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Supporting Information

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

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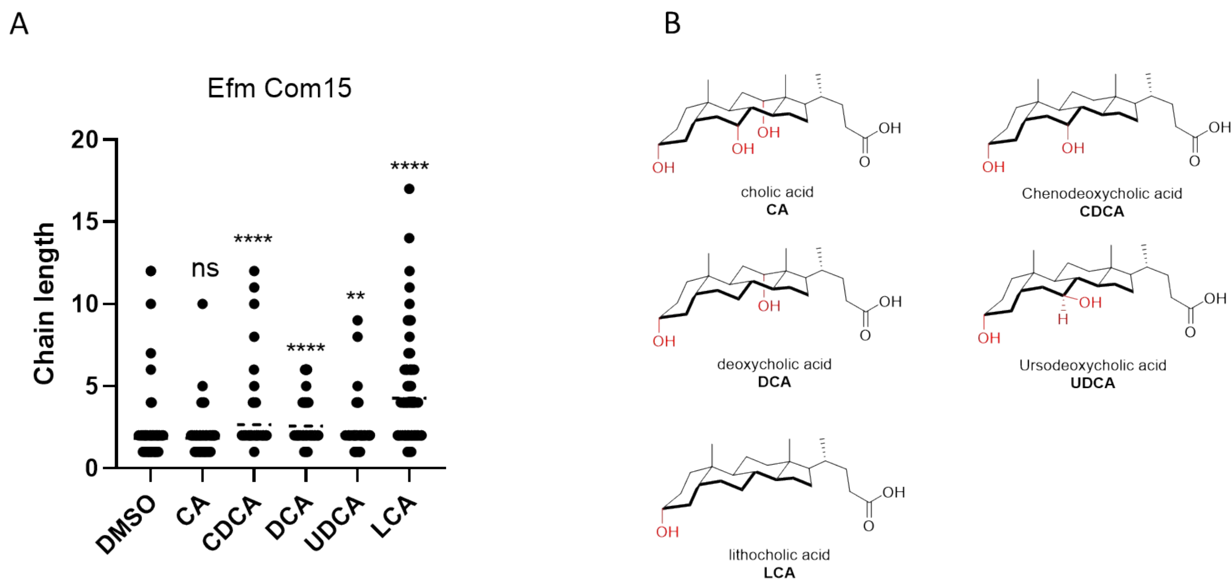
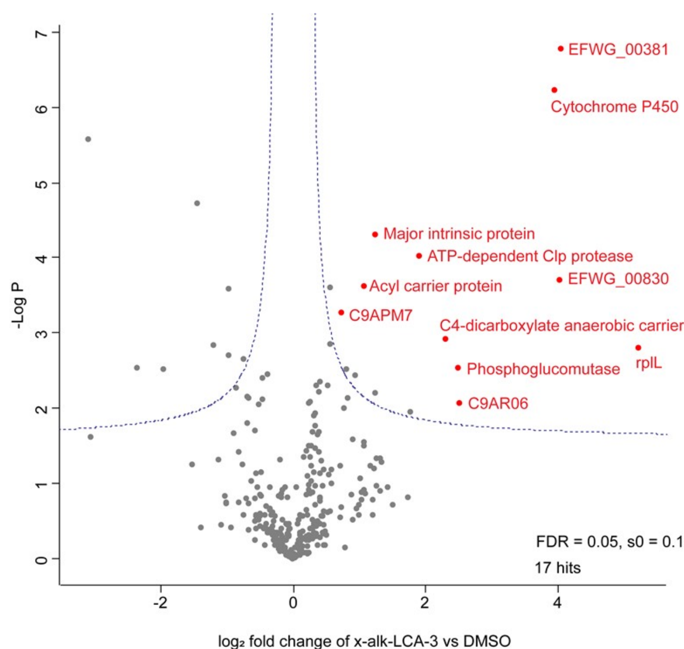
Table of contents	Page

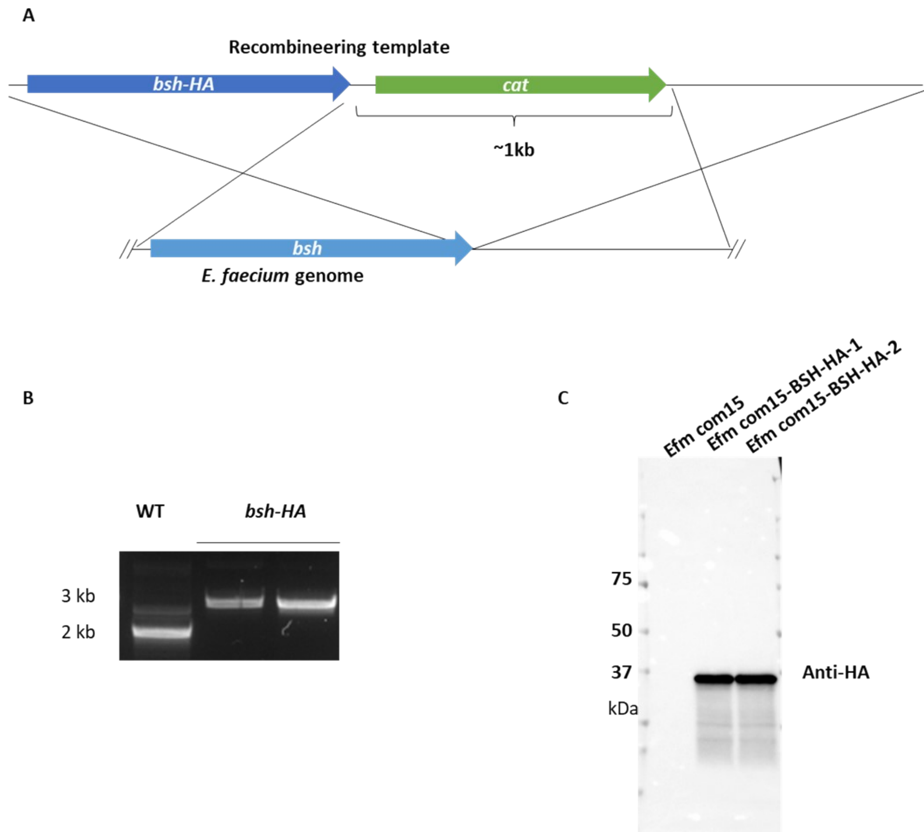
I.	**Supplementary Figure 1-7**	**S2**
II.	**Table S1**	**S8**
II.	**Methods**	**S9**
V.	**References**	**S16**



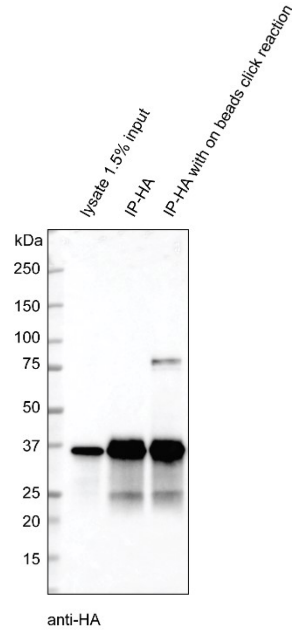
Supplementary Figure 1. Effects of bile acid and LCA chemical probes on the chaining of *E. faecium* Com15. (A) Chaining effects to *E. faecium* Com15 when grown with the presence of DMSO, CA, CDCA, DCA, UCDA or LCA. Statistics were done using Kruskal-Wallis ANOVA with Dunn's correction to compare each condition with DMSO treated cells. Statistical differences are as follows: DMSO vs CA, P > 0.9999; DMSO vs CDCA, P < 0.0001; DMSO vs DCA, P < 0.0001; DMSO vs UDCA, P = 0.0018; DMSO vs LCA, P < 0.0001. (B) Chemical structure of bile acids.



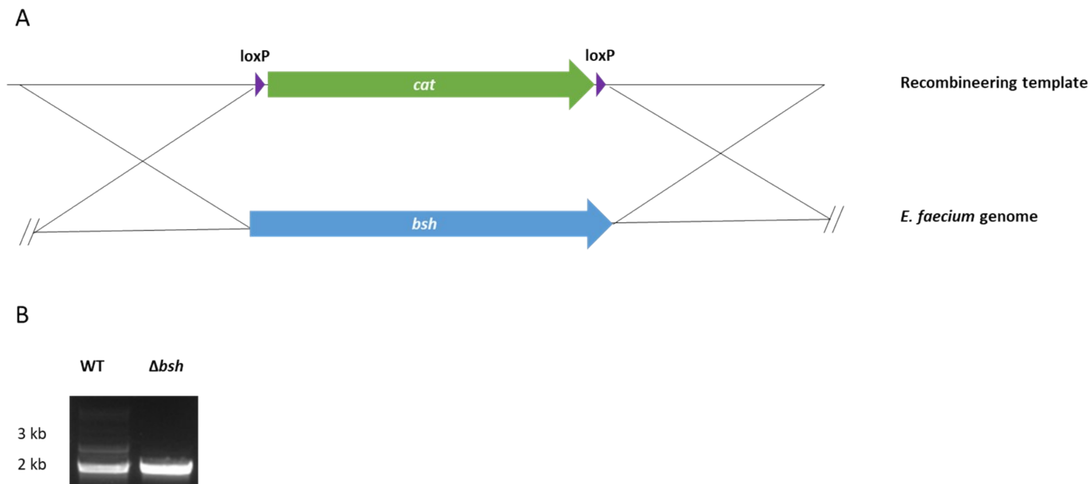
Supplementary Figure 2. LFQ Proteomics analysis of x-alk-LCA-3 modified *E. faecium* proteins. DMSO or x-alk-LCA-3 treated *E. faecium* Com15 cell lysates were reacted with biotin-azide. Enriched proteins were digested and analyzed by Mass Spectrometry.



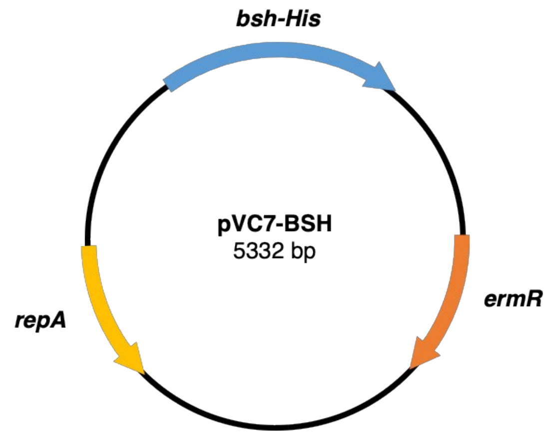
Supplementary Figure 3. Generation of genome encoded BSH-HA *E. faecium* Com15. (A) Scheme of dsDNA recombineering to generate genome encoded HA C-terminally tagged BSH. ~1kb homology arms were generated to target upstream and downstream of the C-terminus of *bsh*. Recombineering mediated insertion was facilitated by chloramphenicol selection. (B) DNA gel showing the PCR of the *bsh* region in wild-type and BSH-HA *E. faecium*. BSH-HA mutants show a gel shift corresponding to the insertion size of the recombineering template. (C) Western blot was performed on cell lysate protein against HA peptide. Protein band corresponding to the size to BSH-HA is present in *E. faecium* Com15 BSH-HA, but not in wild-type cells.



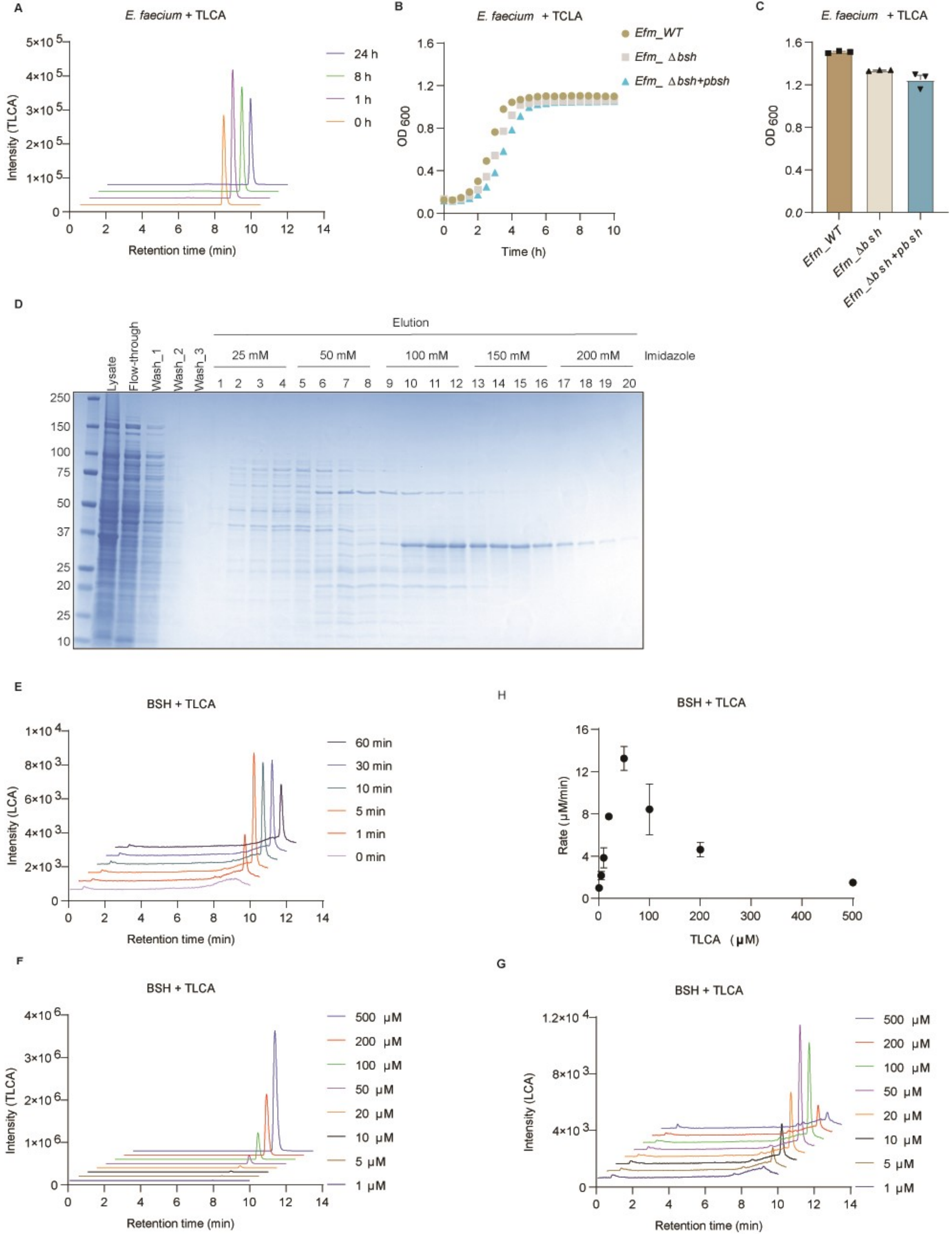
Supplementary Figure 4. Immunoprecipitation of BSH-HA by anti-HA beads. Efm Com15 (BSH-HA) lysate was immunoprecipitated form cell lysates by anti-HA magnetic beads. Potential dimer was observed in the anti-HA blot depending on Cu-catalyzed click reaction ($CuSO_4$, TCEP, TBTA, Rhodamine-azide).



Supplementary Figure 5. Generation of *E. faecium* Com15 Δ*bsh::cat*. (A) Recombineering scheme of *bsh* knockout in *E. faecium* Com15 using dsDNA recombineering template encoding *cat*. Homology arms of ~1kb targeting upstream and downstream regions of *bsh* were generated. LoxP sites flank *cat* to create a ~1kb genetic replacement of *bsh*. (B) DNA gel of PCR performed on the *bsh* region of *E. faecium* Com15. *cat* gene replacement size is nearly identical to the *bsh* gene.



Supplementary Figure 6. *bsh* expression and complementation vector. C-terminally his-tagged *bsh* from *E. faecium* Com15 expression is driven by a constitutive *bacA* promoter. Backbone carries an erythromycin resistance marker.


A
E. faecium + TLCA
Intensity (TLCA)
Retention time (min)
24 h
8 h
1 h
0 h
B
E. faecium + TCLA
OD 600
Time (h)
Efm_WT
Efm_ Δbsh
Efm_ Δbsh+pbsh
C
E. faecium + TLCA
OD 600
Efm_WT
Efm_Δbsh
Efm_Δbsh+pbsh
D
Elution
25 mM
50 mM
100 mM
150 mM
200 mM
Imidazole
Lysate
Flow-through
Wash_1
Wash_2
Wash_3
E
BSH + TLCA
Intensity (LCA)
Retention time (min)
60 min
30 min
10 min
5 min
1 min
0 min
H
BSH + TLCA
Rate (μM/min)
TLCA (μM)
F
BSH + TLCA
Intensity (TLCA)
Retention time (min)
500 μM
200 μM
100 μM
50 μM
20 μM
10 μM
5 μM
1 μM
G
BSH + TLCA
Intensity (LCA)
Retention time (min)

Supplementary Figure 7. Enzymatic activity of *Enterococcus faecium* Com15 bile salt hydrolase (BSH). (A) LC-MS analysis of TLCA from the wild-type *E. faecium* cultured in BHI medium supplemented with 50 μM TLCA after 1, 8, and 24 hours. (B) Bacterial growth curves of wild-type (*Efm_WT*), BSH-deletion (*Efm_Δbsh*) and complementation of BSH (*Efm_Δbsh+pbsh*) *E. faecium* Com15 in BHI medium supplemented with 100 μM TLCA. Data are presented in three replicates as mean ± SEM. (C) Optical density of bacterial cultures in BHI medium supplemented with 100 μM TLCA for 24 h. Data are presented in three replicates as mean ± SEM. (D) Purification and SDS-PAGE analysis of the C-terminal His6-tagged BSH. (E) LC-MS analysis of LCA in the enzymatic reactions containing 50 μM TLCA and 100 nM BSH for 1, 5, 10, 30, 60 min. (F-G) LC-MS analysis of TLCA and LCA in the enzymatic reactions containing TLCA of increasing concentrations (1, 5, 10, 20, 50, 100, 200, 500 μM) and 100 nM BSH for 1 min. (H) Substrate saturation curve of the enzymatic reactions containing TLCA of increasing concentrations (1, 5, 10, 20, 50, 100, 200, 500 μM) and 100 nM BSH for 1 min. Data are presented in three replicates as mean ± SEM.

Table S1 Primers used in this study

Primer	Sequence	Description
oVC265	cgattttataatagagtaattggtatcg	F primer Δbsh::cat recombineering template
oVC266	atcgtttatatattgtcaatacatattcag	R primer Δbsh::cat recombineering template
oVC268	aaaactttggagtaaaatgaacg	F BSH-Ha reombineering template
oVC20	atattgaaacaggagttagaaaaa	R BSH-Ha reombineering template/ screening *bsh* recombineering
oVC338	aatagaaacgcttcttcgtg	F Primer to screen *bsh* recombineering
BSH_F	atattttgaggaggagtatcATGTGTACGTCTATTACTTATG	F primer for *bsh* to generate pBSH
BSH_R	ggtgatggtgATTTATATATTTAATTTGTTGTTTTTCTATAATTG	R primer for *bsh* to generate pBSH
Backone_1_F	atatataaatCACCATCACCATCACCATTAAC	F primer for backbone to generate pBSH
Backbone_1_R	ttttgttcatTTGATATGCCTCCTAAATTTTTATCTAAAG	R primer for backbone to generate pBSH
ery_F	ggcatatcaaATGAACAAAAATATAAAATATTCTCAAAAC	F primer for erthromycin resistance cassette for pBSH
ery_R	acagtcggcattatctcataTTATTTCCTCCCGTTAAATAATAG	R primer for erthromycin resistance cassette for pBSH
backbone_2_F	TATGAGATAATGCCGACTG	F primer for backbone to generate pBSH
backbone_2_R	GATACTCCTCCTCAAAATATTTTTG	R primer for backbone to generate pBSH

Methods

Microscopy and chaining analysis.

Microscopy and bacterial chaining quantification methods were adapted from previous literature.[1,2] In brief, overnight cultures of *E. faecium* Com15 treated with bile acid derivatives were gently mixed via votexing. Cultures were then prepped onto Fisherbrand Superfrost Plus Microscope Slides, covered with Zeiss cover glasses and sealed with Sally Hansen Insta-Dri Nail Color. Light microscopy was performed to quantify chain lengths of *E. faecium* by manually counting the number of cells in each particle, where each particle is defined by a continuous chain of cells. Cell chain lengths were analyzed using Kruskal-Wallis one-way ANOVA test with Dunn's correction on PRISM 9. Imaging was performed at the Rockefeller University Bio-Imaging Resource Center with Zeiss Axioplan 2 upright microscope on objective plan apochromat 63x oil NA 1.4 0.19mm. Images were taken using Hamamatsu High Resolution Digital B/W CCD Camera and acquired using MetaVue version 7.7.0.0.

Label-Free Quantitative proteomics of bile acid reporter in *E. faecium* strain *Com15*

1:50 dilutions of overnight BHI cultures of *E. faecium* strain *Com15* were grown in 20 mL BHI for 3 h at 37 °C with 220 rpm shaking. Cultures were incubated with 25 μM bile acid reporter in DMSO or DMSO as the negative control for another 1 h at 37 °C with 220 rpm shaking. The culture medium was centrifuged at 5000 g for 10 min. Bacteria pellets was re-suspended in 10 mL cold PBS. For in-cell photo-crosslinking experiments, bacteria in 10 mL cold PBS was transferred to petri dish (100 mm X 15 mm) and subjected to UV irradiation at 365 nm on ice for 10 min using a Spectrolinker XL-1000 UV crosslinker (Spectronics) at a distance of 3-5 cm (UV lamp should be warmed-up for 20 min). Bacteria were then harvested and centrifuged 5000 g at 4 °C for 10 min. Pellet was flash-frozen and stored in the -70 degree. Bacteria pellet was thawed and resuspended in B-PER™ Complete Bacterial Protein Extraction Reagent (1 mL, catalog number: 89821) with

1X EDTA-free protease inhibitor cocktail (Roche). Bacteria were then incubated 1 hour at room temperature with gentle rocking. Cell lysates were centrifuged at 16000 g for 20 min to remove cell debris and supernatants were collected. Protein concentration was estimated by BCA assay with BCA Protein Assay Kit (Thermo) and normalized to 1 mg/mL. For each total cell lysates was added with click chemistry reagents as a 10X master mix (az-Biotin: 0.1 mM, 10 mM stock solution in DMSO; tris(2-carboxyethyl)phosphine hydrochloride (TCEP): 1 mM, 50 mM freshly prepared stock solution in dH_2O; tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine (TBTA): 0.1 mM, 2 mM stock in 4:1 *t*-butanol: DMSO; $CuSO_4$: 1 mM, 50 mM freshly prepared stock in dH_2O). Samples were mixed well and incubated at room temperature for 1 h. After incubation, samples were mixed with 4 mL cold methanol and incubated at -20 °C overnight. Protein pellets were centrifuged at 5000 g for 30 min at 4°C, pellets were transferred to 2.0 mL centrifuge tube and were washed with 1 mL cold methanol 3 times. After last wash, pellets were let air dried before being re-solubilized in 250 µl 4% SDS PBS with bath sonication. Solutions were diluted with 750 µl PBS, and incubated with 100 µl PBS-T-washed High Capacity NeutrAvidin agarose (Pierce) (500 µl PBS-T-washed twice, 2500 g X 60 s) at room temperature for 1 h with end-to-end rotation. The agarose was washed with 500 µl 1% SDS PBS 3 times, 500 µl 4M Urea PBS 3 times, and 500 µl PBS 3 times and then reduced with 500 µl 10 mM DTT (Sigma) in PBS for 30 min at 37 °C, and alkylated with 500 µl 50 mM iodoacetamide (Sigma) in PBS for 30 min in dark. 50 µl NH_4HCO_3 (10 mM) was added to the tube. Neutravidin-bound proteins were digested on bead with 400 ng Trypsin/Lys-C mix (Promega) at 37 °C overnight with shaking. Digested peptides were collected (2500 g for 60 s) and lyophilized before being desalted with custom-made stage-tip containing Empore SPE Extraction Disk (3M). Peptides were eluted with 2% acetonitrile, 2% formic acid in dH_2O.

Peptide LC-MS analysis was performed with a reversed phase C18 column coupled to a Q Exactive HF-Orbitrap MS mass spectrometer. A 126-minute gradient increasing from 99% buffer

A (HPLC grade water with 0.1% formic acid) and 1% buffer B (HPLC grade acetonitrile with 0.1% formic acid) to 90% buffer B was used at 300 nL/min. MS spectra was measured at a resolution of 60,000 with full MS scan (400–1500 MW). Label-free quantification of bile acid reporter-labeled proteins was performed in MaxQuant (version 2.1.0.0) as described. The peptides were identified from the MS/MS spectra searched against the database (*Enterococcus faecium* Com15, Proteome ID: UP000005116). Label free quantification experiments in MaxQuant were performed using the built-in label free quantification algorithm. Other parameters were used as preset in the software[3]. The search results from MaxQuant were analyzed by Perseus 2.0.5.0. Briefly, the DMSO replicates and bile acid reporters-labeled sample replicates were grouped correspondingly. The results were cleaned to filter off reverse hits and contaminants. Only proteins that were identified in 3 out of 4 sample replicates and with more than two unique peptides were subjected to subsequent statistical analysis. LFQ intensities were used for measuring protein abundance and logarithmized. Signals that were originally zero were imputed with random numbers from a normal distribution, whose mean and standard deviation were chosen to best simulate low abundance values below the noise level (Replace missing values by normal distribution – Width = 0.3; Shift = 1.8). The summary of proteomics data is shown in the excel file of proteomics summary (methods are adapted from previous literature[4]).

In-gel profiling of x-alk-LCA-3 interacting proteins in *E. faecium* Com 15

1:50 dilutions of overnight BHI cultures of *E. faecium* strain *Com15* were grown in 4 mL BHI for 3 h at 37 °C with 220 rpm shaking. Cultures were incubated with different concentration of x-alk-LCA-3 in DMSO or DMSO as the negative control for another 1 h at 37 °C with 220 rpm shaking. The culture medium was centrifuged at 5000 g for 10 min. Bacteria pellets was re-suspended in 4 mL cold PBS. For in-cell photo-crosslinking experiments, bacteria in 4 mL cold PBS were transferred to petri dish (35 mm) and subjected to UV irradiation at 365 nm on ice for 10 min using a Spectrolinker XL-1000 UV crosslinker (Spectronics) at a distance of 3-5 cm (UV lamp should

be warmed-up for 20 min). Bacteria were then harvested and centrifuged 5000 g at 4 °C for 10 min. Pellet was flash-frozen and stored in the -70 ^{o}C. Bacteria pellet was thawed and resuspended in B-PER™ Complete Bacterial Protein Extraction Reagent (200 µL, catalog number: 89821) with 1X EDTA-free protease inhibitor cocktail (Roche). Bacteria were then incubated 1 hour at room temperature with gentle rocking. Cell lysates were centrifuged at 16000 g for 20 min to remove cell debris and supernatants were collected. Protein concentration was estimated by BCA assay with BCA Protein Assay Kit (Thermo). 100 µg total lysate is brought to 90 µL with buffer and were added 10 µL Click master mix (az-Rhodamine: 0.1 mM, 10 mM stock solution in DMSO; tris(2-carboxyethyl)phosphine hydrochloride (TCEP): 1 mM, 50 mM freshly prepared stock solution in dH_2O; tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine (TBTA): 0.1 mM, 2 mM stock in 4:1 *t*-butanol: DMSO; $CuSO_4$: 1 mM, 50 mM freshly prepared stock in dH_2O). The resulted mixture was incubated at room temp without light for 1 hour. Proteins were precipitated by 4X MeOH and stored in -20 ^{o}C overnight. Samples were then centrifuged (18000 g X 20 min, 4 ^{o}C) and resulted pellet were washed by MeOH for three times. Protein pellet was boiled in protein loading buffer and analyzed by SDS-PAGE.

Genome modification of *bsh* in *E. faecium* Com15.

Genome modifications to generate C-terminally HA tagged *bsh* and E. faecium *bsh* knockout were done via recombineering as previously described[1]. In brief, *E. faecium* Com15 containing pRecT was induced with IPTG and electroporated with dsDNA template containing the desired edit. dsDNA template contains ~1kb homology arms that target the *bsh* locus flanking a chloramphenicol acetyltransferase (*cat*) gene for selection. For *bsh*-HA, an HA tag was inserted at the C-terminus of *bsh*, followed by *cat*. For *E. faecium* Δ*bsh::cat*, loxP sites flanking the *cat* gene was designed with ~1kb homology arms targeting upstream and downstream of *bsh* to replace the *bsh* gene. Accessory loxP sites were added in order to retain the option to remove

the *cat* gene if desired for future studies. Mutants were verified via PCR and sequencing. dsDNA templates were cloned into the pET21 vector and generated via PCR as previously described[1].

Generation of complementation plasmid.

pBSH was generated by cloning the *bsh* into the psrtA1 backbone that already contained a 6His tagged region in the vector, while swapping *cat* for an erythromycin resistance casette[1]. *bsh* expression was driven under the bacA promoter as previously described[1]. pBSH was transformed and propagated in *E. coli* DH5α (New England Biolabs). pBSH was electroporated into *E. faecium* Δ*bsh::cat* to generate the complementation strain as previously described[1].

Validation of BSH-LCA reporter interaction.

1:50 dilutions of overnight BHI cultures of *E. faecium* strain Com15 (EFWG_00531-HA) were grown in 4 mL BHI for 3.5 h at 37 °C with 220 rpm shaking. Cultures were incubated with different concentration of x-alk-LCA-3 in DMSO or DMSO as the negative control for another 0.5 h at 37 °C with 220 rpm shaking. The culture medium was centrifuged at 5000 g for 10 min. Bacteria pellets was re-suspended in 4 mL cold PBS. For in-cell photo-crosslinking experiments, bacteria in 4 mL cold PBS were transferred to petri dish (35 mm) and subjected to UV irradiation at 365 nm on ice for 10 min using a Spectrolinker XL-1000 UV crosslinker (Spectronics) at a distance of 3-5 cm (UV lamp should be warmed-up for 20 min). Bacteria were then harvested and centrifuged 5000 g at 4 °C for 10 min. Pellet was homogenized by Fastprep-24 5G (MP Biomedicals, 6 m/s, 2 cycles, 45 s for each cycle, 20 mM HEPES, 150 mM NaCl, 1% SDS, 1X EDTA-free protease inhibitor cocktail (Roche)). Supernatant after centrifugation (18 000 g, 25 ^{o}C, 10 min) were collected. Protein concentration was estimated by BCA assay with BCA Protein Assay Kit (Thermo).

For in-gel fluorescence labeling, 250 μg of each total cell lysates were immunoprecipitated with 20 μL PBS-T-washed Pierce™ Anti-HA Magnetic Beads (Thermo Scientific, 88836). Samples were mixed well and incubated at 4 °C for 2 h with end-to-end rotation. After samples were washed with 500 μL PBS-T 3 times, 45 μL of PBS-T (PBS with 0.1% Tween-20) was added to each sample. 5 μL of click chemistry reagents as a 10X master mix mentioned above were added to each sample. After incubation at room temperature for 1 h, samples were washed with 500 μL PBS-T 3 times. Samples were boiled with 1X Laemmli buffer at 95 °C for 5 min and analyzed by SDS-PAGE (in-gel fluorescence scanning for labelling, western blot anti-HA for protein loading).

For biotin pull down experiment, to 250 μg of each total cell lysates (endogenous EFWG_00531-HA strain or overexpressed EFWG_00531-His strain), click master mix 10X (Az-biotin: 0.1 mM, 10 mM stock solution in DMSO; tris(2-carboxyethyl)phosphine hydrochloride (TCEP): 1 mM, 50 mM freshly prepared stock solution in dH_2O; tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine (TBTA): 0.1 mM, 2 mM stock in 4:1 t-butanol: DMSO; $CuSO_4$: 1 mM, 50 mM freshly prepared stock in dH_2O) was added. Samples were mixed well and incubated at room temperature for 1 h. MeOH (4X volume) was added to the reaction. The resulted mixture was vortexed and incubated at -20 °C overnight to precipitate the protein. Precipitated proteins were centrifuged at 18000g for 20 min at 4 °C, aqueous layer was then removed. The resulted pellet was washed for 3 times with cold MeOH (18000 g X 10 min). The pellet was then dried and redissolved in 100 μl 4% SDS PBS with bath sonication (2.5 μl solution was kept for "input" before enrichment). The samples were centrifuged at 18000 g for 10 min and supernatant was diluted with PBS to 1% SDS and incubated with 20 μl PBS-T-washed High Capacity NeutrAvidin agarose (Pierce) (500 μl PBS-T-washed twice, 2500 g X 60 s) at room temperature for 1 h with end-to-end rotation. The agarose was then washed with 500 μl 1% SDS PBS 3 times, 500 μl 1M Urea PBS 3 times, and 500 μl PBS 3 times. Samples were boiled with 2X Laemmli buffer 95 °C for 5 min and analyzed by western blot. (methods are adapted from previous literature[4]).

Bacterial growth curve

Bacterial strains were cultured in 3 mL BHI medium at 37 °C with shaking at 220 rpm overnight. They were then diluted to an OD600 value of 0.1 in 200 μL fresh BHI medium containing 100 μM TLCA and grown in a 96-well plate at 37 °C with shaking up to 10 hours. Optical density was measured via endpoint absorbance at 600 nm every 30 min using Biotek Cytation 5.

Analysis of BSH activity in *E. faecium* Com15

Bacterial strains were cultured in 5 mL BHI medium at 37 °C with shaking at 220 rpm overnight. They were then diluted to an OD600 value of 0.1 in fresh BHI medium containing 50 μM or 100 μM TLCA and grown up to 24 hours. 1 mL of bacterial cultures were collected at different time points (0, 1, 8 and 24 hours). Bacteria were pelleted through centrifugation at 20,000 g and 4 °C for 5 min. The supernatants were transferred to new tubes, acidified to a pH value of 1 using 6N HCl, and extracted with equal volume of ethyl acetate. The organic extracts were collected in new tubes and air dried overnight. The dried components were reconstituted in 100 μL 50% MeOH/H2O for LC-MS analysis.

Analysis of *E. faecium* BSH activity *in vitro*

BSH (EFWG_00531) was amplified from *E. faecium* Com15 genomic DNA, and inserted to a pVC7 vector for generating a C-terminal His6-tagged BSH fusion protein. The plasmid was transformed into chemical competent *E. coli* DH5α. Bacteria were cultured in 50 mL LB medium containing 150 μg/mL erythromycin overnight, and then grown in 500 mL LB medium containing erythromycin starting from OD600 of 0.1 for 24 h at 37 °C with shaking at 220 rpm. Bacteria were pelleted, resuspended in 40 mL 1xDPBS buffer (pH 7.5) containing 20 mM imidazole and 0.25 mM tris(2-carboxyethyl)phosphine hydrochloride (TCEP), and lysed by sonication. The lysates were clarified by centrifugation, incubated with 2 mL of Ni-NTA resin at 4 °C for 16 h. The resins

were loaded on a column, washed three times with DPBS buffer, eluted using a gradient of 25 mM to 200 mM imidazole in DPBS buffer. Elution fractions containing pure proteins were combined and dialyzed in DPBS buffer containing 5% w/v glycerol. The dialyzed proteins were concentrated using spin columns and examined by BSA assay to determine protein concentration. Protein aliquots were frozen in liquid nitrogen and stored at -80 °C.

BSH-His6 were firstly incubated in 90 μL DPBS buffer containing 10 mM TCEP and 5% glycerol at 37 °C to yield a final concentration of 100 nM, then mixed with 10 μL TLCA dissolved in DMSO of different concentrations. The reactions were performed at 37 °C for different time before they were stopped by mixing with 100 μL cold methanol and seated on ice. The reaction solutions were centrifugated, transferred to 96-well plates for LC-MS analysis.

LC-MS analysis

Chemicals, bacterial culture extracts and enzymatic reaction solutions were analyzed by 1290 Infinity II LC/MSD system (Agilent technologies) using Poroshell 120 EC-C18 column (2.1 x 50 mm, 4 μM). The flow rate was 0.5 mL/min using 0.05% formic acid in water as mobile phase A and 0.05% formic acid in acetone as mobile phase B at room temperature. The gradient was applied as below: 0-1 min: 5% B, 1-3 min: 5-40% B, 3-9 min: 40-95% B, 9-10 min: 95-5% B. MSD API-ES SIM mode on Sample Target Masses of LCA (m/z = 375.30) and TLCA (m/z = 482.30). All data were collected in negative-ion mode. Extracted intensity and retention time were analyzed by GraphPad Prism 8.

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