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

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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 ultracentrifugation, before re-suspention 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	% proteins from core	No. MV proteins	% proteins from core
											Exponential growth BHI		Stationary growth LB	
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