

Activity-based protein profiling of serine hydrolases and penicillin-binding proteins in *Enterococcus faecium*

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Abstract

Enterococcus faecium is a gut commensal bacterium which is gaining increasing relevance as an opportunistic, nosocomial pathogen. Its high level of intrinsic and acquired antimicrobial resistance is causing a lack of treatment options, particularly for infections with vancomycin-resistant strains, and prioritizes the identification and functional validation of novel druggable targets. Here, we use activity-based protein profiling (ABPP), a chemoproteomics approach using functionalized covalent inhibitors, to detect active serine hydrolases across 11 *E. faecium* and *Enterococcus lactis* strains. Serine hydrolases are a big and diverse enzyme family, that includes known drug targets such as penicillin-binding proteins (PBPs), whereas other subfamilies are underexplored. Comparative gel-based ABPP using Bocillin-FL revealed strain- and growth condition-dependent variations in PBP activities. Profiling with the broadly serine hydrolase-reactive fluorescent probe fluorophosphonate-TMR showed a high similarity across *E. faecium* clade A1 strains, but higher variation across A2 and *E. lactis* strains. To identify these serine hydrolases, we used a biotinylated probe analog allowing for enrichment and identification via liquid chromatography–mass spectrometry. We identified 11 largely uncharacterized targets (α , β -hydrolases, SGNH-hydrolases, phospholipases, and amidases, peptidases) that are druggable and accessible in live vancomycin-resistant *E. faecium* E745 and may possess vital functions that are to be characterized in future studies.

Keywords: vancomycin-resistant enterococci (VRE); chemoproteomics; activity-based probe; fluorophosphonate; Bocillin-FL; *Enterococcus lactis*

Introduction

Although *Enterococcus faecium* can be found in a wide variety of environments including as commensal members of the gut microbiota, the prevalence of clinical cases of antibiotic resistant *E. faecium* has been increasing in the last decades (Treitman et al. 2005, Zhou et al. 2020). *Enterococcus faecium* shows a population structure, which clusters into distinct clades. Previously, the clade structure was divided into clades A and B, where clade A contained clinical, animal, and environmental strains, whereas B contained commensal strains. However, in a recent genome-based study it was established that clade B *E. faecium* strains in fact belong to another species, *Enterococcus lactis* (Belloso Daza et al. 2021). Within clade A there are two subclades called A1 and A2, where most clinical isolates cluster in the A1 clade (van Hal et al. 2021), whereas the A2 clade contains mostly commensal and animal strains.

The success of *E. faecium* as an emerging nosocomial pathogen can in part be explained by its high level of intrinsic antibiotic resistance as well as its pronounced ability to acquire resistance through horizontal gene transfer (Hegstad et al. 2010) and chromosomal mutations. It can survive disinfection procedures (Bhardwaj et al. 2018, Pidot et al. 2018) and take advantage of the dysbiosis of the gut microbiota caused by treatment

with antibiotics, which in turn can lead to it overgrowing the gut and further causing serious infections such as bacteraemia (Edmond et al. 1995) and endocarditis (Murdoch et al. 2009, Paganelli et al. 2016). Of particular concern is the growing prevalence of infections with vancomycin-resistant enterococci (VRE) (Treitman et al. 2005), of which the majority belongs to *E. faecium* (Lee et al. 2019), and for which there is a lack of treatment options.

Promising strategies to improve and expand the existing chemotherapeutic options for clinical management of vancomycin-resistant *E. faecium*, are to increase the susceptibility of bacteria to antibiotic treatment (antibiotic sensitizers) or to block their ability to cause disease (antivirulence agents). For an opportunistic pathogen such as *E. faecium*, whose versatility and adaptability are key to its progression from harmless commensal to priority pathogen (Guzman Prieto et al. 2016), such strategies to modulate the bacteria's virulence and resistance properties appear particularly feasible. Except for enterococcal adhesion, for which the role of virulence factors is well studied (Heikens et al. 2011, Somarajan et al. 2015, Montealegre et al. 2016)—other factors contributing to the pathogenicity of VRE-strains have mostly been deduced from genomic and epidemiological studies showing the acquisition of candidate virulence genes, while their molecu-

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lar function and utility as potential drug targets often remains unexplored.

Emarking on the work to fill this gap, we have here undertaken a chemical biology approach to identify previously un- or under-characterized *E. faecium* enzymes that are druggable, i.e. they can be targeted by small molecule inhibitors. For this purpose, we used activity-based protein profiling (ABPP), which is a chemoproteomic technique that uses electrophilic small molecule probes (so-called activity-based probes or ABPs) that specifically target and covalently react with specific enzyme families (Fig. 1A) (Jesani and Cravatt 2004, Barglow and Cravatt 2007, Speers and Cravatt 2009) in an active-site directed manner allowing for their quantification and identification. One particularly attractive candidate enzyme family is serine hydrolases (SHs), which is one of the largest enzyme classes, all of which share the feature of a catalytic serine in the active site (Bachovchin and Cravatt 2012). SHs can be further subdivided into the superfamilies of serine proteases, lipases, esterases, amidases, peptidases, phospholipases, protein, and glycan hydrolases. These enzymes can be targeted and quantified using fluorophosphonate-ABPs (FP-ABPs) (Simon and Cravatt 2010). Utilizing the same ABPs as in this study, we recently identified 10 previously uncharacterized SHs, the FP-binding hydrolases FphA-J in the Gram-positive pathogen *Staphylococcus aureus*. Whereas the functional characterization of these enzymes is still ongoing, we were able to characterize FphB as a novel virulence factor whose function can be specifically blocked with a small molecule inhibitor (Lentz et al. 2018), and FphH appears to be linked to stress response (Fellner et al. 2023). ABPP studies have also identified SHs in other microorganisms (Ortega et al. 2016, Tallman et al. 2016, Keller et al. 2020, 2023, Babin et al. 2022, Li et al. 2022).

SHs as virulence factors and agents of antibiotic resistance have so far been largely uncharacterized in enterococci. The serine protease E has been, together with gelatinase E, associated with virulence in *Enterococcus faecalis* (Sifri et al. 2002, Engelbert et al. 2004), while in *E. faecium* their roles in virulence are still unclear. In a recent chemoproteomic study where the FP-probe identified a dipeptidyl peptidase homolog in the gut commensal *Bacteroides thetaiotaomicron*, Keller et al. (2023) also performed a bioinformatic prediction of SHs in human gut commensals. This analysis was based on homology with SH-associated Pfam domains and predicted a total number of 36 SHs in *E. faecium* ATCC BAA-472 and 37 in *E. faecalis* V583 (Keller et al. 2023).

A subgroup of SHs are the penicillin-binding proteins (PBPs), which are enzymes taking part in the construction of the peptidoglycan layer of the bacterial cell-wall through their transpeptidase and/or glycosyltransferase activities. These enzymes are inhibited by beta-lactam drugs, such as penicillin, but many bacteria, including *E. faecium*, possess low-affinity PBPs that are not inhibited by β -lactam drugs. In *E. faecium* there are six PBPs (Rice et al. 2009), of these the most widely studied is PBP5, a low-affinity PBP. Certain mutations in the gene of PBP5 are important for ampicillin resistance within this species (Zhang et al. 2012). In *E. faecium*, PBP5 is mainly associated with β -lactam resistance (Arbeloa et al. 2004), although other PBPs have recently been reported to contribute (Djorić et al. 2020). Importantly, PBP5 expression (and thus β -lactam resistance) is inducible and varies with culture and stress conditions (Lebreton et al. 2012, Kellogg et al. 2017). Systematic studies on dynamic activity profiles of the different PBPs in *E. faecium* have not been reported to the best of our knowledge.

Here, we have performed a systematic chemoproteomic study elucidating the dynamic SH activity profiles in *E. faecium*. Using the fluorescent probes FP-rhodamine, that primarily targets alpha-

beta-hydrolases, and the PBP-specific probe Bocillin-FL, we addressed the dynamic activity profiles of a collection of 11 *E. faecium* and *E. lactis* strains in different growth media. Furthermore, we used a biotinylated probe to enrich FP-binding SH from the vancomycin-resistant *E. faecium* E745 and identified a set of 11 largely uncharacterized SHs by LC-MS/MS. The highly conserved activity profiles across clinical strains and growth conditions suggest that these SH fulfil important functions that are yet to be described.

Methods

Growth conditions, bacterial strains, and activity-based probes

All enterococcal strains used in this study are outlined in Supplementary Table S1. Before labelling with biotinylated probes and sample preparation for mass spectrometry, *E. faecium* strain E745 was grown for two consecutive overnight incubations (18–20 h) on BHI agar plates. For fluorescent labelling and profiling all strains were grown on LB, BHI, and blood agar plates in the same way as for proteomics. All incubations were performed at 37°C. Fluorescent labelling was performed with the BOCILLIN™-FL Penicillin (ThermoFisher Scientific) probe targeting PBPs or the ActivX™ TAMRA-FP (ThermoFisher Scientific) probe targeting SHs. For enrichment of SHs, the ActivX™ Desthiobiotin-FP (ThermoFisher Scientific) probe was used.

Live cell labelling of bacteria with fluorescent ABPs and SDS-PAGE analysis

After growth as indicated, bacteria were harvested and suspended to a density of OD₆₀₀ ~20 in either LB (when grown on LB plates) or BHI broth (when grown on BHI and blood agar). The suspensions were then aliquoted to 1.5 ml tubes at a volume of 100 μ l. The chemical probes were added at a concentration of 100 μ M to a final concentration of 1 μ M in the bacterial suspension. The bacterial suspensions were incubated with probe for 45 min at 37°C and shaking at 300 rpm.

After incubation, samples were transferred to 0.5 ml screw-cap tubes with around 60 μ l 0.1 mm glass beads (BioSpec Products) and 33 μ l 4X SDS-PAGE buffer and lysed by bead-beating (3 \times 30 s at full speed with 1-min pause in-between). Samples were centrifuged at 5000 g, 4°C for 5 min preceded by boiling at 95°C for 10 min. Profiling of enzymes bound to the probes was done by separating proteins on NuPAGE 4%–12% Bis-Tris SDS-PAGE gels (ThermoFisher Scientific) for 1 h 50 min at 120 V. Imaging was done on a Typhoon 5 (Cytiva Lifesciences) in the Cy3-channel. All labelling experiments were repeated twice. For one of the replicates of each growth conditions gels were stained with Invitrogen™ SimplyBlue™ Safestain (ThermoFisher Scientific) according to the manufacturer's protocol, with overnight destaining, for visualizing total protein content and ensuring equal loading.

FP-biotin labelling and sample preparation for mass spectrometry

Strain E745 was grown as indicated above in three independent culture replicates (i.e. on separate plates), that were harvested and resuspended to an OD₆₀₀ ~20 in BHI. One ml of each of these biological replicates was aliquoted to one sample tube and one negative control. For each biological replicate, 10 μ l FP-biotin probe (200 μ M stock in DMSO) was added to achieve a final concentration of 2 μ M. For unlabelled controls, 10 μ l DMSO was added. The bacterial suspension was incubated with the probe solution for 60 min at 37°C, shaking at 700 rpm.

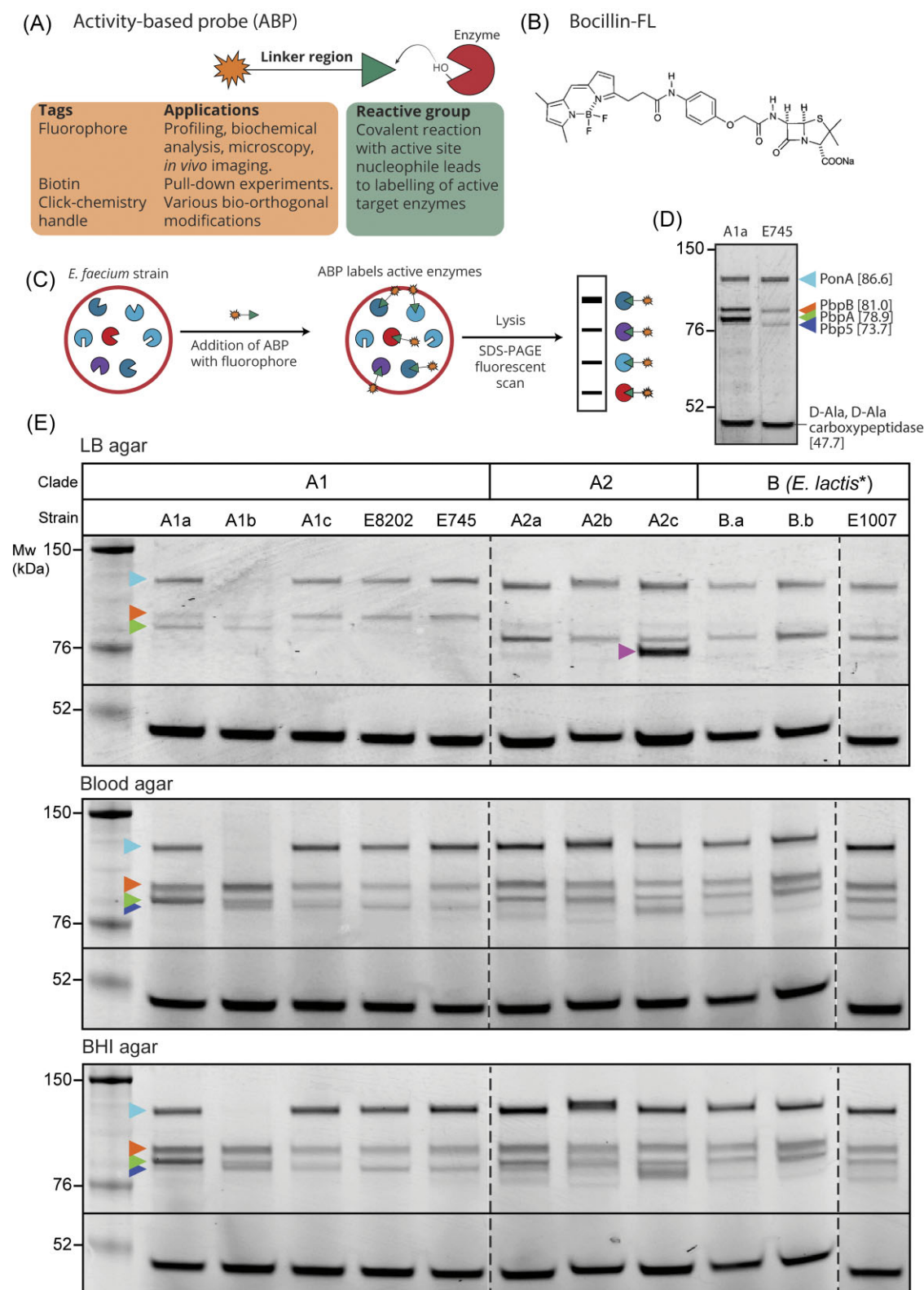


Figure 1. ABPP of PBPs in *E. faecium*. (A) Schematic overview of an exemplary activity-based probe, (B) chemical structure of the fluorescent probe Bocillin-FL, (C) schematic overview of the experimental workflow, in which live bacteria were labelled with Bocillin-FL, before lysis and SDS-PAGE analysis of the labelled proteome of the labelling procedure for profiling of enzymatic activities, and (D) Bocillin-FL-labelling profiles of the two exemplary strains A1a and E745 as analysed by fluorescence scanning following SDS-PAGE analysis. Band annotation was done according to LC-MS/MS analysis of extracted gel fragments and are marked with arrowheads. Sizes of proteins given in square brackets, (E) comparative ABPP analysis of different *E. faecium* clade A1, A2, or *E. lactis* strains that were labelled with Bocillin-FL after growth on different solid media. Arrowheads are used to mark bands on the gel. Dashed lines indicates that the profile of E1007 has been moved digitally from the middle of the gel to the B/*E. lactis* part of the gel for easier comparison to the other B/*E. lactis* strains. The gels are representative of two independent experiments.

After incubation bacteria were spun down at 10 000 *g*, 4°C, 5 min, discarding the supernatant and resuspending the cells in 1.2 ml RIPA lysis buffer (50 mM Tris, 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, and 1% Triton X-100) with 10% SDS. The samples were transferred to 2 ml screw cap tubes with 100 µl 0.1 mm glass beads and lysed by bead beating as previously described. Samples were then centrifuged at 10 000 *g*, 5 min, 4°C. Supernatant was transferred to protein-low-binding tubes, and protein concentration in samples was determined using Qubit (ThermoFisher Scientific). Prior to sample preparation for mass spectrometry samples were frozen at –80°C. The continued sample preparation was performed according to the same procedure as described in Uddin et al. (2023).

Before LC–MS/MS, the sample volumes were decreased to 100 µl by evaporation in a vacuum concentrator and samples were acidified with Trifluoroacetic acid to 0.5%–1% final concentration. Samples were then concentrated and cleaned using Omix C18 pipette tips (A57003100, Agilent). Purified peptide samples were dried in a vacuum concentrator and dissolved in 15 µl 0.1% formic acid. Protein concentration was measured with a Nanodrop ONE spectrophotometer (ThermoScientific™).

Liquid chromatography–mass spectrometry analysis

A total of 0.25 µg peptide from each sample were loaded onto a ThermoFisher Scientific EASY-nLC1200 system and EASY-Spray column (C18, 2 µm, 100 Å, 50 µm, 50 cm). Peptides were fractionated using a 5%–80% acetonitrile gradient in 0.1% formic acid over 60 min at a flow rate of 300 nl/min. The separated peptides were analysed using an Orbitrap Exploris 480 mass spectrometer (ThermoFisher Scientific). Data was collected in data-dependent mode using a Top40 method. Data was searched against a nonredundant protein sequence database of *E. faecium* E745 (obtained from NCBI bioproject PRJNA295268) using the Proteome Discoverer 2.5. An abundance ratio was calculated based on the average abundance across the replicates of the FP-biotin-enriched samples against the abundance in the controls. Proteins with an abundance ratio below 1.5 were filtered out. Proteins only found in one of the three replicates of the FP-biotin enriched samples were also filtered out. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (Perez-Riverol et al. 2021) partner repository with the dataset identifier PXD048798.

Data analysis

For prediction of features based on the protein sequences various online tools were used. For investigating the presence of signal peptides SignalP-6.0 (Teufel et al. 2022) was used, DeepTHMM (Hallgren et al. 2022) for looking at possible transmembrane domains, PSORTb v.3.0 (Yu et al. 2010) for predicting location and Interpro (Paysan-Lafosse et al. 2023) for looking at domains, also transmembrane domains, and other features to the sequences. BLASTp (Altschul et al. 1990) was used to identify human, *E. faecalis*, and *S. aureus* homologues of the newly identified *E. faecium* proteins. Full-length protein sequences were blasted against the nonredundant protein database of *Homo sapiens* (taxid: 9606), *S. aureus* USA300 (taxid: 1458279), and *E. faecalis* OG1RF (taxid: 474186), respectively. Partial sequences were not considered.

Results

PBP activity profiling

Using the fluorescent, commercially available ABP Bocillin-FL (structure in Fig. 1B) we first aimed to profile PBPs in 11 different *E.*

faecium strains across clade A1, A2, and B/E. *lactis* (Supplementary Table S1) under three different growth conditions. Bacteria were grown on LB agar as a standard yeast-derived medium, and blood agar and BHI agar as more physiologically relevant media including host components. The live bacteria were then labelled with Bocillin-FL, lysed and the extracts including labelled proteins were analysed by SDS-PAGE (Fig. 1D and E). Most of the bands visualized in the gel after fluorescence scanning are between 76 and 150 kDa (Fig. 1E) matching the expected size range of the six known PBPs (Fig. 1D and E) (PonA, PbpZ, PbpF, PbpA, and PbpB) between 73.7 and 6.7 kDa. One additional band of an approximate size < 2 kDa was also evident (Supplementary Fig. S1), which did not correspond to the size of any of the six known PBPs in *E. faecium* (Supplementary Table S2). Although some PBPs are very similar in size (e.g. PonA and PbpZ are only 0.1 kDa different, and PbpA, PbpB, and PbpF are all ranging within 2.1 kDa) and may appear as one band in the gel, we excised gel fragments around the fluorescent bands for proteomic analysis by LC–MS/MS in an attempt to assign the bands to their corresponding PBP targets (Supplementary Dataset S1). In most gel samples peptide fragments corresponding to more than one PBP were identified, and therefore the total number of identified peptide sequences [peptide spectral matches (#PSMs)] for a particular protein was compared. According to this assignment in order of decreasing band size, the top band most likely corresponds to PonA (light blue arrowhead, Fig. 1D), followed by PbpB (orange arrowhead), and PbpA (green arrowhead), although, the MS-analysis also revealed high levels of the peptides derived from the similarly sized PbpB in the latter sample. Pbp5, which is the smallest PBP with a size of 73.7 kDa, likely corresponds to the generally faint band at around 76 kDa (Fig. 1D and E, dark blue arrowhead). Neither PbpF, nor PbpZ were detected as the dominant protein in either of the samples. Gel extraction and LC–MS/MS analysis of the band of <52 kDa size suggests that this band corresponds to a serine-type D-Ala D-Ala carboxypeptidase of 47.7 kDa size (WP_002296298.1). This enzyme is encoded by the *ddcP* gene and has been previously demonstrated to be crucial for high-level resistance to ampicillin (Zhang et al. 2012). Zhang et al. (2012) already predicted this carboxypeptidase to be a low-molecular weight PBP and our current work further supports the notion that this enzyme is a 7th PBP in *E. faecium*.

A comparison of Bocillin-FL labelling profiles across different media conditions revealed a high degree of similarity upon growth on BHI and blood agar, whereas on LB, the least favourable growth medium, fewer bands could be detected (Fig. 1E, see Supplementary Fig. S2 for total protein staining of the gels that confirm even loading across the samples). However, certain observations oppose this general trend. For instance, a band of ~76 kDa, i.e. not observed for other strains, is the most intensely labelled band in strain A2a upon growth on LB (Fig. 1E, purple arrowhead). The most notable clade-to-clade differences in PBP activity profiles were observed for the band putatively corresponding to PbpB upon growth on LB media (orange arrowhead, Fig. 1E, LB agar), which had a higher intensity in clinical clade A1 strains, but which was nearly absent in the nonclinical strains from clade A2 and B/E. *lactis*. Under the other culturing conditions, PbpB seems to be equally active in all strains. On the other hand, the band most likely corresponding to PbpA, is less pronounced in the clinical A1 strains (green arrowhead Fig. 1E, LB agar), compared to the nonclinical strains when grown on LB agar. There are also strain-specific labelling differences, e.g. for the 76-kDa band that likely corresponds to Pbp5, which is present in all clade A1 strains except A1a when grown on blood agar or BHI. The topmost band, likely PonA, appears to be nearly absent in strain A1b compared

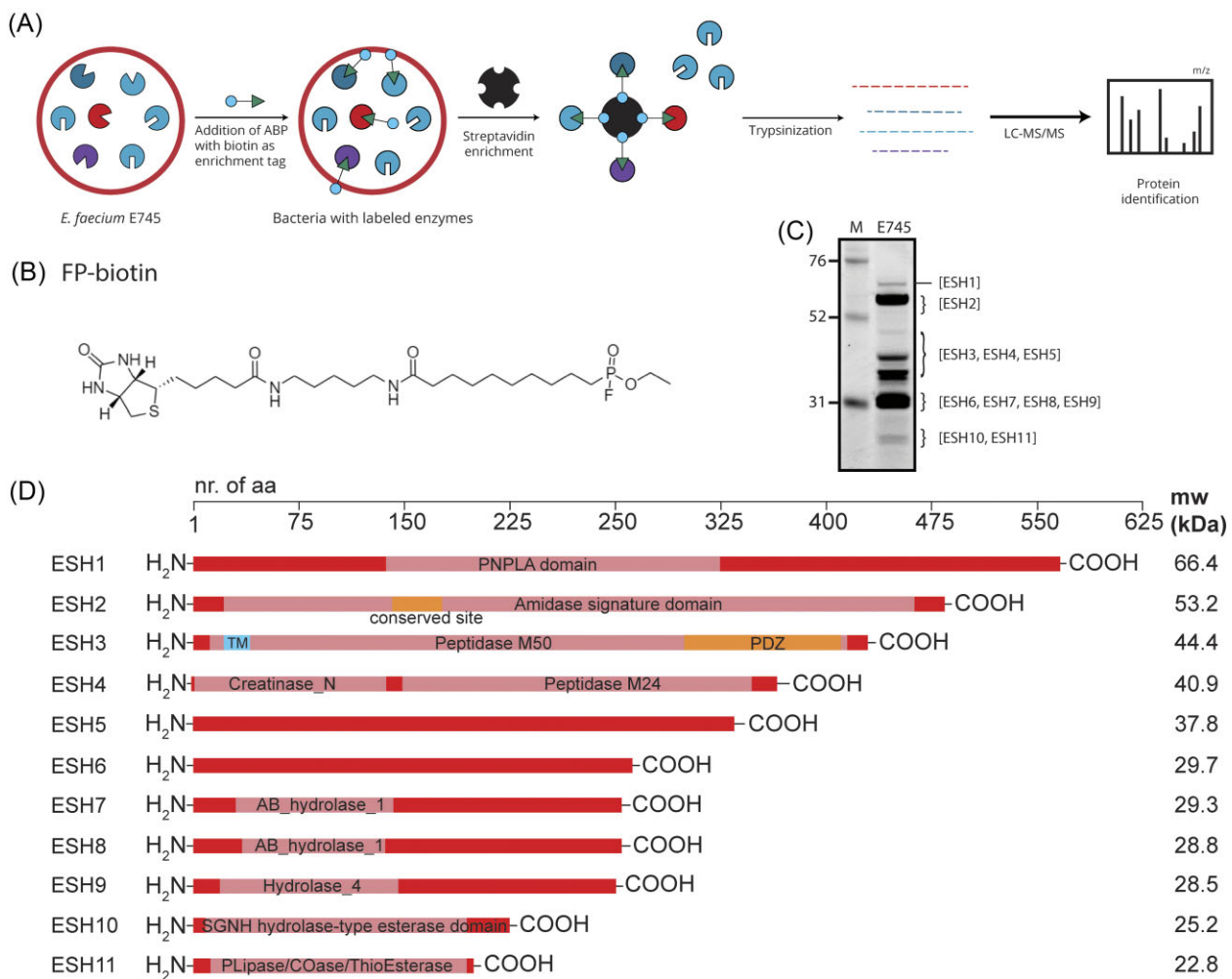


Figure 3. Identification of SHs using a biotinylated FP probe. (A) Schematic overview of the experimental workflow for identification of *E. faecium* E745 SHs including FP-biotin labelling, streptavidin-based enrichment, and proteomic analysis by LC-MS/MS, (B) chemical structure of FP-biotin, (C) suggested, but not validated, placement of identified proteins on the gel-based protein profiles we see after labelling with FP-TMR in strain E745, and (D) size and protein domain overview of 11 putative *E. faecium* SHs targeted and enriched by FP-biotin. Domain prediction by Interpro, and TM predicted by DeepTHMM.

Table 1. Overview of identified SHs in *E. faecium* E745. Refer to [Supplementary Dataset S2](#) for additional information and Fig. 3(D) for domain information.

Gene name	Functional annotation (putative)	Predicted location (PSORTb)	% Homology with <i>H. sapiens</i> protein	% Homolog with <i>S. aureus</i> protein	% Homology with <i>E. faecalis</i> protein	MW (kDa)
ESH1	Patatin-like phospholipase	Cytoplasm	40%	–	–	66.4
ESH2	<i>nylA</i>	Cytoplasm	42%	25% (<i>GatA</i>)	63%	53.2
ESH3	<i>htrA</i>	Unknown	40% (<i>HtrA2-p2</i>)	38%	65%	44.4
ESH4	<i>pepQ_2</i>	Cytoplasm	33%	44%	68%	40.9
ESH5	<i>mccF</i>	Cytoplasm	–	–	–	37.8
ESH6	<i>frmB</i>	Cytoplasm	–	36% (<i>FphF</i>)	72%	29.7
ESH7		Cytoplasm	–	–	49%	29.3
ESH8		Cytoplasm	30%	26%	43%	28.8
ESH9	<i>est</i>	Cytoplasm	–	43% (<i>FphH</i>)	40%	28.5
ESH10		Unknown	–	–	–	25.2
ESH11	<i>mhqD</i>	Cytoplasm	–	–	–	22.8

