Enterococcus faecium produces membrane vesicles containing virulence factors and antimicrobial resistance related proteins

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ARTICLE INFO

ABSTRACT

Enterococcus faecium is a commensal but also a bacteremia causing pathogen, which is inherently resistant to several antimicrobials and has a great ability to acquire new traits. Bacterial membrane vesicles (MVs) are increasingly recognized as a mode of cell-free communication and a way to deliver virulence factors and/or antimicrobial resistance determinants. These features make MVs interesting research targets in research on critical hospital pathogens. This study describes for the first time that *E. faecium* strains produce MVs. It presents a morphological as well as a proteomic analysis of MVs isolated from four different, clinically relevant *E. faecium* strains grown under two different conditions and identifies MV-associated proteins in all of them. Interestingly, 11 virulence factors are found among the MV-associated proteins, including biofilm-promoting proteins and extracellular matrix-binding proteins, which may aid in enterococcal colonization. Additionally, 11 antimicrobial resistance-related proteins were MV-associated. Among those, all proteins encoded by the *vanA*-cluster of a vancomycin resistant strain were found to be MV-associated. This implies that *E. faecium* MVs may be utilized by the bacterium to release proteins promoting virulence, pathogenicity and antimicrobial resistance.
Significance: Enterococcal infections, especially bacteremia and endocarditis, are challenging to treat because *E. faecium* have acquired resistance to multiple classes of antimicrobials, including ampicillin, aminoglycosides, and glycopeptides. Thus, research on different modes of enterococcal pathogenicity is warranted. This study utilized a proteomic approach to identify MV-associated proteins of different nosocomial *E. faecium* strains representing four clinically relevant sequence types (STs), namely ST17, ST18, ST78, and ST192. The presented data suggest that *E. faecium* MVs are involved in virulence and antimicrobial resistance.

1. Introduction

Enterococci are Gram-positive, ubiquitous, facultative anaerobic cocci. They are known to survive hostile conditions such as a saline environment and wide temperature ranges and also for their ability to persist long-term in the hospital environment [1]. *Enterococcus faecalis* and *Enterococcus faecium* naturally colonize the human gut as commensals. However, *E. faecium* in particular has undergone a pronounced transition towards a multi-drug resistant pathogen. The most common infection caused by *E. faecium* is urinary tract infection, but they may also cause life-threatening infections such as endocarditis and bacteremia, especially in debilitated patients [2]. The genetic clade structure

of *E. faecium* is characterized by a distinct split of commensal lineage (clade B) and hospital-associated lineage (clade A1) [3]. The nosocomial A1 clade includes sequence types (STs) of the clonal complex 17 (CC17), a globally spread genetic complex characterized by ampicillin resistance, possession of a pathogenicity island and association with hospital outbreaks [4].

Extracellular vesicles are suggested as a mechanism for cell-free intercellular communication across all domains of life. They are crucial components of the bacterial secretome, as these 20–200 nm sized spheres contain lipopolysaccharides, soluble membrane-associated proteins, virulence factors and nucleic acids [5, 6].

Bacterial membrane vesicles were first described in the Gram-

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negative *Escherichia coli* in the 1960s [7, 8], and later in several other Gram-negative species such as *Shigella* sp. [9], *Salmonella* sp. [10], and *Vibrio* sp. [11]. In Gram-negative bacteria, the vesicles are called outer-membrane vesicles (OMVs), as they derive from the outer membrane (OM). The OMVs contain OM components as well as inner membrane constituents and cytoplasmic elements. The role of OMVs in bacterial physiology and pathogenesis, stress responses, biofilm formation as well as secretion and delivery of biomolecules has been demonstrated [12]. The mechanism of vesiculogenesis is poorly understood but seems to involve phospholipid accumulation in the outer leaflet of the outer membrane, whereupon vesicles pinch off from the outer membrane among Gram-negative bacteria [13].

It used to be a long-standing assumption that the thick cell wall of Gram-positive bacteria precluded the existence of vesicles, as they could not escape such a barrier. Gram-positive MVs were discovered in a study from the early 1990s in *Bacillus cereus* and *Bacillus subtilis* [14], but not further characterized for the next 20 years. Finally, in 2009 MVs were described in *Staphylococcus aureus* [15] and have since gained increased attention, i.e. in *Bacillus anthracis* [16], *Mycobacterium tuberculosis* [17] and others, as reviewed by Brown et al. [6]. MVs are key players in host-pathogen interactions, as they can cause disease without the living bacterial cell [18] and may induce strong host responses [19].

MV production in enterococci has not been described previously. The aim of this study was therefore to explore the potential of MVs release from *E. faecium*. In addition, we investigated whether different cultural conditions and strain backgrounds may account for variation in proteinaceous cargo. Four strains representing different, clinically important sequence types (STs) within CC17, the major disease causing clonal complex [20]: ST17, ST18, ST78, and ST192 respectively, were therefore chosen for the study. To the best of our knowledge, this is the first report describing MV release by clinical strains of enterococci and their proteomics-based characterization using an in-solution approach.

2. Materials and methods

2.1. Strains and growth conditions

In the present study, the *E. faecium* strains DO (PRJNA71), E155 (PRJNA192879), K59-68 (in-house sequenced, under submission) and K60-39 (PRJNA407052), representing ST 18, 17, 78 and 192, respectively, were used. They are hospital isolates either from the US or Norway. Their properties and additional information are presented in Table 1.

E. faecium strains were routinely cultured on brain heart infusion (BHI) or Luria Bertani (LB) agar or in liquid BHI or LB at 37 °C.

2.2. Isolation of membrane vesicles

Vesicles were isolated as described for *S. aureus* [16, 21] with a few modifications.

First, to isolate vesicles from bacteria mainly in a viable state, cultures in mid-exponential growth phase grown in nutrient-rich BHI were used (Fig. S1A). Therefore, 1 L of BHI broth were inoculated with enterococci grown overnight in BHI broth (1100) and grown at 37 °C with shaking (230 rpm) to mid-exponential phase (optical density at 600 nm [OD_{600}], approximately 1.5). The cultures were centrifuged at 6000 ×*g* for 30 min with a JLA 9.1000 rotor Beckman Instruments Inc., USA) and the supernatant was transferred to a clean Erlenmeyer flask and filtered through a 0.22 μm pore size filter (Merck Millipore, USA). The obtained bacterial-free filtrate was concentrated using Amicon tubes (Merck Millipore, USA, cut off 100 kDa) in a "Beckman" centrifuge at 4000 ×*g* for 30 min at 4 °C and the concentrate was ultracentrifuged using a SW 40-TI rotor (20,000 x *g* for 3 h at 4 °C) to pellet MVs. The MVs were washed in phosphate-buffered saline (PBS) followed by another ultra-centrifugation, before re-suspension in PBS. Purified MVs were stored at −80 °C until further analysis. Purified MVs were streaked onto BHI agar

Table 1
Strain characteristics and morphological characterization of MVs.

Strain	Accession	Phylogeny	Isolation site	Country of origin	Ref.	Genes involved in resistance	Genome size in bp	Proteome size in number of coding sequences	Size of MVs in nm ± SD	Charge (ζ potential)	No. MV proteins Exponential growth BHI	% proteins from core	No. MV proteins Stationary growth LB	% proteins from core
DO	PRJNA71	ST18 (line 18)	Blood-stream isolate	Texas, USA	[98]	*aph(3')-III, ermB, msrC, tetM*	2,848,380	2721	42 ± 18	−3,43 ± 0.15	445	88	660	88
E155	PRJNA192879	ST17 (line 17)	human feces	Chicago, USA	[99]	*aac(6')-Ie-aph(2'')-Ia, aph (3')-III, dfrG, ermB, msrC, vanR-A, vanS-A, vanH-A, vanA, vanX-A, vanY-A, vanZ-A, vanR-B, vanS-B, vanY-B vanW-B, vanH-B, vanB, vanX-B*	3,066,426	3013	37 ± 23	−19.63 ± 1.33	362	90	674	91
K59-68	Under submission to PRJNA407052	ST78 (line 78)	Blood-stream isolate	Tromsø, Norway	[100]	*aph(3')-III, ermB, lnuB, msrC*	3,370,597	3272	83 ± 29	−21.57 ± 1.45	162	88	424	89
K60-39	PRJNA407052	ST192 (line 78)	Blood-stream isolate	Oslo, Norway	[100]	*aac(6')-Ie-aph(2'')-Ia, aph (3')-III, ermB, msrC, tetM*	3,071,612	2879	51 ± 21	−24.90 ± 1.60	605	88	754	90

to confirm the sterility of isolated MVs.

Second, in order to isolate vesicles from bacteria under stress, bacterial stationary phase cultures grown in growth-limiting LB were used (Fig. S1B). For this, the above-described procedure was applied to enterococcal overnight cultures grown in LB.

2.3. Density gradient centrifugation (DGC)

DGC was performed in order to fractionate and purify the MVs further. Therefore, the MV sample was mixed with an equal volume of ice-cold 60% OptiPrep (Sigma-Aldrich, Germany; 60% stock) to obtain a 30% solution. The 30% solution was transferred into the bottom of an ultracentrifugation tube (ultraClear™, 5 mL, 13 × 51 mm, Beckman Instruments Inc., USA) and 2 mL of 25% (w/v), 1 mL of 5% (w/v) OptiPrep solution and PBS were added. Centrifugation was carried out at $100000 \times g$ for 3 h at 4 °C (rotor SW 50.1, Beckman Instruments Inc., USA) in slow acceleration and deceleration mode to prevent disturbance in the various density layers and achieve MV ring formation (see example in Fig. S2A). After centrifugation, the sample was divided into 200 μL fractions, which were transferred into clean Eppendorf tubes. Aliquots of the fractions were analyzed by SDS PAGE (see example in Fig. S2B) and electron microscopy.

To remove the OptiPrep solution, the fractions containing MVs were pooled and concentrated by ultrafiltration (10 kDa molecular weight cut-off, Vivaspin 20, Sartorius, Germany) at $4000 \times g$ for 30 min at 4 °C and re-suspended in 200 μL PBS.

2.4. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Protein profiling of isolated MVs from all the four strains of enterococci was determined by SDS-PAGE using standard molecular stain. Briefly, 15 μL of MV sample were mixed with 5.75 μL 4× NuPage LDS sample buffer (NuPage Novex 4–12%; Invitrogen, Life Technologies, USA) as well as 2.3 μL of dithiothreitol (DTT) and heated for 10 min at 70 °C prior to electrophoresis. Samples were separated by 12% NuPage Bis-Tris SDS-PAGE gel, and the gels were fixed in fixation solution (methanol, acetic acid (50/50 v/v)) for 1 h at room temperature under gentle agitation. The gels were then stained with Coomassie solution (50% methanol, 10% acetic acid, 0.05% Coomassie brilliant blue R-250, Thermo Fisher Scientific USA) for 1 h and excess dye was removed through incubation in de-stain solution (H$_2$O, methanol, acetic acid (50/40/10 v/v/v)) until bands were visible.

2.5. Transmission electron microscopy (TEM) analysis

Five μL of purified MVs were applied to Formvar coated 75 mesh copper grids (Electron Microscopy Science, USA) and incubated for 5 min. The sample was washed four times with double distilled water and negatively stained with 9 parts of 2% methylcellulose and 1 part of 3% uranyl acetate for 2 min on ice. The excess stain was removed and samples were dried at room temperature. The samples were then visualized with a JEOL JEM 1010 transmission electron microscope (JEOL, Japan) operated at 80 kV.

2.6. Atomic force microscopy (AFM) analysis

AFM analysis of *E. faecium* strains DO and E155 cultivated on LB agar were performed as described previously [22]. A loop of bacterial cells was suspended in ultrapure water and placed on a freshly cleaved mica surface, incubated at room temperature and ultimately blotted dry before being placed into a desiccator. Imaging was carried out using a Nanoscope V Atomic Force Microscope (Bruker AXS, Germany).

2.7. Particle size distribution and measurement of zeta potential

The effective diameter and size distribution of the purified MVs were determined by dynamic light scattering (DLS, also known as photon correlation spectroscopy) (Nicomp Submicron Particle Sizer 370, PSS Nicomp Particle Sizing Systems, USA), at an angle of 90° with a 632 nm laser as described in an earlier study [23]. Briefly, MVs were diluted in PBS in a particle-free environment to a count rate of 250–350 kHz at room temperature. Analyses were run in a vesicle mode data calculated as intensity weighted distribution from three measuring cycles (10 min cycle, 3 cycles) The zeta (ζ) potential of vesicles was determined using Zetasizer Nano Z (Malvern Instruments, United Kingdom). Three parallels were determined for each vesicle suspension.

2.8. Measurement of MV-derived protein per CFU

The protein content of isolated and purified MVs from bacterial culture (2 L) was measured by Bradford assay (Bio Rad, USA). For each strain, CFUs per mL were assessed by plating dilution series on blood agar plates and counting CFUs. With these two values, MV-derived protein in attogram (ag) per CFU was calculated from these two values.

2.9. Lipid staining of purified vesicles

The purified membrane vesicles were stained for 1 h with lipid-specific dye (5 μM of DiD, Thermo Fisher Scientific, USA). The stained vesicles were then ultra-centrifuged at a speed of $100,000 \times g$ for 1 h at 4 °C. The pellets were re-suspended in 20 μL PBS mounted on a glass slide and examined using a confocal microscope (Leica Microsystems CMS GmbH, Germany), with an excitation and emission spectra for DiD at λ_{638nm} and λ_{700nm}, respectively.

2.10. Detection of peptidoglycan

ELISA was used to assess the presence of peptidoglycan, which was exposed by incubating the membrane vesicles and bacteria with 25 mM HCl for 1 h at room temperature under shaking. Microtiter plates (Maxisorb, Thermo Fischer Scientific, USA) were coated with membrane vesicles or bacteria in coating buffer (100 mM Sodium carbonate/bicarbonate, pH 9.6) at 4 °C overnight. After 4 washes with PBS-T (PBS with 0.01% Tween), plates were blocked with 0.05% BSA for 2 h at 37 °C and washed. To evaluate the presence of peptidoglycan in the samples, mouse anti-bacterial peptidoglycan monoclonal antibody (IgG, MAB995, Chemicon, Merck Millipore, USA; 1:1000) was added, and the plate incubated for 2 h at 37 °C. Excess primary antibody was removed by additional washing with PBS-T, before it was detected with peroxidase-conjugated rabbit anti-mouse immunoglobulin (DAKO, Agilent, USA; 1:500) for 20 min at 37 °C. Thereafter, tetramethylbenzidine (3, 3′, 5, 5′-TMB, Sigma Aldrich, Germany) was added as peroxidase substrate, and the reaction was stopped with sulfuric acid upon color change. Absorption was measured at 450 nm.

2.11. MV-associated protein identification by mass spectrometry

The protein concentration within the MVs was quantified, and 20 μg MV proteins were re-suspended in 8 M urea. The sample was reduced by adding 20 mM DTT and alkylated with 40 mM iodoacetamide. Alkylation was quenched by 10 mM DTT. Proteins were digested 4 h by 1:100 (w/w) lysyl endopeptidase (Wako Biochemicals, USA). The sample was diluted to 1 M urea and digested overnight by 1/20 (w/w) trypsin (V511A, Promega, USA). OMIX C18 tips (Varian Inc., USA) were used for sample cleanup and concentration. Peptide mixtures containing 0.1% formic acid were loaded onto an EASY-nLC1000 system (Thermo Fisher Scientific, USA) and EASY-Spray column (C18, 2 μm, 100 Å, 50 μm, 50 cm). Peptides were fractionated using a 2–100% acetonitrile gradient in 0.1% formic acid over 50 min at a flow rate of

250 nL/min. The separated peptides were analyzed using a Q-Exactive mass spectrometer (Thermo Fisher Scientific, USA). Data were collected in data-dependent mode using a Top10 method. The raw data were processed using the Proteome Discoverer 2.1 software. The fragmentation spectra were searched against the predicted proteome from the whole genome sequence (WGS) data of the strain itself using the Sequest HT program. Peptide mass tolerances used in the search were 10 ppm, and fragment mass tolerance was 0.02 Da. Peptide ions were filtered using a false discovery rate (FDR) set to 5% for protein identifications.

The MV-associated proteins were identified in 3 biological replicates of MV derived from bacteria in the exponential growth phase in BHI, and are represented as one in the results table without restrictions (Supplementary table S1). For MV derived from bacteria in the stationary growth phase in LB one biological replicate is described in the result table (Supplementary table S1).

2.12. Bioinformatic characterization of identified MV proteins

To evaluate MV-associated proteins, the entire proteomes were classified into core and strain(s)-specific. The core proteome was assigned with the proteins shared by all four strains whereas the strain(s)-specific proteome had proteins found only in one or few strains. All proteins were clustered using CD-HIT with default settings [24]. Homologous clusters possessing at least one protein from each strain were categorized into core proteins, if not as strain(s)-specific proteins.

Overlapping protein content among MVs isolated from four different strains was demonstrated using Venny 2.0 [25]. These results were shown individually for proteomes of MVs isolated after growth in LB to stationary phase and in BHI to mid-exponential phase.

Prediction of signal peptide cleavage sites was performed in SignalP 4.1 [26] and PrediSi [27] on all proteins identified in the MV proteomes. The subcellular localization of identified proteins was predicted using PSORTb version 3.0 [28]. Gene-term enrichment analysis was performed with DAVID Functional Annotation Tool [29] on proteins with an exponentially modified protein abundance index (empai) above 1.

Selected virulence factors and vaccine candidates were searched for using BLAST [30].

Antimicrobial resistance genes were identified within the respective genomes of the bacterial strains using ResFinder 3.0 [31] with an ID threshold of 90% and a minimum length of 60%. Genes with < 100% ID threshold and 100% minimum length are indicated in Table 2. Identified genes are *aph(3′)-III, aac(6′)-aph(2″); vanR-A, vanS-A, vanH-A, van-A, vanX-A, vanY-A, vanZ-A, vanR-B, vanS-B, vanY-B, vanW-B, vanH-B, vanB, vanX-B; ermB, lnuB, msrC, tetM* and *dfrG*. The corresponding proteins were searched for in the MV proteome in order to identify MV-associated antimicrobial resistance determinants.

3. Results

3.1. Clinical E. faecium strains produce MVs

This study explored whether four different clinical *E. faecium* strains, belonging to distinct hospital adapted high-risk STs, were able to release membrane vesicles in vitro. Notably, the growth rates of the examined strains at the measured intervals showed only minor variation (Fig. S1).

MVs were isolated from *E. faecium* strains, which were grown in BHI until mid-exponential phase (OD_{600} approximately 1.5). To ensure purity of the vesicles from cellular debris and protein aggregates, MVs were further purified and fractionated by gradient centrifugation (Fig. S2A). The purified MVs were analyzed by TEM where the presence of vesicle-like circular shape structures was confirmed (Fig. 1A). Similar structures were detected for the other three examined strains (results not shown). AFM was conducted as a complementary approach and

revealed release of MVs by the bacteria to the surrounding environment (Fig. 1B). To measure the size distribution of larger populations of MVs from the four *E. faecium* strains, dynamic light scattering (DLS) was employed. While all strains generated MVs of various sizes, the average size of enterococcal MVs ranged from 37 ± 23 nm in the E155 strain to 83 ± 29 nm in the K59–68 strain (Fig. 1C and Table 1). Furthermore, the size measured by DLS corresponded to the results obtained from TEM images (Fig. 1A–C, Table 1).

Based on protein content, the amount of MVs produced by the four strains ranged from 1.2 ag/CFU in strain K59-68 to 14.6 ag/CFU in strain E155 (Fig. 1D).

To gain insight into the interaction of the MVs in the colloidal system and gain a better understanding of their physiochemical properties (aggregation, flocculation or dispersion), the membrane (ζ-) potential, of the MVs was measured. The ζ-potential was calculated upon measuring particles electrophoretic mobility. The *E. faecium* MV surface charge in all examined strains was found to be negative in PBS and reproducible (Table 1) demonstrating a high quality of the MV isolation.

To further characterize the vesicle properties, the purified MVs of strain E155 were stained with a lipophilic fluorescent dye (DiD) for detection of lipids. The confocal microscopy analysis of the stained MVs confirmed the presence of lipids in MVs (Fig. 2A). The presence of peptidoglycan in the isolated MVs from all four strains was evaluated using a competitive ELISA approach. Interestingly, peptidoglycan could be detected in *E. faecium*-derived MVs from all examined strains. *E. faecium* cells were included as a positive peptidoglycan control for this particular assay (Fig. 2B).

3.2. Different cultural conditions and strain backgrounds account for variation in enterococcal MV proteinaceous cargo

To investigate whether the MV protein cargo is strain dependent and influenced by different conditions, the four strains were grown to exponential phase in a nutritious media, BHI, and to stationary phase in a growth-limiting media, LB. MVs were isolated and purified further by gradient centrifugation, and proteomic analysis was performed using an in-solution approach.

Proteomic analyses demonstrated variability in the number of identified proteins among MV-associated proteins isolated from the four selected strains grown in BHI and LB (Supplementary Tables 1–8).

Comparison of shared versus unique proteins revealed that, in exponential growth in BHI, a lower number (19.2%) of MV-associated proteins was shared than in stationary growth in LB (36.9%), as illustrated in Fig. 3. MVs from K60-39 showed the highest number of total MV-associated proteins and consequently also possess most unique MV-associated proteins under both conditions. In contrast, MVs from K59-68 showed a lower number of total vesicle associated proteins and accordingly the lowest number of unique proteins.

Based on the sequence similarity of the entire proteome encoded by all four genomes, homologous proteins present in all four were classified as core and the remaining as accessory. Accordingly, the MV-associated proteins were categorized based on their occurrence either in core or accessory proteome. Notably, it was found that 88–91% of the MV-associated proteins are from the core proteome (Table 1).

The predicted cellular localization of proteins encoded by the whole genome based on WGS data and the origin of MV-associated proteins was predicted by PSORTb. The predicted localization of proteins showed a common pattern among strains and MV-associated proteins originating from two different conditions (Fig. 4). As expected, most of the MV-associated proteins were predicted as either cytoplasmic or part of the cytoplasmic membrane proteins. A minor number of cell wall or extracellular bound proteins were also predicted in the derived MVs.

SignalP and PrediSi were applied to predict the presence of signal peptides. Although the number of identified proteins varied substantially between strains and conditions, the percentage of putative

Table 2
Presence of virulence factors and antibiotic resistance related proteins associated with MVs.

Virulence factor and functional description		WGS				MV							
		Tag in genome				stationary phase MV (LB)				exponential phase MV (BHI)			
		DO	E155	K60–39	K59–68	DO	E155	K60–39	K59–68	DO	E155	K60–39	K59–68
AtlA	Major autolysin, biofilm formation	Q3Y3J6	E155_00939	7_02408	6_02630	✓	✓	✓	✓	✓	✓	✓	✓
Acm	Collagen binding adhesion, MSCRAMM	Q3XXN7	E155_00761	7_02160	6_02383	✓	✓	✓	✓	X	X	X	✓
CapD	Capsular polysaccharide biosynthesis protein, adhesion	Q3XXA5	E155_00870	7_00899	6_00915	✓	✓	✓	X	✓	✓	✓	✓
CcpA	Catabolite control protein A, growth, virulence	I3U3N8	E155_00210	7_01900	6_02068	✓	✓	✓	X	✓	✓	✓	✓
Esp	Enterococcal surface protein, biofilm formation	-	-	-	6_02803	-	-	-	✓	-	-	-	-
Fnm	Fibronectin binding protein, adhesion	Q3XYE6	E155_00278	7_01343	6_01354	X	✓	✓	X	✓	✓	✓	X
PilA2	Pilus subunit protein A	Q3Y0Y4	-	7_02894	6_03276	X	-	✓	X	✓	-	X	X
PrpA	Prolinrich protein A, binding to extracellular matrix proteins	Q3XZP6	-	7_01420	6_01433	✓	-	X	X	✓	-	X	X
PtsD	Enzyme IID subunit of phosphotransferase stystem, intestinal colonization determinant	Q3Y1Z0	E155_00486	7_00634	6_00710	X	X	X	X	✓	✓	X	X
SagA	Secreted antigen A, biofilm formation	I3U595	E155_00236	7_02462	6_02688	✓	✓	✓	✓	✓	✓	✓	✓
Scm	Collagen adhesion, MSCRAMM	I3U5K9	-	7_02636	6_02858	✓	-	X	✓	✓	-	X	✓
Vaccine candidate													
PsaA	Metal binding lipoprotein	Q3Y2A0	E155_02566	7_00432	6_00512	✓	✓	✓	✓	✓	✓	✓	X
PBP5	Penicillin binding protein	Q3XZN6	E155_02659	7_01431	6_01444	✓	✓	✓	✓	✓	✓	✓	✓
LysM	Peptidoglycan binding protein	Q3Y2A3	E155_01532	7_00435	6_00515	✓	✓	✓	✓	✓	✓	✓	✓
DdcP	D,D-carboxypeptidase	I3U3E0	E155_02369	7_01824	6_01978	✓	✓	✓	✓	✓	✓	✓	✓
PpiC	Peptidyl-prolyl-cis-trans-isomerase	Q3Y2Y5	E155_01118	7_02348	6_02568	✓	✓	✓	✓	✓	✓	✓	✓
Antibiotic resistance related proteins													
Aac6'-Ie-Aph2″-Ia	Aminoglycoside resistance	-	E155_02844	-	6_03206	-	X	-	✓	-	✓	-	X
Aph3′-III	Aminoglycoside resistance	Q3Y248	E155_02752	7_03002	6_03355	✓	✓	X	✓	✓	✓	X	X
VanR-A	Glycopeptide resistance	-	E155_02402	-	-	-	✓	-	-	-	-	-	-
VanS-A	Glycopeptide resistance	-	E155_02403	-	-	-	✓	-	-	-	-	-	-
VanH-A	Glycopeptide resistance	-	E155_02496	-	-	-	✓	-	-	-	-	-	-
VanA	Glycopeptide reistance	-	E155_02495	-	-	-	✓	-	-	-	✓	-	-
VanX-A	Glycopeptide resistance	-	E155_02494	-	-	-	✓	-	-	-	-	-	-
VanY-A	Glycopeptide resistance	-	E155_02850	-	-	-	✓	-	-	-	✓	-	-
VanZ-A	Glycopeptide resistance	-	E155_02851	-	-	-	✓	-	-	-	✓	-	-
VanS-B	Glycopeptide resistance	-	E155_02765 (ID: 96.49%)	-	-	-	✓	-	-	-	-	-	-
ErmB	Macrolide resistance	Q3Y241	E155_02538	7_03003	6_03356	X	✓	✓	X	X	X	X	X

MSCRAMM - Microbial Surface Component Recognizing Adhesive Matrix Molecules, ✓ presence, X absence, - not within genome.

secreted proteins was comparable (Fig. 5). In all strains 4–5% of the ORFs encode secreted proteins in the whole genome proteomes, while 10–22% secreted proteins were predicted in MVs derived from exponential growth phase in BHI, and 5–12% secreted proteins in MVs derived from stationary growth phase in LB. The highest number of secreted (22%) and extracellular (4%) proteins was associated with MV proteinaceous cargo obtained from K59–68 grown in BHI to the mid-exponential phase.

Gene functional enrichment analyses for strain DO identified 10 functional annotation clusters in MV-associated proteins from exponential growth conditions in BHI and 16 functional annotation clusters in MV-associated proteins from stationary growth phase in LB. The difference in cluster number is probably due to the lower protein number in exponential growth phase (for empai > 1 $n_{protein}$ = 138) compared to stationary growth phase (for empai > 1 $n_{protein}$ = 258). The analysis showed that ribosomal, ATP synthesis, and membrane proteins were enriched in both exponential and stationary growth phase derived MVs in DO. The gene ontology enrichment analysis is illustrated in Fig. 6.

3.3. Presence of virulence factors, vaccine candidates, and antimicrobial resistance-related proteins among the E. faecium MV-associated proteins

Fifteen distinct E. faecium genes encoding virulence factors, associated with biofilm formation and adherence, were identified within the genomes of the 4 examined strains: atlA [32], acm [33, 34], bepA [35], capD [36], ccpA [37, 38], ecbA [39], esp [40], fnm [41], pilA2 [42], pilB/ebp_fm [43], ptsD [44], prpA [45], sagA [46–48], scm [49] and sgrA [39]. gelE [50] was also searched for but was absent in the selected genomes.

Presence or absence of selected virulence factors associated with the E. faecium-derived MVs is indicated in Table 2. Proteomic analysis demonstrated the absence of BepA, EcbA, PilB/Ebp_fm and SgrA in the purified MVs. Interestingly, the two biofilm determinants, namely AtlA and SagA, were found to be associated with MVs of all tested strains under both conditions. The adhesins Acm and CapD as well as the catabolite protein CcpA were also associated with purified MVs of all examined strains. Esp, an enterococcal surface protein enhancing biofilm formation, Fnm, a fibronectin-binding protein, PilA2, a pilus protein, PrpA, an extracellular matrix binding protein, PtsD, a phosphotransferase system protein, and the adhesin Scm, were associated with MVs from some of the examined strains.

The search was expanded to further include the enterococcal bacteriocins EntA, EntP, EntB, EntL50 [51], EntQ [52], Bac32 [53] and
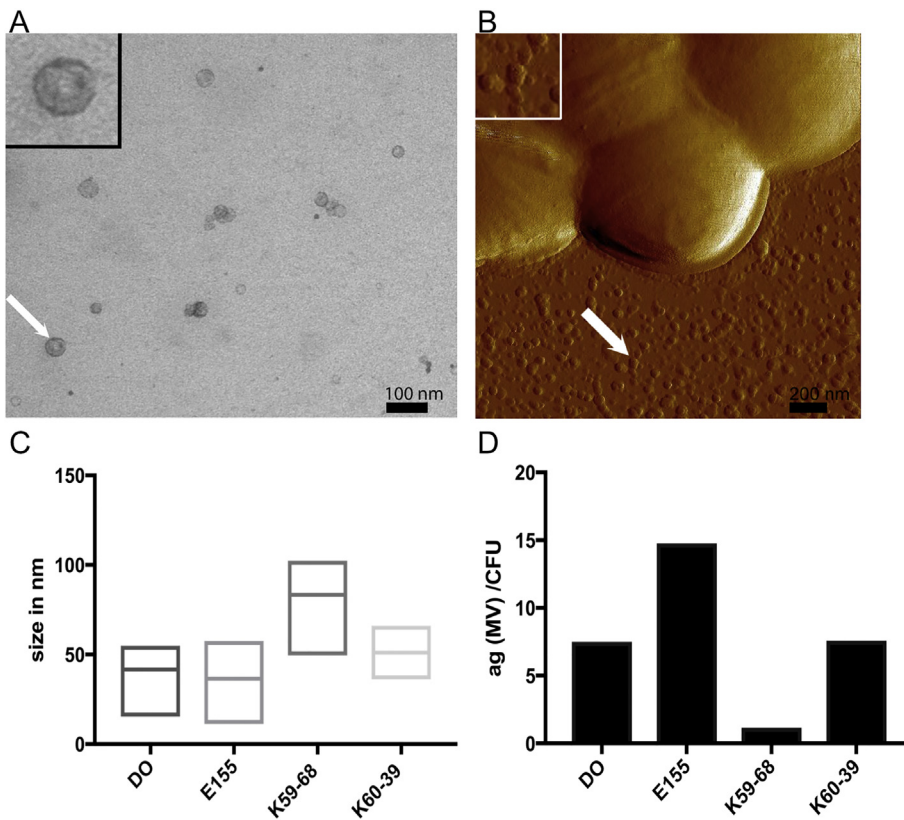
Fig. 1. *E. faecium* releases spherical MVs *in vitro*. A) TEM of MVs isolated from *E. faecium* E155 (the inset in the upper left corner shows the vesicle indicated by an arrow 2 times magnified). B) AFM of *E. faecium* E155 surrounded by MVs (the inset in the upper left corner shows the vesicle indicated by an arrow 2 times magnified). C) Abundance of the various sizes of purified MV from DO, E155, K59-68 and K60-39 grown to exponential phase in BHI as determined by DLS (mean in nm ± SD). D) MV-derived protein per CFU in DO, E155, K59-68 and K60-39 in cultures grown in BHI to OD$_{600}$ approximately 1.5.

Bac43 [54]. Out of those, only the gene *entA* was present in all the four genomes used in this study, but EntA was not found to be associated with the *E. faecium* derived MVs.

As previous studies have identified putative enterococcal vaccine targets, the following candidates were searched for in the vesicular proteome: PsaA, AdcA [55], PBP5, LysM, DdcP, PpiC [56] and SagA [48] (Table 2). PsaA and AdcA are both metal binding lipoproteins [55], and PsaA was found associated with MVs from all four strains. PBP5, LysM, DdcP, and PpiC are putative surface-exposed proteins, which are associated with peptidoglycan [56], and all four were found within all the examined MV samples. Additionally, SagA, which was present in all MV samples, was not only described as a virulence factor [46, 47], but also as the first promising vaccine candidate in *E. faecium*, as it exhibited protective properties in a mouse model [48].

Furthermore, resistance genes were identified in the four genomes using ResFinder [31], and subsequently, the corresponding proteins were searched for in the MV proteomes (Table 2). The protein Aph3′-III, a kanamycin kinase conveying resistance to aminoglycosides, was found in MV samples in both conditions. Aac6′-Ie-Aph2″-Ia, a

bifunctional protein conferring broad-spectrum resistance to aminoglycosides such as gentamicin through N- and O-acetylation and phosphorylation of hydroxyl groups [57], was associated with DO-, E155- and K59-68-derived MVs, isolated from BHI and/or LB cultures. ErmB, conferring macrolide, such as erythromycin, resistance, was associated with E155- and K60-39-derived MVs grown in LB to the stationary phase.

All proteins encoded by the *vanA*-cluster that contribute to glycopeptide resistance [58] were associated with E155-derived MVs grown in LB to the stationary growth phase: VanR-A, VanS-A, VanH-A, VanA, VanX-A, VanY-A, VanZ-A. A subset of those was also present in MVs isolated from exponential growth phase. Additionally, VanS-B encoded by the *vanB*-cluster was found associated with E155-derived MVs grown to the stationary growth phase. However, the E155 genome also encodes the rest of the genes of the *vanB*-cluster: *vanR-B*, *vanY-B*, *vanW-B*, *vanH-B*, *vanB* and *vanX-B*, but they were absent in the MVs (Table 2).

The macrolide resistance genes *lnuB* (K60-39) and *msrC* (all 4 strains) were present in the respective genomes but absent in the MVs. This also accounts for *tetM* (DO and K59-68) and *dfrG* (E155).
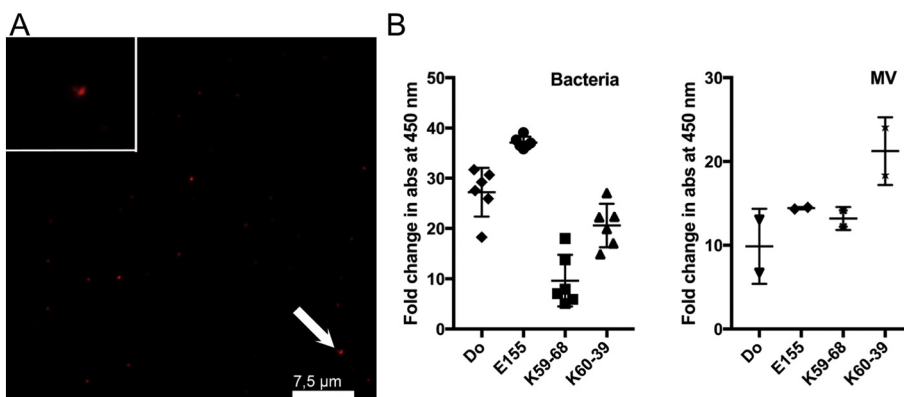

Fig. 2. *E. faecium* MVs contain lipids and peptidoglycan. A) Confocal microscopy of MVs stained with lipid-tracer dye (DiD shown in red, the inset in the upper left corner shows the vesicle indicated by an arrow 2 times magnified). B) Fold changes in absorbance using anti-peptidoglycan antibodies in ELISA to detect peptidoglycan in bacteria and MVs isolated for the four strains at mid-exponential growth phase in BHI compared to the control (uncoated well). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
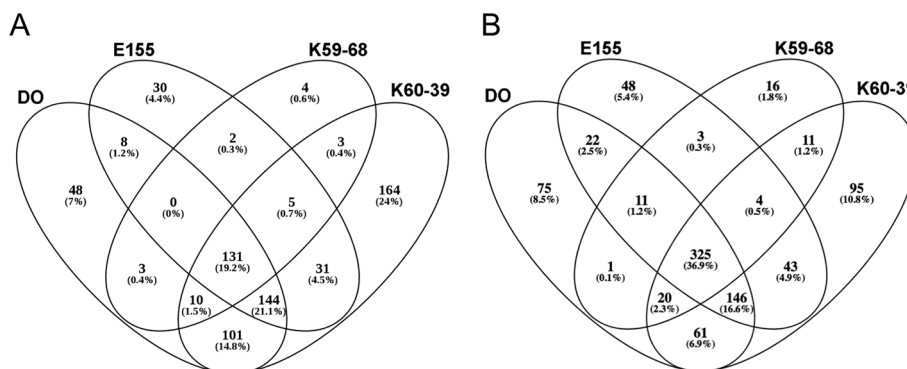
Fig. 3. Four-set Venn diagram representing the number of shared and unique MV-associated proteins of MVs isolated from *E. faecium* cultures. A) Mid-exponential phase growth in BHI, B) Stationary phase growth in LB. Numbers of shared or unique proteins are given in bold, percentage in brackets.
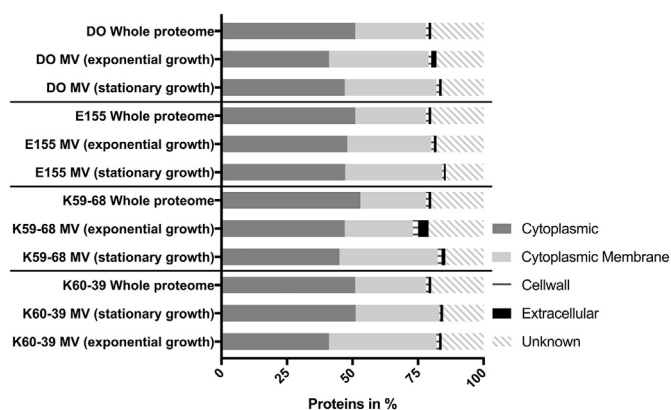
Fig. 4. Localization prediction of proteins in whole genome proteomes and in proteomes of MVs derived from two different conditions. Subcellular localization prediction was done in PsortB and is illustrated in proportional numbers.
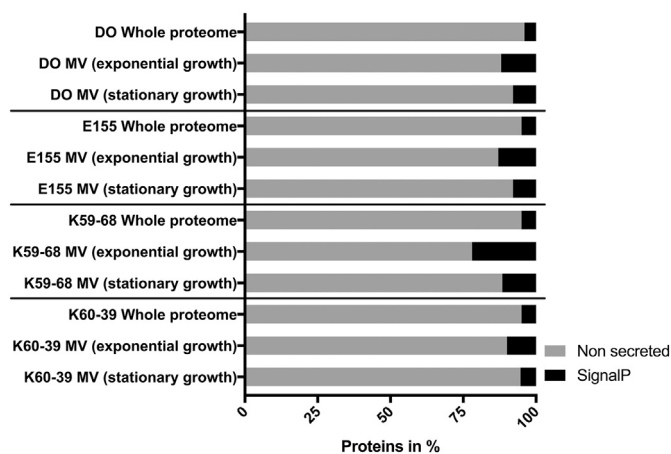
Fig. 5. Proportion of proteins with a signal peptide in *E. faecium* whole genome proteomes and MV associated proteomes from two different conditions. Signal peptide prediction was done by SignalP.

4. Discussion

All cells investigated so far have been found to release vesicles, indicating a universal phenomenon. The release of bacterial membrane vesicles and their functional importance, including both offense and defense, has been widely studied in Gram-negative bacteria [59]. In recent years, vesicle research has also increased in Gram-positive bacteria such as *Listeria* [60], *Bacillus* [16], *Staphylococcus* [61], *Lactobacillus* [62], *Streptococcus* [63] and *Clostridium* [64]. Here we provide the

first report describing MVs released by *E. faecium*.

Rivera et al. [16] observed spherically shaped vesicles with the size of 50–300 nm in *B. anthracis*. Similarly, MV isolated from *Listeria, Staphylococcus, Lactobacillus, Streptococcus,* and *Clostridium* ranged from 20 to 400 nm [60, 61, 63, 65]. The morphology of MVs secreted by *E. faecium* was found to be similar to the above-mentioned MVs from Gram-positive bacteria, both in terms of size and appearance (Fig. 1, Table 1). We also quantified the vesicles based on protein measurement through Bradford reagent and found similar MV amounts produced by the four *E. faecium* strains. However, a drawback of these types of measurement approaches is the lack of a universal standard procedure for MV quantification. Until now, vesicular proteins have been quantified using protein-based (SDS-PAGE staining, Bradford reagent or bicinchoninic-acid assay BCA) and lipid-based (lipid probes such as FM4–64) approaches. However, all these methods have both strengths and weaknesses [66]. Moreover, for the proteomic analysis assessment of the purity of the MV sample is utmost important. Contaminating artifacts may co-pellet in the ultracentrifugation step of MV isolation. However, density gradient centrifugation was included in our study, which improves the purity of the MV sample [66, 67].

As previously described in Gram-negative bacteria [68] and the Gram-positive bacterium *Clostridium perfringens* [64], peptidoglycan was confirmed to be associated with enterococcal vesicles, through use of an antibody with specificity for the three-dimensional polymer complex structure of peptidoglycan. In addition, the MVs from all four strains exhibited negative surface charge, based on ζ-potential. The surface charge of *Pseudomonas aeruginosa* and *B. anthracis* derived MVs was also reported to be negative [16, 69] and in *P. aeruginosa* the ζ-potential was more negative in stationary growth phase derived vesicles compared to exponential growth phase [69]. Gram-positive and Gram-negative bacterial cells are negatively charged, too. It has been suggested that the charge is critical to understand the interaction between MVs and the bacterial cells [69] and the selectivity of this interaction since it was found that MVs are more likely to interact with bacterial cells of the parent species than of other genera [70].

Besides the morphological characterization, a mass spectrometry based approach was used to gain insight into the proteomic profile of the isolated MVs. The overall proteomic pattern within MVs was similar regarding the percentage of proteins that derived from the core genome, their origin of localization, and whether they had a signal peptide or not (Figs. 3–5). However, different numbers of MV-associated proteins identified by proteomics were found among the four examined strains and under varying conditions (Table 1, Fig. 3). Among Gram-positive bacteria substantially varying numbers of MV-associated proteins have been described previously, from 431 MV-associated proteins in *C. perfringens* grown in Trypticase-peptone-glucose broth [65] to 36 MV-associated proteins in *B. anthracis* 34F2 in BHI [16]. The growth medium affects the gene expression in bacteria which subsequently alters the amount and/or content of vesicles released into the
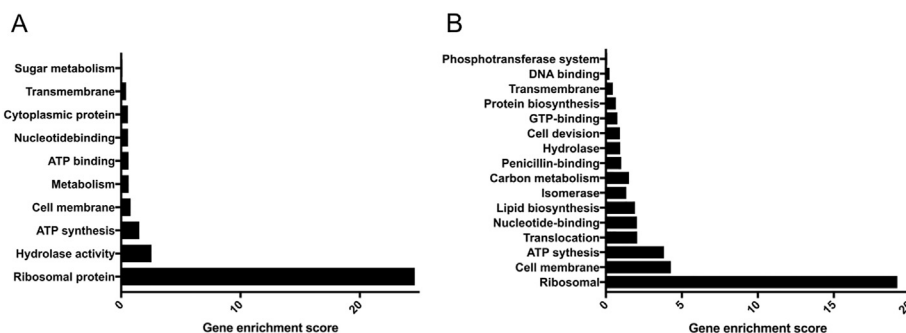
Fig. 6. Distribution of DO MV-associated proteins from two different conditions compared to the whole genome proteome of DO in gene ontology enrichment analysis. Proteome of MVs derived in exponential growth in BHI is shown in A) and stationary growth in LB in B).

growth medium [71]. Similarly, Bager et al. found significant changes in OMV production and protein composition upon altering medium composition in *Gallibacterium anatis* [72]. Moreover, a recent study on the proteome of *Pseudomonas putida* KT2440 by Choi et al. demonstrated that OMV production in LB media was three-fold higher than in two different minimal media containing succinate and benzoate [73]. Other previous studies identified a higher number of vesicular proteins in *P. aeruginosa* in LB compared to a more nutrient rich media [74–76]. Likewise, in our study, we observed a higher number of different proteins in MVs derived from *E. faecium* grown to stationary growth phase in nutrient-limiting LB compared to exponential growth in nutrient rich BHI. Under nutrient limitation and stationary growth, we expect the bacteria suffer more stress, which might explain more different vesicular proteins.

In the presented study we found a high abundance of cytoplasmic proteins, especially ribosomal proteins, which is similar to the number of cytoplasmic proteins encoded by the genome (Figs. 4, 6). This is in agreement with previous studies of MVs derived from Gram-positive bacteria [15, 19, 61]. This implicates, that the MV content represents basically the composition of the parent cell, even though final conclusions would require protein quantification rather than identification. We furthermore found, that high numbers of MV-associated proteins lack a secretion signal, which is in line with a previous study on *S. aureus* [21] as well as *Campylobacter jejuni* [77] and suggests that MVs may play an important role in the delivery of effector molecules, which lack a secretion signal.

As previous studies have shown that MVs of Gram-positive bacteria contain virulence factors [15, 16, 60, 78], serve as vaccine candidates [79] and are contributing to antimicrobial resistance [80], in silico methods were used to assess whether such factors are also associated with *E. faecium* MVs. Indeed, we identified 11 known virulence factors among the MV-associated proteins, including adhesins (CapD and PrpA), collagen-binding proteins (Acm and Scm), and fibronectin-binding protein (Fnm). These virulence factors are of particular importance for enterococcal adhesion, colonization and tissue invasion [34, 36, 41, 45, 49]. The association of collagen-binding proteins, as well as the fibronectin-binding protein and adhesins with MVs may suggest a role of the vesicles in enterococcal adhesion and colonization.

E. faecium utilizes biofilm formation as a survival strategy, which enables protection and persistence [81, 82]. Previous studies demonstrated that OMVs contribute to biofilm formation and stability [83, 84]. The virulence factors AtlA, Esp and SagA that contribute to enterococcal biofilm formation [32, 40, 46–48] were found to be MV-associated. This may suggest that *E. faecium* MVs can contribute to biofilm formation.

As highly stable, non-infectious, non-replicative particles vesicles are interesting vaccine candidates. Furthermore, they contain major immunogenic proteins of the MV producing bacterium that can act as antigens and thus can elicit responses in both arms of the immune system. Additionally, they display adjuvant activity (reviewed in [79,

85]). The first OMV-based vaccine to be licensed for human use was the meningococcal serogroup B vaccine 4CMenB against the Gram-negative *Neisseria meningitidis* [86]. Also in the Gram-positive *Streptococcus pneumoniae* [87] and *S. aureus* [21, 88], the protective effect of immunization with MVs was recently shown. For enterococci, there are to date no available vaccines, but there has been research towards identifying components for a vaccine to prevent *E. faecium* infection [48, 55, 56]. Here we found several previously described vaccine candidates to be associated with MVs (Table 2). However, whether enterococcal MVs can be used as vaccines remains to be investigated.

Special interest has been drawn to *E. faecium* due to their multidrug resistance and high genomic plasticity, which allows them to steadily acquire new resistance determinants. In the Gram-negative *E. coli* it was shown that OMVs not only have a protective role against membrane-active antimicrobials [80], but also that certain antimicrobials increase the production of OMVs and OMV-associated Shiga toxin [89, 90]. Also resistance-genes were found to be transferred via OMVs [91]. Furthermore, OMVs of β-lactamase resistant *Haemophilus influenzae* and *Moraxella catarrhalis* were shown to protect *Streptococcus pyogenes* against amoxicillin [92]. Interestingly, our study demonstrates that both the ribosome methylase ErmB, the aminoglycoside modifying enzymes Aac6′-Ie-Aph2″-Ia and Aph3′-III as well as all proteins of the *vanA* vancomycin resistance cluster can be MV-associated in *E. faecium*. All these proteins mainly function inside the enterococcal cell. Thus they could potentially be active in creating antimicrobial resistance if they can be delivered to a recipient cell by the MVs. However, low empai values of the *vanA*-cluster encoded proteins, ErmB, Aac6′-Ie-Aph2″-Ia and Aph3′-III under the conditions studied here, suggest that these MVs do not have a great potential to cause antimicrobial resistance. Still, MV production and thus resistance-conferring proteins could be more abundant upon induction through antimicrobial stress as seen in MVs from *Stenotrophomonas maltophilia* [93] and *E. coli* [89] and then be presented in high enough concentrations to a recipient cell to confer an antimicrobial effect. This implicates that enterococcal MVs may play a role in antimicrobial resistance, not only for *E. faecium* itself but also for the bacterial community.

The mechanism of vesiculogenesis has not been conclusively clarified in Gram-positive bacteria. Recently, prophage-triggered MV formation was described and it has been postulated that phage endolysins may confer MV escape by locally digesting the cell wall [94]. Interestingly, by using PHASTER tool [95], we detected phage genes within the genomes of DO, K59–68 and K60-39, but not in E155 (results not shown). Thus, prophages may play a role in the release of MV from certain strains. Apart from prophage-triggered MV formation, it has been suggested, that the vesicles passage the peptidoglycan layer with the aid of cell wall modifying enzymes such as peptidoglycan muramidases/ hydrolases, which are part of the Gram-positive type 4 secretion system (T4SS) [6, 15]. In the genomes of the four strains, we found T4SS using T346Hunter [96] (data not shown), suggesting that these strains might use components of this system for vesicle release. However, the mechanism for MV release in enterococci remains elusive.

5. Conclusion

A proteomic approach was used to describe the protein cargo of membrane vesicles derived from four different, clinically relevant *E. faecium* strains under varying conditions. It was found that the MV-associated proteome is strain and condition dependent. The most interesting MV-associated proteins were the virulence factors and antimicrobial resistance related proteins. It is thus tempting to speculate that enterococcal MVs play an important role in infection and antimicrobial resistance. Our findings highlight the potential of bacterial MVs for research on bacterial pathogenesis.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jprot.2018.05.017.

Data availability

The raw mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE [97] partner repository with the dataset identifier PXD008801.

Conflict of interest

The authors declare no potential conflict of interest.

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