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Chemical proteomic analysis of bile acid-protein targets in *Enterococcus faecium*†

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Bile acids are important gut microbiota metabolites that regulate both host and microbial functions. To identify the direct protein targets of bile acids in *Enterococcus*, we synthesized and validated the activity of a lithocholic acid (LCA) photoaffinity reporter, x-alk-LCA-3. Chemical proteomics of x-alk-LCA-3 in *E. faecium* Com15 reveals many candidate LCA-interacting proteins, which are involved in cell wall synthesis, transcriptional regulation and metabolism. To validate the utility of bile acid photoaffinity labeling, we characterized a putative bile salt hydrolase (BSH) crosslinked by x-alk-LCA-3, and demonstrated that this BSH was effective in converting taurolithocholic acid (TLCA) to LCA in *E. faecium* and *in vitro*. Chemical proteomics should afford new opportunities to characterize bile acid-protein targets and mechanisms of action in the future.

Introduction

Human microbiota generate structurally diverse small molecules that play important roles in the regulation of host physiology,^{1,2} as well as serve as signal molecules to gut commensals and pathogens.³ Bile acids are one class of important gut microbiota metabolites. Primary bile acids are synthesized in the liver from cholesterol, and modified with glycine or taurine to form conjugated bile acids that are stored in the gall bladder. These conjugated bile acids can then be secreted into intestine and metabolized (*e.g.*, deconjugation, oxidation and reduction) by gut microbiota to form structurally diverse secondary bile acids.⁴ These bile acids can directly regulate host physiology through engaging host proteins, such as nuclear receptors^{5,6} and GPCRs.^{7,8} In addition, bile acids are important signaling molecules to regulate gut microbiota.^{9,10} As biological detergents, bile salts may induce membrane damage and affect cellular homeostasis of intestinal bacteria.⁹ Moreover, bile acids can directly regulate pathogen virulence.^{11–14} However, the direct identification of bile acid-protein targets and mechanisms of action in bacteria has been challenging.

The development of bile acid-based chemical probes has provided important new tools to characterize novel interacting proteins and enzymes. For example, activity-based probes for

bile acid hydrolases (BSH) with different reactive warheads such as acyloxyethylketone,¹⁵ α -fluoromethyl ketone¹⁶ and β -lactam or acrylamide¹⁷ have been developed to profile the gut microbiota-associated bile salt hydrolase activity. Alternatively, photoaffinity reporters for bile acids and other microbiota metabolites can be employed to characterize other protein targets in mammalian cells and microbes.¹⁸ Of note, photoaffinity reporters were developed for primary bile acid cholic acid and enabled the identification of cholic acid-binding proteins in mammalian cell lines¹⁹ and *E. coli*.²⁰ In addition, our chemoproteomic analysis in *Salmonella* revealed anti-infective bile acids such as chenodeoxycholic acid can bind and inactivate a transcriptional regulator of *Salmonella* virulence HilD.²¹ Furthermore, chemical proteomic analysis of bile acid targets in *Clostridium difficile* showed that lithocholic acid (LCA) can bind and modulate the function of a stress response transcriptional regulator BapR.²² In this study, we employ bile acid photoaffinity chemical proteomics to investigate their protein targets in *Enterococcus faecium* (Fig. 1A), a prominent commensal and drug-resistant bacterial species in humans.

E. faecium and *E. faecalis* are common commensals in humans, but are also prevalent causes of hospital-acquired infections.^{23,24} The primary location of enterococci in humans is small and large intestine, where the bacteria encounter high concentration of bile acids ranging from low micromolar to low millimolar. Thus, the ability of these intestinal bacteria to adapt to bile is important for their survival and colonization in intestine. A previous study demonstrated that sublethal concentration of bile exposure increases the survival of *E. faecalis* to the bile challenge.²⁵ To understand the molecular mechanisms responsible for bile acid responses, transcriptome^{26–28} and proteome^{29–31} studies of bile-treated *E. faecalis*

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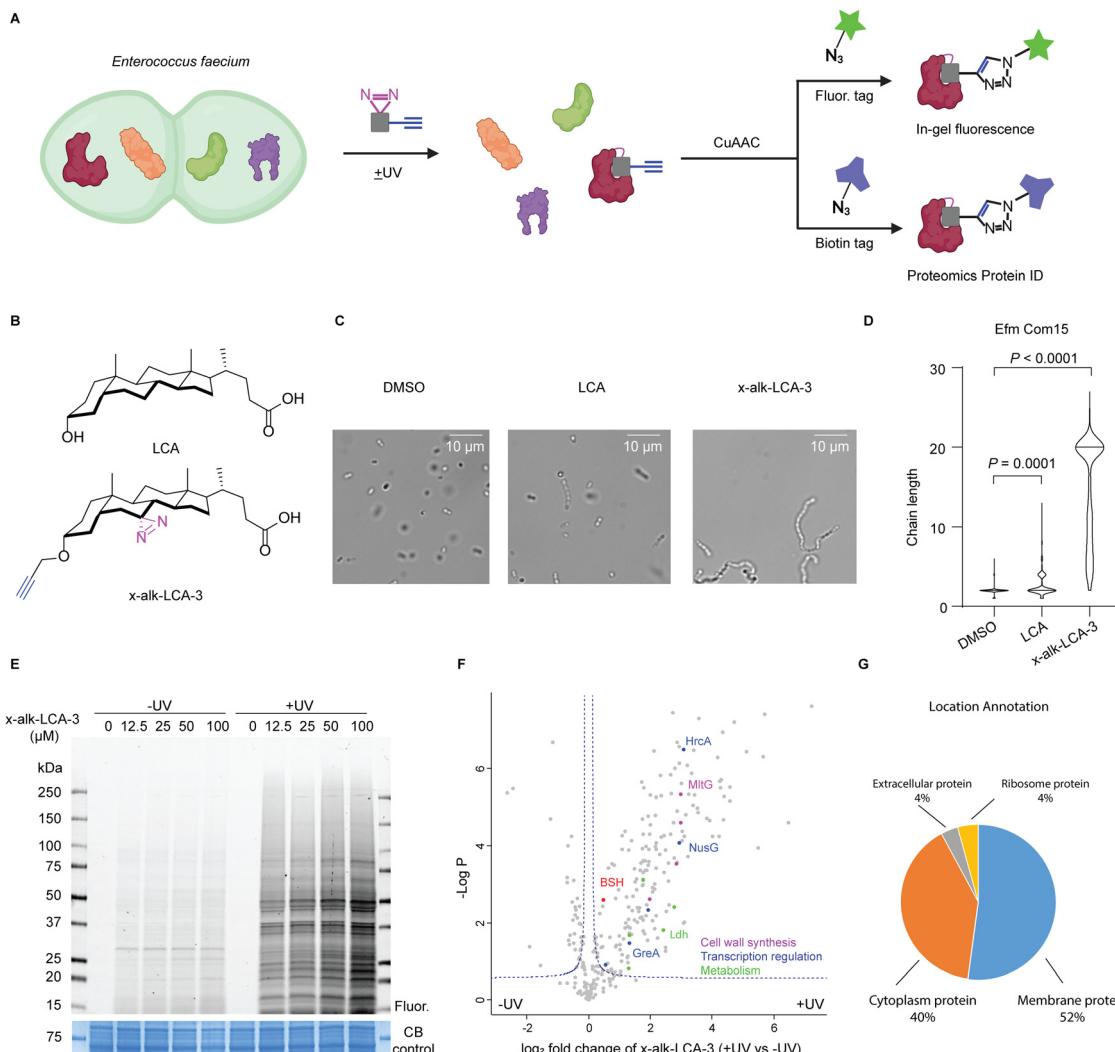


Fig. 1 Chemical proteomic analysis of x-alk-LCA-3 interacting proteins in *Enterococcus faecium*. (A) Schematic for imaging and protein ID of x-alk-LCA-3 interacting protein in *E. faecium*. *E. faecium* was treated with x-alk-LCA-3 reporter and irradiated with or without 365 nm UV light. Bacteria lysate was reacted with rhodamine-azide for imaging and biotin-azide for proteomics. (B) Chemical structure of LCA and x-alk-LCA-3. (C) Light microscopy of *E. faecium* Com15 after being grown in the presence of DMSO, LCA or x-alk-LCA-3 chemical reporter. Chaining is observed in LCA and x-alk-LCA-3 treated bacteria. (D) Comparisons of chaining in *E. faecium* when inoculated in the presence of LCA or x-alk-LCA-3. *E. faecium* Com15 chain lengths quantified by picking at least 300 particles. Statistical analysis was done using Kruskal-Wallis ANOVA with Dunn's correction to compare DMSO treated cells with multiple molecule-treated conditions. (E) x-alk-LCA-3 photo-crosslinks *E. faecium* proteins with UV- and dose-dependent manners. DMSO or x-alk-LCA-3 treated *E. faecium* Com15 lysates were reacted with rhodamine-azide. Resulted proteins were separated by SDS-PAGE and visualized by in-gel fluorescence and Coomassie blue (CB) staining. (F) LFQ Proteomics analysis of x-alk-LCA-3 photo-crosslinked *E. faecium* proteins. Representative proteins were coloured according to their function. (G) Location annotation of significant protein hits in (F).

were carried out, which revealed the up-regulation of fatty acid and phospholipid pathways. In addition, screening of transposon mutagenesis of *E. faecium* and *E. faecalis* identified key genes required for bile acid resistance,^{32,33} such as *gltK8*, a glutamate/aspartate permease and *sagA*, which encodes a secreted peptidoglycan hydrolase.³⁴ In addition, LCA has been reported to induce diplococci-chaining and increased biofilm formation in vancomycin-resistant strains of *E. faecium*.³⁵ These studies revealed key pathways involved in *Enterococcus* responses to bile acids, but did not identify specific bile acid-binding protein targets.

To identify the bile acid-interacting proteins in *E. faecium*, we employed a LCA photoaffinity reporter for chemoproteomic studies. Our proteomic analysis in *E. faecium* Com15 revealed

many candidate bile-interacting proteins, including key metabolic enzymes, transcriptional regulators and cell wall synthesis factors. We validated these LCA reporter proteomic studies by biochemical characterization of a *E. faecium* Com15 bile salt hydrolase (BSH), demonstrating this approach can directly reveal bile acid-interacting proteins and enzymes.

Results and discussion

Chemical proteomics of LCA protein targets in *E. faecium* Com15

We first evaluated the activity of five free bile acids on *E. faecium* Com15, a well-characterized commensal strain. Bacteria were

grown overnight with DMSO or bile acids and then harvested for microscopy analysis. Consistent with previous studies,³⁵ LCA most effectively induce chaining of *E. faecium* (Fig. S1, ESI†). Importantly, our LCA photoaffinity reporter (x-alk-LCA-3) (Fig. 1B) showed similar and even slightly higher activity than LCA in *E. faecium* diplococci chaining (Fig. 1C and D). We therefore used x-alk-LCA-3 to perform chemical proteomics in *E. faecium*.

To identify LCA-interacting proteins in *E. faecium*, bacteria were treated with different concentrations of x-alk-LCA-3 for 1 hour, followed by UV irradiation. As shown in Fig. 1E, x-alk-LCA-3 photo-crosslinks proteins in dose- and UV-dependent manners. Since 25 μ M of x-alk-LCA-3 significantly induces chaining of *E. faecium* Com15, we used this concentration for chemical proteomics. Without UV irradiation, x-alk-LCA-3 enriched 17 protein hits compared to DMSO control, which suggests x-alk-LCA-3 may covalently modify these proteins. These potential covalent modified proteins include enzymes, such as cytochrome P450, phosphoglucomutase, ATP-dependent Clp protease, and transporter and carrier, such as major intrinsic protein and C4-dicarboxylate anaerobic carrier (Fig. S2, ESI†). To identify proteins which non-covalently interact with x-alk-LCA-3, we compare protein hits under UV irradiation to those without UV irradiation. As shown in the volcano plot (Fig. 1F), 182 protein hits are enriched with UV-dependent manner. These proteins are located in the cell membrane (52%), cytoplasm (40%), ribosome (4%) and extracellular region (4%) (Fig. 1G). Further analysis reveals different types of proteins are enriched, including: (i) metabolic enzymes, such as Ldh which is L-lactate dehydrogenase, (ii) transcription regulation related proteins, such as transcription termination protein NusG, (iii) cell wall synthesis related protein, such as endolytic murein transglycosylase MltG. Since LCA induces chaining of *E. faecium*, it was

proposed that LCA may interact and interfere with the function of cell wall synthesis-related proteins, which can be further validated in the future. Meanwhile, a putative bile salt hydro-lase (BSH, EFWG_00531) was also enriched in our proteomics dataset. BSH in *E. faecium* shares 82.35% identity with a reported highly active BSH from *Enterococcus faecalis*.^{36,37} BSHs hydrolyse taurine- or glycine-conjugated bile acids to free bile acids. The deconjugation step is the gateway reaction for following transformations of primary bile acids to divergent secondary bile acids. There is no reported BSHs from *E. faecium* Com15, therefore, we decided to validate the interaction of this potential BSH with LCA.

Validation of *E. faecium* bile salt hydrolase LCA target

To validate BSH's interaction with LCA, we generated a C-terminally HA-tagged *bsh* construct in the *E. faecium* genome using dsDNA recombineering methods we previously reported³⁸ (Fig. S3A, ESI†). Transformants were selected on chloramphenicol plates and assayed for *bsh*-HA and *cat* insertion via PCR 1 kb upstream and downstream of the *bsh* gene. Gel shifts were observed corresponding to the insertion size of about 800 bp when compared to the wild-type, indicating correct insertion of HA at the C-terminus of BSH (Fig. S3B, ESI†). Nanopore sequencing of the PCR products further validated the correct insertion into the genome. To validate if BSH-HA is properly being expressed, western blot analysis was performed on wild-type and BSH-HA *E. faecium*. A single band appears at the predicted size of BSH-HA in *E. faecium* Com15 BSH-HA but not in wild-type bacteria, indicating BSH-HA is being expressed in these cells (Fig. S3C, ESI†). *E. faecium* BSH-HA was treated with x-alk-LCA-3. After UV irradiation, bacteria were lysed and reacted with fluorescence or biotin tags through Click Chemistry (Fig. 1A). We found that x-alk-LCA-3 photo-crosslinks BSH-HA in a UV-dependent

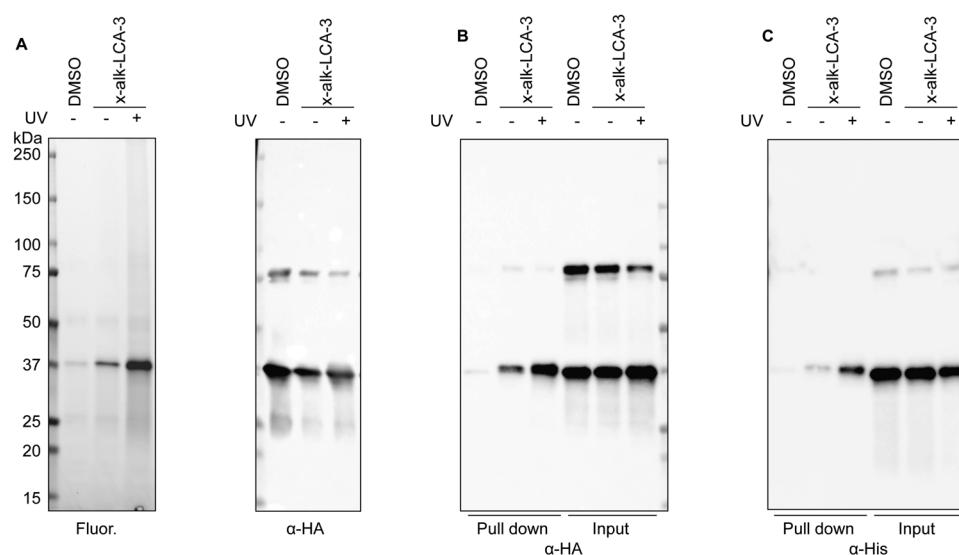


Fig. 2 Validation of BSH (EFWG_00531) and LCA interaction. (A) BSH-HA was immunoprecipitated from bacterial lysates and reacted with rhodamine-azide tag. In-gel fluorescence imaging shows the photocross-linking of BSH-HA anti-HA immunoblotting indicates the protein loading. (B) Cell lysates were reacted with biotin-azide, followed by enrichment with the streptavidin beads. Pull-down and input proteins were detected by anti-HA immunoblotting. (C) BSH-His6 was expressed in Efm_ΔBSH strain. BSH and x-alk-LCA-3 interaction was validated by western blot.



manner (Fig. 2A). BSHs usually form homo-tetramer assembly in solution³⁹ which is also confirmed by the crystal structure.^{16,37} Interestingly, we found potential dimer in the anti-HA blot for BSH-HA strain (Fig. 2A–C), but the formation of potential dimer depends on Cu-catalyzed click reaction (Fig. S4, ESI†). Importantly, we found the chromosomally expressed BSH-HA is pulled down by x-alk-LCA-3 in a UV-dependent manner (Fig. 2B). Pull-down of the overexpressed BSH-His6 by x-alk-LCA-3 further confirms the interaction between BSH and x-alk-LCA-3 (Fig. 2C).

Activity of bile salt hydrolase in *E. faecium*

To examine if conjugated secondary bile acids are able to be transformed to free bile acids by this putative BSH (Fig. 3A), we cultured *E. faecium* with taurolithocholic acid (TLCA). First, wild-type bacteria were grown in BHI media supplemented with TLCA up to 24 h. At different time points, bacterial cultures were collected for extracting bile acids from the supernatants. Liquid chromatography-mass spectrometry (LC-MS) was used to determine the formation of LCA. We could detect LCA after 8 hours of culturing *E. faecium* with TLCA, which indicates the transformation of TLCA in *E. faecium* (Fig. 3B and Fig. S7A, ESI†). To evaluate the specific activity of BSH in *E. faecium*, we performed knockout of *bsh* using dsDNA recombineering. *E. faecium* *bsh* was replaced with *cat* flanked by *loxP* sites to

generate *E. faecium* Com15 Δ *bsh*:*cat* (*Efm* Δ *bsh*) (Fig. S5A, ESI†). Gene replacement of *bsh* was confirmed via sanger sequencing of the PCR product. We cultured wild-type (*Efm* WT) and BSH knock-out (*Efm* Δ *bsh*) strains in TLCA supplemented BHI media, separately. We observed the loss of capability in converting TLCA to LCA by *Efm* Δ *bsh* strain (Fig. 3C). In addition, we transformed a plasmid in *Efm* Δ *bsh* that is able to express a C-terminus His6-tagged BSH (*Efm* Δ *bsh* + *pbsh*, Fig. S6, ESI†). Importantly, complementation of BSH enzyme in *Efm* Δ *bsh* recovered the capability and produced more LCA than the wild-type *E. faecium* (Fig. 3C). The incapability of producing LCA by *Efm* Δ *bsh* and recovery of LCA production in *Efm* Δ *bsh* + *pbsh* are not caused by defective growth of respective strains (Fig. S7B and C, ESI†).

Biochemical activity of *E. faecium* bile salt hydrolase *in vitro*

To evaluate the enzymatic activity of *E. faecium* BSH, we expressed and purified recombinant BSH for *in vitro* studies (Fig. 3A). The C-terminal His6-tagged BSH was purified as a 37 kDa protein (Fig. S7D, ESI†). Incubation of *E. faecium* BSH with TLCA in PBS buffer at pH 7.5 and 37 °C resulted in formation of LCA after 1 minute (Fig. S7E, ESI†) and total conversion of TLCA in 30 minutes (Fig. 3D and E). Next, we performed enzyme kinetics analysis of the deconjugation

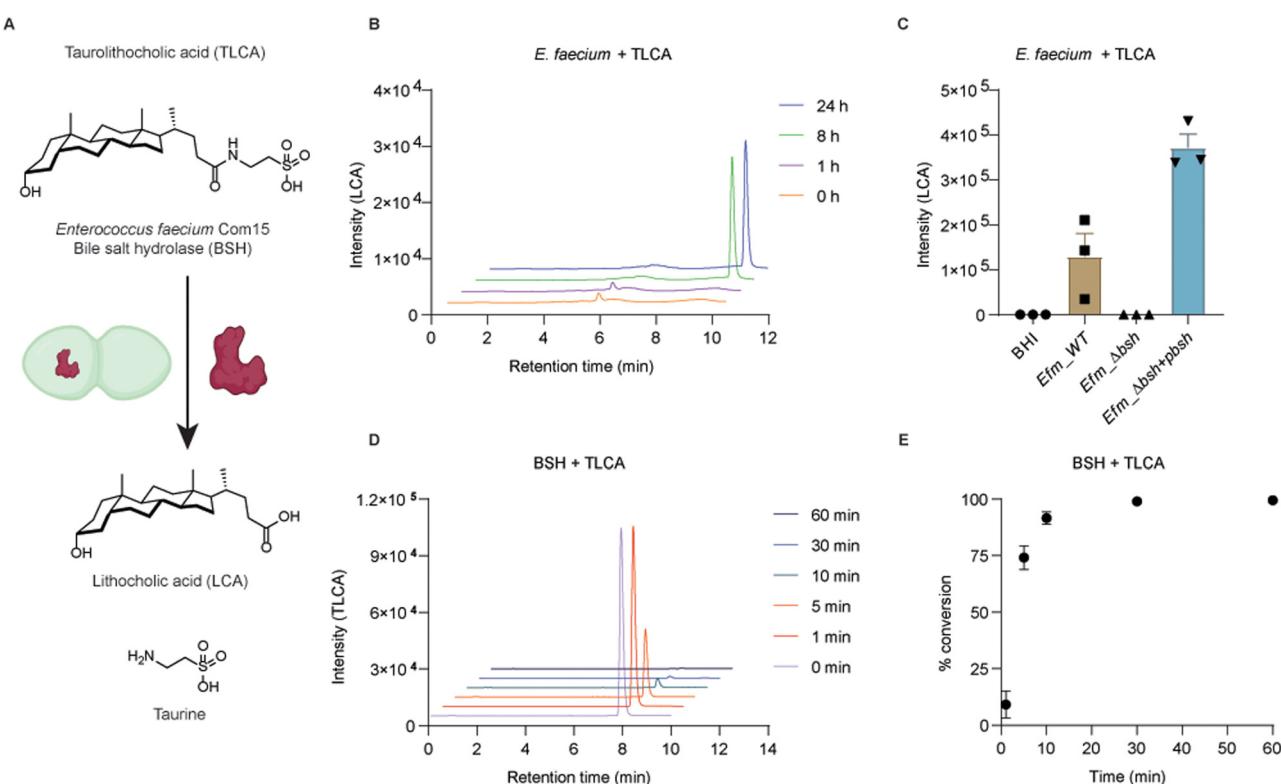


Fig. 3 Enzymatic activity of *E. faecium* Com15 bile salt hydrolase (BSH). (A) Schematic description of enzymatic deconjugation of taurolithocholic acid (TLCA). (B) Determination of LCA produced in wild-type *E. faecium* culture. BHI culture medium supplemented with 50 μ M TLCA was examined after 1 h, 8 h and 24 h respectively. (C) Determination of LCA produced in wild-type (*Efm* WT), BSH-deletion (*Efm* Δ *bsh*) and complementation of BSH (*Efm* Δ *bsh* + *pbsh*) *E. faecium* cultures. Bacteria were cultured in BHI medium supplemented with 100 μ M TLCA for 24 h. (D) Determination of TLCA deconjugated by BSH. 50 μ M TLCA was incubated with 100 nM BSH for 1, 5, 10, 30, 60 min. (E) Enzymatic deconjugation of TLCA is reported as percentage conversion. Data are presented in three replicates as mean \pm SEM.



reaction. TLCA of increasing concentrations (1, 5, 10, 20, 50, 100, 200, 500 μ M) were incubated with 100 nM BSH for 1 min. The input of TLCA and production of LCA were examined by LC-MS (Fig. S7F and G, ESI †). The substrate saturation curve (Fig. S7H, ESI †) revealed an approximate maximum enzyme velocity (V_{max}) value of 13.2 μ M min $^{-1}$ and K_m value of around 15 μ M. It is of interest to note that high concentrations of TLCA decrease the enzymatic activity of *E. faecium* BSH. This effect was also reported in a previous characterized BSH from *Bacteroides fragilis*.⁴⁰ These experiments demonstrate x-alk-LCA-3 crosslinked *E. faecium* Com15 (EFWG_00531) bile salt hydrolase that can convert TLCA to free secondary bile acid LCA.

Conclusions

Bile acids are a group of important metabolites that modulate human physiology and activity of other microbes. Identification of bile acid-interacting proteins in host cells and microbes is important for understanding the mechanisms of host-microbiota interactions. In this study, we employed an active bile acid photoaffinity reporter (x-alk-LCA-3) for chemical proteomic analysis of LCA-interacting proteins in *Enterococcus*. These studies identify many candidate LCA-interacting proteins, including a putative bile salt hydrolase that was validated in *E. faecium* by gene deletion, overexpression and *in vitro* with purified recombinant enzyme using activity assays. Our study highlights the utility of chemical proteomics for investigating the protein targets of microbiota metabolites.

Author contributions

H. C. H conceived the project. X. Y. designed and synthesized the bile acid reporter, performed proteomics and validated the BSH-bile acid interaction. V. C. tested the activity of bile acids and bile acid reporter and generated mutated strains of *E. faecium*. X. Z. evaluated the activity of *E. faecium* BSH. H. C. H, X. Y., X. Z. and V. C. wrote the manuscript.

Conflicts of interest

There are no conflicts to declare.

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