Bile Acids Are Substrates for Amine N-Acyl Transferase Activity by Bile Salt Hydrolase

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Bile Acids Are Substrates for Amine N-Acyl Transferase Activity by Bile Salt Hydrolase

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Bacteria in the gastrointestinal tract (GI) produce a variety of amino acid bile acid amidates that impact host-mediated metabolic processes; however, the bacterial gene(s) responsible for their production remain unknown. Herein, we report that bile salt hydrolase (BSH) possesses dual functions, including a new role in bile acid metabolism by functioning as an amine N-acyl transferase that conjugates amines to form bacterial bile acid amidates (BBAAs). To characterize this new amine N-acyl transferase role for BSH, we used pharmacological inhibition of BSH, heterologous expression of bsh in *Escherichia coli*, and generated a bsh knockout and knockin in *Bacteroides fragilis* to demonstrate that BSH is necessary and sufficient for BBAA production. Lastly, we report that BBAAs activate host ligand-activated transcription factors including the farnesoid X receptor, pregnane X receptor, constitutive androstane receptor, and the aryl hydrocarbon receptor. These new findings expand our understanding and appreciation for the important roles that bacteria play in shaping the bile acid metabolic network.
Introduction

Bile acids are host-microbiota co-metabolites that facilitate dietary lipid absorption and regulate lipid, glucose, and xenobiotic metabolism\(^1^\)\(^-\)\(^3\). In humans and rodents, the primary bile acids cholic acid (CA) and chenodeoxycholic acid (CDCA) are synthesized in the liver from cholesterol. To facilitate their elimination, CA and CDCA are conjugated with glycine or taurine in a two-step reaction by bile acid-CoA synthetase (BACS) and bile acid:CoA N-acyl transferase (BAAT, Fig. 1a)\(^4\). Conjugated primary bile acids, predominantly glycocholic acid (GCA) and taurocholic acid (TCA), are stored in the gallbladder until the consumption of a meal triggers secretion into the duodenum. Bile salt hydrolase (BSH), an enzyme commonly found in bacteria inhabiting the small intestine and colon, hydrolyzes the amide linkage of conjugated bile acids (Fig. 1b)\(^5\). Post-hydrolysis, bacteria further metabolize the bile acid backbone to generate secondary bile acids such as deoxycholic acid (DCA) and lithocholic acid (LCA), which are reabsorbed, conjugated, and recycled by the host\(^6\)\(^,\)\(^7\). BSH activity has been associated with colonization resistance to \(C.\) \(difficile\)\(^8\), weight gain\(^9\), and regulation of circadian rhythm\(^10\).

Furthermore, the addition of a recombinant BSH cocktail changes the pathogenesis of \(C.\) \(difficile\)\(^11\), which was previously attributed to BSH deconjugation activity. Recent studies demonstrated that bacteria, in addition to the host, have bile acid amine \(N\)-acyl transferase activity, with dozens of bacterially conjugated amino acid amidates identified\(^12^-\)\(^16\). The mechanism for these conjugation reactions is still unknown. Furthermore, bacterial bile acid amidates (BBAAs) are abundant in the gastrointestinal tract (GI) and have been associated with human inflammatory bowel disease\(^14\). Although bacterial re-conjugation of bile acids shapes the bile acid pool, how this impacts host-microbiota communication is not yet understood. Some BBAAs activate host bile acid nuclear receptors like the farnesoid X receptor (FXR) and pregnane X receptor (PXR)\(^12\)\(^,\)\(^14\), but an assessment of other receptors has not been completed. We demonstrate herein that bacterial BSHs reamidate unconjugated bile acids to produce BBAAs in addition to their known role in hydrolyzing conjugated bile acids (Fig. 1c-d).
In addition, we report that unique collections of BBAAs activate host ligand-activated receptors to potentially mediate host-microbiota communication.

**Figure 1. Amidation and deamidation reactions of bile acids.**

(a) Bile acid-CoA:amino acid N-acyl transferase (BAAT) activity with cholic acid (CA)-CoA as a representative bile acid modified by the host. (b) Deconjugation reaction by bacterial bile salt hydrolase (BSH) using taurocholic acid (TCA) as a representative conjugated bile acid. (c) New BSH amino acid N-acyl transferase activity characterized in this manuscript, with the conjugated bile acid TCA as an example. This activity leads to the biosynthesis of bacterially-conjugated bile acid amidates (BBAAs). (d) New BSH activity also leads to the biosynthesis of BBAAs from unconjugated bile acid, represented as CA, via a currently unknown mechanism.
Characterization of the bile acid amine N-acyl transferase activity of BSH

Diverse bacteria from the human GI produce BBAAs\textsuperscript{13,14}. We therefore set out to discover the gene(s) responsible for the production of BBAAs. We report here that BSH has amine N-acyl transferase activity. It was recently demonstrated that gut bacteria produce cholic acid amidates conjugated with phenylalanine, tyrosine, and leucine\textsuperscript{17}. Additionally, in a screen of 202 human-associated bacterial isolates, production of BBAAs from a mixture of CA and DCA was reported\textsuperscript{14}. Others found that of 72 bacterial isolates screened, 25 strains were able to conjugate DCA, CDCA, or CA to produce at least one BBAA\textsuperscript{13}. We compared the genomes of the isolates involved in these two studies with their production of BBAAs to identify possible candidate gene(s) responsible for BBAA production. We found that the bile salt hydrolase (bsh) gene was significantly associated ($p = 9.4\text{e}-06$, phylogenetic linear regression) with BBAA production (Fig. 2a, Extended Data Fig. 1). The association is exemplified in the genera *Bifidobacterium* and *Enterococcus*, which are efficient producers of BBAAs and also widely harbor the *bsh* gene, while *Fusobacterium*, which lack the *bsh* gene, do not make BBAAs.

Overall, with few exceptions, bacterial isolates that produce at least one BBAA have a *bsh* homolog.

To further explore the involvement of *bsh* and other genes in bile acid conjugation, we performed RNA sequencing of *Bifidobacterium longum* NCTC 11818 treated with 100 μM unconjugated bile acids (CA and DCA, equimolar). Global transcriptional profiling identified 1,929 genes of which 89 were differentially expressed ($p\text{-adj} \leq 0.05$ and log2FC $\leq -1.5$ or $\geq 1.5$), including *bsh* which was significantly upregulated (log2FC $= 2.14$, $p\text{-adj} = 6.52\text{E}-85$) (Fig. 2b; Supplementary Table S1). We also validated the RNA sequencing results with RT-qPCR, in which *bsh* expression was increased 2.7-fold ($p = 0.0026$) (Fig. 2c). Previously, *bsh* was reported to be upregulated in response to a mixture of conjugated and unconjugated bile acids, but the response was attributed solely to the presence of conjugated bile acids\textsuperscript{18,19}. Therefore,
we sought next to examine the possibility that BSH has amine N-acyl transferase activity with unconjugated bile acids as the substrate.

Figure 2. Bacterial bile salt hydrolase (bsh) is associated with bile acid conjugation to amino acids. (a) Correlation between presence or absence of the bsh gene encoding BSH, with the ability of the bacterium to produce BBAAs. (b) Differential gene expression analysis of *Bifidobacterium longum* NCTC 11818 supplemented with 200 μM each of CA and DCA. (c) RT-qPCR to validate upregulation of the bsh gene with 200 μM CA and DCA treatment, equimolar. Expression of bsh was compared to vehicle using the $2^{-\Delta\Delta Ct}$ method normalized to the reference
gene *ldhl* and significant differences were determined using t-test (*p* = 0.0026**). Replicates of n=5, error bars represent SD.

**BSH is necessary for bacterial N-acyl transferase activity**

We hypothesized that pharmacologic inhibition of the BSH enzyme would eliminate the production of BBAAs from unconjugated bile acids. The pan BSH inhibitor gut restricted–7 (GR–7)²⁰ was used to inhibit BSH of *Bifidobacterium longum* NCTC 11818 treated with 100 μM CA and DCA, equimolar. We monitored the production of BBAAs after 4, 6, 8, 10, 12, 14, 16 and 24 hours of incubation with and without GR-7. Incubation with 100 μM GR-7 resulted in a significant decrease in the production of BBAAs, particularly phenylalanine- and alanine-conjugates, as compared to vehicle-treated controls (Fig. 3a). GR-7 did not inhibit the rate of bacterial growth during the exponential phase (Fig. 3b). At this dose, GR-7 did not completely eliminate BBAA production, possibly due to incomplete inhibition of BSH.

Given that *Bifidobacterium* strains are difficult to manipulate genetically²¹, we investigated the necessity of BSH using *Bacteroides fragilis* NCTC 9343, which has been extensively studied for secondary bile acid metabolism and is more compatible with genetic manipulation. We generated a complete *bsh* deletion strain (Δ*bsh*) and another strain that complemented the *bsh* knockout in trans (Δ*bsh* bsh⁺). We then treated the bacterial strains with 100 μM CA or 100 μM TCA for 16 hrs to assess whether differences could be observed in BBAA production by BSH from unconjugated or conjugated bile acid substrates. Deletion of *bsh* eliminated the production of BBAAs from both CA and TCA, while complementation rescued BBAA production (Fig. 3c). These observations support our hypothesis that BSH has amine N-acyl transferase activity.

To test whether BSH is sufficient for the production of BBAAs, we cloned the *bsh* gene from *Bifidobacterium longum* NCTC 9343 into the pET-28b(+) vector (Bl/BSH) and overexpressed in *Escherichia coli* BL21(DE3). Heterologous expression of the enzyme in *E. coli*, which lacks endogenous *bsh*, confers amine N-acyl transferase activity. Incubation of the
E. coli-BlBSH with 100 µM CA or 100 µM TCA produced BBAAs while the untransformed E. coli did not produce BBAAs (Fig. 3d). The 100 µM CA and TCA concentrations are well below the millimolar concentrations observed in humans.

Next, we purified BlBSH and assayed for BBAA production from 100 µM CA or TCA and a 250 µM equimolar mixture of amino acids. Both CA and TCA treatment yielded BBAAs but not all amino acids were conjugated, demonstrating substrate selectivity by BSH. The large proportion of alanine and serine conjugates produced by B. fragilis (Fig. 3c), BlBSH-expressing E. coli (Fig. 3d), and purified BlBSH suggest that the enzyme prefers amino acids with smaller side chains. However, the greater diversity of BBAAs produced by purified BlBSH in vitro (Fig. 3e) compared to BlBSH-expressing E. coli (Fig. 3d) is likely a result of the different amino acid profiles in E. coli (alanine ~100x higher concentration than serine)\(^\text{22}\). Although we observed BSH amine N-acyl transferase activity with both CA and TCA, the conjugation mechanism is not yet understood. In the case of conjugation with TCA, a CA-based intermediate formed during the loss of taurine may be able to react with the other amino acids in the reaction mixture. However, the CA reaction must include either a step to activate the CA carboxylate or an activated enzyme intermediate for coupling to occur. Investigation of the BSH conjugation mechanism is required to fully understand the activity and specificity.
Figure 3. Bacterial bile salt hydrolase is necessary and sufficient for bile acid N-acyl transfer. (a) The pan-BSH inhibitor GR-7 attenuates conjugated bile acid production in culture of *B. longum* NCTC 9343. Points indicate mean concentration of bacterial bile acid amidate (BBAA) quantified by targeted LC-MS/MS and vertical lines represent SD, n=3 per treatment group. (b) *B. longum* growth after treatment with 100 µM GR-7 for 24 hours. Thick lines indicate mean OD$_{600}$ and thin lines denote SD. Growth was completed in triplicate. (c) BSH-knockout in *B. fragilis* ablates the aminotransferase activity observed in wild-type *B. fragilis* supplemented with 100 µM CA or TCA. (d) Transformation of *E. coli* with a B/BSH expression vector induces production of BBAAAs from 100 µM CA or TCA. (e) Purified B/BSH enzyme synthesizes BBAAAs in vitro. For (c-e), bar heights represent the concentration of each BBAA measured in that sample, with adjacent bars representing the four biological replicates.

**BBAAs signal through human ligand-activated transcription factors**

To assess the potential that host-microbiota communication is facilitated through BBAAs, we tested whether BBAAs could act as agonists for host ligand-activated transcription factors. FXR
is an essential transcription factor in human bile acid metabolism, regulating the expression of bile acid biosynthetic enzymes such as cholesterol 7α-hydroxylase (CYP7A1) and transporters such as the bile salt export pump (BSEP)\textsuperscript{23}. Many bile acids are FXR agonists including, in descending order of potency, CDCA, DCA, LCA, and CA\textsuperscript{24}. Other bile acid nuclear receptors, namely PXR, the vitamin D receptor (VDR), and the constitutive androstane receptor (CAR), function to biotransform and eliminate toxic LCA and LCA derivatives\textsuperscript{24}. Certain transcription factors are not considered to be bile acid receptors but have some bile acid ligands. Although the aryl hydrocarbon receptor (AHR) is known for its involvement in the metabolism and clearance of environmental pollutants and other aromatic compounds\textsuperscript{25}, CDCA is reportedly an AHR ligand\textsuperscript{26} and AHR activation in rats upregulates \textit{Cyp7a1}\textsuperscript{27}. Therefore, we screened 33 BBAAs using a luciferase-based transcriptional activation assay to measure agonism of human FXR, VDR, CAR3, PXR, and AHR at a concentration of 50 µM (Fig. 4a). FXR was activated by 7 BBAAs (\(p<0.0001\)), 5 of which are amino acid conjugates of CDCA (Glu-CDCA, Ile-CDCA, Leu-CDCA, Met-CDCA, and Trp-CDCA). A preference for CDCA conjugates likely reflects the known potency of CDCA as an FXR agonist, whose activity was largely unaffected by the addition of these amino acids. Although others have observed FXR activation due to Phe-CA and Tyr-CA\textsuperscript{17}, our data did not support this finding at a 50 µM concentration. We demonstrated significant PXR activation by Phe-DCA, Glu-CA, Glu-DCA, and Glu-UDCA, providing additional evidence for the preference for glutamate-conjugated BBAAs seen previously\textsuperscript{14}. Six BBAAs also significantly activated CAR (Glu-CDCA, His-CA, Ile-CDCA, Ile-UDCA, Leu-UDCA, and Phe-DCA), but the largest fold-change was a modest 2.05 by Ile-UDCA. However, we discovered that AHR was activated by nearly all tested conjugated and unconjugated bile acids, with the strongest agonists being Glu-CA, Glu-UDCA, Leu-CA, Phe-DCA, Trp-CDCAC, and Trp-DCA. Based on their activation of multiple receptors in the luciferase assays, Glu-CA and Glu-CDCA were selected for further assessment of host ligand activated receptor activation. We treated organoids cultured from human ileocytes with 50 µM or 100 µM Glu-CA or Glu-CDCA.
and measured the expression of FXR, CAR, PXR, and AHR target genes (Fig. 4b). Although Glu-CA activated both PXR and AHR in luciferase reporter cells, in organoids Glu-CA only induced the transcription of the AHR target gene CYP1A1 at 100 µM ($p=0.00011$). Similarly, Glu-CDCA did not increase the expression of CYP2B6, despite activating CAR3 via luciferase assay. There are no known bile acid ligands for CAR, which agrees with our observations of weak or no activation by BBAAs in vitro and in ileal organoids. Glu-CDCA also did not alter the expression of FXR target genes (FGF19, SHP, BSEP, IBABP, or OSta/β) in organoids despite demonstrating FXR activation in vitro (Extended Data Fig. 2). The discrepancies between luciferase assay and organoid activation may represent differences in metabolism of the supplemented bile acids in these separate systems or background activation of each receptor. However, treatment with 100 µM Glu-CDCA upregulated both CYP3A4 ($p=0.0003$) and CYP1A1 ($p<0.0001$), suggesting that Glu-CDCA acts as an agonist for PXR and AHR. Glutamate-conjugated bile acids were also discovered to be human PXR agonists$^{14}$, which lends additional support to their physiological relevance in vivo. We observed the near ubiquitous activation of AHR by both bacterially-conjugated and conventional bile acids, suggesting that bile acids are an additional class of non-aromatic AHR ligands. Therefore, BBAAs may unexpectedly impact the metabolism of substrates, drugs, and carcinogens regulated by PXR and AHR.
Figure 4. Regulation of human ligand-activated transcription factors by BBAAs. (a) Transcription factor activation by 50 µM dose of conventional and bacterially-conjugated bile acids measured by luciferase reporter assays for human FXR, VDR, CAR, PXR, and AHR. Differences between treatments and vehicle-treated control were determined using ANOVA with Dunnett’s post-hoc test (p<0.05*), with replicates of n=3. Relative expression of target gene transcripts for (b) PXR (CYP3A4), (c) AHR (CYP1A1), (d) FXR (FGF19), and (e) CAR (CYP2B6). Error bars represent SD. Biological replicates were n=6 for vehicle and n=3 for treatments. Differences between treatments and vehicle were determined using one-way ANOVA with Dunnett’s post-hoc test (p<0.001***, p<0.0001****).

Conclusion

We identified a new amine N-acyl transferase activity for BSH that produces BBAAs. These BBAAs facilitate communication between the microbiota and host through the activation of the human ligand-activated transcription factors PXR and AHR. As there are hundreds of different bile salt hydrolases found in microbiota that synthesize different bile acid amidates, BSHs may be instrumental in fine tuning different messages that promote health or disease. Historically, BSH knockout and inhibition has been associated with modulation of inflammation, cancer, and liver disease without knowledge of BSH amine N-acyl transferase activity. Future studies must re-evaluate the contribution of BBAA production on disease outcomes related to BSH activity.
References


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Methods

Correlation of BSH and Ability to Produce BBAAs

To check for the association of BSH and BBAA production, we leveraged LC-MS/MS data from two published datasets involving 202 Human Microbiome Project (HMP) bacterial isolates in Gentry et al\textsuperscript{1} and 69 representative gut bacteria members in Lucas et al\textsuperscript{2}. We combined the two datasets. For comparison between bacteria from the studies, we normalized the dataset based on the proportion of BBAAs detected from the total number of BBAAs tested in each study. Next, we downloaded the assembled genomes of the bacteria from the NCBI genome database. Phylogenetic tree was obtained from the NCBI taxonomy database using taxids in the study. Using Prokka\textsuperscript{3}, we annotated the genomes and checked for the presence or absence of the \textit{bsh} gene. Briefly, Prokka uses multiple databases (Uniprot, RefSeq, and Pfam) in a hierarchical manner to annotate coding sequences within the genome. Genomes with coding sequences annotated as choloylglycine hydrolase (cbh) or having its domain were deemed as having the \textit{bsh} gene or its homolog, while genomes lacking these annotations were deemed lacking the gene. To test for correlation, we used \textit{phylolm}\textsuperscript{4} package in R to fit a following phylogenetic linear regression model:

\[
BBAAs \sim bsh + 1
\]

where,

\textit{BBAAs} is the vector of proportions of the BBAAs detected for bacterium in each study,

\textit{bsh} is the vector of presence or absence of \textit{bsh} gene

All the analyses and visualization were performed in RStudio.

RNA Sequencing Sample Preparation and Analysis

\textit{Bifidobacterium longum} subsp. \textit{longum} NCTC 11818 was acquired from ATCC (Manassas, VA).

30 µL of an overnight culture of \textit{B. longum} was inoculated into 3 mL of BHI broth (BD Difco)
supplemented with 0.05% w/v cysteine, 5 µg/mL hemin, and 1 µg/mL vitamin K (Sigma).

Equivalent volumes of 1M NaOH (control) or cholic acid and deoxycholic acid in 1 M NaOH at a final concentration of 200 µM were added. Cultures were grown to mid-exponential phase (~6 h) at 37°C in an anaerobic chamber filled with 20% CO₂ and 5% H₂ in N₂ gas. 4 mL of RNAprotect (Qiagen) was added to 2 mL culture and incubated for 5 min at RT. Samples were centrifuged at 5000 x g for 5 min at 4°C and the bacterial pellets were flash frozen in liquid nitrogen and stored at -80°C. Pellets were resuspended in 1 mL TRIzol Reagent and transferred to Lysing Matrix E tubes for homogenization at 2x20 sec cycles at 6500 x g. 200 µL of chloroform was added, vortexed for 15 sec, and incubated at RT for 10 min. Samples were centrifuged for 15 min at 16,000 x g, 4°C, and 500 µL upper aqueous phase was transferred to a fresh tube containing 500 µL of 100% ethanol. Samples were then transferred to a Purelink spin column, centrifuging for 15 sec at 12,000 x g to elute flowthrough. Columns were washed with 350 µL Wash buffer I and removed by centrifugation for 15 sec at 12,000 x g. Contaminating DNA was degraded by adding 80 µL of DNase solution (10 µL DNase in 1x reaction buffer) and incubating at RT for 15 min. A second wash with Wash buffer I was then performed. Columns were washed twice with 500 µL Wash buffer II, eluting each time by centrifugation for 15 sec at 12,000 x g. Purified RNA was eluted with 30 µL RNase-free water by centrifugation for 1 min at 12,000 x g into a fresh tube.

RNA samples were submitted to Microbial Genome Sequencing Center (Pittsburgh, PA) for sequencing. Samples were DNase treated with Invitrogen DNase (RNAse free). Library preparation was performed using Illumina’s Stranded Total RNA Prep Ligation with Ribo-Zero Plus Kit and 10bp IDT for Illumina indices. Sequencing was done on a NextSeq2000 to yield 2x50bp reads. Demultiplexing, quality control, and adapter trimming were performed with bcl-convert (v3.9.3).

For the analysis, the complete reference genome and annotation of *Bifidobacterium longum* NCTC 11818 were obtained from the NCBI Assembly database. Obtained
demultiplexed reads were checked for quality and filtered using fastp (v0.12.4). Filtered reads were aligned with the reference genome using bowtie2 (v2.2.5). Obtained alignments were sorted using samtools (v1.10). Qualimap (v2.2.2) was used to check for the quality of alignments. featureCounts (v2.0.1) was used to generate counts of reads uniquely mapped to annotated genes. These raw counts were then normalized and analyzed for differential gene expression using the DESeq2 package (v1.35.0) in RStudio.

qScript cDNA SuperMix (Quantabio, Beverly, MA) was used for the reverse transcription of 250 ng RNA template in a 20 µL reaction for RT-qPCR. Primer sequences were designed using PrimerBlast for *B. longum bsh* and lactate dehydrogenase (*ldhl*) (Supplementary Table S2). RT-qPCR was performed with 1 µL cDNA template and 0.4 µM of each primer on a QuantStudio3 using fast cycling parameters with PowerUp SYBR detection reagent.

**Bifidobacterium longum BSH Inhibition**

*Bifidobacterium longum* subsp. *longum* NCTC 11818 was acquired from ATCC (Manassas, VA).

30 µL of an overnight culture of *B. longum* was inoculated into 3 mL of BHI broth (BD Difco) supplemented with 0.05% w/v cysteine, 5 µg/mL hemin, and 1 µg/mL vitamin K (Sigma). 2 µL of the overnight culture was added to 196 µL fresh supplemented-BHI media. 1 µL of 1M NaOH (vehicle) or 20 mM cholic acid and deoxycholic acid in 1M NaOH at a final concentration of 100 µM were added to the plate. To inhibit BSH, 1 µL of 20 mM GR-7 in DMSO was added to the treatment wells to a final concentration of 100 µM. Equivalent volume of DMSO was added to the control wells. To monitor the production of BBAAs, samples were taken out at 4, 6, 8, 10, 14, 16 and 24 hrs after incubation, and flash frozen in liquid nitrogen until extraction for LC-MS/MS quantification of bile acids.
Construction of B. fragilis NCTC 9343 Δbsh Mutant

A 1,797 bp DNA fragment containing the N-terminal region of the BSH gene was PCR amplified from the B. fragilis NCTC 9343 using primers BSH-NT-FOR and BSH-NT-REV. The N-terminal fragment was cloned into the SphI/BamHI sites of pFD516 vector. Then, a 1,734 DNA fragment containing the C-terminal region was PCR amplified with primers BSH-CT-FOR and BSH-CT-REV2 and cloned into the SacI site of pFD516. The new construct, pER363, contains a deletion of 765 bp DNA fragment from the bsh gene. A 3,533 bp BglII/SalI DNA fragment from pER-363 containing the Δbsh construct was cloned into the BamHI/SalI sites of the suicide vector for allelic replacement in Bacteroides, pLGB13 to construct pER-366. pER-366 was mobilized from E. coli S17-1 λpir into B. fragilis NCTC 9343 by biparental mating as described previously.

Transconjugants were selected on BHIS plus 0.5% yeast extract plates containing 200 μg/mL gentamycin and 10 μg/mL erythromycin. Four transconjugant colonies were grown overnight on BHIS broth with 200 μg/mL gentamycin (erythromycin was omitted). Ten μL of an overnight culture was spread on BHIS with 0.5% yeast extract plates containing 200 μg/mL gentamycin and 100 ng/mL anhydrotetracycline. Transconjugant colonies were streaked on fresh BHIS with 0.5% yeast extract plates containing 200 μg/mL gentamycin and 100 ng/mL anhydrotetracycline. PCR amplification using BSH-mutcheck-FOR and BSH-mutcheck-REV primers was used to identify chromosomal double-crossed over genetic recombination. The B. fragilis NCTC 9343 Δbsh mutant isolates (BER-279) were also tested for loss of erythromycin resistance and respiratory test.

Genetic Complementation of Δbsh Mutant

pER-300 containing bsh operon in the pNBU2-bla-ermGb vector was mobilized into BER-279 by biparental mating. Transconjugants were selected on BHIS plus 0.5% yeast extract plates containing 200 μg/mL gentamycin and 10 μg/mL erythromycin (BER-280).
Heterologous Expression and Purification of Bifidobacterium longum BSH in E. coli

The gene for *Bifidobacterium longum* BSH (NC_015067.1, codon-optimized) cloned into pET-28b(+) using NdeI and XhoI restriction sites was synthesized by GenScript. The resulting plasmid was transformed into *Escherichia coli* Tuner (DE3) cells (Novagen) via electroporation at 2500 V and positive transformants were selected on LB media containing 50 µg/mL kanamycin. Expression strains were grown in yeast extract media (45 g yeast extract (Research Products Int.), 1.6 g KH₂PO₄ (Research Products Int.), 13.0 g K₂HPO₄ (Research Products Int.), and 1% glycerol (v/v) (Sigma Aldrich) per 1 L) at 37°C to late exponential phase (OD₆₀₀ = 0.8). The cells were cooled to 18°C before induction with 0.1 mM IPTG (Research Products Int.) and allowed to express BSH for 18-20 hours prior to harvesting by centrifugation (4000 x g. at 4°C, 20 min). The resulting cell pellets were frozen at -80°C until use.

The cells were resuspended in buffer A (50 mM Tris (Research Products Int.), 300 mM NaCl (Research Products Int.), 20 mM imidazole (Sigma Aldrich), pH 7.4) with protease inhibitors benzamidine HCl (Research Products Int.) and Pefabloc ®SC (SigmaAldrich) and disrupted via sonication (QSonica Sonicator Q500) for 6 min on ice (30 second pulse, 30 second pause, at 60% amplitude). The resulting lysate was centrifuged at 130,000 x g in a Beckman Optima L-90X ultracentrifuge at 4°C for 1 hour.

The following purification steps were performed at 4°C. Supernatant was applied to a HisPur Ni-column (Fisher Scientific) pre-equilibrated with buffer A. The column was washed with 20 column volumes of buffer, then the BSH protein was eluted with 5 column volumes of buffer B (50 mM Tris, 300 mM NaCl, 250 mM imidazole, pH 7.4). The eluent was dialyzed into the final storage buffer (50 mM Tris, 50 mM NaCl, 5% Glycerol (v/v), pH 7.0) overnight at 4°C. The resulting protein solutions were concentrated using Amicon® Ultra 15 mL centrifugal filters (Millipore) with a molecular weight cutoff of 10 kDa, flash frozen in liquid nitrogen, and stored at -80°C.
In Vitro Purified Enzyme Assay

Purified B/BSH enzyme was diluted to 10 mg/mL in phosphate buffer (pH 7.0). A 5 mM equimolar mixture of 20 amino acids was prepared in H$_2$O (L-alanine, L-arginine, L-asparagine, L-aspartic acid, L-cysteine, L-glutamine, L-glutamic acid, glycine, L-histidine, L-isoleucine, L-leucine, L-lysine, L-methionine, L-phenylalanine, L-proline, L-serine, L-threonine, L-tryptophan, L-tyrosine, L-taurine, and L-valine). Each reaction mixture contained 178 µL phosphate buffer (pH 6.0), 10 µL of 5 mM amino acids, 2 µL of 100 mM bile acid (CA or TCA in DMSO) or vehicle (DMSO), and 10 µL of the 10 mg/mL enzyme. The reaction mixture was incubated for 90 minutes at 37°C. Reaction was stopped by adding 800 µL ice-cold 100% methanol containing 0.5 µM deuterated bile acid internal standards.

Extraction and LC-MS/MS Quantification of Bile Acids

Following growth, 200 µL of bacterial culture was lysed by three freeze-thaw cycles in liquid nitrogen. 150 µL of each lysate was transferred to a 1 mL capacity 96-well plate, and 600 µL of ice-cold 100% HPLC-grade methanol containing 0.5 µM deuterated bile acid internal standards was added and mixed thoroughly by pipetting. The plate was then incubated on ice for 20 minutes. Samples were sonicated for 10 mins in a sonicating water bath and then centrifuged for 15 min at 2000 rpm at 4°C. 200 µL of each supernatant was transferred to a fresh 1.5 mL microcentrifuge tube and dried using a speedvac. Dried extracts were resuspended in 200 µL HPLC-grade methanol and sonicated for 10 mins before transfer to autosampler vials for LC-MS/MS.

Quantitative LC-MS/MS analyses were performed using a Waters ACQUITY UPLC system coupled with a Waters Xevo TQ-S Triple Quadrupole mass spectrometer (Waters, Milford, MA). Chromatographic separation was achieved using an ACQUITY BEH C8 (2.1 × 100 mm, 1.7 µm) UPLC column (Waters, Milford, MA) heated to 60°C. Samples (1 µL) were
injected into the column and eluted with 90% mobile phase A (1 mM ammonium acetate in 9% acetonitrile, pH 4.15) and 10% mobile phase B (1:1 acetonitrile:isopropanol) for 0.1 min. Mobile phase B was increased in consecutive linear gradients from 10% to 35% over 9.15 min, 35% to 85% B over 2.25 min, and 85% to 100% B over 0.3 min. 100% B was then held for 0.6 min followed by a linear gradient to 10% B over 0.1 min and held for 2.5 min, for a total of 15 min. The flow rate was modified throughout the run starting with 300 µL/min for 9.25 min, then increasing to 325 µL/min over 2.25 min and 500 µL/min over 0.6 min. The flow rate was held at 500 µL/min for 0.3 min, then reduced to 300 µL/min over 0.4 min and held for 2.2 min. MS analyses were carried out using electrospray ionization in positive ion mode using the following settings: capillary voltage 3.5 kV, cone voltage 20 V, source temperature 150°C, desolvation temperature 300°C, desolvation gas flow 540 L/h, and cone gas flow 150 L/h. BBAAs were identified by MRM transitions from parent [M+H]+ ions to daughter [M+H]+ ions corresponding to the amino acid fragment (Supplementary Table S3). Peak areas for each conjugated BA were normalized to peak areas from deuterated internal standards of GCA-d4 or GDCA-d4, matching the bile acid. Quantification was performed against a standard curve of authentic standards at 8 concentrations ranging from 7.8125 nM to 1 µM.

Cell Based Reporter Assays

Human Farnesoid X Receptor (hFXR, Cat # IB00601), Vitamin D receptor (hVDR, IB00701), Constitutive Androstane Receptor variant 3 (hCAR3, IB00901), Pregnane X Receptor (hPXR, IB07001) and Aryl Hydrocarbon Receptor (hAhR, IB06001) Reporter Assay kits were from INDIGO Biosciences, Inc. (Indigo Biosciences, State College, PA, USA). Agonism assays were performed with test compounds at a single concentration (50 µM) with a concurrent dose-response of reference ligands following the manufacturer’s instructions. Briefly, each reporter assay cell suspension was thawed by the addition of cell recovery medium (CRM) and one hundred microliters of the cell suspension were used per well. One hundred microliters of
compound screening medium (CSM) supplemented with test compounds were added and incubated at 37°C, 5% CO₂ for 24 hours. The next day, medium was removed, detection reagents were added, and luminescence was measured (Synergy™ H4 Hybrid Multi-Mode Microplate Reader). Data was expressed relative to vehicle-treated cells and log10-transformed for visualization. Differences between treatments were determined using ANOVA followed by Dunnett’s post-hoc test (JMP Pro 15, SAS Institute, Cary NC). Significant differences were determined when \( p < 0.05 \). Non-linear regression and EC50 calculations were performed with Prism 9.0 (GraphPad Software, Inc., San Diego, CA).

**Enteroid Model**

Normal (de-identified) human small intestinal samples were established and cultured in high Wnt containing media (Human colonoid medium) as previously described\(^1\). For treatments, enteroids were treated with 50 µM and 100 µM Glu-CA or Glu-CDCA for 18 hours, media was removed, and enteroids were washed twice and collected in PBS. Matrigel and PBS were carefully removed and enteroids were lysed and extracted using Pure Link RNA mini kit, according to manufacturer’s instructions. qScript cDNA SuperMix (Quantabio, Beverly, MA) was used for the reverse transcription of 500 ng RNA template in a 40 µL reaction for RT-qPCR. RT-qPCR was performed with 1 µL cDNA template and 0.4 µM of each primer (Supplementary Table S2) on a QuantStudio 3 using fast cycling parameters with PowerUp SYBR detection reagent.


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Author Contributions

BR, SLC, and ADP conceived the project. PCD and ECG provided standards, suggestions and feedback for the duration of the project. MAG and JVH performed luciferase reporter assays and edited the manuscript. ERR and JPC generated bsh mutants. SS and YMS performed organoid experiments. NH and EEW helped with BSH purification and provided suggestions and feedback for the duration of the project. TY, KWK and FJG helped with bile acid analysis and interpretation. IK and JEB provided data analysis and interpretation. DD and SH synthesized BBAA standards. BR, SLC, NH, EEW, PCD, and ADP wrote the manuscript. All authors edited and approved the final manuscript.

Data Availability Statement

RNA-sequencing datasets are deposited in NCBI SRA Database under bioproject: PRJNA878764. The code used for analysis and generation of figures for RNA-seq are uploaded to https://zenodo.org/record/7065468. Bile acid quantification data will be deposited in GNPS.
Competing Interests Statement

PCD is an advisor to Cybele and a co-founder and advisor for Ometa and Enveda with prior approval by UC San Diego. JVH is co-founder and Chief Scientific Officer with INDIGO Biosciences, Inc. with prior approval from Penn State University.

The other authors declare no competing interests.

Additional Information

Supplementary Information is available for this paper.

Correspondence and requests for materials should be addressed to Andrew Patterson (adp117@psu.edu).
Extended Data Figure 1. Complete taxonomy-based correlation between bsh presence in human-associated bacteria and BBAA production.
Extended Data Figure 2. RT-qPCR of additional FXR target gene and receptor transcripts.

(a) Relative mRNA expression of FXR and the FXR target genes short heterodimeric partner (SHP), bile salt export protein (BSEP), organic solute transporter-α (OSTα) and -β (OSTβ), and ileal bile acid binding protein (IBABP). (b) Relative mRNA expression of PXR and CAR.

Differences between treatments and vehicle were determined using one-way ANOVA with Dunnett’s post-hoc test (p < 0.05*).
Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementalTablesS2S3.pdf
- SupplementaryTableS1.csv
- SupplementalFigures.pdf