Identification and Characterization of a Bile Salt Hydrolase from *Lactobacillus salivarius* for Development of Novel Alternatives to Antibiotic Growth Promoters

Zhong Wang, a,b Ximin Zeng, a Yiming Mo, a Katie Smith, a Yuming Guo, b and Jun Lin a

Department of Animal Science, University of Tennessee, Knoxville, Tennessee, USA, a and State Key Laboratory of Animal Nutrition, College of Animal Science and Technology, China Agricultural University, Beijing, China b

Antibiotic growth promoters (AGPs) have been used as feed additives to improve average body weight gain and feed efficiency in food animals for more than 5 decades. However, there is a worldwide trend to limit AGP use to protect food safety and public health, which raises an urgent need to discover effective alternatives to AGPs. The growth-promoting effect of AGPs has been shown to be highly correlated with the decreased activity of intestinal bile salt hydrolase (BSH), an enzyme that is produced by various gut microflora and involved in host lipid metabolism. Thus, BSH inhibitors are likely promising feed additives to AGPs to improve animal growth performance. In this study, the genome of *Lactobacillus salivarius* NRRL B-30514, a BSH-producing strain isolated from chicken, was sequenced by a 454 GS FLX sequencer. A BSH gene identified by genome analysis was cloned and expressed in an *Escherichia coli* expression system for enzymatic analyses. The BSH displayed efficient hydrolysis activity for both glycoconjugated and tauroconjugated bile salts, with slightly higher catalytic efficiencies (K_m/Kcat) on glycoconjugated bile salts. The optimal pH and temperature for the BSH activity were 5.5 and 41°C, respectively. Examination of a panel of dietary compounds using the purified BSH identified some potent BSH inhibitors, in which copper and zinc have been recently demonstrated to promote feed digestion and body weight gain in different food animals. In sum, this study identified and characterized a BSH with broad substrate specificity from a chicken *L. salivarius* strain and established a solid platform for us to discover novel BSH inhibitors, the promising feed additives to replace AGPs for enhancing the productivity and sustainability of food animals.

A ntitibiotic growth promoters (AGPs) are defined as a group of antibiotics used in feed at subtherapeutic levels to improve average daily weight gain and feed efficiency in agricultural animals (8, 14). This husbandry technique has been practiced since the 1950s, and the net benefit of antibiotic feeding to food animals is still measurable now. Although it has been believed that the low dosages of AGPs used for growth promotion are an unquantified hazard, recent epidemiological studies strongly suggest that use of AGPs is associated with the emergence of drug-resistant bacteria that may be transmitted to humans, posing a significant threat to public health (25,31). Therefore, Denmark banned all AGPs in 1998 and European Union member nations also banned all AGPs in 2006. To date, there is a worldwide trend of limiting AGP use in food animals (8,14, 25). However, it has been shown that AGP bans had a negative impact on animal health and productivity in some countries (5). Thus, ending the use of AGPs creates challenges for the animal feed and feed additive industries. Effective alternatives to AGPs are urgently needed to maintain current animal production levels without threatening public health.

Understanding the mode of action of AGPs is critical for developing effective and novel AGP replacements. Given the antibacterial nature of AGPs and the important role of intestinal microbiota in host physiology, it is widely accepted that the growth-promoting effect of AGPs is primarily mediated through altered microbiota in the animal intestine (8, 14, 21). Therefore, examination of microbial diversity and dynamics in response to AGP treatment would facilitate the development of microbiota-based strategies for growth promotion. With the aid of culture-independent molecular approaches, investigations of the effect of AGPs on intestinal microbiota have been initiated in different food animals, including poultry and swine, which greatly improved our understanding of intestinal microbiota changes in response to AGPs (21). However, in previous studies, the relevance of an observed microbial structure shift to animal growth was weak, primarily due to the lack of a growth-promoting effect of AGPs in the experimental system (21). Therefore, to clearly reveal the relationship between AGP usage and intestinal microbiota, it is imperative to obtain high-quality, growth-relevant intestinal samples for microbial ecology study by simulating industrial conditions for the observation of growth promotion in an experimental system (21).

Although the definitive relationship between AGP usage and the complex microbial ecology for optimal growth promotion is still largely unknown, previous animal studies (13, 15, 18, 19) have consistently shown that the growth-promoting effect of AGPs was highly correlated with the decreased activity of bile salt hydrolase (BSH), an enzyme produced by various commensal bacteria in the intestine (4, 8). Consistent with these findings, independent chicken studies also showed that AGP usage significantly reduced the population of *Lactobacillus* species, the major BSH-producing

Received 14 August 2012 Accepted 4 October 2012
Published ahead of print 12 October 2012
Address correspondence to Jun Lin, jlin6@utk.edu.
Supplemental material for this article may be found at http://aem.asm.org/
Copyright © 2012, American Society for Microbiology. All Rights Reserved.  
doi:10.1128/AEM.02519-12
commensals in the intestine; in particular, Lactobacillus salivarius, the dominant lactic acid bacterium present in the chicken intestine, was reduced in response to AGP treatment (9, 10, 15, 19, 32). The function of BSH is to catalyze conjugated bile salts to unconjugated bile salts; the conjugated bile salts are more efficient than unconjugated molecules in the emulsification of dietary lipids and the formation of micelles (4). Thus, the conjugated bile salts are needed to maintain efficient lipid digestion and the absorption of fatty acids. Therefore, the decreased intestinal BSH activity in the AGP-treated animals would increase the relative abundance of conjugated bile salts, consequently promoting lipid metabolism and energy harvest and enhancing feed efficiency and body weight gain in food animals. Based on these observations, inhibition of BSH activity using specific inhibitors is likely a promising approach to improve the growth performance of food animals.

To test the above hypothesis and to achieve the goal of developing BSH inhibitor-based feed additives as alternatives to AGPs for improving both the quantity and the safety of our food supply, it is critical to develop an effective system for screening desired BSH inhibitors. In this study, the genome of L. salivarius strain NRRL B-30514, a BSH-producing commensal from chicken intestine (29), was sequenced by a 454 GS FLX sequencer. A BSH gene identified by genome analysis was cloned and expressed in an Escherichia coli expression system. The purified BSH was subjected to various enzymatic analyses and was also used for screening of BSH inhibitors. Copper and zinc, the two BSH inhibitors demonstrated in this study, have recently been used at high dietary levels for growth promotion in different food animals with unclear mechanisms (17). Thus, the findings from this study strongly support our hypothesis and establish an effective system for screening novel BSH inhibitors in the future.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. Lactobacillus salivarius NRRL B-30514, previously isolated from chicken intestine (29), was routinely grown in de Man-Rogosa-Sharpe (MRS) broth or on agar (Difco Laboratories, Detroit, MI) at 37°C under anaerobic conditions, which were generated by an AnaeroGen gas pack (Becton, Dickinson Company) in an enclosed jar. Escherichia coli strain BL21(DE3) (Novagen) was used as a host for gene expression. The plasmid vector pET-21b(+) (Novagen) was used for expressing His-tagged recombinant protein. Escherichia coli strains were grown routinely in Luria-Bertani (LB) broth, with shaking (250 rpm), or on agar supplemented with ampicillin (100 μg/ml) at 37°C overnight.

Detection of bacterial BSH activity using plate assay. The BSH activity of L. salivarius NRRL B-30514 was detected by using a plate assay as described previously (7), with slight modifications. Briefly, the fresh over-night cultures were streaked on MRS agar supplemented with 0.5% (wt/vol) taurodeoxycholic acid (Sigma) and 0.035% (wt/vol) CaCl2. The plates were then incubated for 5 days at 37°C under anaerobic conditions. The BSH activity was indicated by the formation of opaque halos of precipitated unconjugated bile acids surrounding the colonies on the bile-salt-containing MRS agar plate.

Genome sequencing and analysis of L. salivarius NRRL B-30514. The 454 GS FLX pyrosequencing technology (24) was used to sequence the L. salivarius NRRL B-30514 genome. The genomic DNA from L. salivarius NRRL B-30514 was extracted using the UltraClean microbial DNA isolation kit (MoBio) and size fractionated into 400- to 500-bp fragments, which subsequently were blunt-end-repaired, ligated to specific adaptors, immobilized and amplified on the DNA capture beads, and then sequenced using the PicoTiter Plate in the 454-FLX instrument, according to FLX Titanium protocol. A quarter of the plate was used for the genome sequencing of this strain. The generated reads were de novo assembled into contigs using 454 Newbler Assembler software. The assembled genome sequence draft was analyzed by the RAST server (Rapid Annotation using Subsystem Technology; http://rast.nmpdr.org/) for automatic annotation (3).

Sequence analysis of BSH. The BLASTP program from the National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov/) was used for nucleotide and amino acid sequence analyses. To determine the phylogenetic relationship between the identified BSH from L. salivarius NRRL B-30514 and the BSH from other bacteria, multiple sequence alignment was performed using the ClustalW program in MEGA 4.0 (20). To identify the conserved amino acids that may play a critical role in BSH activity, multiple sequence alignment of BSH enzymes from different bacterial strains was performed using the ClustalW2 program (http://www.ebi.ac.uk/Tools/clustalw2/index.html).

Production and purification of rBSH. A histidine-tagged recombinant BSH (rBSH) was produced in E. coli by using the pET-21b(+) vector (Novagen). Briefly, the bsh target gene was PCR amplified from L. salivarius NRRL B-30514 using purified genomic DNA as a template and the primer pairs bshF (5’-CCGCGATCATGTTGTAACGACGTTACCTTT-3’) and bshR (5’-CGCGTCGAGATTTCACTTTATATTATTTGT-3’); underlined sequence, BamHI site) and bshF (5’-CCGCGATCATGTTGTAACGACGTTACCTTTATATTATTTGT-3’; underlined sequence, XhoI site). Cloning, expression, and purification of rBSH peptides were performed using the procedure described in our previous studies (22, 23). The plasmid pET21b-BSH in the E. coli BL21(DE3) clone (JL885) producing rBSH was sequenced, and no mutations in the coding sequence of bsh were detected. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with a 12% (wt/vol) polyacrylamide separating gel was performed to monitor the production and purification of the rBSH. The purified rBSH was finally dialyzed against phosphate-buffered saline (PBS) buffer containing 10% glycerol (pH 7.0) and stored in a freezer at −80°C prior to use. Protein concentration was measured by a bichinchoninic acid (BCA) protein assay kit (Pierce).

BSH activity assay. BSH activity was determined with a two-step procedure by estimating the amounts of amino acids liberated from conjugated bile salts as described previously (30), with slight modifications. In the first step, 178 μl of reaction buffer (0.1 M sodium-phosphate, pH 6.0), 10 μl of purified rBSH (1 μg/μl), 10 μl of a specific conjugated bile acid (100 mM), and 2 μl of 1 M dithiothreitol (DTT) were mixed gently and incubated at 37°C for 30 min. A 50-μl aliquot of the reaction mixture was then immediately mixed with 50 μl of 15% (wt/vol) trichloroacetic acid to stop the reaction, followed by centrifugation for 5 min at 12,000 × g at room temperature to remove the precipitate. The supernatant was used in the second step, in which 50 μl of the supernatant was thoroughly mixed with 950 μl of ninhydrin reagent mix (0.25 ml of 1% [wt/vol] ninhydrin in 0.5 M sodium-citrate buffer [pH 5.5], 0.6 ml of glyceral, and 0.1 ml of 0.5 M sodium-citrate buffer [pH 5.5]). The reaction mix was incubated in a boiled water bath for 14 min, followed by cooling in ice water for 3 min. The absorbance at 570 nm (A570) was measured using a Smart Spec Plus spectrophotometer (Bio-Rad). A control without BSH added was set up in each independent experiment. Sodium salts of various glycoconjugated and tauroconjugated bile acids were purchased from Sigma and used in this study. A standard curve was performed for each assay by using either glycine or taurine. Experiments were carried out in triplicate. BSH activity was expressed as μmol of amino acids released from the substrate per minute per mg of BSH.

A kinetic study of the rBSH for a specific conjugated bile acid was performed by measuring the reaction rate (V) with substrate concentrations ranging from 0 to 8 mM (0, 1, 2, 4, 6, and 8 mM). An assay of each concentration was run in triplicate. Reaction mixtures contained 10 μg of rBSH enzymes. Initial velocities (V0) of hydrolysis were measured for 10 min with five time points (1, 3, 5, 7, and 10 min after reaction initiation). The Michaelis–Menten constant (Km) and maximum velocity for the reaction (Vmax) were calculated from a Lineweaver-Burk plot. The Km was defined as Vmax/[E], in which [E] is the molar concentration of rBSH. The
in the 1,923,653-bp genome assembled from 108 contigs. Two
tomato annotation revealed 1,878 coding sequences and 66 RNAs
statistics detailed in Table S1 in the supplemental material). Au-
tated 238,829 reads with a total of 96,071,065 bases (sequencing
genomic DNA of this strain was sequenced using a 454 GS FLX
cholate (Fig. 1). To rapidly identify potential
zones on MRS plate supplemented with sodium taurodeoxy-
demonstrated by typical colonies with surrounding precipitation
(29), displayed a BSH ability to hydrolyze conjugated bile salts, as
MRS agar plate (A) or an MRS agar plate containing 0.5% taurodeoxy-
JX120369, respectively.
RESULTS
Effect of pH and temperature on the activity of rBSH. The tempera-
ture dependence of the enzyme activity was measured by assaying the
rBSH in 0.1 M sodium phosphate buffer (pH 6.0) at various temperatures
ranging from 20 to 75°C. The optimal pH of the rBSH was determined at
pH values ranging from 3.0 to 8.0 using 0.1 M sodium citrate-citrate acid
buffer (pH 3.0 to 5.4) or 0.1 M sodium phosphate buffer (pH 6.0 to 8.0).
All assays were performed using the standard procedure described above
with glycocholic acid as the substrate. The experiments were performed in
triplicate.
Effect of dietary compounds on the activity of BSH. The following
compounds, which have been used as feed additives in food animals, were
used in a BSH activity assay at a final concentration of 5 mM: CuCl2,
ZnCl2, MnCl2, FeCl3, CaCl2, MgCl2, CoCl2, KCl, NaCl, KI, FeSO4, FeCl3,
MnSO4, ZnSO4, NaSO4, MgSO4, NaSeO3, KIO3, NaHCO3, and ascorbic
acid (vitamin C). Prior to the addition of substrate sodium glycocholate,
the rBSH was incubated with or without a specific compound for 30 min
at 37°C. The standard enzyme assay was performed as described above to
determine the residual enzyme activity. A control without a compound
added was set up in each independent experiment. All assays were per-
formed in triplicate. Percentage of inhibition was calculated by dividing
the inhibited activity (mean activity of the control minus mean residual
activity of the presence of a compound) relative to the mean activity of the
control and then multiplied by 100.
Nucleotide sequence accession numbers. The genome sequence of
L. salivarius NRRL B-30514 was deposited into GenBank under BioProject
number PRJNA167612. The identified bsh1 and bsh2 genes have been
deposited into GenBank under the accession numbers JX120368 and
JX120369, respectively.
Identification of putative BSH genes in the L. salivarius NRRL
B-30514 genome. L. salivarius NRRL B-30514, a chicken isolate
(29), displayed a BSH ability to hydrolyze conjugated bile salts, as
demonstrated by typical colonies with surrounding precipitation
zones on MRS plate supplemented with sodium taurodeoxy-
cholate (Fig. 1). To rapidly identify potential bsh genes, the
genomic DNA of this strain was sequenced using a 454 GS FLX
sequencer with Titanium series reagents. The sequencing gener-
ated 238,829 reads with a total of 96,071,065 bases (sequencing
statistics detailed in Table S1 in the supplemental material). Au-
tomatic annotation revealed 1,878 coding sequences and 66 RNAs
in the 1,923,653-bp genome assembled from 108 contigs. Two
putative bsh genes, bsh1 and bsh2, were identified in contigs 107
and 7, respectively. Specifically, bsh1 (nucleotides 109583 to
110557 of contig 107) encodes a putative 324-amino-acid (aa)
choloylglycine hydrolase while bsh2 (nucleotides 4566 to 5543 of
contig 7) encodes a 325-aa choloylglycine hydrolase in the same
enzyme family (EC 3.5.1.24).
Homology and phylogenetic analyses of identified BSH. The deduced amino acid sequences of the bsh1 and bsh2 genes identified in
L. salivarius NRRL B-30514 were aligned with the BSH of other bacteria. As shown in Fig. S1 in the supplemental material,
the identified BSH1 and BSH2 enzymes share only a 24% aa iden-
tity. The BSH1 enzyme is highly homologous to the megaplasmid-
borne BSH1 enzyme of L. salivarius strain UCC118 (98% aa iden-
tity) (6) and L. salivarius strain JCM1046 (96% aa identity) (12).
The BSH2 enzyme identified in this study shared a high homology
with the chromosomal BSH2 enzyme of L. salivarius
UCC118 (99% aa identity) (6). In addition, a genome comparison
between the L. salivarius NRRL B-30514 and UCC118 strains (6)
showed identical or similar genomic organization surrounding
the bsh1 and bsh2 genes (data not shown), suggesting that the
identified bsh1 gene is carried by a megaplasmid while the bsh2
gene is in a chromosome.
Both the BSH1 and BSH2 enzymes are phylogenetically distant
from the BSH enzymes identified in other bacteria (see Fig. S1 in
the supplemental material). Specifically, the BSH1 enzyme shared
low homology to the BSH enzymes of Lactobacillus acidophilus
strain PF01 (35% aa identity), L. acidophilus strain NCFM (45% aa
identity), Lactobacillus gasseri (35% aa identity), Lactobacillus
johnsonii (42% aa identity), Lactobacillus plantarum (53% aa
identity), Bifidobacterium bifidum (32% aa identity), Bifidobacterium
longum (31% aa identity), Enterococcus faecium (54% aa identity),
Listeria monocytogenes (55%), Clostridium perfringens (36%), and
Bacillus sphaericus strain PVA (31%). Despite the significant se-
quence variation among the identified BSH1 and BSH2 enzymes
and the other BSH enzymes from various bacterial species, the
BSH enzymes contain all conserved, catalytically important
amino residues in the active site of BSH (Cys-2, Arg-16, Asp-19,
and the other BSH enzymes from various bacterial species, the
BSH enzymes contain all conserved, catalytically important
amino residues in the active site of BSH (Cys-2, Arg-16, Asp-19,
appeared universal presence of bsh1 homologs in L. salivarius
(12), we subsequently targeted bsh1 in L. salivarius
Multiple alignment of the amino acid sequences of BSH from various bacteria. The sequences were aligned with ClustalW (http://www.ebi.ac.uk/Tools/clustalw). Identical amino acids are marked by an asterisk, and conserved and semiconserved substitutions are marked by two dots and by a single dot, respectively. The shaded residues are conserved amino acids implicated in the active site. Six proposed active sites (Cys-2, Arg-16, Asp-19, Asn-79, Asn-171, and Arg-224) are highlighted in white on a black background.

Wang et al. 8798 aem.asm.org Applied and Environmental Microbiology on March 27, 2021 by guest http://aem.asm.org/ Downloaded from
NRRL B-30514 for cloning and production in an E. coli expression system. A fragment bearing the full length of bsh1 was PCR amplified from L. salivarius NRRL B-30514 and cloned into the pET-21b(+) vector, which subsequently was transformed into an E. coli BL21(DE3) host strain for production of recombinant BSH (rBSH). As shown in Fig. 3A, upon induction by 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG), the recombinant E. coli construct showed significant production of an additional protein with a molecular mass of 37 kDa on SDS-PAGE (Fig. 3A, lane 2), consistent with the calculated molecular mass from the deduced amino acid sequence of the rBSH. The high purity of the C-terminal His-tagged rBSH was subsequently obtained from the E. coli culture using one-step Ni-nitrilotriacetic acid agarose affinity chromatography (Fig. 3A, lane 3). Approximately 15 mg of the rBSH was consistently purified from 1 liter of induced culture.

Biochemical characteristics of the L. salivarius rBSH. The purified rBSH enzyme showed a broad substrate range and could efficiently hydrolyze both glycoconjugated and tauroconjugated bile salts (Table 1). Of the six major bile salts tested, the rBSH displayed the highest hydrolysis activity for glycochenodexychocholic acid (defined as 100% activity). However, the rBSH did not exhibit an obvious preference for the other two glycoconjugated bile salts (glycocholic acid and glycodexyocholic acid) compared to the other three tauroconjugated bile salts (taurocholic acid, taurodexyocholic acid, and taurochenodexyocholic acid) (Table 1); the relative activity of the rBSH for these five bile salts ranged from 22.3% to 47.9%. Based on the $K_m$ value, the rBSH exhibited a generally higher affinity for the glycoconjugated bile salts than that for the tauroconjugated bile salts (Table 1). The $k_{cat}/K_m$ value, the indicator of catalytic efficiency, showed that the glycoconjugated bile salts are the preferred substrates of the rBSH; however, the $k_{cat}/K_m$ difference between the glycoconjugated and tauroconjugated bile salts was less than 2-fold (Table 1).

The pH optimum of the rBSH activity ranged from pH 5.0 to 6.0, with the maximum activity observed at pH 5.4 (Fig. 3B). The rBSH enzyme activity at pH values above 7.0 and below 4.0 rapidly declined. Regarding temperature effects, the highest rBSH activity was observed at approximately 41°C (Fig. 3C). The rBSH exhibited stable activity at temperatures ranging from 35°C to 55°C. However, a significant decrease in enzyme activity was observed at temperatures higher than 55°C; the rBSH enzyme lost about 76% and 90% of its original activity after a 30-min incubation at 60°C and 70°C, respectively.

Identification of dietary compounds inhibitory to the activity of rBSH. Different compounds that are used as dietary supplements in animal feeds were selected for testing their inhibitory effects on the activity of the rBSH. As shown in Table 2, CuCl$_2$, CuSO$_4$, FeSO$_4$, CoCl$_2$, NaSeO$_3$, NaIO$_4$, and KIO$_3$ displayed the most potent inhibitory effect (>90% inhibition). Lower inhibition (70% to 89%) was observed for ZnCl$_2$, MnCl$_2$, MnSO$_4$, FeCl$_3$, and KIO$_3$. Interestingly, citric acid enhanced the catalytic activity of the rBSH by 38.9%. We also observed that inhibition of the rBSH activity by the compounds is in a dose-dependent manner (data not shown). It is important to mention that the final concentration of each tested compound in the reaction mix (5 mM) is determined by the need of the standard in vitro BSH assay, which does not mean that the same concentration is required to exert a similar magnitude of the inhibitory effect on the BSH present in the intestine. Specifically, the short reaction time (30 min) and the presence of large quantities of highly purified BSH enzymes (10 μg
in a 200-µl reaction mix) require the use of a 5 mM concentration of the compound to achieve optimal resolution of this quantitative assay.

DISCUSSION

The precise mechanisms of AGPs are still largely unknown, which has hampered the development of effective alternatives to AGPs. It has been widely accepted that AGPs may improve feed efficiency and body weight gain primarily by reshaping microbial diversity and relative abundance in the intestine to achieve optimal microbiota for growth (8, 14, 21). Several products, such as organic acids, probiotics, and prebiotics, have been used to alter intestinal microbiota for improved animal health and production (8). However, very limited data are available to justify the choice of specific bacterial species or products for such microbiota manipulation.

Understanding the effect of AGP on gut microbiota is critical but still challenging for elucidating the mode of action of AGP clearly (21). In 1987, Feighner and Dashkevicz (13) first observed the relationship between the AGP-induced growth promotion and the reduced intestinal BSH activity in poultry and proposed that specific inhibitors for BSH may promote weight gain and feed conversion in livestock. However, since then, there is no published information concerning relevant efforts for the identification of BSH inhibitors, likely due to the limited understanding and capability of BSH chemistry. In contrast, AGP studies from different research groups continued to show the tight inverse relationship between the AGP-induced growth promotion and BSH activity (%)

<table>
<thead>
<tr>
<th>Compound</th>
<th>% inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>CuCl2</td>
<td>98.1</td>
</tr>
<tr>
<td>ZnCl2</td>
<td>68.3</td>
</tr>
<tr>
<td>MnCl2</td>
<td>68.1</td>
</tr>
<tr>
<td>FeCl3</td>
<td>73.0</td>
</tr>
<tr>
<td>KCl</td>
<td>25.9</td>
</tr>
<tr>
<td>NaCl</td>
<td>27.7</td>
</tr>
<tr>
<td>MgCl2</td>
<td>25.7</td>
</tr>
<tr>
<td>NaI</td>
<td>31.8</td>
</tr>
<tr>
<td>KI</td>
<td>36.8</td>
</tr>
<tr>
<td>NaIO3</td>
<td>96.4</td>
</tr>
<tr>
<td>CoCl2</td>
<td>95.9</td>
</tr>
<tr>
<td>CaCl2</td>
<td>22.4</td>
</tr>
<tr>
<td>CuSO4</td>
<td>91.7</td>
</tr>
<tr>
<td>ZnSO4</td>
<td>89.5</td>
</tr>
<tr>
<td>MnSO4</td>
<td>83.1</td>
</tr>
<tr>
<td>FeSO4</td>
<td>96.1</td>
</tr>
<tr>
<td>Na2SeO3</td>
<td>93.1</td>
</tr>
<tr>
<td>NaNO3</td>
<td>27.7</td>
</tr>
<tr>
<td>MgNO3</td>
<td>31.3</td>
</tr>
<tr>
<td>NaHCO3</td>
<td>86.4</td>
</tr>
<tr>
<td>KIO4</td>
<td>96.5</td>
</tr>
<tr>
<td>FeCl3</td>
<td>38.9</td>
</tr>
<tr>
<td>ZnCl2</td>
<td>20.6</td>
</tr>
<tr>
<td>Vitamin C</td>
<td>21.8</td>
</tr>
</tbody>
</table>

To achieve optimal resolution for all compounds with the quantitative BSH activity assay, 5 mM each chemical compound was used in the reaction mix, which includes 10 µg of purified BSH. The procedure is detailed in Materials and Methods.

This study revealed a unique feature of the BSH1 enzyme identified in L. salivarius NRRL B-30514 from this study showed limited amino acid variations compared to Bsh1UCC118 and Bsh1JCM1046 (Fig. 2; see also Fig. S1 in the supplemental material), the NRRL B-30514 BSH1 enzyme clearly showed broad substrate specificity. Notably, we performed a delicate kinetic assay of the BSH1 enzyme for the six tested conjugated bile salts in this study, which further demonstrates that the BSH1 enzyme showed similar catalytic efficiency for different bile salts, with slightly higher catalytic efficiencies ($k_{cat}/K_m$) on glycoconjugated bile salts (Table 1). It has been suggested that the 8-aa deletion next to Asn-171 in the BSH1 enzyme makes this enzyme an ideal candidate for screening inhibitors targeting a wide range of BSH enzymes, regardless of their substrate specificities (12). However, since both the Bsh1JCM1046 and BSH1 enzymes identified in this study do not have the 8-aa deletion but still display different hydrolysis activity against different substrates, other unique amino acids or regions would also have a significant impact on the function of BSH. Future structure and comparative sequence analyses of the BSH enzymes from L. salivarius would be critical for identifying key residues contributing to catalytic efficiency and substrate selectivity.

In this study, we also examined the effect of a panel of feed additives on the activity of the BSH. The nature of the broad substrate specificity of the L. salivarius BSH1 enzyme makes this BSH an ideal candidate for screening inhibitors targeting a wide range of BSH enzymes, regardless of their substrate specificities. The inhibitory effect of copper and zinc on the BSH is of particular interest. Recently, copper and/or zinc have been used at high concentrations (up to 250 ppm for copper and 3,000 ppm for zinc) to aid in feed efficiency and growth promotion in poultry (1, 11, 26) and swine (2, 16, 17, 28). To date, there is a lack of scientific

### Table 1 Activity and kinetics of rBSH for different bile salts

<table>
<thead>
<tr>
<th>Substrate</th>
<th>BSH activity (µmol/min/mg)</th>
<th>Relative activity (%)</th>
<th>$K_m$ (mM)</th>
<th>$k_{cat}$ (min⁻¹)</th>
<th>$k_{cat}/K_m$ (min⁻¹·mM⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GCA</td>
<td>7.7 ± 0.48</td>
<td>41.9</td>
<td>1.71</td>
<td>532</td>
<td>311</td>
</tr>
<tr>
<td>GDCA</td>
<td>4.0 ± 0.54</td>
<td>22.3</td>
<td>1.15</td>
<td>382</td>
<td>332</td>
</tr>
<tr>
<td>GCDCA</td>
<td>17.7 ± 1.18</td>
<td>100</td>
<td>2.48</td>
<td>938</td>
<td>378</td>
</tr>
<tr>
<td>TCA</td>
<td>5.6 ± 0.33</td>
<td>31.4</td>
<td>3.21</td>
<td>585</td>
<td>182</td>
</tr>
<tr>
<td>TDCA</td>
<td>8.5 ± 2.26</td>
<td>47.9</td>
<td>3.19</td>
<td>806</td>
<td>252</td>
</tr>
<tr>
<td>TCDCA</td>
<td>8.0 ± 2.37</td>
<td>45.1</td>
<td>2.53</td>
<td>510</td>
<td>201</td>
</tr>
</tbody>
</table>

$^a$The following six bile salts were tested: glycocholic acid (GCA), glycodeoxycholic acid (GDCA), glycochenodeoxycholic acid (GCDCA), taurocholic acid (TCA), taurochenodeoxycholic acid (TDCA), and taurodeoxycholic acid (TCDCA).

### Table 2 Effect of different dietary compounds on BSH activity

<table>
<thead>
<tr>
<th>Compound</th>
<th>% inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>CuCl2</td>
<td>98.1</td>
</tr>
<tr>
<td>ZnCl2</td>
<td>68.3</td>
</tr>
<tr>
<td>MnCl2</td>
<td>68.1</td>
</tr>
<tr>
<td>FeCl3</td>
<td>73.0</td>
</tr>
<tr>
<td>KCl</td>
<td>25.9</td>
</tr>
<tr>
<td>NaCl</td>
<td>27.7</td>
</tr>
<tr>
<td>MgCl2</td>
<td>25.7</td>
</tr>
<tr>
<td>NaI</td>
<td>31.8</td>
</tr>
<tr>
<td>KI</td>
<td>36.8</td>
</tr>
<tr>
<td>NaIO3</td>
<td>96.4</td>
</tr>
<tr>
<td>CoCl2</td>
<td>95.9</td>
</tr>
<tr>
<td>CaCl2</td>
<td>22.4</td>
</tr>
<tr>
<td>CuSO4</td>
<td>91.7</td>
</tr>
<tr>
<td>ZnSO4</td>
<td>89.5</td>
</tr>
<tr>
<td>MnSO4</td>
<td>83.1</td>
</tr>
<tr>
<td>FeSO4</td>
<td>96.1</td>
</tr>
<tr>
<td>Na2SeO3</td>
<td>93.1</td>
</tr>
<tr>
<td>NaNO3</td>
<td>27.7</td>
</tr>
<tr>
<td>MgNO3</td>
<td>31.3</td>
</tr>
<tr>
<td>NaHCO3</td>
<td>86.4</td>
</tr>
<tr>
<td>KIO4</td>
<td>96.5</td>
</tr>
<tr>
<td>FeCl3</td>
<td>38.9</td>
</tr>
<tr>
<td>ZnCl2</td>
<td>20.6</td>
</tr>
<tr>
<td>Vitamin C</td>
<td>21.8</td>
</tr>
</tbody>
</table>

$^a$To achieve optimal resolution for all compounds with the quantitative BSH activity assay, 5 mM each chemical compound was used in the reaction mix, which includes 10 µg of purified BSH. The procedure is detailed in Materials and Methods.
evidence to explain why copper and zinc function as AGP alternatives at elevated concentrations (17). It has been suggested that the potential mechanisms of growth-promoting effects of high dietary levels of copper and zinc are attributed to their antimicrobial activities (28). However, there is little evidence showing that the concentrations of copper and zinc in the intestine are high enough to exert a similar pattern of antimicrobial activity as AGPs. In addition, additive growth-promoting effects of high dietary levels of copper and AGP demonstrated other modes of action of high-level copper and zinc (17). The biochemical data presented here suggest that the elevated concentrations of copper and/or zinc in feed may exert a further inhibitory effect on the activity of intestinal BSH, consequently leading to enhanced lipid metabolism and host energy harvest. Notably, there are several potential problems with long-term use of high doses of copper or zinc in animal feed, such as copper/zinc toxicity and environmental contamination (17). Therefore, novel BSH inhibitors with low toxicity and minimal environmental impacts should be identified in the future. Such BSH inhibitors should effectively inhibit BSH enzymes present in different niches in the intestine. In particular, to effectively inhibit intracellular or membrane-associated BSH, the desired BSH inhibitors should be able to transport through the cell envelope efficiently in a manner similar to many clinical antibiotics. In addition, given that intestinal conditions are much more complex than an in vitro experimental system, in vivo stability and bioavailability are other important issues that must be addressed for the development of potent and practically useful BSH inhibitors.

In addition to copper and zinc, some other compounds also displayed a great inhibitory effect on the rBSH, such as a panel of iodine compounds (sodium periodate, sodium iodate, and potassium iodate) (Table 2). However, the feasibility of these BSH inhibitors as potential alternatives to AGPs must be cautiously evaluated and justified. Specific feed additives could have multiple, profound effects on the host, particularly when used at elevated concentrations. For example, iodine compounds are constituents of thyroid hormones. Thus, high dietary levels of iodine may greatly enhance the basal metabolic rate, consequently leading to decreased body weight despite its dietary levels of iodine for bile salt hydrolase-active Lactobacillus spp. Appl. Environ. Microbiol. 55:11–16.


