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Role of membrane vesicles in the transmission of vancomycin resistance in *Enterococcus faecium*

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Clonal transmission and horizontal gene transfer (HGT) contribute to the spread of vancomycin-resistant enterococci (VRE) in global healthcare. Our study investigated vesiduction, a HGT mechanism via membrane vesicles (MVs), for *vanA* and *vanB* genes that determine vancomycin resistance. We isolated MVs for VRE of different sequence types (STs) and analysed them by nanoparticle tracking analysis. Selected MV samples were subjected to DNA sequence analysis. In resistance transfer experiments, vancomycin-susceptible enterococci were exposed to MVs and bacterial supernatants of VRE. Compared to bacteria grown in lysogeny broth (MVs/LB), cultivation under vancomycin stress (MVs/VAN) resulted in increased particle concentrations of up to 139-fold (ST80). As a key finding, we could show that VRE isolates of ST80 and ST117 produced remarkably more vesicles at subinhibitory antibiotic concentrations (approx. 9.2×10^{11} particles/ml for ST80 and 2.4×10^{11} particles/ml for ST117) than enterococci of other STs (range between 1.8×10^{10} and 5.3×10^{10} particles/ml). In those MV samples, the respective resistance genes *vanA* and *vanB* were completely verifiable using sequence analysis. Nevertheless, no vancomycin resistance transfer via MVs to vancomycin-susceptible *Enterococcus faecium* was phenotypically detectable. However, our results outline the potential of future research on ST-specific MV properties, promising new insights into VRE mechanisms.

A major increase in deaths related to antimicrobial resistance is predicted worldwide in the upcoming years[1]. Hence, adequate treatment of bacterial infections poses a challenge to global healthcare[2]. In this context, the World Health Organisation published a list of bacteria for which research and development of new antibiotics are urgently needed. Here, the list of 'high-priority pathogens', containing increasingly drug-resistant bacteria, included vancomycin-resistant *Enterococcus faecium* (*E. faecium*) (VRE)[3].

Enterococci are Gram-positive bacteria that colonise the human intestine. They are also frequently found in the environment and can survive hostile growth conditions[4]. In addition, enterococci, in particular *E. faecalis* and *E. faecium*, are common causes of infections in the clinical setting comprising endocarditis, bloodstream, catheter-associated and surgical site infections. Moreover, VRE bacteraemia tends to have a worse outcome than that caused by vancomycin-susceptible enterococci (VSE)[5]. Current data show rising trends towards VRE in Europe[6]. In Germany, this led to an increase in the national burden of disease due to VRE bloodstream infections[7].

The spread of VRE within healthcare settings is attributed to both clonal transmission and horizontal gene transfer (HGT) events[8]. So far, various genotypes of vancomycin resistance have been described, with VanA- and VanB-types being the most relevant[9]. Complementing the known HGT mechanisms of transformation, conjugation, and transduction, bacterial membrane vesicles (BMVs) have been described as vectors for gene transfer in recent years, introducing a fourth pathway, termed vesiduction[10].

BMVs were first discovered as outer membrane vesicles (OMVs) in Gram-negative bacteria[11]. Distinctly delayed, they were also found in microorganisms with cell walls, such as Gram-positive bacteria[12], designated as membrane vesicles (MVs). Consequently, MVs still leave many questions unanswered and offer great research potential. BMVs appear to be relevant in a variety of areas, such as communication, immunological processes, biofilm formation, toxicity, and gene transfer[13,14], e.g. the transfer of antibiotic resistance genes. The latter has already been validated for OMVs from *Escherichia coli*[15–17], *Klebsiella pneumoniae*[18], *Acinetobacter baylyi*[19] and *Acinetobacter baumannii*[20,21].

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Regarding *E. faecium,* the release of MVs has just recently been confirmed[22]. So far, the potential of MVs as a vector for HGT remains hypothetical. Therefore, our study aims to elucidate for the first time whether MVs, released by different VRE, transfer vancomycin resistance to VSE in vitro.

Results

Characteristics of VRE isolates and released MVs

Vancomycin resistance of all investigated isolates was confirmed both phenotypically and genotypically (Table 1).

Characterisation of MVs in terms of particle size and concentration via nanoparticle tracking analysis (NTA) revealed differences between MV samples depending on multilocus sequence typing (MLST) sequence types (STs) and cultivation conditions. The most frequently measured particle size ± standard error (SE) ranged from 69.1 ± 2.0 nm for MVs released by ST721 after cultivation in lysogeny broth (LB) (MVs/LB) up to 92.3 ± 5.5 nm (MVs/LB of ST80) (Fig. 1a). Results expanded by the mean particle sizes can be found in Supplementary Figure S1. Size distributions of MVs released by ST80 and ST117 cultured in LB supplemented with vancomycin at subinhibitory concentrations (MVs/VAN) are shown in Fig. 2.

The mean particle concentration ± SE of MV samples varied between $3.48 \times 10^9 \pm 1.88 \times 10^8$ particles/ml (MVs/LB of ST192) and $9.17 \times 10^{11} \pm 3.50 \times 10^{10}$ particles/ml (MVs/VAN of ST80) (Fig. 1b). We recorded higher particle concentrations under vancomycin stress for all STs, with the most striking increase for ST80 (139-fold from $6.60 \times 10^9 \pm 4.11 \times 10^8$ particles/ml for MVs/LB to $9.17 \times 10^{11} \pm 3.50 \times 10^{10}$ for MVs/VAN) and ST117 (35-fold from $6.99 \times 10^9 \pm 2.25 \times 10^8$ for MVs/LB to $2.44 \times 10^{11} \pm 6.76 \times 10^9$ for MVs/VAN).

PCR of MVs

PCR of MVs/VAN released by ST80 and ST117, respectively, revealed clearly detectable DNA bands in samples prior to DNase treatment. After removing extravesicular DNA by DNase treatment and subsequent heat lysis of MVs, only weak bands were found consistently in samples MVs/VAN of ST80, while weak bands were only irregularly detectable in samples MVs/VAN of ST117, indicating very low amounts of DNA near or below the detection limit. Figure 3 exemplifies representative samples of MVs/VAN derived from ST80 with weak bands and from ST117 without visible bands.

Whole genome sequencing (WGS) of MVs

After DNase treatment of MVs/VAN derived from VRE of ST80 and ST117 with subsequent DNA isolation, double-stranded (ds) DNA concentrations amounted to 0.029 ng/µl (ST80) and 0.040 ng/µl (ST117), which was too low for subsequent WGS. Analysis of WGS of DNA isolated from untreated MVs/VAN of VRE isolates ST80 and ST117 showed that for ST80, *vanB* was detected on the chromosome while *vanA* was located on a plasmid in the ST117 isolate and MV DNA. Pairwise alignments of sequences from both MVs and their respective donor isolates showed that the *van* cassettes were complete and identical.

Exposure of VSE to MVs and bacterial supernatant

For exposure experiments, we determined the mean colony forming units (CFU) ± standard deviation (SD) of *E. faecium* ATCC 6057 under our specific test parameters at $4.65*10^7 \pm 3.29*10^6$ CFU/ml.

After coincubation of both MVs/VAN released from VRE isolates ST80 and ST117 with vancomycin-susceptible *E. faecium* ATCC 6057 at an MV-bacteria ratio of 1,000 and 10,000, no bacterial growth on VRE selective agar was visible indicating no phenotypical detectable *van* resistance inclusion. Controls on Columbia blood agar showed *E. faecium* typical growth.

Similar to the MV experiments, after exposure to supernatants, no growth on VRE selective agar but on Columbia blood agar was observed.

Discussion

In the hospital setting, VRE pose a threat to particularly vulnerable patient groups, as adequate treatment is only possible using last-resort antibiotics. Due to increasing incidences and resistance, new therapeutic and infection prevention approaches are urgently needed. Our study aimed to assess the risk associated with VRE-derived MVs as a potential vehicle for *van* gene transmission.

	MIC vancomycin [µg/ml]	MIC teicoplanin [µg/ml]	*van*-resistance (eazyplex® VRE)
E. faecalis **ATCC 29,212**	4	1.5	-
E. faecium **ATCC 6057**	0.5	1.5	-
E. faecium **ST80**	>256	1.5	*vanB*
E. faecium **ST117**	>256	32	*vanA*
E. faecium **ST192**	>256	2	*vanB*
E. faecium **ST203**	>256	>256	*vanA*
E. faecium **ST721**	>256	>256	*vanA*
E. faecium **ST1489**	>256	2	*vanB*

Table 1. Phenotypic and genotypic vancomycin resistance of VRE isolates and reference strains.

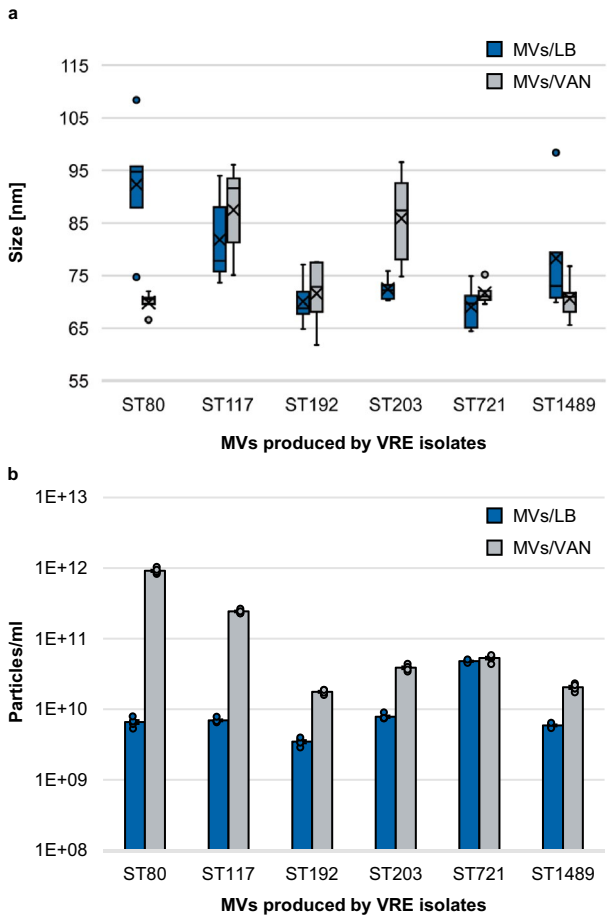

Figure 1. Particle sizes (**a**) and concentrations (**b**) of representative membrane vesicle (MV) samples derived from vancomycin-resistant enterococci (VRE). MVs were isolated from VRE of sequence types (STs) ST80, ST117, ST192, ST203, ST721 and ST1489 defined by multilocus sequence typing cultivated either in lysogeny broth (LB) (MVs/LB) or in LB supplemented with vancomycin (MVs/VAN). Nanoparticle tracking analysis (NTA) measurements consisted of five measurement cycles lasting 60 s each. The mean value of the most frequently measured particle sizes (**a**) is represented by a cross and the median by a line. Whiskers indicate the maximum and minimum values provided they lie within 1.5-fold of the interquartile range, depicted by the box. Dots visualise outliers. The quartile calculation included the median. Particle concentrations (**b**) are given as mean ± standard error. The dots mark the concentrations determined in the individual measuring cycles. For error analysis, the standard evaluation of the NanoSight NTA 3.4 software was used.

We were able to successfully prepare MVs from all investigated VRE isolates. After coincubation of VSE with VRE-derived supernatants and MV samples, no phenotypically effective transfer of vancomycin resistance was observed neither by vesiduction nor by transformation. This is remarkable since at least WGS of DNase-untreated MV samples showed that all *van* resistance-determining genes were present and intact. However, our PCR experiments provide indications that only very small DNA quantities near or below the PCR detection limit were present intravesicularly, hampering vesiduction of vancomycin resistance. Nonetheless, literature has shown that many BMVs contain DNA, albeit conceivably in smaller quantities than extravesicular BMV-associated DNA[23]. Also, the fusion of MVs and vesiductants might be hindered due to species or genotype specificity or cell wall properties that completely differ from those of Gram-negative bacteria. However, fusion of MVs and bacterial cells has already been detected in the Gram-positive *Bacillus subtilis*[24] and as HGT via MVs in *Ruminococcus* species[25], assuming resistance transfer is not being prevented by the peptidoglycan layer of Gram-positive bacteria. Investigations regarding the gene transfer ability of BMVs did not inevitably lead to a confirmation[26–28], suggesting that vesiduction is a complex process which is not yet fully understood. Partly, resistance was selectively transferred[29], assuming mechanisms for which certain conditions need to be fulfilled[21].

NTA characterisation of MVs could confirm previous findings, including reported particle sizes[22, 30, 31]. We observed an increased vesicle release under vancomycin stress conditions that has already been witnessed for MVs from *E. faecium*[30]. The reason is suspected to be a weakening of the cell wall caused by vancomycin below inhibitory concentrations[30, 32]. Interestingly, with the help of NTA, we were able to show for the first time that there are intra-species differences regarding the MV release of VRE depending on the ST. VRE of the genotypes ST80 and ST117 release significantly more vesicles under vancomycin stress compared to VRE of other STs

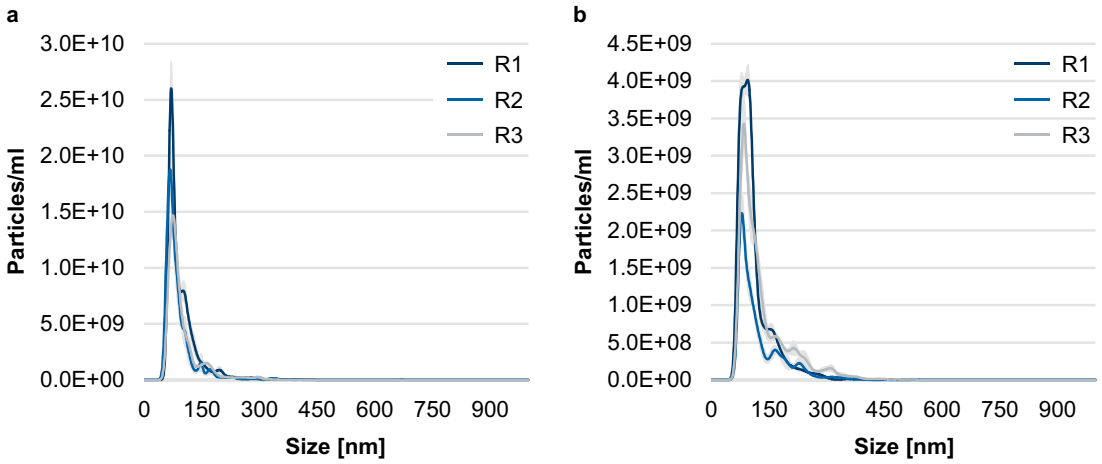

Figure 2. | Exemplary size distribution of representative membrane vesicle (MV) samples derived from vancomycin-resistant enterococci (VRE). MVs were gained under vancomycin stress (MVs/VAN) produced by VRE isolates of sequence types (STs) ST80 (**a**) and ST117 (**b**). In both cases (**a**, **b**), three biological replicates (R1–R3) were examined by nanoparticle tracking analysis (NTA) in five measurement cycles of 60 s each. The results are presented as mean ± standard error, shown as shading. For error analysis, the standard evaluation of the NanoSight NTA 3.4 software was used.

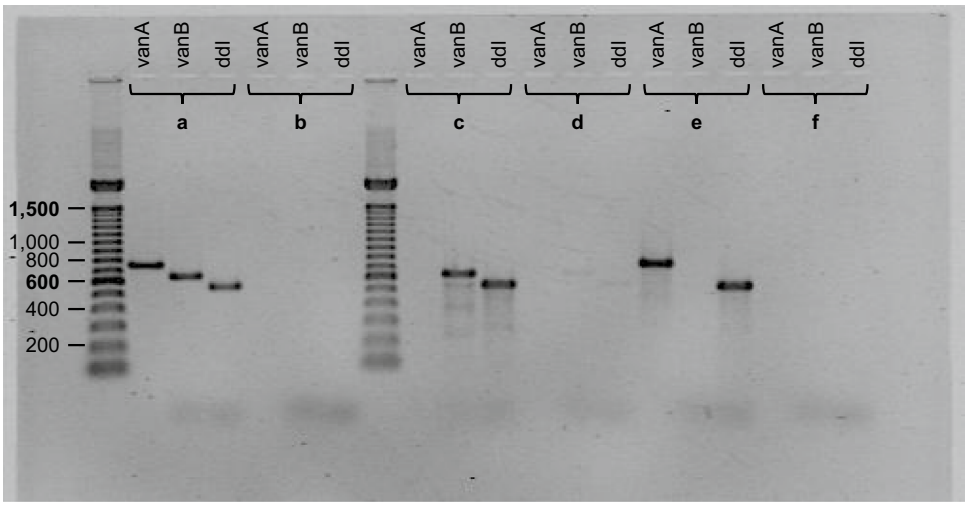

Figure 3. Resistance-encoding DNA fragments of membrane vesicle (MV) samples. MVs were gained under vancomycin stress (MVs/VAN) produced by vancomycin-resistant enterococci (VRE) isolates of sequence types (STs) ST80 and ST117. (**a**) bacterial DNA as a positive control, (**b**) water as a negative control, (**c**) MVs/VAN of isolate ST80, (**d**) MVs/VAN of isolate ST80 post 8 U DNase I treatment, (**e**) MVs/VAN of isolate ST117, and (**f**) MVs/VAN of isolate ST117 post 8 U DNase I treatment. The size of the DNA fragments [bp] is indicated on the left (gel cropped at the lower edge; for full-length gel see Supplementary Figure S2).

(Fig. 1b). These STs may have properties that could provide a selection advantage depending on the enterococcal MV function, hitherto rarely studied. In accordance with this, ST80 and ST117 are currently responsible for the majority of invasive VRE infections in Germany[33]. In several regions, ST117 was reported to be strikingly dominant[34–37], but no distinct reason, like specific virulence factors[35] or colonisation persistence[38], could yet be identified. The increased virulence potential of ST80- and ST117-positive VRE might be due to the elevated MV release itself. MVs have been shown to be crucial modulators of inflammatory responses in a wide range of host tissues[39] that can substantially contribute to a dysregulated host response. Hence, the enlarged MV quantities might enable ST80- and ST117-positive VRE to contribute to the severity of human infection and inflammation. This would open up new infection prevention options, namely MV-based vaccine development[40]. Since MVs are native antigen-presenting particles harbouring a high immunogenic capacity and lacking replicating potential, they seem to be the perfect target structures for such approaches. These have already been successful in controlling Gram-negative pathogens, e.g. meningococcal B strains[39].

We would like to address the limitations of our work. First, we used only a single VRE isolate representing each MLST ST. Hence, there might be differences regarding strain and MV characteristics. However, we

deliberately chose investigated isolates that are representative of isolates connected to cluster or outbreak events[41]. Second, characterisation using NTA identifies particles that do not necessarily represent vesicles. But, due to distinct peaks in the NTA measurements, it can be assumed that the samples consist predominantly of MVs. Third, as we inconsistently detected weak DNA bands after PCR of DNase-treated MV samples, we cannot rule out artificial measurements, e.g. due to incomplete DNA degradation. Fourth, with the help of the chosen methods, information about vesicle integrity is not provided. Nevertheless, we have no reasonable indication of any integrity disturbance as we followed published protocols and avoided MV treatments redundant for our research[20].

Summarising, we noticed differences between the MV characteristics depending on the ST of VRE. Especially, enterococci assigned to ST80 and ST117 released markedly more vesicles under vancomycin stress than other isolates investigated. Further research is necessary to uncover ST-specific differences in vesiculation to better understand host–pathogen interactions in terms of invasive VRE infections, their therapy and infection prevention approaches.

Materials and methods

Bacterial isolates and growth conditions

Six different VRE isolates defined by MLST (ST80, ST117, ST192, ST203, ST721, and ST1489) were used for MV preparation. The isolates originated from a surveillance sample collection of the University Hospital Münster, Germany, and all of them were derived from routine anal swab samples. Based on previous investigations by Correa-Martinez et al. addressing the spread of VRE in the hospital environment[41], representative isolates were selected for the respective STs. *E. faecalis* ATCC 29212 and *E. faecium* ATCC 6057 served as vancomycin-susceptible reference strains; *E. faecium* ATCC 6057 was also used as a potential vesiductant.

For general cultivation purposes, all bacteria were grown on Columbia blood agar (Thermo Scientific, Waltham, USA) and incubated at 37 °C for approximately 24 h.

They were further cultured either in LB (Carl Roth, Karlsruhe, Germany) or on LB agar (Carl Roth) to investigate vesiculation under stressful growth conditions[22].

Determination of phenotypical and genotypical resistance of bacterial isolates

Phenotypic resistance was identified by detecting the minimum inhibitory concentrations (MICs). Vancomycin and teicoplanin test strips (Liofilchem, Roseto degli Abruzzi, Italy) were placed on Mueller–Hinton agar (Thermo Scientific) to which a bacterial suspension in 0.9% NaCl (B. Braun, Melsungen, Germany) with a McFarland value of 0.5, measured by using the Densimat (Biomérieux, Marcy-l'Étoile, France) densitometer, was applied. After incubation at 35 °C for 24 h, MICs were compared to the clinical breakpoints given by the European Committee on Antimicrobial Susceptibility Testing at its latest version[42]. The genotypic resistance of every isolate was confirmed using the eazyplex® VRE platform (AmplexDiagnostics, Gars Bahnhof, Germany).

MV preparation

MV preparation from VRE isolates was carried out as previously described with slight modifications[16]. Bacterial isolates were grown in 500 ml LB at 37 °C with shaking at 180 rpm until stationary phase (16 h). After centrifugation (5,525 g, 4 °C, 20 min) in the Allegra™ 25R centrifuge with a TS-5.1–500 rotor (Beckman Coulter, Brea, USA), supernatants were passed through a 0.22 µm sterile filter (Merck, Darmstadt, Germany). MVs were pelleted by ultracentrifugation (235,000 g, 4 °C, 2 h) using the Optima™ XPN-80 ultracentrifuge with a Type 45 Ti rotor (Beckman Coulter) and subsequently resuspended in 20 mM TRIS–HCl buffer (pH 8.0). For additional preparation of MVs under vancomycin stress (MVs/VAN), LB of the main culture was supplemented with 30 µg/ml vancomycin (Demo Pharmaceuticals, Hallbergmoos, Germany and Hikma Pharmaceuticals, London, UK), which is in the range of therapeutic serum concentrations[43]. Sterility was tested by spreading 10 µl of sample on Columbia blood agar and incubating it at 37 °C for at least 24 h. MVs were stored at 4 °C until further use.

Characterisation of MVs via NTA

MVs were further characterised by using the NanoSight NS300 (Malvern Panalytical, Malvern, UK) with a 488 nm laser for NTA to determine particle size and concentration. Prior to application of NTA, the samples were diluted in phosphate buffered saline (PBS) (pH 7.4) (Sigma-Aldrich, St. Louis, USA). Measurements were performed according to the manufacturer's recommendations and consisted of five runs lasting 60 s each at the following settings: camera level 16, temperature 25 °C, syringe pump speed 30 and detection threshold 5. Data were analysed using version 3.4.4 of NanoSight NTA 3.4 software (Malvern Panalytical). When reporting results, the program's standard evaluation was used. Exemplifying our overall experiments, we characterised three biological replicates of MVs/VAN derived from ST80 and ST117 (Fig. 2).

PCR of MVs

A standard PCR was used to determine whether MVs/VAN of ST80 and ST117 contained DNA fragments of *vanA*, *vanB* or *ddl*. *Ddl*, as a species-specific gene for *E. faecium*, served as a control. Samples were additionally treated with DNase to degrade extravesicular DNA. Two different test conditions were conducted, each in 100 µl total reaction volume. Vesicles were incubated with 4 U DNase I (New England Biolabs, Ipswich, USA) for 10 min at 37 °C and separately with 8 U DNase I for 30 min at 37 °C. DNase I was inactivated at 75 °C for 10 min. Samples with initial extravesicular dsDNA concentrations over 45 ng/µl were diluted in 20 mM TRIS–HCl buffer (pH 8.0) prior to DNase treatment. For this purpose, dsDNA concentration was determined using the Qubit™ 1X dsDNA High Sensitivity (HS) Assay Kit (Invitrogen, Waltham, USA) following the application instructions. Relative fluorescence units were measured by the DS-11 FX + Spectrophotometer/Fluorometer (DeNovix, Wilmington, USA).

MVs were heat-lysed at 95 °C for 10 min prior to PCR. For each reaction, the PCR master mix consisted of 10.17 µl Milli-Q® water (Merck), 12.5 µl REDTaq® ReadyMix™ (Sigma-Aldrich), and 0.67 µl of forward and reverse primers (Sigma-Aldrich) each (initial concentration of 30 µM). Primers (Table 2) were either used from the literature (*vanA*[44], *ddl*[45]) or designed using Primer-BLAST[46] (*vanB*; accession number AF550667.1). 2 µl of sample in a total reaction volume of 25 µl underwent the following PCR protocol in the T100™ Thermal Cycler (Bio-Rad, Hercules, USA): initial denaturation at 94 °C for 3 min, 30 PCR cycles (denaturation at 94 °C for 1 min, primer annealing at 54 °C for 1 min and elongation at 72 °C for 1 min), and final elongation at 72 °C for 7 min[47]. Milli-Q® water served as a negative control, while bacterial DNA of VRE isolates functioned as a positive control. For bacterial DNA extraction, a single VRE colony was suspended in 5% Chelex® 100 (Bio-Rad) and incubated at 100 °C for 10 min. After centrifugation (13,400 g, 19 °C, 3 min) using the Centrifuge 5418 R with the FA-45-18-11 rotor (Eppendorf, Hamburg, Germany), the supernatant was removed and used as PCR template. PCR reaction products were subsequently transferred to a 1.5% agarose gel (Carl Roth) and separated at 80 V for 2 h, according to PCR product length, in 0.5 TBE running buffer (Merck) in the DNA Sub Cell (Bio-Rad). A 100 bp DNA ladder (Invitrogen) was used as size marker. The gels were stained in 0.5 µg/ml concentrated ethidium bromide solution (Bio-Rad) and analysed with the Gel Doc™ 2000 (Bio-Rad).

WGS of VRE isolates and MVs

WGS of the donor isolates was performed on the MiSeq platform (Illumina, San Diego, USA) using a Nextera XT library according to the manufacturer's recommendations. The resulting reads were de novo assembled using Velvet v1.1.04 and trimmed to an average quality of 30 in a window of 20.

DNA was isolated from MVs/VAN of VRE isolates ST80 and ST117, both untreated and post 4 U DNase I treatment, using the Monarch® Genomic DNA Purification Kit (New England Biolabs). The protocol for genomic DNA purification from Gram-positive bacteria was applied according to the manufacturer's instructions, omitting the lysozyme treatment. For WGS, samples were adjusted to dsDNA concentrations above 20 ng/µl and not sequenced if below.

WGS of the DNA isolated from the untreated MVs/VAN of ST80 and ST117 was performed using the Pacific Biosciences Sequel Ile platform (Pacific Biosciences, Menlo Park, USA). For sequencing on the platform, a library was created with the SMRTbell Express Template Prep Kit 2.0 (Pacific Biosciences) according to the manufacturer's recommendations. For de novo assembly, the resulting HiFi long-reads were prepared using the microbial assembly pipeline within the SMRT Link software version 11 (Pacific Biosciences).

The assembled contigs of both VRE isolates and MVs were screened for the presence of the *vanA* and *vanB* cassettes using the AMRFinderPlus[48] implementation in Ridom SeqSphere+[49]. The contigs containing the *van* cassettes were extracted from the assemblies of MVs and annotated using Bakta v1.7.0[50]. Subsequently, the annotated contigs were pairwise aligned with the assemblies of their respective donor isolates using Mauve v2.4.0[51]. The resulting alignments were manually examined for sequence identity and completeness of the *van* cassettes.

CFU determination of VSE

To estimate the number of living bacteria of the potential vesiductant *E. faecium* ATCC 6057 under our specific test parameters, an overnight grown culture (16 h, 37 °C, 180 rpm) was adjusted to an OD_{600} of 0.1. OD_{600} was ascertained by the DS-11 FX + Spectrophotometer/Fluorometer (DeNovix). After serial dilution in PBS, 50 µl were plated on Columbia blood agar by using glass beads. After 24 h of incubation at 37 °C, CFU/ml was calculated as the mean ± SD of three dilution levels. The experiment was repeated three times.

Preparation of sterile bacterial supernatants

Liquid cultures of VRE isolates ST80 and ST117 were grown overnight (16 h) in LB at 37 °C with shaking at 180 rpm. Bacteria were removed by centrifugation (5,525 g, 4 °C, 5 min) in the Allegra™ 25R centrifuge with a TS-5.1–500 rotor (Beckman Coulter), supernatants were collected and sterile filtered using a 0.2 µm syringe filter (Corning, Corning, USA). A sterile control was set up on LB agar.

Exposure of VSE to MVs and bacterial supernatant

Exposure experiments were performed with MVs/VAN and supernatants of VRE isolates ST80 and ST117 adapted from previously published methods[16,21]. MVs were used for exposure within the first eight days of storage, whereas the supernatants were freshly prepared prior to the experiment. Initially, a single colony of strain *E. faecium* ATCC 6057 was cultivated overnight (16 h) in LB at 37 °C with shaking at 180 rpm. The OD_{600}

Gene	Primer pair [a]	Sequence (5'–3')	PCR product length [bp]	Source
vanA	$vanA_1$	GGGAAAACGACAATTGC	732	[44]
	$vanA_2$	GTACAATGCGGCCGTTA		
vanB	$vanB_1$	TTCGATCCGCACTACATCGG	648	This study
	$vanB_2$	GCCTTTTTCCGGCTCGTTTT		
ddl	ddl_1	GAGACATTGAATATGCCTTATG	560	[45]
	ddl_2	AAAAAGAAATCGCACCG		

Table 2. Oligonucleotide primers. [a] $_1$Forward primer; $_2$Reverse primer.

of the bacterial culture was adjusted to 0.1 by dilution in LB broth. Exposure of VSE to MVs was performed at two concentrations: 1,000 and 10,000 particles per bacterium respectively. 500 µl LB, 100 µl bacterial culture and MVs at the respective concentrations were combined. The mixture was replenished with 20 mM TRIS–HCl buffer (pH 8.0) to a total volume of 1 ml.

For exposure to supernatants, 100 µl adjusted bacterial culture was combined with 900 µl supernatant. After incubation at 37 °C for 4 h without shaking, followed by 4 h with shaking at 180 rpm, 24 ml of fresh LB was added to the culture. After overnight cultivation (16 h) at 37 °C with shaking at 180 rpm, bacteria were pelleted by centrifugation (1,500 g, 25 °C, 2 min) in the Allegra™ 25R centrifuge with a TS-5.1–500 rotor (Beckman Coulter) and resuspended in 1 ml LB. 100 µl each were plated five times on VRE selective agar (Bio-Rad) by using glass beads. The bacteria were further serially diluted in PBS and spread on Columbia blood agar. Each experiment was repeated twice.

Ethical approval

Human participants were not involved in the present study. A statement of the ethical committee approving the experiments was therefore not included.

Data availability

Whole genome sequencing data of analysed VRE genomes were submitted to NCBI database (BioProject ID: PRJNA980408; https://www.ncbi.nlm.nih.gov/bioproject/PRJNA980408).

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Author contributions

The project was conceptualised by S.K. J.L., J.S.S. and S.K. developed the methodology, J.L. and K.Lv.W. conducted the investigations and J.L., N.S. and S.K. contributed to the data analysis. The original draft was written by J.L. and S.K. and revised from J.S.S., N.S., A.M. and K.Lv.W. A.M. and S.K. provided the required resources. Supervision and project administration was carried out by S.K. with support from J.S.S. All authors reviewed and approved the final version of the manuscript.

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Competing interests

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Additional information

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