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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.

Embarking 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) (Jessani 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, phospolipases, 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 OD_{600} ~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 µl. The chemical probes were added at a concentration of 100 µM to a final concentration of 1 µM 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 µl 0.1 mm glass beads (BioSpec Products) and 33 µl 4X SDS-PAGE buffer and lysed by bead-beating (3 × 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 OD_{600} ~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 µl FP-biotin probe (200 µM stock in DMSO) was added to achieve a final concentration of 2 µM. For unlabelled controls, 10 µl DMSO was added. The bacterial suspension was incubated with the probe solution for 60 min at 37°C, shaking at 700 rpm.

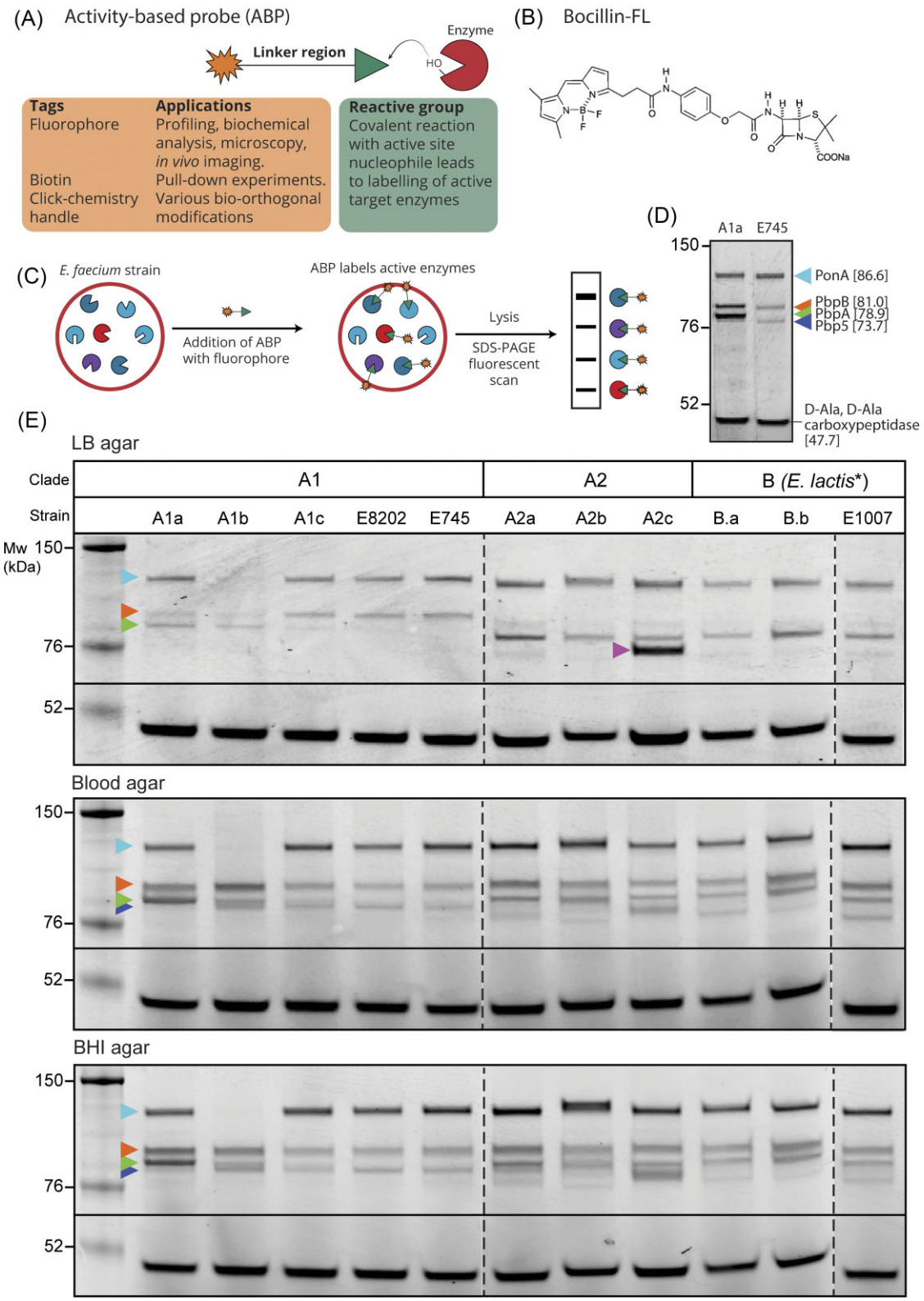

Figure 1. ABPP of PBPs in *E. faecium*. (A) Schematic overview of an exemplary activity-based probe, (B) chemical structure of the fluorescent probe Bocillin-FL, (C) schematic overview of the experimental workflow, in which live bacteria were labelled with Bocillin-FL, before lysis and SDS-PAGE analysis 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, PpbZ, 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

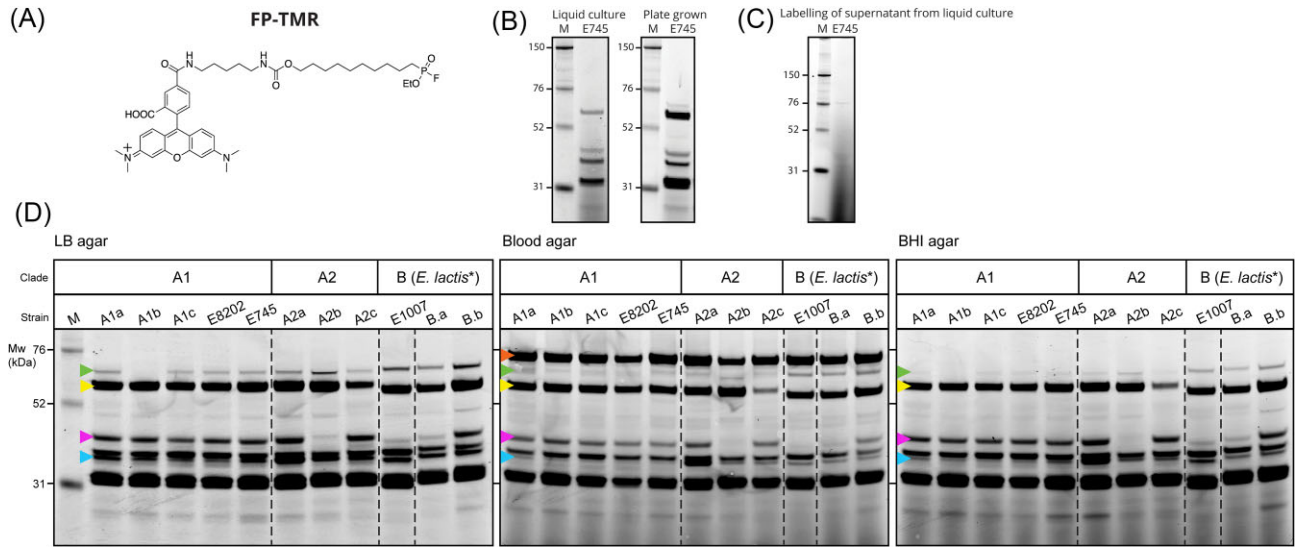

Figure 2. ABPP of *E. faecium* SHs using FP probes. (A) Chemical structure of the fluorescent ABP FP-TMR probe used to target SHs in our samples, (B) comparing the enzymatic expression pattern of SHs in Efm strain E745 after pelleting cells grown in BHI liquid culture, and cells grown on BHI agar plates, (C) expression profile after labelling in the supernatant of the liquid culture, and (D) comparative ABPP analysis of different *E. faecium* clade A1, A2, or *E. lactis* strains that were labelled with FP-TMR after growth on different solid media. The 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.

to the other strains under all growth conditions (light blue arrowhead, Fig. 1E). Having completed the systematic profiling of PBPs activities across *E. faecium*/*E. lactis* strains, we decided to expand the scope and explore the broader SH families.

SH activity profiling

For simple detection of SHs, we started out using the well-characterized, fluorescent ABP FP-TMR (structure in Fig. 2A) in an otherwise analogous experimental setup. Initially, we focused on strain E745 and compared the profiles obtained after growth on solid versus liquid BHI media. The band profiles were very similar (Fig. 2B), but the intensities were higher in samples harvested from solid media that could be adjusted to a higher cell density (OD600 ~20) compared to the liquid culture samples that reached an OD600 ~3 after overnight culture. There were also no, or few, labelled bands present in the supernatant of the liquid culture (Fig. 2C), which could be because the amount of secreted SHs could be too low for detection. Based on these results we decided to perform a more extensive comparative profiling of our *E. faecium* strain collection upon growth on agar plates, to reach the desired level of intensity of the band profiles.

We generally observed little variation in the FP-TMR labelling profiles between strains and media conditions (Fig. 2D, see Supplementary Fig. S2 for total protein staining controls). The five strains that are from clinical samples and belong to clade A1 show identical activity profiles, except for the highest molecular weight band missing in strain A1b after growth on LB and BHI (green arrowhead in Fig. 2C, LB agar and BHI agar). The biggest difference can be observed among the strains belonging to clade A2 and B/*E. lactis* where a ~40 kDa band is much weaker in the strains E1007, A2b, and B.a compared to other A1 and A2 strains. (pink arrowhead Fig. 2D). Moreover, there seems to be a size difference in the thick band at ~60 kDa (yellow arrowhead) in the strains belonging to clade B that either suggests a size/sequence difference of the target enzyme in *E. lactis* compared to *E. faecium* or a different identity. There is a strong band around 76 kDa in the profiles where blood agar was the growth medium (orange arrowhead, Fig. 2D, blood agar), i.e. not present after growth on LB or BHI agar. Since a similar band was observed when profiling various bacterial species on blood agar, we believe that this is an artefact resulting from bacterial growth on blood agar plates rather than a bacterial SH activity.

Intrigued by the conserved SH activity patterns which may be indicative of important physiological functions, we proceeded to identify the molecular targets of the FP-probes in *E. faecium*.

Identification of SHs

To identify SHs in *E. faecium*, E745 cells grown on BHI agar were labelled with the probe analogue FP-biotin, lysed and probe-labelled targets were enriched using a streptavidin-resin prior to LC–MS/MS analysis (Fig. 3B). The resulting dataset was compared against a control dataset where cells were not labelled with FP-biotin but otherwise treated analogously. We identified 14 targets, which were present in all enriched replicates at a higher abundance than in FP-treated samples compared to negative controls (>1.5). Nine of these were identified based on two or more unique peptides, whereas five were based on only one unique peptide. Removing targets identified based on only one unique peptide is common practice, but it has been argued that this can result in loss of, e.g. smaller proteins with fewer tryptic digestion sites or proteins with low concentration, and that true positives could be filtered out (Higdon 2006). Following this consideration and encouraged by the high protein false discovery rate confidence for all hits, we decided to keep those hits based on one unique peptide, which were annotated as SHs, but to remove the hits annotated only as hypothetical proteins, leaving 11 target candidates (Table 1). The hypothetical proteins also did not contain any predicted domains that could indicate them as SHs. Information about all 14 hits can be found in Supplementary Dataset S2.

The putative *E. faecium* SHs (ESHs) identified through enrichment with FP-biotin have a size range of 22.8–66.4 kDa (201 and 566 amino acids) with most enzymes being in the range of 20–

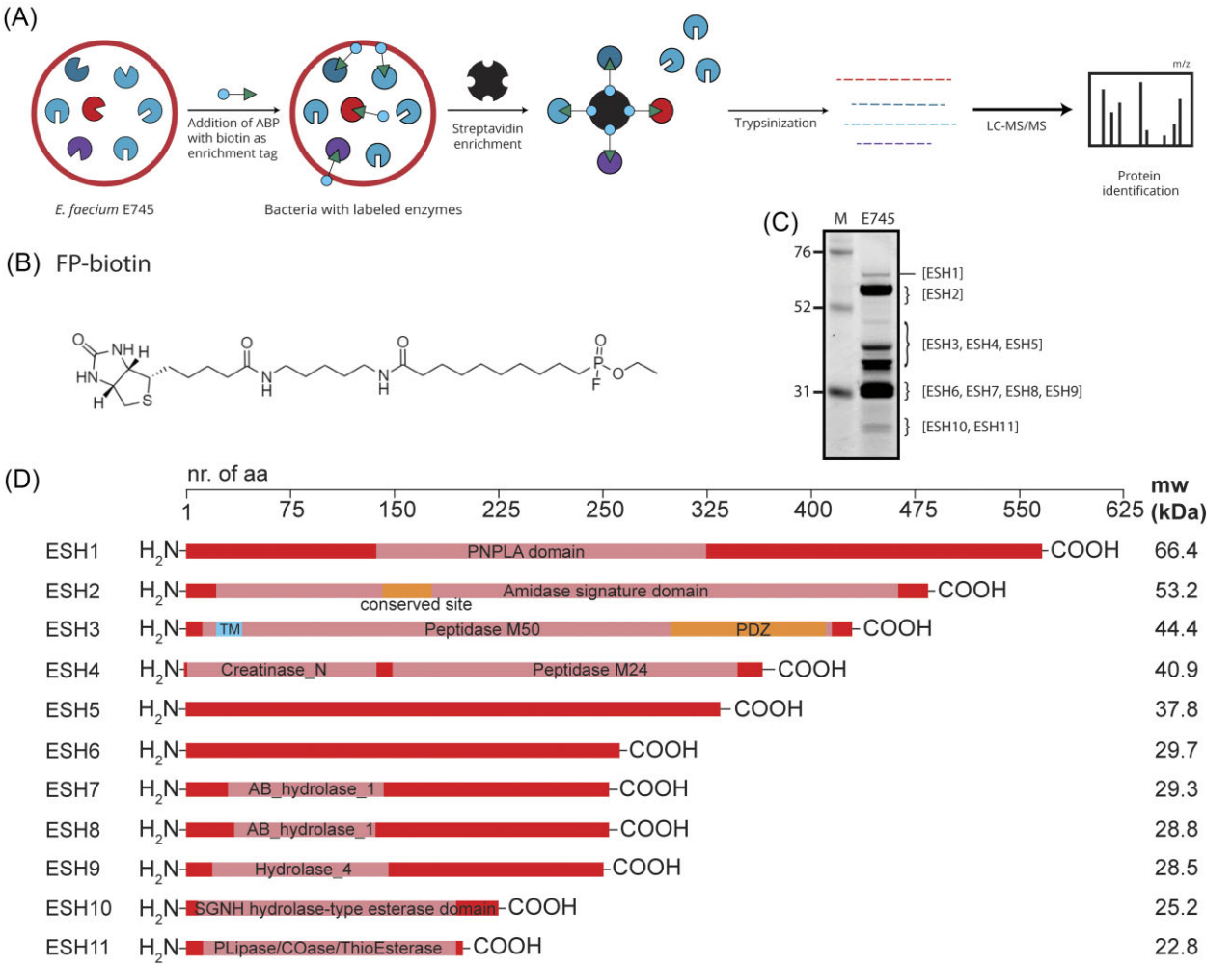

Figure 3. Identification of SHs using a biotinylated FP probe. (A) Schematic overview of the experimental workflow for identification of *E. faecium* E745 SHs including FP-biotin labelling, streptavidin-based enrichment, and proteomic analysis by LC–MS/MS, (B) chemical structure of FP-biotin, (C) suggested, 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 *H. sapiens* protein	% Homolog with *S. aureus* protein	% Homology with *E. faecalis* protein	MW (kDa)
ESH1		Patatin-like phospholipase	Cytoplasm	40%	–	–	66.4
ESH2	*nylA*	Amidase	Cytoplasm	42%	25% (GatA)	63%	53.2
ESH3	htrA	Trypsin-like peptidase	Unknown	40% (HtrA2-p2)	38%	65%	44.4
ESH4	*pepQ_2*	Xaa-Pro peptidase family protein	Cytoplasm	33%	44%	68%	40.9
ESH5	*mccF*	LD-carboxypeptidase	Cytoplasm	–	–	–	37.8
ESH6	*frmB*	Esterase	Cytoplasm	–	36% (FphF)	72%	29.7
ESH7		Hydrolase	Cytoplasm	–		49%	29.3
ESH8		Hydrolase	Cytoplasm	30%	26%	43%	28.8
ESH9	*est*	Hydrolase	Cytoplasm	–	43% (FphH)	40%	28.5
ESH10		Hydrolase	Unknown	–	–	–	25.2
ESH11	*mhqD*	Hydrolase	Cytoplasm	–	–	–	22.8

30 kDa. In Fig. 3(C), we have proposed the location of the identified targets based on their molecular size, on the gel-based profile of strain E745, obtained with the analogous, fluorescent probe FP-TMR. However, to validate the exact match of the targets to the band profiles, it is necessary to profile transposon mutants to all our protein targets, an extensive work, i.e. ongoing using a *mariner*-based transposon mutant library of strain E745 (Zhang et al. 2017).

Four of the targets were annotated as alpha/beta hydrolases, one amidase, one esterase, two patatin-like phospholipase/SGNH hydrolase, and three peptidases/proteases (domain information is shown in Fig. 3D). Eight ESHs had homologs in *E. faecalis* OG1RF and seven had homologs in *S. aureus* USA300. Only five of the *E. faecium* SHs have in-between 33% and 42% identity with human enzymes, whereas six have homology with *S. aureus* and seven with *E. faecalis* proteins (Table 1, Supplementary Dataset S2). For all identified enzymes a cytosolic location is predicted, except for ESH3 and ESH10 where the location is unknown. The absence of secreted enzymes in the dataset might be explained by technical reasons since the culture supernatant was not analysed further in the chemoproteomic workflow.

Discussion

In this study we are reporting on the profiling and identification of PBPs, which are well-known targets of antibiotics, and SHs, a diverse group of enzymes which is undercharacterized in *E. faecium*, especially in relation to pathogenicity.

A comparative gel-based analysis of the SH and PBP-activity profiles across a set of 11 different *E. faecium* strains across clade A1, A2, and B/*E. lactis* showed high levels of conservation across strains and growth conditions tested. For PBPs, the largest clade-to-clade variations were observed under LB growth conditions for the bands corresponding putatively to PbpB and PbpA. We observed higher labelling of PbpB in the clinical A1 strains and higher PbpA activities in the commensals (A2 and *E. lactis*). Why this pattern is only seen for LB media, the least favourable growth media, remains to be determined. We also observed other more strain-specific differences, e.g. a unique high-intensity band in strain A2c absent in all other strains, and the absence of (putative) PonA in strain A1b. Since all six known PBPs are present in the genome of strain A1b, the absence of this band might be explained by reduced levels of gene expression compared to other strains, post-transcriptional or post-translational modification of its activity, or a lower binding affinity of the A1b protein to the probe. Moreover, the strains tested show variations in the *pbp*5 gene, and the clade A1 strains have more mutations leading to their ampicillin resistance (Supplementary Table S1). These variations could lead to a reduced binding affinity for the Bocillin-probe and could account for some of the strain-specific differences in labelling of Pbp5. It cannot be ruled out either that changes in permeability/accessibility of the targets to the fluorescent ABPs could affect the labelling outcome across the different conditions. However, if different expression levels indeed contribute to the observed strain- and condition-dependent combinations of PBP activity profiles it is intriguing to speculate that these differences may lead to qualitative changes in cell wall composition that merit further investigation.

Profiling of SH activities using FP-TMR revealed a high degree of conservation in enzyme activity profiles across different strains, particularly of clade A1, and similarities across different experimental conditions suggesting that these enzymes may have vital roles for bacterial physiology that bacteria may also rely upon during infection. To identify these enzymes, we labelled SHs in *E. faecium* E745 with FP-biotin leading to enrichment and chemoproteomic identification of 11 putative SHs. Since most of these enzymes have not been characterized previously and are poorly annotated (several of them lacking gene name annotation), we are referring to them as ESH1-ESH11 for '*Enterococcus faecium serine hydrolase*'. Although the PBPs are a subgroup of the SHs, no PBPs were enriched in the LC–MS/MS analysis after labelling with FP-biotin. Even though FP is described as a SH probe, it has the highest affinity to SHs possessing the catalytic triad motif (Ser-His-Asp/Glu), which is not found in PBPs. The fact that no PBPs were enriched is therefore not unexpected, and also in line with previous studies in other bacteria (*S. aureus* and *S. epidermidis*) (Lentz et al. 2018, Keller et al. 2020). Similarly, in a study on *M. tuberculosis* only 1 out of 78 FP-enriched enzymes was described as a 'possible penicillin-binding protein' (Ortega et al. 2016).

Out of the 11 ESHs identified, two are related to the two FP-binding hydrolases FphF (36% identity with ESH6) and FphH (36% identity with ESH9) that we identified previously by ABPP in *S. aureus* (Lentz et al. 2018). We demonstrated that recombinant FphH has carboxylesterase and lipase activity and studies with *fph*H-transposon mutant suggested a role in bacterial stress response in *S. aureus* (Fellner et al. 2023). FphH is well conserved across *Bacillales* where the gene, commonly referred to as *yvaK* in *B. subtilis*, is in the *secG-yvaK(fph*H*)-rnr-smpB-ssr*A gene cluster, several genes of which play important roles in the ribosome rescue system. This cluster is in a shorter form (without *ssrA*) also conserved across *Lactobacillales*, which *E. faecium* belongs to. It remains unclear if or how FphH (or YvaK or ESH9) might be linked to the function of the other genes of this cluster and we previously hypothesized a role in post-translational modifications (Fellner et al. 2023). In *E. faecium*, neither of these enzymes have been characterized to the best of our knowledge.

ESH1 (WP_060811677.1) and ESH10 (WP_002288769.1) belong to the SGNH/GDSL hydrolase family members, which are found in a wide variety of bacteria and have diverse functions (Akoh et al. 2004). Subfamilies have been reported to contribute to bacterial virulence in both Gram-positive and Gram-negative bacteria (Anderson et al. 2022). More specifically, ESH1 belongs to the patatin-like phospholipase (PLP) protein subfamily of SGNH hydrolases that are known to cleave phospholipid ester-bonds creating polyunsaturated fatty acids. Several studies on PLPs in different bacteria indicate a role in virulence (Wilson 2018). For instance, ExoU in *Pseudomonas aeruginosa* is a PLP, i.e. cytotoxic to the host cells (Phillips et al. 2003, Anderson et al. 2015). In addition, PLPs have been found to be cytotoxic enzymes expressed by *Rickettsia typhi* (Borgo et al. 2022) and *Legionella pneumophilae* (Aurass et al. 2006, Lang 2011). In contrast to those enzymes, ESH1 is not predicted to be secreted suggesting that it may fulfil a different function.

ESH3 (*htr*A) is annotated as a trypsin-like peptidase domain containing protein. There have been reports of trypsin-like proteases from *Bacteroides gingivalis* being capable of degrading collagen, and therefore being a possible virulence factor (Sorsa et al. 1987). The 37.8-kDa hydrolase ESH5 (*mcc*F) was annotated as an LD-carboxypeptidase. LD-carboxypeptidases are responsible for recycling the peptidoglycan cell wall layer. In *Acinetobacter baumannii* absence of a novel L, D-carboxypeptidase, ElsL, impairs the structural integrity of the cell wall and causes toxicity to the cell due to a buildup of dead-end intermediate compounds (Dai et al. 2021). In the gut commensal *B. thetaiotaomicron* BT4193, a homolog of the human dipeptidyl peptidase 4, was found to be important for envelope integrity (Keller et al. 2023). However, in this case the

authors determined that this function was not dependent on proteolytic activity.

These examples show that there is an untapped potential related to these newly identified SHs for understanding their roles in bacterial physiology and pathogenesis and, not least, for exploration as potential drug targets. However, from a target discovery perspective it is interesting to note that one of the enriched enzymes, ESH11 (*MhqD*), could be essential, since no viable transposon mutants with an insert into this gene could be created when the E745 transposon mutant library was established (Zhang et al. 2017). Importantly, ESH11 as well as the other enzymes have now been identified through their interaction with covalent small molecule ABPs, which not only validates them as druggable but also documents their accessibility to chemical manipulation in live cells making them attractive drug target candidates. The generally low degree of homology with human orthologs is an additional benefit when considering these enzymes as potential antimicrobial drug target candidates for which human off-target activity would not be desired. Given the increasing toolset of reactive electrophile scaffolds for tailored covalent inhibitors targeting specific SHs (Böttcher and Seiber 2008, Staub and Seiber 2008, Adibekian et al. 2011, Zuhl et al. 2012, Camara et al. 2015, Lentz et al. 2016, Chen et al. 2019, Babin et al. 2022, Bakker et al. 2023, Uddin et al. 2023) potentially combined with incorporation of peptidic specificity elements generated by experimental evolution during phage display (Chen et al. 2021) will make it possible to design specific inhibitors and probes for this underexplored class of *E. faecium* targets. Such inhibitors may be used as tool compounds in conjunction with genetic tools for functional studies aimed at elucidating the physiological function of these enzymes and may also represent lead compounds for the development of urgently needed treatment options for VRE infections.

Conclusion

This work is a starting point for functional characterization of SHs and their evaluation as possible therapeutic targets. The relatively similar SH activity profiles across different strains and growth conditions suggest a high degree of conservation and putative biological relevance that will make these enzymes attractive as putative drug targets. Studies addressing biochemical function and physiological role of these enzymes are currently ongoing. Importantly, our chemoproteomic approach in living cells validates these targets as both druggable and accessible to small molecule inhibitors. In addition, the systematic assessment of PBP-activity profiles using Bocillin-FL also revealed strain and growth-condition-dependent differences that warrant future investigations on their impact on cell wall structure and function and antibiotic sensitivity.

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Author contributions

Jeanette S. Grunnvåg (Investigation, Visualization, Writing – original draft, Writing – review & editing), Kristin Hegstad (Conceptualization, Funding acquisition, Supervision, Writing – review & editing), and Christian S. Lentz (Conceptualization, Funding acquisition, Supervision, Writing – review & editing)

Supplementary data

Supplementary data is available at *FEMSMC Journal* online.

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