of its tauryl product. Biochemistry **44:**5739-5748.
764 50. **Saarela, M., G. Mogensen, R. Fondén, J. Mättö, and T. Mattila-Sandholm.** 2000.
765 Probiotic bacteria: safety, functional and technological properties. J Biotechnol **84:**197-215.
766 51. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual,
767 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
768 52. **Schmid, M. B., and J. R. Roth.** 1987. Gene location affects expression level in *Salmonella*
769 *typhimurium*. J Bacteriol **169:**2872-2875.

53. **Sridevi, N., S. Srivastava, B. M. Khan, and A. A. Prabhune.** 2009. Characterization of the smallest dimeric bile salt hydrolase from a thermophile *Brevibacillus* sp. Extremophiles **13:**363-370.

54. **Stellwag, E. J., and P. B. Hylemon.** 1976. Purification and characterization of bile salt hydrolase from *Bacteroides fragilis* subsp. *fragilis*. Biochim Biophys Acta **452:**165-176.

55. **Tamura, K., J. Dudley, M. Nei, and S. Kumar.** 2007. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Mol Biol Evol **24:**1596-9.

56. **Tanaka, H., H. Hashiba, J. Kok, and I. Mierau.** 2000. Bile salt hydrolase of *Bifidobacterium longum*-biochemical and genetic characterization. Appl Environ Microbiol **66:**2502-2512.

57. **Tannock, G. W., M. P. Dashkevicz, and S. D. Feighner.** 1989. Lactobacilli and bile salt hydrolase in the murine intestinal tract. Appl Environ Microbiol **55:**1848-1851.

58. **Van Eldere, J.** 1999. Chemical transformations of bile salts by the intestinal microflora, p. 312-337. *In* G. W. Tannock (ed.), Medical importance of the normal microflora. Kluwer Academic Publishers, Dordrecht, The Netherlands.

59. **van Pijkeren, J.-P., C. Canchaya, K. A. Ryan, Y. Li, M. J. Claesson, B. Sheil, L. Steidler, L. O'Mahony, G. F. Fitzgerald, D. van Sinderen, and P. W. O'Toole.** 2006. Comparative and functional analysis of sortase-dependent proteins in the predicted secretome of *Lactobacillus salivarius* UCC118. Appl Environ Microbiol **72:**4143-4153.

60. **Ventura, M., C. Canchaya, V. Bernini, E. Altermann, R. Barrangou, S. McGrath, M. J. Claesson, Y. Li, S. Leahy, C. D. Walker, R. Zink, E. Neviani, J. Steele, J. Broadbent, T. R. Klaenhammer, G. F. Fitzgerald, W. O'Toole P, and D. van Sinderen.** 2006. Comparative genomics and transcriptional analysis of prophages identified in the genomes of *Lactobacillus gasseri*, *Lactobacillus salivarius*, and *Lactobacillus casei*. Appl Environ Microbiol **72:**3130-3146.

61. **Watson, D., R. D. Sleator, C. Hill, and C. G. Gahan.** 2008. Enhancing bile tolerance improves survival and persistence of *Bifidobacterium* and *Lactococcus* in the murine gastrointestinal tract. BMC Microbiol **8:**176.

62. **Whitehead, K., J. Versalovic, S. Roos, and R. A. Britton.** 2008. Genomic and genetic characterization of the bile stress response of probiotic *Lactobacillus reuteri* ATCC 55730. Appl Environ Microbiol **74:**1812-1819.

63. **Yuan, J., B. Wang, Z. Sun, X. Bo, X. Yuan, X. He, H. Zhao, X. Du, F. Wang, Z. Jiang, L. Zhang, L. Jia, Y. Wang, K. Wei, J. Wang, X. Zhang, Y. Sun, L. Huang, and M. Zeng.** 2008. Analysis of host-inducing proteome changes in *Bifidobacterium longum* NCC2705 grown *in vivo*. J Proteome Res **7:**375-385.

64. **Zaidi, A. H., P. J. Bakkes, J. Lubelski, H. Agustiandari, O. P. Kuipers, and A. J. M. Driessen.** 2008. The ABC-type multidrug resistance transporter LmrCD is responsible for an extrusion-based mechanism of bile acid resistance in *Lactococcus lactis*. J Bacteriol **190:**7357-7366.

811 Figure legends

812

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

817

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

824

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

829

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

833

834 Fig. 5. Disruption of *bsh1* (LSL_1801) in *L. salivarius* LS201

835 (A) Southern hybridization analysis of insertional inactivation of the *bsh1* gene in *L. salivarius*
836 LS201. LS201 and LS201Δ*bsh1* genomic DNA were digested with *Spe*I (Lane 1-2) and hybridized
837 with a labeled 582-bp amplicon of LSL_1801 (primers FF027 and FF028) as a probe. M, labeled
838 DNA marker; 1, *L. salivarius* LS201 (derivative of *L. salivarius* UCC118 cured of pSF118-20; 2, *L.*
839 *salivarius* LS201Δ*bsh1*. DNA sizes are indicated by arrows.

840 (B) Schematic representation of the relevant regions of the LS201 and LS201Δ*bsh1* genomes.
841 Heavy lines represent megaplasmid DNA; thin lines represent plasmid DNA. *bsh1* (LSL_1801) is
842 presented by the arrow, and the grey box is the *bsh1* internal fragment corresponding to the
843 hybridization probe. *Spe*I sites are indicated

844

845 Fig. 6. BSH1 and BSH2 contribute to bile resistance in *L. salivarius*.

846 (A) Survival of *L. salivarius* LS201 in the presence (open symbols) or absence (closed symbols) of
847 0.2 % porcine bile. Big squares, *L. salivarius* LS201; triangles, *L. salivarius* LS201Δ*bsh1*; cycles,
848 *L. salivarius* LS201Δ*lacZ*; diamonds, *L. salivarius* LS201Δ*bsh1*(pLS219 [pLS209+*118bsh1*]);
849 small squares, *L. salivarius* LS201Δ*bsh1*(pLS209)

850 (B) Survival of *L. salivarius* JCM1046 in the presence (open symbols) or absence (closed symbols)
851 of 0.1 % porcine bile. Squares, wild type *L. salivarius* JCM1046; triangles, *L. salivarius*
852 JCM1046Δ*bsh1*

853 (C) Disruption of the *bsh1*gene of LS201 reduces survival during murine intestinal tract transit.
854 Grey bars, *L. salivarius* LS201Δ*bsh1*; white bars, *L. salivarius* LS201Δ*lacZ*.

855

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

857 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		
L. salivarius		
UCC118	Ileocaecal 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-hsd*RMS-*mcr*BC) φ80*lacZ*ΔM15 Δ*lac*X74 *rec*A1 *ara*Δ139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L(StrR) *end*A1 *nup*G	Invitrogen
Rosetta BL21(DE3)pLysS	F- *ompT hsdS*$_B$ ($r_B^- m_B^-$) *gal dcm* (DE3) pLysS (CmR)	Invitrogen
Lactococcus lactis		
LL108	Strain with *repA* gene integrated in chromosome	(35)
Plasmids		
pORI19	Em^r Ori$^+$ RepA$^-$ lacZ' 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, *Lactobacillus* gene cloning vector, a 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 1002–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-6 × His-tag fusion	OPPF[c]
pEB118	Amp^r, derivative of pOPINE for expression of C-His-tagged *118bsh1*	This work
pEB1046	Amp^r, derivative of pOPINE for expression of C-His-tagged *1046bsh1*	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)

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*bsh1*	This study	-	-	3	4.0	0.1
	JCM1046Δ*bsh1*	This study	-	+ (p)	6	>20	>5.0

TDCA, sodium taurodeoxycholate hydrate; GDCA, sodium glycodeoxycholate; MIC, minimm inhibitory concentration; -, 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; >, 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		LS201Δ*bsh*		LS201		LS201Δ*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 μM (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

888 Supplemental material

889

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

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

899

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

901

902 Table S1. Primers used in this study

903

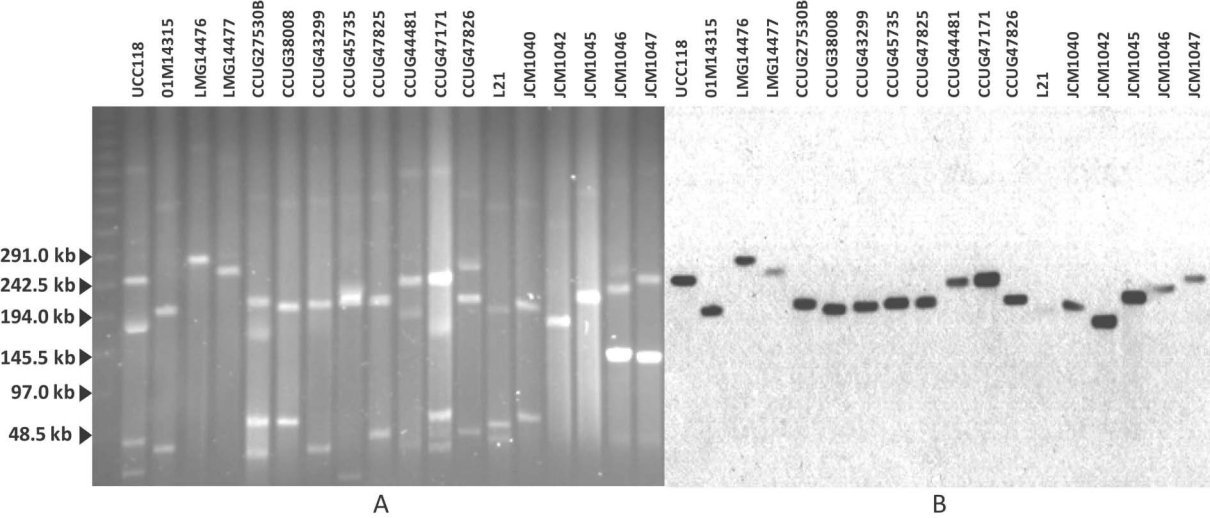
904 Table S2. Genes significantly up/down-regulated by porcine bile extract (expression ratio
905 ≥ 2-fold, $p < 0.05$)

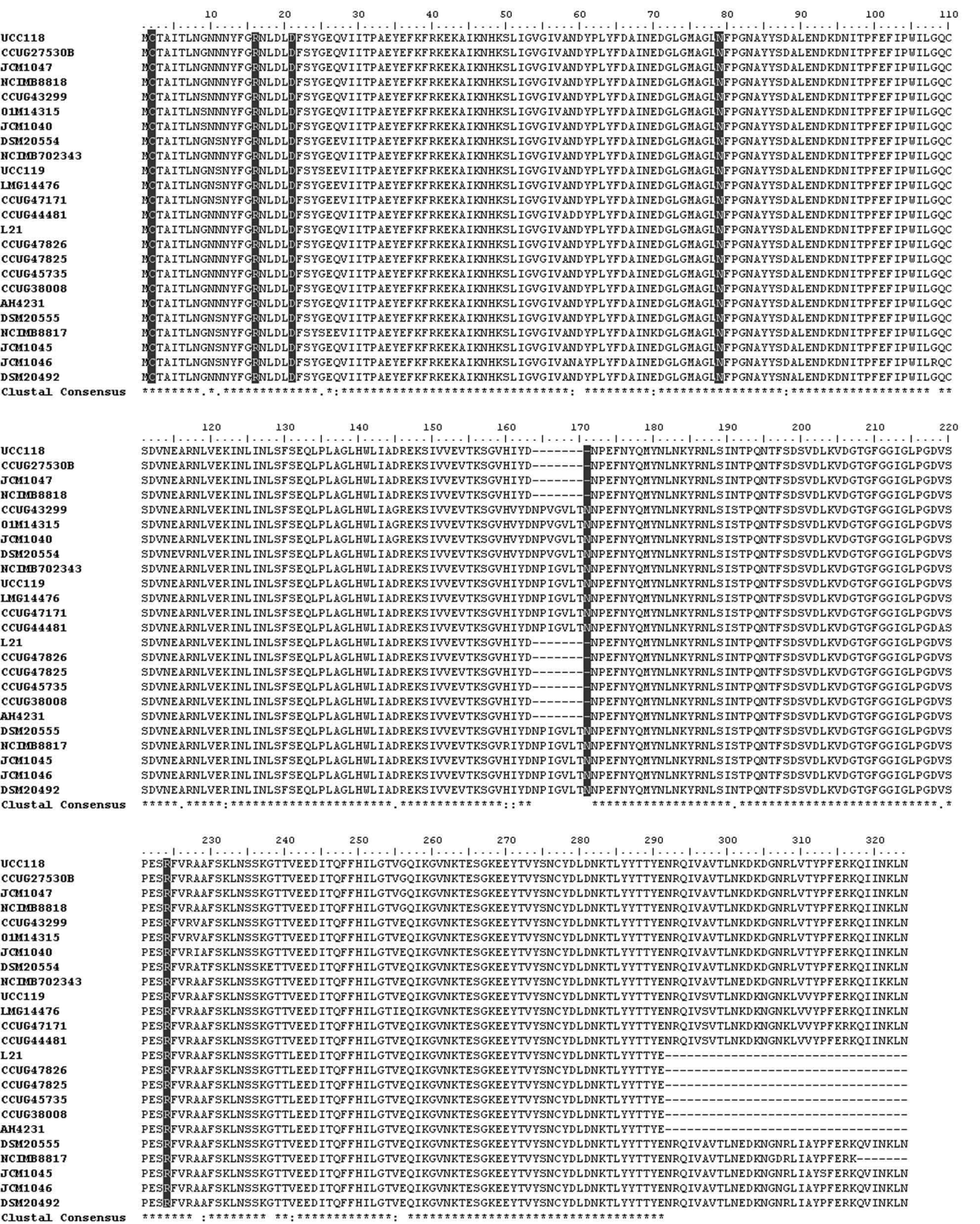
906

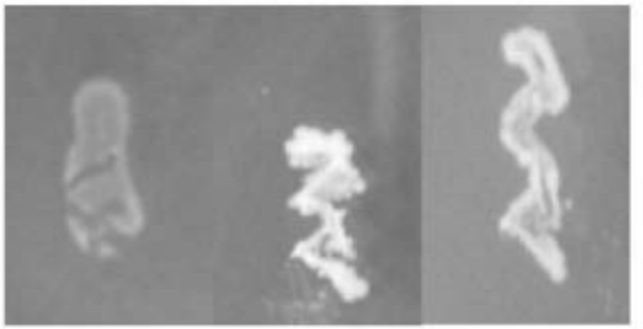
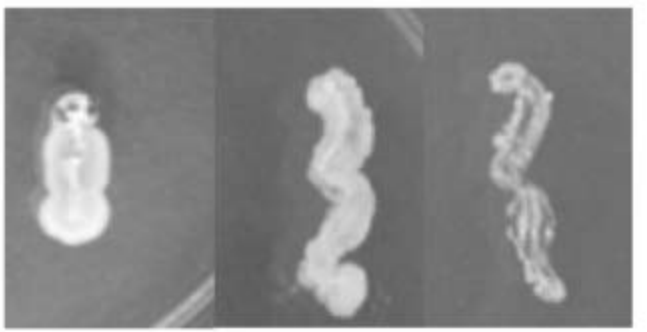
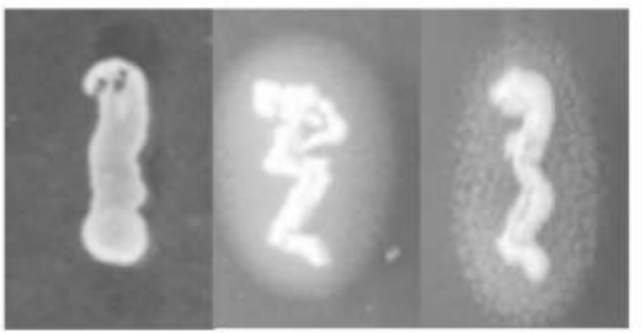
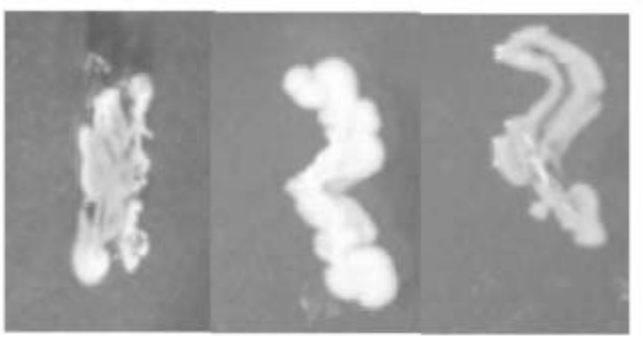
907 Table S3. Genes significantly induced by cholate (expression ratio ≥ 2-fold, $p < 0.05$,
908 ArrayExpress accession no.XXX)

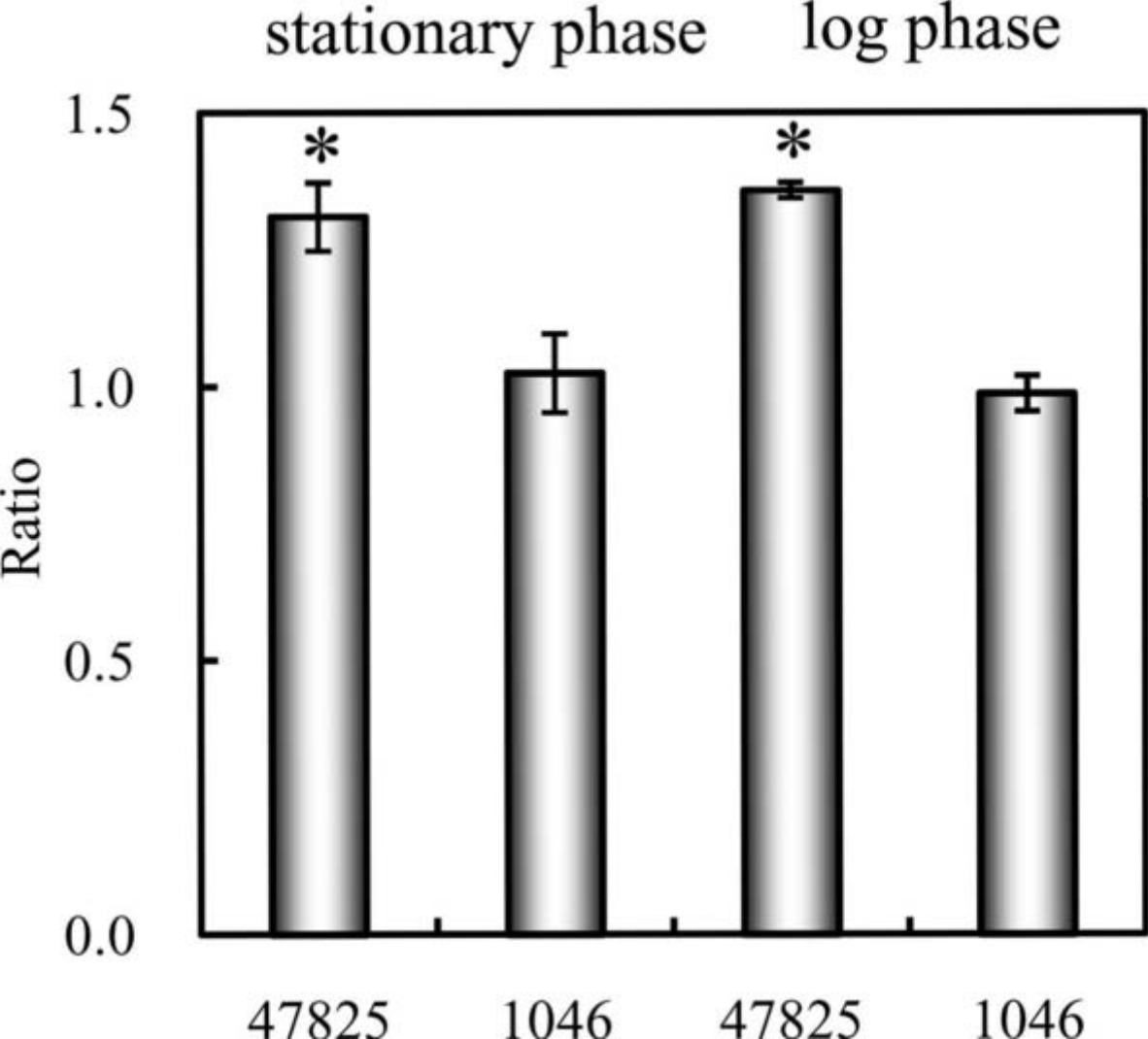
909

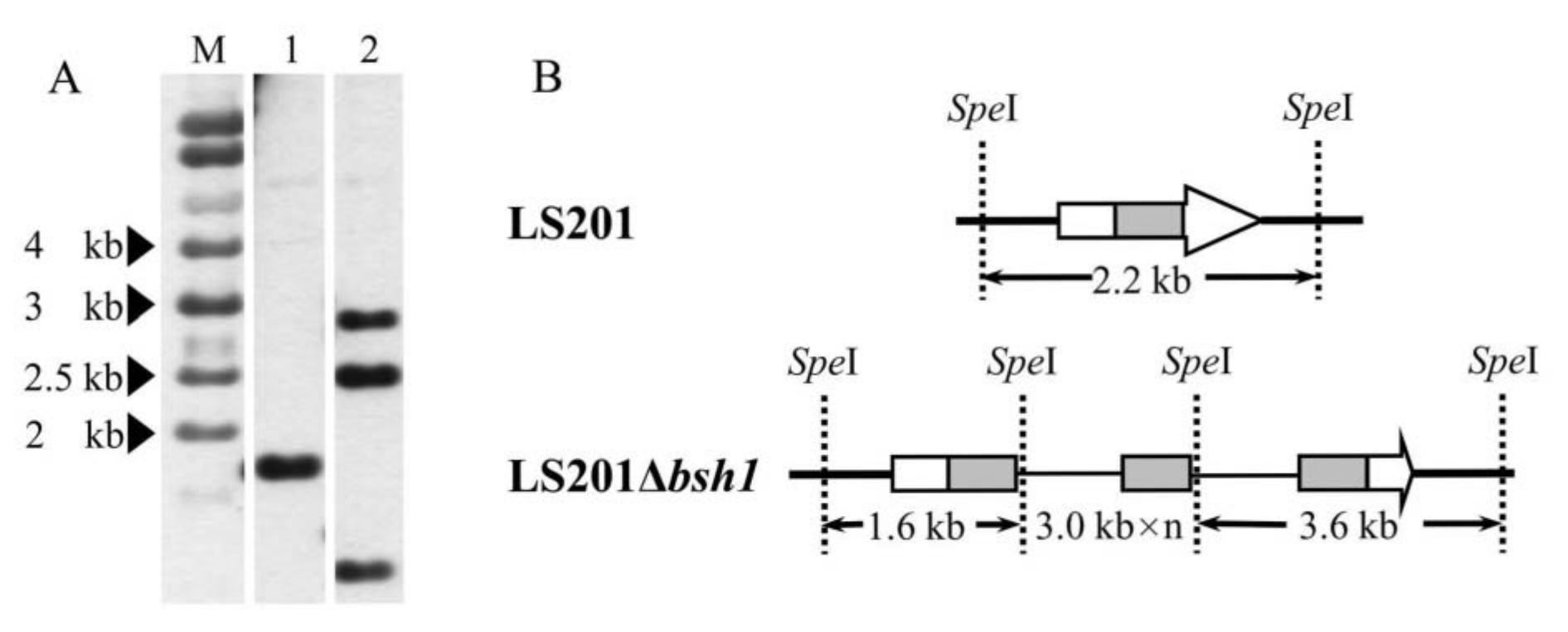
910

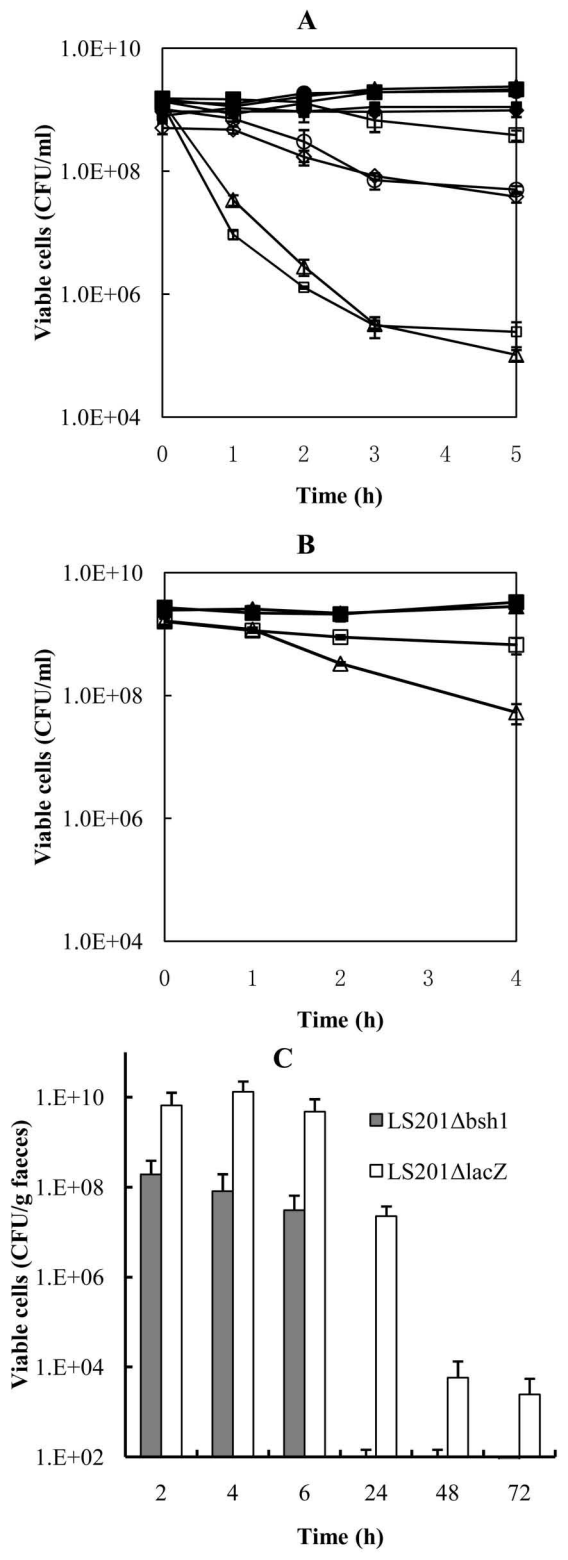

UCC118
01M14315
LMG14476
LMG14477
CCUG27530B
CCUG38008
CCUG43299
CCUG45735
CCUG47825
CCUG44481
CCUG47171
CCUG47826
L21
JCM1040
JCM1042
JCM1045
JCM1046
JCM1047
291.0 kb
242.5 kb
194.0 kb
145.5 kb
97.0 kb
48.5 kb
A
B

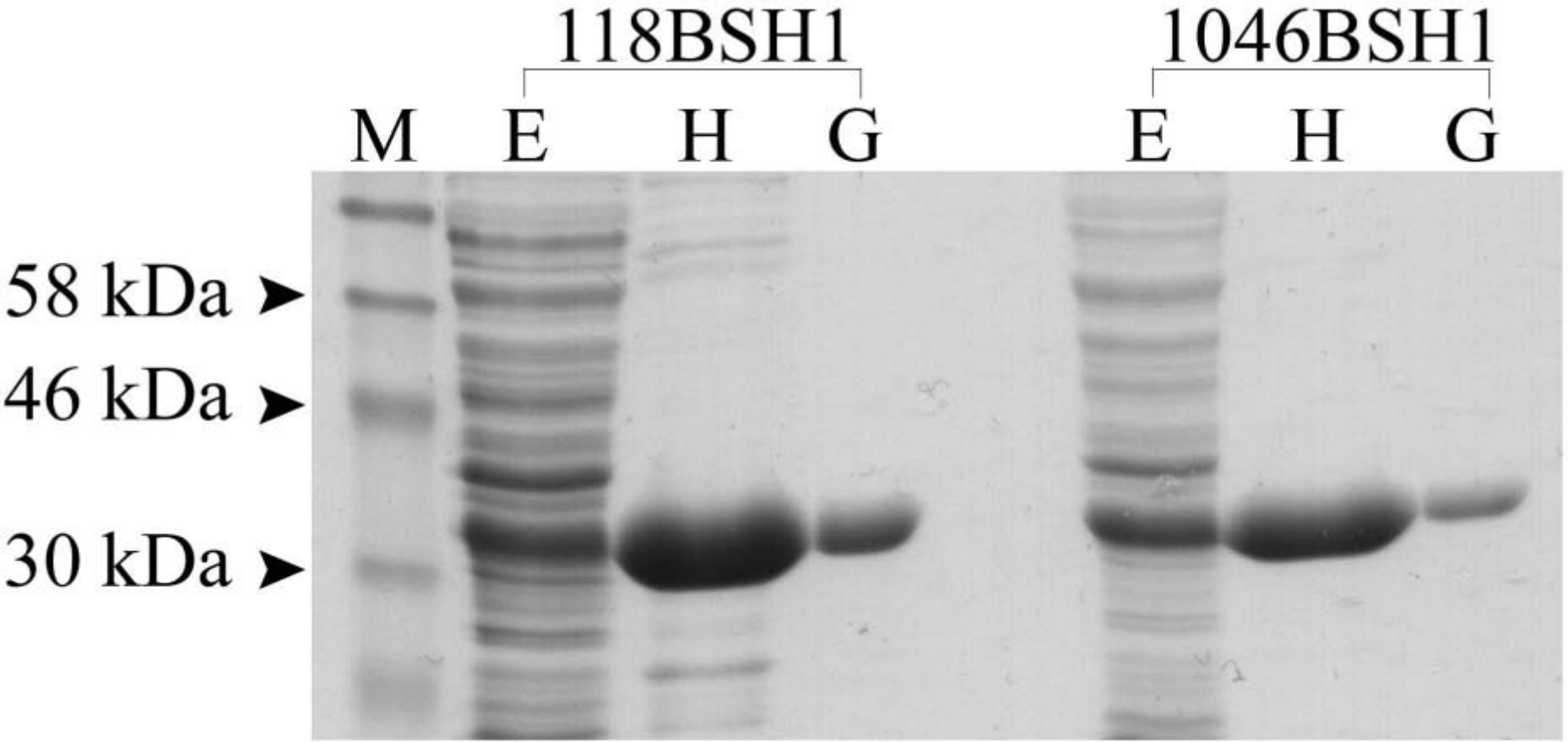


Strain	BSH1 Group	MRS	TDCA	GDCA	BSH activity TDCA	BSH activity GDCA
UCC118	A				-/+, w	-
CCUG47825	B				-	-
JCM1046	C				+, p	+, p
NCIMB8816	D				+, w	-

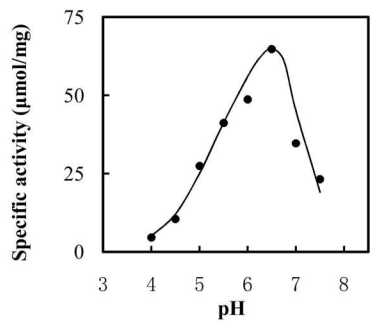

stationary phase
log phase
1.5
1.0
0.5
0.0
Ratio
*
*
47825
1046
47825
1046

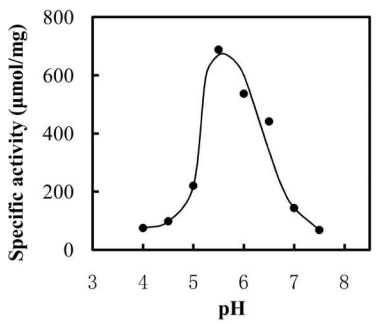

A
M
1
2
4 kb
3 kb
2.5 kb
2 kb
B
LS201
SpeI
SpeI
2.2 kb
LS201Δbsh1
SpeI
SpeI
SpeI
SpeI
1.6 kb
3.0 kb×n
3.6 kb

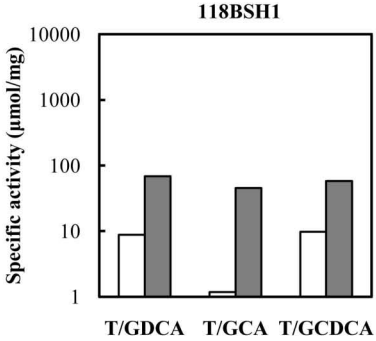

A
1.0E+10
1.0E+08
1.0E+06
1.0E+04
Viable cells (CFU/ml)
0
1
2
3
4
5
Time (h)
B
1.0E+10
1.0E+08
1.0E+06
1.0E+04
Viable cells (CFU/ml)
0
1
2
3
4
Time (h)
C
1.E+10
1.E+08
1.E+06
1.E+04
1.E+02
Viable cells (CFU/g faeces)
LS201Δbsh1
LS201ΔlacZ
2
4
6
24
48
72
Time (h)

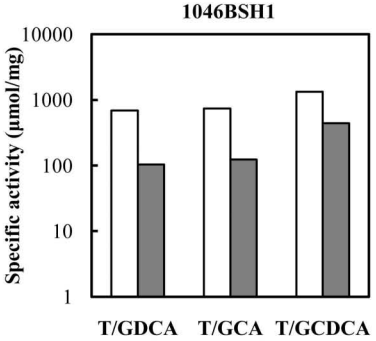

118BSH1
1046BSH1
M
E
H
G
E
H
G
58 kDa
46 kDa
30 kDa

A

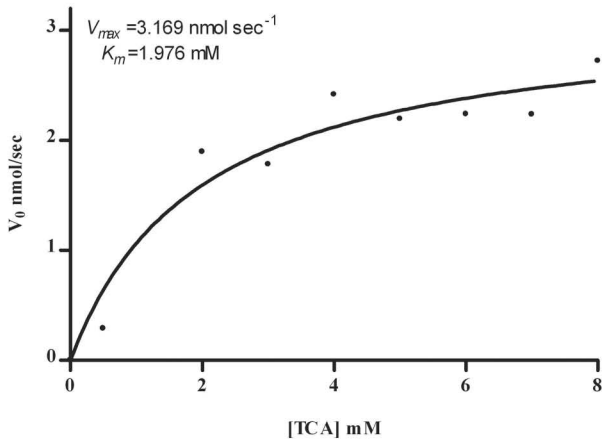

118BSH1
Specific activity (μmol/mg)
75
50
25
0
3
4
5
6
7
8
pH

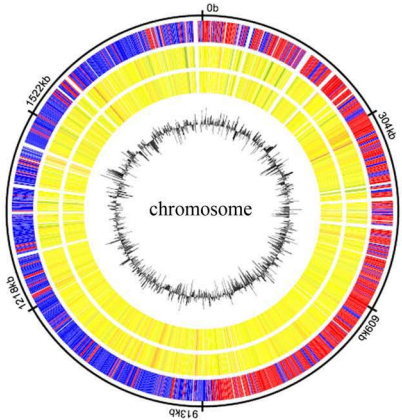

1046BSH1
Specific activity (μmol/mg)
800
600
400
200
0
3
4
5
6
7
8
pH

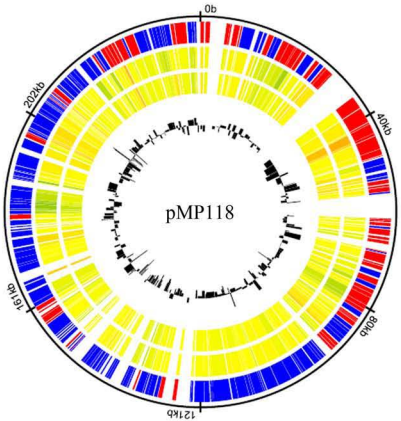

118BSH1
Specific activity (μmol/mg)
10000
1000
100
10
1
T/GDCA
T/GCA
T/GCDCA


1046BSH1
Specific activity (μmol/mg)
10000
1000
100
10
1
T/GDCA
T/GCA
T/GCDCA

B

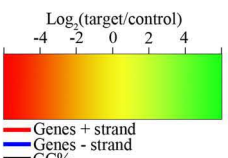

V_{max} =3.169 nmol sec^{-1}
K_m=1.976 mM
V_0 nmol/sec
3
2
1
0
0
2
4
6
8
[TCA] mM

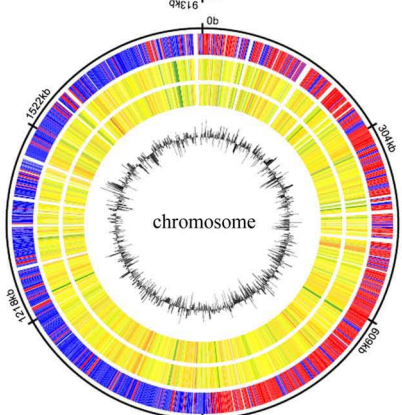


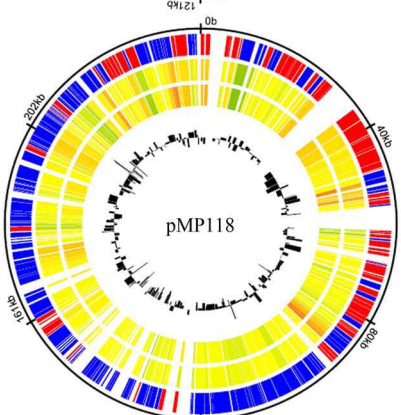


bile transcriptome

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







cholate transcriptome