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Genetic Characterization and Gene Expression of Bile Salt Hydrolase (*bsh*) from *Lactobacillus reuteri* CRL 1098, a Probiotic Strain

Research Article

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Abstract

Intestinal microbes containing the bile salt hydrolase (BSH) enzyme, releases free BA plus aminoacids from conjugated BA. BSH activity trigger scholesterol consumption in liver to synthesize BA *de novo* leading to consequential cholesterol lowering. *Lactobacillus* (*L.*)*reuteri* CRL 1098 is a probiotic bacterium with a proven hypocholesterolemic effect associated to its ability to hydrolyze BA. In this work we characterized the bile salt hydrolase (*bsh*) operon of CRL 1098 strain as a single open reading frame of 978 nucleotides that encodes a predicted protein of 325 amino acids, with a calculated mass of 36098.1 Da and a theoretical pI of 4.81. Moreover, deduced BSH protein had high similarity with BSHs of other *L. reuteri* strain and also exhibited similarity to the Pencillin V amidases of *Listeria* and *Bacillus* strains. Five catalytically important amino acids were highly conserved in *Lactobacillus, Enterococcus* and *Bifidobacterium* strains while four amino acid motifs around these active sites, were only partially conserved. After the *bsh* gene product was expressed in the heterologous host *Lactococcus lactis* NZ9000. The activity was specific towards bile acids but not against alternative substrates. Finally, a significant up-regulation of the *bsh* gene was observed at pH 5.2 (optimal pH of BSH activity).

Our studies suggest that BSHs would have an important but so far unknown role in the physiology and lifestyle of *L. reuteri* strains. The present work would be useful to unravel the ecological role of the BSH and to deepen their influence in the reduction of blood cholesterol levels.

Keywords: *Lactobacillus reuteri*; Bile Salt Hydrolase; Molecular Cloning; Gene Expression.

Introduction

In vertebrates, Bile Acids (BA) play an essential role in lipid digestion, acting as a detergent that emulsifies and solubilizes dietary lipids and lipid-soluble vitamins. BA are synthesized from cholesterol, conjugated with taurine or glycine in the liver and secreted into the duodenum. Intestinal microbes, containing the Bile Salt Hydrolase (BSH) enzyme, release the free BA plus amino acids from conjugated BA.

BSHs and also penicillin V amidases (PVA) (EC 3.5.1.11) belong to the choloylglycine hydrolase family of enzymes and have been classified as N-terminal nucleophilic hydrolases with an N-terminal cysteine residue [1]. BSH activity has been described among a wide variety of genus, including *Lactobacillus* [2-7], *Bifidobacterium* [8-10], *Clostridium* (C). [11], *Listeria* [12], and *Enterococcus* [13]. Additionally, a number of *bsh* genes from *Lactobacillus plantarum* [14,6], *Lactobacillus johnsonii* [2], *Lactobacillus fermentum* [3], *Lactobacillus casei* [15] and *Bifidobacterium* [16-18] strains have been cloned and characterized. Interestingly, the presence and genetic organization of *bsh* genes are highly variable and also, several strains possess more than one BSH homologo, which in most cases are not identical [19].

Probiotics with BSH activity have received much attention on account of its influence on serum cholesterol lowering. BA deconjugation enhances its excretion through the feces and could lead to serum cholesterol reduction by increasing its demand for *de novo* synthesis of BA [5, 20, 21]. Therefore, the World Health Organization (WHO) recommended BSH activity as one of the

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main criteria to propose a microorganism as probiotic along with their ability to resist the harsh gut environment and to colonize gastrointestinal epithelia [22].

L. reuteri CRL 1098 is a probiotic bacterium with a proven hypocholesterolemic effect closely associated to its ability to hydrolyze BA [20, 23, 24]. Moreover, this strain displays a moderate immune stimulant effect [25] and ability to produce corrinoids with cobalamin activity [26, 27]. BSH enzymes from *L. reuteri* strains are and their relation to cholesterol reduction have been previously documented [24, 28]; however, data on its *bsh* gene are still poor. Therefore, the aim of this study was to identify, clone and characterize the *bsh* gene of *L. reuteri* CRL 1098, and to investigate the functionality of the *bsh* gene product against BA and alternative substrates. Finally, the regulation of *bsh* at different pH was examined by Real Time-PCR.

Materials and Methods

Bacterial strains and growth conditions

L. reuteri strain CRL 1098 was grown at 37°C in MRS broth [29]. The heterologous-expression host *Lactococcus* (*Lac.*) *lactis* NZ9000 was grown at 30°C in M17 broth (Biokar Diagnostics, Beauvais Cedex – France) supplemented with 0.5% glucose (w/v) [M17-Glu 0.5%], without aeration. *Escherichia* (*E.*) *coli* DH10β was grown at 37°C on Luria Bertani (LB) medium in aerobic conditions. When necessary, 1 mM or 10 mM of conjugated BA (Sigma Aldrich, St. Louis, MO, USA), antibiotics and others reagents were added to the media. For *Lac.lactis strain* development, 5 µg/ml erythromycin (Erm) was used; 100 µg/ml ampicillin, 40 mg/ml X-gaL, 100 mM isopropil-β-D-1-tiogalactopiranósido (IPTG), 15 µg/ml chloramphenicol (Cmp) or 10 µg/ml tetracycline (Tet) were used to development of *E. coli* DH10β.

DNA manipulations

Genomic DNA was extracted from *L. reuteri* strain following the procedure previously described [30]. Plasmid DNA was isolated from *E. coli* on a small scale, using the alkaline lysis method [31]. Plasmid DNA isolation and electroporation technique used for *Lac. lactis* NZ9000 were performed as described by Lambert et al. [6]. Purification of DNA fragments from agarose gels was performed using the PCR AccuPrep® Purification Kit (Bionner, Korea). Restriction endonucleases, Taq DNA polymerase, T4 DNA ligase and Klenow enzyme were used as indicated by the manufacturers (Promega and Invitrogen, Buenos Aires, Argentina). Primers were synthetized from Invitrogen.

Cloning *bsh* gene

PCR primers from *bsh* gene of *L. reuteri* DSM20016 (WP_003668136.1) were designed using Primer3 Software to determine the *bsh* gene sequence of CRL 1098 strain. PCR amplification reactions was conducted in a Thermal Cycler System MyCycler™ using genomic DNA of CRL 1098 strain as template and following a protocol previously described [32] except that the annealing step was performed at 55°C for 1 min.

The PCR product was cloned in TOPO®-TA Cloning (Invitrogen, Breda, The Netherlands). The recombinant plasmid, named pBTR1 was propagated into calcium chloride competent cells of the intermediate cloning hosts *E. coli* DH10β using the heat shock method [33]. Recombinant cells were recovered in LB medium with ampicillin and were determined using universal primers T3 (5'GCAATTAACCCTCACTAAAGG3') and T7 (5'TAATACGACTCACTATAGGG3') at the Servicio de Diagnóstico Genético de Animales Domésticos (GAD), Facultad de CiencíasVeterinarias, Universidad Nacional de La Plata.

Sequence analysis

The nucleotide and the predicted amino acid sequences of BSH of *L. reuteri* CRL 1098 were analyzed and compared with reference sequences available in GenBank (EMBL) by BLAST program at the website of the NCBI (http://www.ncbi.nlm.nih.gov). Promoter sequences were predicted by using a Neural Network Promoter Prediction program (NNPP), versión 2.2 (http://www.fruitfly.org/seq_tools/promoter.html), while the secondary structure of the putative transcription termination sequence was predicted using http://transterm.cbcb.umd.edu. Multiple amino acid sequence alignments and phylogenetic tree were carried out using ClustalW software package. The nucleotide sequence of the *bsh* gene from *L. reuteri* CRL 1098 was submitted to the GenBank database under accession number FJ006722.1.

Heterologous expression of *bsh* gene

The *bsh* gene of *L. reuteri* CRL 1098 was expressed in the GRAS organism (Generally Recognized as Safe) *Lac. lactis* NZ9000. The *bsh* gene was recovered from the pBTR1 as a 1.2-kb *Bam*HI-E*co*RV fragment and cloned into the *Bam* HI-*Sal*I sites of plasmid pSA3 (inactivating the *tet* gene). The recombinant plasmid pBTR3 was purified from Cmp-resistant, Tet-sensitive *E. coli* cells and used to transform *Lac. lactis* NZ9000. Electrocompetent cells prepared as previously described [6] were exposed to a single electrical pulse delivered by a Gene-Pulser (Bio-Rad Laboratories, Richmond, California) set at 25 µF and normally at 2.0 kV. Recombinant cells of *Lac. lactis* NZ9000 (pBTR3) were recovered in M17-Glu 0.5% broth with Erm.

BSH activity and alternative acylase functionality

Stationary phase cells (DO_{600nm}: 2) of *Lac. lactis* NZ9000-pBTR3 were harvested by centrifugation (8.600 x g, 10 min) at room temperature and cell pellets were washed twice with equal volume of saline solution (NaCl 0.85% wt/vol). Then, cells were concentrated 5-fold in 100 mM sodium acetate buffer (pH 5.2) containing 1 mM dithiothreitol (DTT) and 1 g of zirconium beads and mechanically disrupted in a Mini-Beadbeater-8TM Cell Disrupter using 7 treatments of 1 min interspaced by 1 min in ice. Following centrifugation, cells-free extracts (CFE) were obtained and kept at -20 °C until use. The reaction mixture (1 ml) contained 10 mM of conjugated BA glycodeoxycolic acid (GDCA), glycocolic acid (GCA), taurodeoxycholic acid (TDCA) and, taurocholic acid (TCA), penicillin V, ampicillin, ketocaproyl-homoserine lactone, or oxooctanoyl-homoserine lactone (Sigma, Zwijndrecht, The Netherlands), 100 mM sodium acetate buffer (pH 5.2), 1 mM DTT and 100 µl of CFE. The reaction was incubated 6 h at 37°C, after which the hydrolase activity was inactivated by the addition of 500 µl of 20% wt/vol trichloroacetic acid. Amino

acids liberated were measured with the o-phthalaldehyde (OPA) method [34] and results were expressed as mmol of amino acid released/µg protein. CFE of *L. reuteri* CRL 1098 and *Lac. lactis* NZ9000-pSA3 cells, obtained as described above, were used as positive and negative controls, respectively.

RNA isolation

RNA isolation from *L. reuteri* CRL 1098 was performed as previously described with some modifications [35]. An overnight culture of CRL 1098 strain was inoculated in fresh MRS broth (1% v/v) with 1 mM of GDCA at a fixed pH (4.5, 5.2 and 6.5). The cells were incubated until mid-exponential phase (OD560 nm 0.9) and harvested by centrifugation at 3500 x g for 10 min followed by cell pellet suspension in ice-cold TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5). The cell suspension was transferred to a 2-ml screw-cap microcentrifuge tube containing 0.8 g of zirconium beads, 0.18 g of macaloid, 50 µl of 10% (wt/vol) SDS, and 500 µl of water-saturated phenol-chloroform (1:1). Cells were mechanically disrupted in a Mini-Beadbeater-8TM Cell Disrupter using 7 treatments of 1 min interspaced by 1 min ion ice. Subsequently, the RNA was purified from the upper aqueous phase of the cell extract by phenol-chloroform extraction, precipitated with absolute ethanol, washed with 70% ethanol and resuspended in MilliQ water. The RNA obtained was stored in aliquots at –70 °C until further use.

Real-time PCR assays

RNA samples were treated with the RNase-free DNase I set (Tecnolab, Buenos Aires, Argentina) according to the manufacturer's recommendations. The absence of DNA in the samples was confirmed by PCR. For reverse transcriptase PCR (RT-PCR) analysis, cDNA was synthesized from RNA by using SuperScript II reverse transcriptase (Invitrogen, Breda, The Netherlands) following the manufacturer's protocol. The resulting cDNA samples were used for Real-Time PCR amplification. Specific primers for bsh gene (RT-BSHF: 5'GGTTGGGATGGCAGGCCTTAATTT3' and RT-BSHR: 5'AACTTCTTCGCTTCAGCAACCGAG3') and housekeeping genes primers were designed to have melting temperatures of 60 to 65°C and amplicon sizes around 130 bp. RecA primers were used as internal gene control to normalize the amount of RNA added and the relative expression was calculated using the $2^{-\Delta\Delta CT}$ method [36] where $\Delta\Delta CT = (CT^{bsh} - CT^{recA})_{BA} - (CT^{bsh} - CT^{recA})_{Control}$. Cells growing in MRS broth at pH 6.5 were used as reference condition. The experiments were performed in an IQ iCycler (Biorad, Hercules, CA, USA) using the double stranded DNA intercalating fluorescent agent SYBR green for product detection. Each well contained SYBR green Master Mix, 0.05 µM of each primer, and 30 ng/µl of template. Control PCRs were included to detect background contamination (no-template control) and remaining chromosomal DNA (RT reactions in which Superscript II was omitted). PCR specificity and product detection were checked post-amplification by examining the dissociation curves (melting curve) of the PCR products.

Data analysis

Data analysis was carried out using the software InfoStat 2014, FCA, Universidad Nacional de Córdoba, Argentina. URL http://www.infostat.com ar. Analysis of variance (ANOVA) followed by Tukey's multiple range tests were used to study any significant difference between means with a significance level of $P < 0.05$. All data were presented as mean ± standard deviation.

Results and Discussion

Amplification and nucleotide sequence analysis of the *L. reuteri* CRL 1098 *bsh* gene

The *bsh* gene of *L. reuteri* CRL 1098 was amplified using PCR primers (F1-BSH: 5'GCCCAAAGTTCAAGGACAAGCAGA3' and R2-BSH: 5'CCACGCAATCGCAACTGAAGTAT3') designed based on the *bsh* gene encoding sequences of DSM 20016 strain (WP_003668136.1). The sequenced fragment contains a single open reading frame of 978 nucleotides (Supplemental material) bounded by a methionine start codon ATG and a TAA translation termination codon. A potential promoter-type structure is located from 22 to 72 nucleotides upstream of the ATG codon as well as a palindromic DNA sequence capable of forming a stem-loop structure downstream of the structural gene. In fact, a putative rho-independent type transcription terminator sequence (AG = -14.1 kcal/mol) followed by a T rich region was recognized 20 nucleotides downstream of the stop codon (Supplemental material), indicating that the *bsh* gene of CRL 1098 strain is transcribed as a monocistronic unit. While polycistronic *bsh* transcripts have been reported from *L. johnsonii* [37] and *B. longum* [18], monocistronic *bsh* transcripts are more common features in many other bacterial groups [1]. The encoded polypeptide (325 amino acids) has a calculated mass of 36098.1 Da, with a predicted pI of 4.81. The deduced amino acid sequence of the N terminal of the protein does not resemble a signal sequence typical for secretory proteins, suggesting an intracellular location of the BSH enzyme. BLAST analysis of the predicted amino acid sequence revealed that BSH of CRL 1098 is identical to *L. reuteri* DSM 20016 (WP_003668136.1) and share highest sequence identity (between 97 and 98%) with the BSHs of *L. reuteri* TD1 (WP_019254138.1), *L. reuteri* 100-23 (WP_003665850), and *L. reuteri* ATCC 53608 (WP_003674287) and identities over 55% were also found with the BSH enzymes of several *Lactobacillus* and *Enterococcus* strains.

On the other hand, a conserved domain belonging to the superfamiliy of penicillin V acylase (PVA) (Accession: cd00542) was detected in BSH from CRL 1098 strain. PVA is an enzyme that catalyzes the hydrolysis of penicillin V to yield 6-APA and has an N-terminal nucleophilic cysteine, as do other members of the Ntn hydrolase family to which PVA belongs.

Multiple sequence alignment of the deduced amino acid sequences between BSH of CRL 1098 strain and other BSH and PVAs from *Bacillus* and *Listeria* strains was performed. As shown in Figure. 1 five catalytically important amino acids residues (C, D, N, N, and R) are highly conserved in CRL 1098 strain while four amino acid motifs (YFGRNXD, NEXGLXXAGLNF, VXVLTNNPXF, and SXSRFVRXAF), located around the active site were partially conserved. The Cys-2 becomes a catalytic centre after removal of the initiation formyl methionine by an autoproteolytic process, which is one of the common features of the Ntn hydrolase superfamily [19]. The thiol (SH) group of Cys-2 has been shown to be essential for BSH catalysis [1, 38]. However, the active sites showed little different between BSH and PVAs examined. For

Figure 1. CLUSTAL alignment of BSHs

```
                                10        20        30        40        50        60
                                |         |         |         |         |         |
AAR39435.1|          --MCTGVRFSDDEGNMYFGRNLDWSFSYGETILVTPRGYQYDYEYGAEGKSE-PNAVIGV
WP_032743741.1       --MCTGVRFSDDEGNTYFGRNLDWSFSYGETILVTPRGYHYDTVFGASGKAK-PNAVIGV
ACH81023.1           --MCTSVIYTAG--DYYFGRNLDLEVNLGQEVVITPRNKTLEFREMPNLEH--HYAIIGM
WP_004897162.1       --MCTSIVYSSNN-HHYFGRNLDLEISFGEHPVITPRNYEFQYRKLPNKKA--KYAMVGM
UNK_211317           --MCTSIIFSPK--DHYFGRNLDLEITFGQQVVITPRNYTFKFRKMPSLKK--HYAMIGI
UNK_211315           --MCTSITYVTS--DHYFGRNFDYEISYNEVVTVTPRNYKLNFRKVNDLDT--HYAMIGI
UNK_211316           --MCTSITYTTN--DHYFGRNLDLEVSYNEQVTITPRNYPFVYRKVPKQES--HYAIIGM
AAA22654.1           MLGCSSLSIRTTDDKSLFARTMDFTMEPDSKVIIVPRNYGIRLLEKENVVINNSYAFVGM
CAC98525.1           --MCTSFVLETLDGKHLLSRTMDFAFILEANPTISPRNYGWKSSTDGANYIN-RYAFVGA
                           *:..          .     :. * .:*     .          :  **.          *.:*
Consensus            MLMCTSI4YST22GDHYFGRNLDLE2SYGE2V2ITPRNY2F22RK3PNLK3NNHYAMIGM

                                70        80        90        100       110       120
                                |         |         |         |         |         |
AAR39435.1           GVVMTDRPMYFDCANEHGLAIAGLNFPGYASFAHEPVEGTENVATFEFPLWVARNFDSVD
WP_032743741.1       GVVMADRPMYFDCANEHGLAIAGLNFPGYASFVHEPVEGTENVATFEFPLWVARNFDSVD
ACH81023.1           SIVRDDYPLYFDGVNEKGVGMAGLNFDGPAHYFPV-QEGKDNIASFELVPYILAAASSVA
WP_004897162.1       AIVEDNYPLYFDASNEEGLGIAGLNFDGPCHYFPE-VSGKNNVTPFELIPYLLSQYTTVA
UNK_211317           SLDMDDYPLYFDATNEKGLGMAGLNYPGNATYYEE-KENKDNIASFEFIPWILGQCSTIS
UNK_211315           AAGIADYPLYYDATNEKGLSMAGLNFSGYADYKEI-QEGKDNVSPFEFIPWILGQCSTVG
UNK_211316           AAVVDNYPLYYDATNEKGLSMAGLNFPGNAHFFDE-DPEKTNITPFEFIPWILSQCKNVE
AAA22654.1           GSTDITSPVLYDGVNEKGLMGAMLYYATFATYADEPKKGTTGINPVYVISQVLGNCVTVD
CAC98525.1           G-RELDKYIFADGLNEEGLSCASLYLPGEAVYAPAPVEGKINLAPQEFLLWLLGTCATIK
                     .           :    *   * * .*:   *    *       .   :          .     .:.    .    :        .:
Consensus            G3VMDDYPLYFDATNEKGL2MAGLNFPGYAHY2PEPVEGKDN2APFEFIPWILGQCSTVD

                                130       140       150       160       170       180
                                |         |         |         |         |         |
AAR39435.1           EVEEALKNVTLVSQVVPGQ-QESLLHWFIGDGT-RSIVVEQMADGMHVHHDDVDVLTNQP
WP_032743741.1       EVEEALRNVTLVSQIVPGQ-QESLLHWFIGDGK-RSIVVEQMADGMHVHHDDVDVLTNQP
ACH81023.1           EAKKLLSNANIANINFSDKLQAAPLHWIIADKTGASVTVESTAKGLNVYDNPVGVLTNNP
WP_004897162.1       EVKEALKSVNLVKINFSEKLQLSPLHWLMADKTGESIVVESTLSGLHVYDNPVHVLTNNP
UNK_211317           EVKDLLSRINIADLNFSEKMQASSLHWLIADKTGTSLVVETDKDGMHIYDNPVGCLTNNP
UNK_211315           EAKKLLKNINLANINYSDELPLSPLHWLLADKE-KSIVIESMKDGLHIYDNPVGVLTNNP
UNK_211316           EARTLLADINLVNINFSEELPLSPLHWILADQE-TSIVIESVAEGLKIYDNPIGVLTNNP
AAA22654.1           DVIEKLTSYTLLNEANIILGFAPPLHYTFTDASGESIVIEPDKTGITIHRKTIGVMTNSP
CAC98525.1           DVEAKLSVINLVDQPVPLLGITTPLHWIFTDKSGRCVVIEPTETSLRIKENPVGVMTNTP
                     : .      *    .:   .            .  ** : : *       . :.: *      . :   :   .   :    :** *
Consensus            EVKELL2NINLVNINFSEKLQ2SPLHW2IADKTGRSIVVES2ADGLHIYDNPVGVLTNNP

                                190       200       210       220       230       240
                                |         |         |         |         |         |
AAR39435.1           TFDFHMENLRNYMCVSNEMAEPTTWGKAELSAWGAGVSMHGIPGDVSSPSRFVRVAYTNT
WP_032743741.1       TFDFHMENLRNYMCVSNEMAEPTSWGKASLTAWGAGVGMHGIPGDVSSPSRFVRVAYTNA
ACH81023.1           EFPRQLLNLSNYRSIAPANPANVFAPNVDLPVYSRGLGTHFLPGGMDSESRFVKATFTKM
WP_004897162.1       EFPGQLSNLANYSNIAPSQPKNTLVPGVDLNLYSRGLGTHFLPGGMDSASRFVKVAFVRA
UNK_211317           QFPKQLFNLNNYADVSPKMPKNNFSDKVNMAGYSRGLGSHNLPGGMDSESRFVRVAFNKF
UNK_211315           SFDYQLFNLNNYRVLSSENPKNNFSNQISLNAYSRGMGGIGLPGDLSSVSRFVKATFTKL
UNK_211316           TFDYQLFNLNNYRSVSPTTPKNHFSDELDLSVYSRGMGGLGLPGDLSSASRFVKVAFTKL
AAA22654.1           GYEWHQTNLRAYIGVTPNPPQDIMMGDLDLTPFGQGAGGLGLPGDFTPSARFLRVAYWKK
CAC98525.1           RIEWHIENLRNYTGLQATQLAPVKFGEYMAKPFSQGTGTSKLPGGYTPPERFVRAAYLKE
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                     :  **    *  :                     :.   *  .      : **.    .  **: :. : :  .
Consensus            TFD3QL2NLRNYR3VSPEMPKNTFSGKVDL3AYSRGLG2HGLPGDMSSPSRFVRVAFTK2

                          250       260       270       280       290       300
                           |         |         |         |         |         |
AAR39435.1           HYPQQNNEAANVSRLFHTLVSVQMVDGMSKMGNGQFERTLFTSGYSGKTNTYYMNTYEDP
WP_032743741.1       HYPQQNDEAANVSRLFHTLGSVQMVDGMAKMGNGQFERTLFTSGYSSKTNTYYMNTYDDP
ACH81023.1           HAPVGNSEVENITNYFHILQSVEQQKGLDEVAPNTFEYTIYSDGSNLKKGIFYYKTYENS
WP_004897162.1       HSPQGNNELSSVTNYFHILHSVEQPKGTDEVGPNSYEYTIYSDGTNLETGTFYYTNYENN
UNK_211317           NAPIAETEEENIDTYFHILHSVEQQKGLDEVGPNSFEYTIYSDGTNLDKGIFYYTTYSNK
UNK_211315           NSVSGDSESESISQFFHILGSVEQQKGLCDVGDGKYEYTIYSSCCNVDKGIYYYRTYEDS
UNK_211316           NSVKATTEQESVSQFFHILKSVEQQKGLCDVGNGKFEYTIYSSCCNVDKGIYYYTTYTNS
AAA22654.1           YTEKAKNETEGVTNLFHILSSVNIPKGVVLTNEGKTDYTIYTSAMCAQSKNYYFKLYDNS
CAC98525.1           NIIKAKNEEEAITNVWYVLNSVRIPNGAVIKESGDPDFTQYVASMCSESKTYYFTSYENN
                         *   :   : :  * **.   . *        .   :  * :       . .  :*    *  :
Consensus            2SP2ANNE2ENV2N2FHIL2SVEQQKGLDEVG2GKFEYTIYSSG4NL2KG2YYYTTYENS

                        310        320     330
                         |          |       |
AAR39435.1           AIRSFAMSDFDMD-SSELITAD----------------
WP_032743741.1       AIRSYAMADYDMD-SSELISVAR---------------
ACH81023.1           QINAVDMHKEDLE-ASELITYPVQNKQIINQQN------
WP_004897162.1       QINAIELNKENLN-GDELIDYKLIEKQTINYQN------
UNK_211317           QINVVDMNKEDLD-SSNLITYDMLDKTKFNHQN------
UNK_211315           QITAIDMNKEDLD-SHKLISYPIIEKQQIKYIN------
UNK_211316           QINAVDMTKEDLD-STELLTYPLIEEEQINYVN------
AAA22654.1           RISAVSLMAENLN-SQDLITFEWDRKQDIKQLNQVNVMS
CAC98525.1           QINSVTLSKEVIENMKEPTTYQVDKVQNVNKLV------
                     *         :     : :    .
Consensus            QINAVDMNKEDLDNSSELITYP2IEKQQINYQNQVNVMS
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Protein sequences were obtained from the National Center for Biotechnology Information site (http://www.ncbi.nlm.nih.gov/). Alignments were performed by using CLUSTAL OMEGA (http://www.ebi.ac.uk/Tools/msa/clustalo/). Five proposed active sites (C, D, N, N, R) and four amino acid motifs, (YFGRNXD, NEXGLXXXGLNF, VXVLTNNPXF, and SXSRFVRXAF) are indicated by dark grey and grey shadow boxes, respectively. Identical amino-acids are marked by an asterisk, conserved substitutions are marked by two dots, and semi-conserved substitutions are marked by a single dot. AAR39435.1: *Bifidobacterium (Bif.) bifidum*, WP_032743741.1: *Bif. longum*, WP_002313629.1: *Enterococcus (Ent.) faecium*, WP_019723927.1: *Ent.mundtii*, WP_004897162.1: *Lactobacillus (L.) johnsonii*, ACH81023.1: *L. reuteri* CRL 1098, WP_003546965.1: *L. acidophilus*. AAA22654.1: *Bacillus sphaericus*, CAC98525.1: *Listeria monocytogenes*.

example, the catalytic residue of Asn82 was replaced by Tyr82 in PVAs from *Bacillus* and *Listeria* strains. In addition, phylogenetic tree analysis (Figure 2) revealed that the *bsh* genes in *L. reuteri* strains were highly conserved but differed from the BSH enzymes from other genera and PVA from of *Bacillus* and *Listeria* strains.

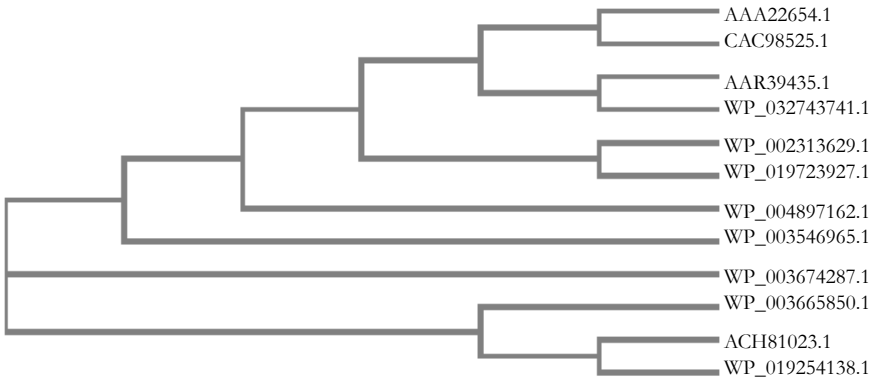
Although the physiological role of the BSH has not yet been clearly established, the high level of conservation supports an important role of this gene in the physiology and lifestyle of *L. reuteri* strains.

BSH expression in a heterologous host

Substrate specificity of BSH

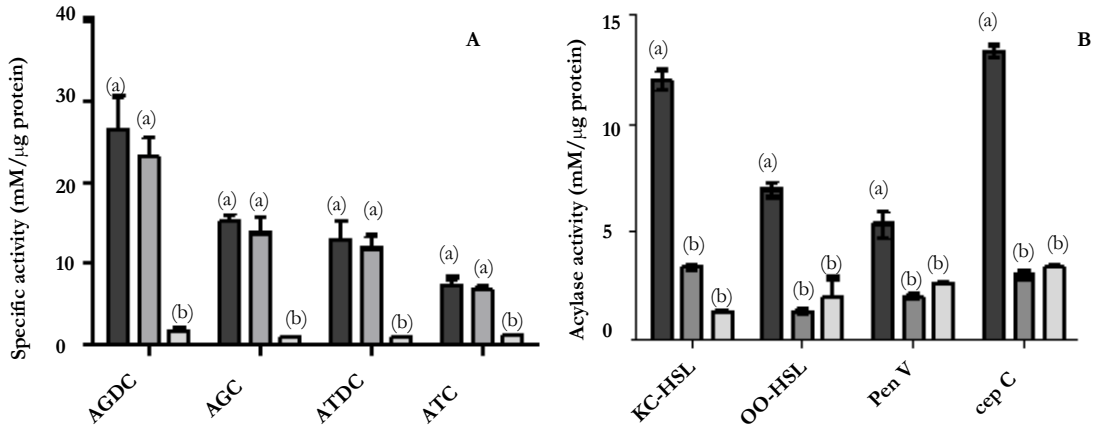
The substrate specificity of the BSH of *L. reuteri* CRL 1098 was determined in enzyme assays with the four major human bile salts [19]. As shown in Figure 3 A, the enzymes exhibited a preference for glycine-conjugated BA over taurine-conjugated forms. Both CRL 1098 strain as the recombinant *Lac. lactis* NZ9000 cells, expressing the *bsh* gene showed activity against all BA tested, being the enzyme in both cases more active on GDCA. The highest affinity observed by the glycoconjugated substrates is consistent with our previous results [39, 38], as well as for numerous choloylglycine hydrolase enzymes described in the literature [19], which could be related to the higher toxicity of glycine-conjugated BA respect to taurine-conjugated BA [6]. Although the precise mechanism is unknown, the capability to hydrolyze BA may contribute to the survival and persistence of bacterial strains in the intestinal tract, as was previously suggested by many authors [19, 39, 1]. Since deconjugated BAs display reduced solubility compared to their conjugated counterparts, especially at lower pH values, BA hydrolysis may lead to precipitation of the BA and thus may reduce the damage caused by these compounds. In this sense, a recent study reported that expression of BSH improved BA tolerance of *Lac. lactis*, however excessive production of BA micelles by BSH activity inhibited cell growth [40].

Figure 2. Phylogenetic tree illustrated the relationship among BSHs from *Lactobacillus* and PVA from *Bacillus sphaericus* and *Listeria monocitogenes*.



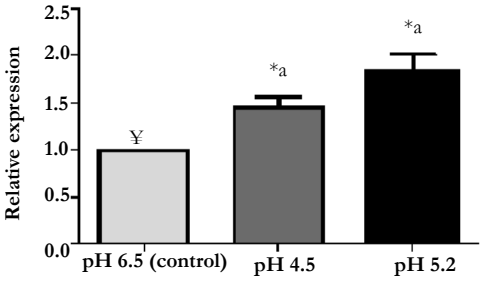
AAA22654.1: *Bacillus sphaericus*, CAC98525.1: *Listeria monocytogenes*, AAR39435.1: *Bifidobacterium (Bif.) bifidum*, WP_032743741.1: *Bif. longum*, WP_002313629.1: *Enterococcus (Ent.) faecium*, WP_019723927.1: *Ent.mundtii*, WP_004897162.1: *Lactobacillus (L.) johnsonii*, WP_003546965.1: *L. acidophilus*, WP_003674287.1: *L. reuteri* ATCC 53608, WP_003665850.1: *L. reuteri* 100-23, ACH81023.1:*L. reuteri* CRL 1098, WP_019254138.1: *L. reuteri* TD1.

Figure 3. BSH activity and alternative acylase functionality of CFE of *L. reuteri* CRL 1098, *Lac. lactis* NZ 9000-pBTR3 and *Lac. lactis* NZ 9000-pSA3 against conjugated BA and alternative substrates.



Hydrolase activity were measured in CFE by determining the amount of amino acids liberated by the BSH enzyme from conjugated BA (GDCA, GCA, TDCA and, TCA) and penicillin V (penV), ketocaproyl-homoserine lactone (KC-HSL), oxooctanoyl-homoserine lactone (OO-HSL), cephalosporin C (cep C). Variables with the same superscript letter show no significant differences between them ($P < 0.05$).

Figure 4. Relative expression of *bsh* gene in *L. reuteri* CRL 1098 cells grown in MRS broth at different pHs in the presence of GDCA.



***RecA* primers were used as internal gene control to normalize the amount of RNA added and the relative expression was calculated using the $2^{-\Delta\Delta CT}$ method.**

¥ Cells growing in MRS broth at pH 6.5 were using as reference condition and an arbitrary value of 1 was assigned. * Indicate significant difference with the control; and the variables with the same letter show no significant differences between them ($P < 0.05$).

Alternative acilase activity

The BSH protein of CRL 1098 shares some sequence homology (30.83 % of identity at amino acid level) with the experimentally verified PVA of *List. monocytogenes* EGDe (Figure 1), which are in turn related to β-lactam acylases and to acyl-homoserine lactone acylases, which play a key role in *quorum* sensing-dependent gene regulation in Gram-negative bacteria [1]. However, despite some BSHs share significant sequence homology with some of these enzymes and the type of bond target is identical; the structures of the respective substrates differ considerably. Thus, the hydrolysis ability of CRL 1098 strain and the heterologous host (*Lac. lactis* NZ9000-pBTR3) was evaluated on different substrates: penicillin V, ampicillin, ketocaproyl-homoserine lactone and oxooctanoyl-homoserine lactone. As shown in (Figure3) B, *L.reuteri* CRL 1098 showed activity against all the substrates assayed, especially on ketocaproyl-homoserin lactone. However, the hydrolysis observed could not be related to the presence of *bsh* gene since the heterologous strain (*Lac. lactis* NZ9000-pBTR3) - showed detectable activity only toward the conjugated BA, confirming the specificity of BSH enzyme codified by *bsh* gene cloned. Lambert *et al.* [6] reported some acylase activity by BSH2, BSH3 and BSH4 of *L. plantarum* WCFS1 toward two types of acyl-homoserine lactones, indicating that these enzymes has a broad substrate specificity. However, these enzymes displayed very low hydrolysis activity against BA. In contrast, the presence of BSH1 appeared to correlate exclusively with the capability to hydrolyze BA and not the others studied substrates.

Regulation of *bsh* gene

In a previous study we reported an up-regulation of the *bsh* gene when exponential phase cells of *L. reuteri* CRL 1098 were growing in the presence of GDCA [41]. The BSH of *L. reuteri* CRL 1098 is active in a range of pH from 4.5 to 5.5, with an optimum at pH 5.2 [38]. In this study, the regulation of *bsh* gene of *L. reuteri* CRL 1098 in the presence of GDCA and at different pH values was evaluated using quantitative Real Time-PCR. As shown in (Figure 4), an increase in *bsh* gene expression levels of 1.34 ± 0.12 and 1.85 ± 0.18 at pH 4.5 and 5.2, respectively, compared to pH 6.5 (control condition) was observed. Koskenniemi *et al.* [42] and Duary *et al.* [43] reported the strong up regulation of *bsh* genes upon bile exposure. Moreover, Bron *et al.* [44], applying DNA microarrays, reported an over-expression in *bsh1* gene and a significant reduction in *bsh3* gene in *L. plantarum* WCFS1 when cells were grown in porcine bile. On the contrary, Lambert *et al.* [6] reported that the expression of the four *bsh* genes of *L. plantarum* WCFS1 was not induced as a consequence of the exposure to porcine bile. Thus, the BSH regulation at genomic, transcriptomic or proteomic level strongly fluctuates according to the studied microorganisms. However, until now, the influence of pH on the gene expression of *bsh* has not been described.

In the present work, the identification, cloning and characterization of the *bsh* gene of *L. reuteri* CRL 1098 is reported. It was found that the *bsh* gene is organized as a single operon and is present as a single copy in the *L. reuteri* CRL 1098 and others *L. reuteri* genomes. The *bsh* gene with its putative promotor region was cloned and expressed in the heterologous host *Lac. lactis* NZ9000. Hydrolysis activity was specific towards BA, in particular GDCA, but not against alternative substrates, confirming the functionality and specificity of the cloned gene. Finally, a significant up-regulation of the *bsh* gene was observed at pH 5.2. Our studies suggest that BSHs would have an important but so far unknown role in the physiology and lifestyle of *L. reuteri* strains. The present work would be useful for future investigations in order to deepen the influence of the BSH in the reduction of blood cholesterol levels in humans and animals and unravel its ecological role.

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