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ORIGINAL RESEARCH

Characterization of a Tigecycline-, Linezolid- and Vancomycin-Resistant Clinical *Enteroccoccus faecium* Isolate, Carrying *vanA* and *vanB* Genes

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ABSTRACT

Introduction: Increasing incidence of *Enterococcus faecium* resistant to key antimicrobials used in therapy of hospitalized patients is a worrisome phenomenon observed worldwide. Our aim was to characterize a tigecycline-, linezolid- and vancomycin-resistant *E. faecium* isolate with the *vanA* and *vanB* genes, originating from a hematoma of a patient hospitalized in an intensive care unit in Poland.

Methods: Antimicrobial susceptibility (a broad panel) was tested using gradient tests with predefined antibiotic concentrations. The complete genome sequence was obtained from a mixed assembly of Illumina MiSeq and Oxford Nanopore's MinION reads. The genome was analyzed with appropriate tools available at the Center for Genomic Epidemiology, PubMLST and GenBank. Transferability of oxazolidinone, tigecycline and vancomycin resistance genes was investigated by conjugation, followed by PCR screen of transconjugants for antimicrobial resistance genes and plasmid *rep* genes characteristic for the donor and genomic sequencing of selected transconjugants.

Results: The isolate was resistant to most antimicrobials tested; susceptibility to daptomycin, erythromycin and chloramphenicol was significantly reduced, and only oritavancin retained the full activity. The isolate represented sequence type 18 (ST18) and carried *vanA*, *vanB*, *poxtA*, *fexB*, *tet*(L), *tet*(M), *aac(6')-aph(2'')*, *ant(6)-Ia* and *ant(6')-Ii*. The *vanA*, *poxtA* and *tet*(M) genes located on ~ 40-kb plasmids were transferable by conjugation yielding transconjugants resistant to vancomycin, linezolid and tigecycline. The substitutions in LiaS, putative histidine kinase, SulP, putative sulfate transporter, RpoB and RpoC were potential determinants of an elevated daptomycin MIC. Comparative analyses of the studied isolate with *E. faecium* isolates from other countries revealed its similarity to ST18 isolates from Ireland and Uganda from human infections.

Conclusions: We provide the detailed characteristics of the genomic determinants of antimicrobial resistance of a clinical *E. faecium* demonstrating the concomitant presence of both

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vanA and *vanB* and resistance to vancomycin, linezolid, tigecycline and several other compounds and decreased daptomycin susceptibility. This isolate is a striking example of an accumulation of resistance determinants involving various mechanisms by a single hospital strain.

Keywords: *Enterococcus faecium*; HAI; LRE; Plasmid; VRE

Key Summary Points

Why carry out this study?

Enterococcus faecium is one of the two clinically important enterococcal species, causing an increasing number of hospital-acquired infections (HAIs). Although isolates with both *vanA* and *vanB* vancomycin-resistance determinants as well as isolates resistant to "last resort" drugs linezolid and tigecycline have been reported, they remain rare or even very rare, and this combination of resistance phenotypes is very unusual.

The study was performed on a clinical *E. faecium* isolate demonstrating a concomitant resistance to vancomycin, linezolid and tigecycline and reduced susceptibility to daptomycin. We aimed at answering the question of what the character and possible origins of resistance determinants to antimicrobials in this isolate were and what the possibility of their further horizontal dissemination.

What was learned from the study?

We established the complete genome of the isolate, and on this basis we identified it as a representative of sequence type 18, typical for hospital meroclone of *E. faecium*. The *vanA* determinant was located in Tn*1546* in a 39.5-kb transferable plasmid while *vanB* resided in Tn*1549* on a chromosome. Linezolid and tigecycline resistance was due to the acquisition of a 42.4-kb transferable plasmid, carrying *poxtA* and *tet*(M)/*tet*(L) genes.

Although *E. faecium* is well known for accumulation of various antimicrobial resistance determinants, the studied isolate was a particularly striking example of this phenomenon. Genomic analyses revealed different mechanisms beyond acquisition of a plethora of antimicrobial resistance determinants, in several cases facilitated by mobile genetic elements.

Appearance of a strain with extremely limited treatment options and ability to further disseminate genes conferring resistance to glycopeptides, linezolid and tigecycline are particularly worrisome.

INTRODUCTION

Enterococci, usually harmless human and animal commensals, are also causative agents of serious hospital-associated infections (HAIs). Their intrinsic and acquired resistance to several antimicrobials often limits treatment options. This is particularly observed for *Enterococcus faecium*, a species currently demonstrating an increasing prevalence in HAIs [1]. Strains of *E. faecium* infecting hospitalized patients almost exclusively belong to a large meroclone, initially described as clonal complex 17 (CC17) [2], based on the multilocus sequence typing (MLST) approach [3]. CC17 was later split into two major lineages, 17/18 and 78, named from their main sequence types (STs) [4]. One of important early adaptations of CC17 to the hospital environment was development of resistance to aminopenicillins and ciprofloxacin [2], followed by acquisition of high-level resistance to aminoglycosides (HLAR) and glycopeptides [5]. The spread of vancomycin-resistant *E. faecium* (VR*Efm*), which is typically resistant to many other antimicrobial agents, is considered a significant epidemiological threat, and such pathogens belong to the so-called ESKAPE group, responsible for most HAIs worldwide [6]. The World Health Organization (WHO) recently listed VR*Efm* as the highest

priority Gram-positive pathogen for which new antibiotics are urgently needed [7]. Some antimicrobials, such as linezolid, daptomycin and tigecycline, are used as last-line drugs against VR*Efm*; however, resistance to these compounds has been increasingly reported [8].

The most common vancomycin-resistance phenotypes include VanA (vancomycin and teicoplanin resistance) and VanB (vancomycin resistance and teicoplanin susceptibility), determined by the *vanA* and *vanB* operons, respectively [9]. The Tn*1546*-type transposons, usually carried on Inc18 and RepA_N family conjugative plasmids, constitute basic genetic elements responsible for dissemination of *vanA* operons among clinical enterococci [10, 11], while the *vanB* gene clusters are usually present on the bacterial chromosome, most commonly within the Tn*1549*-Tn*5382* integrative conjugative elements [12, 13]. Linezolid resistance mechanisms observed in enterococci include mutational changes in 23S rRNA and ribosomal proteins L3 and L4, as well as acquisition of resistance genes, including *cfr*, *cfr*(B), *cfr*(D), *optrA* and *poxtA* [14–17]. Sporadically described tigecycline resistance is associated in enterococci with ribosomal genes mutations and an overexpression of *tet*(M) and *tet*(L) genes resulting from an increased copy number of plasmids carrying these genes [18–20]. Daptomycin resistance is also still rare in enterococcal HAIs and may appear because of mutations in cell envelope stress response regulatory pathways and genes involved in phospholipid metabolism [8]. Here, we report a clinical vancomycin-, linezolid- and tigecycline-resistant *E. faecium* isolate carrying both *vanA* and *vanB* determinants. Since, to our knowledge, no such multiresistant *vanA*-*vanB E. faecium* have been identified, we further analyzed this isolate by phenotypic tests and whole-genome sequencing (WGS).

METHODS

Bacterial Isolate and Patient Data

The 4995/20 isolate of *E. faecium* was obtained by the National Reference Centre for Susceptibility Testing (NRCST) in 2020 for verification of the resistance mechanisms. The isolate used in the current study was obtained during a routine national surveillance activity of the NRCST under the mandate of the Ministry of Health according to the national legislation relevant to human infections and infectious diseases. The study was performed in a retrospective manner with anonymization of patient data; thus, ethical approval and informed consent were not required. The strain was cultured from an infected hematoma and intra-abdominal abscess from a patient (age range, 30–39 years) who, prior to the isolation of 4995/20, had been hospitalized for > 2 months, first in cardiology, then in the intensive care unit (ICU), underwent surgery and again, in the time of strain isolation, was in the ICU. During hospitalization the patient received treatment with linezolid and tigecycline as well as piperacillin/tazobactam, metronidazol, meropenem, imipenem, cefuroxime, ciprofloxacin, levofloxacin, amikacin, colistin and trimethoprim/sulfamethoxazole but not vancomycin or daptomycin. The patient had no history of travel, hospitalization or long-term care facility stay for 6 months prior to the admission. No other *E. faecium* isolates with the concomitant resistance to vancomycin, tigecycline and linezolid were observed in the hospital.

Antibiotic Susceptibility Testing

Antimicrobial susceptibility was tested using the gradient tests with predefined antibiotic concentrations (Liofilchem, Roseto degli Abruzzi, Italy; BioMérieux, Marcy-l'Etoile, France), ComASP™ Oritavancin Test (Liofilchem, Roseto degli Abruzzi, Italy) and broth microdilution method for streptomycin (ISO 20776–1 standard). The results were interpreted according to the EUCAST clinical breakpoints [21] and the ecological cut-off (ECOFF) values (http://mic.eucast.org/Eucast2/, last accessed 12th January 2022). *Enterococcus faecalis* ATCC 29212 and *Staphylococcus aureus* ATCC 29213 were used as controls.

PCR-Based Detection of Antimicrobial Resistance and Plasmid-Specific Genes

Bacterial DNA was isolated using the Genomic DNA Prep Plus kit (A&A Biotechnology, Gdansk, Poland), and the detection of *vanA*, *vanB*, *aac(6′)-aph(2″)*, *poxtA*, *tet*(M), *tet*(L) and plasmid *rep* genes $repUS15_{DO3}$, $rep2_{pRE25}$, $repUS1_{pVEF1}$, $rep18a_{p200B}$, $rep29_{AUS0085p4}$, rep_{p4995_5}, $repUS12_{pUB110}$, rep_{p4995_6} and $rep14a_{AUS0085p5}$ was performed by PCR [22–27] in 4995/20 and/or its transconjugants with appropriate controls from the laboratory collection. Primers specific for rep_{p4995_5} and rep_{p4995_6} were designed in the current study (sequences available upon request).

Genome Sequencing and Analysis

The total DNA of 4995/20 was obtained using the Genomic Mini AX Bacteria Kit (A&A Biotechnology, Gdynia, Poland), and WGS was carried out as an external service (Genomed S.A., Warsaw, Poland) on the Illumina MiSeq Platform with the PE300 mode (Illumina Inc., San Diego, CA) and Oxford Nanopore's MinION (Oxford Nanopore Technologies, UK). Reads were trimmed with Cutadapt v 3.1 [28] and used in a mixed assembly with Unicycler v 0.4.7., which also provided a relative approximate sequencing depth for chromosomes and plasmids [29]. Complete genomic sequences were annotated with PROKKA 1.11 [30] and supplementary manual BLASTx analyses (https://blast.ncbi.nlm.nih.gov/; last accessed on the 17th of May 2023). The assembled genome was screened using services available at the Center for Genomic Epidemiology (CGE; http://www.genomicepidemiology.org; last accessed on 17 May 2023) for genes encoding antibiotic resistance (ResFinder 4.1, LRE-Finder 1.0), virulence factors (VirulenceFinder 2.0) and plasmid Rep (PlasmidFinder 2.1). For the in silico MLST, the https://pubmlst.org/organisms/enterococcus-faecium database was used (last accessed on 9 August 2023) [31]. Conjugation transfer-associated regions were analyzed by oriTFinder and MOBScan [32, 33]. The Geneious Prime v.2022.1.1 software (Biomatters, Auckland, New Zealand) was used for sequence alignments and comparisons and for reference-guided assembly of sequencing reads with the complete genome of 4995/20 as a reference. Plasmid and transposon sequences were visualized using the BLAST Ring Image Generator (BRIG, http://brig.sourceforge.net; 7 November 2022 date last accessed) [34]. Visualization of sequence comparisons was done with Artemis Comparison Tool (ACT) [35]. Searches for *E. faecium* isolates related to 4995/20 were done using the Pathogen Detection website of GenBank (https://www.ncbi.nlm.nih.gov/pathogens/; accessed on the 9th August 2023). The core-genome MLST (cgMLST) profiles were obtained using the https://pubmlst.org/organisms/enterococcus-faecium database and analyzed with GrapeTree v 1.5.0 [36]. For all the software, default parameters were used.

Conjugation

Mating experiments were performed with the *E. faecalis* OG1RF and *E. faecium* 64/3 recipients according to the procedure developed for strains with a low transfer efficiency [37]. Transconjugants were selected on BHI agar with fusidic acid (25.0 mg/l) supplemented with vancomycin (VAN, 32.0 mg/l), linezolid (LZD, 4.0 and 6.0 mg/l), tigecycline (TGC, 2.0 mg/l) or tetracycline (TET, 16.0 mg/l). Selected transconjugants were confirmed by the PFGE analysis [38] and tested for the presence of *vanA*, *vanB*, *aac(6′)-aph(2″)*, *poxtA*, *tet*(M), *tet*(L) and all nine *rep* genes present in 4995/20 using PCR. Representative transconjugants were then analyzed by establishing their susceptibility to vancomycin, teicoplanin, linezolid, chloramphenicol, tigecycline, tetracycline, streptomycin and gentamicin, and by WGS.

Accession Numbers

The assembled sequences of 4995/20 and its transconjugants have been deposited at DDBJ/ENA/GenBank in the BioProjects PRJNA766534 and PRJNA1002867, respectively.

RESULTS

Antimicrobial Susceptibility of 4995/20, General Features of its Genome and Relationships with Other Isolates of *E. faecium*

The 4995/20 *E. faecium* isolate showed resistance or reduced susceptibility to almost all compounds tested (Table 1). The PCR analysis, a standard procedure for all VRE in the NRCST, demonstrated the presence of both *vanA* and *vanB*. Mixed assembly of Illumina and MinION reads yielded seven circular replicons, including a chromosome and six plasmids (Table 2); in addition, two putative free phages (40.0 kb and 36.2 kb) were observed (data not shown). Altogether, the genome contained 3145 genes with 2928 protein-coding sequences, 6 complete rRNAs operons, 69 tRNAs genes, 4 noncoding RNA (ncRNA) genes and 126 pseudogenes. In MLST, this isolate represented ST18 of the 17/18 lineage. ResFinder revealed the presence of the *aac(6′)-aph(2″), ant(6)-Ia, aac(6′)-Ii*, *msr*(C); *tet*(L), *tet*(M), *poxtA, fexB; vanA* and *vanB* resistance genes, in concordance with observed antimicrobial resistance phenotypes and the previous PCR results for *vanA* and *vanB*. The isolate also contained genes of virulence-associated factors such as collagen adhesin (*acm*), endocarditis antigen A ($efaA_{Efm}$), enterococcal surface protein (esp_{Efm}) and hyaluronidase (hyl_{Efm}), as established by the VirulenceFinder analysis, and six plasmid replication genes, including $rep2_{\text{pRE25}}$ (in two plasmids), $rep14a_{\text{AUS0085p5}}$-like, $rep29_{\text{AUS0085p4}}$, $repUS1_{\text{pVEF1}}$, $repUS12_{\text{pUB110}}$ and $repUS15_{\text{DO3}}$, found using PlasmidFinder. Analyses with the Pathogen Detection system revealed that 4995/20 belonged to the PDS000100071 SNP group together with six isolates from Ireland, three isolates from Uganda and three isolates of an unreported origin (Supplementary Table 1). The isolates from Ireland and Uganda were obtained from human infections. All 12 isolates carried *dfrG*, absent from 4995/20, and they all had *tet*(M). In addition, the isolates from Uganda were also positive for *tet*(L). The isolates from Ireland and the isolates of an unknown origin had *vanA*; none of the PDS000100071.5 group except 4995/20 showed the presence of *vanB, poxtA* or *fexB*. The cgMLST revealed the closest linkage of 4995/20 with the EFM0469 isolate of unknown origin while isolates from Ireland and Uganda formed two separate, more distant clusters (Supplementary Fig. 1).

Vancomycin Resistance, Tn*1546* and Tn*1549* Transposons and Their Localization

The *vanA* gene was located within the truncated Tn*1546*-type transposon of the B5-type [39] on the p4995_3 plasmid (Fig. 1). The 12.6-kb segment of p4995_3 plasmid, flanked by two direct IS*1216* copies and encompassing the B5-Tn*1546* with the downstream *prgN*, $repUS1_{\text{pVEF1}}$ and *parA* genes, differed only by a single nucleotide substitution from the region present on the p1207_4 plasmid from the Polish *E. faecalis* clinical isolate 1207/04 [40]. This segment likely represents a composite transposon or even more likely a pseudo-compound transposon, PCT, typically associated with IS*1216* in enterococci [41]. This 12.6-kb putative PCT remains unique for Polish enterococci (GenBank query on the 10th of August 2023). The carrier of this presumable PCT, the p4995_3 plasmid, was 39.5 kb in size and harbored 52 probable protein-coding genes in total, including three *rep* genes, $rep2_{\text{pRE25}}$ (Inc18), $repUS1_{\text{pVEF1}}$ (Inc18) and $repUS12_{\text{pUB110}}$ (Rep1). The 5.2-kb region of p4995_3 encompassing *prgP*, $rep2_{\text{pRE25}}$, *prgN*, IS*1062* and a part of an ORF of unknown function was almost identical to its counterpart in the prototype plasmid pRE25 [42] (Supplementary Fig. 2), differing by the presence of five SNPs. No plasmids with a structure corresponding to p4995_3 were reported as yet.

The *vanB* operon was located on the chromosome in the Tn*1549*-type transposon, 33,813 bp in size (Supplementary Fig. 3). Tn*1549* in 4995/20 shared the structure with the original Tn*1549* transposon [43] but demonstrated the presence of several substitutions and 1–2 bp indels in the *vanB* operon, resulting in 98.7% identity in this region. The transposon integration site was located in a

Table 1 Antimicrobial susceptibility profile of the 4995/20 isolate of *E. faecium*

Compound	MIC (mg/l)	EUCAST breakpoint (R)	ECOFF[a]/ TECOFF[b]/PK/ PD[c]	Interpretation[d]	Determinant
Vancomycin	> 256	> 4		R	*vanA, vanB*
Teicoplanin	256	> 2		R	*vanA*
Dalbavancin	> 256	n/a	> 0.25[c]	R	*vanA*
Oritavancin	0.06	n/a	> 0.06[a,f]	S	n/p
Telavancin	2	n/a	> 0.5[a]	R	*vanA*
Linezolid	8	> 4		R	*poxtA*
Tedizolid	1.5	n/a	> 1[a]	R	*poxtA*
Tigecycline	2	> 0.25		R	[*tet*(M), *tet*(L)][e]
Tetracycline	48	n/a	> 4[a]	R	[*tet*(M), *tet*(L)][e]
Doxycycline	32	n/a	> 0.5[a]	R	[*tet*(M), *tet*(L)][e]
Minocycline	2	n/a	> 0.5[b]	R	[*tet*(M), *tet*(L)][e]
Omadacycline	0.5	n/a	> 0.25[a]	R	[*tet*(M), *tet*(L)][e]
Eravacycline	1.5	> 0.125		R	[*tet*(M), *tet*(L)][e]
Penicillin	> 256	n/a	> 16[a]	R	n/d
Ampicillin	> 256	> 8		R	n/d
Imipenem	> 64	> 4		R	n/d
Gentamicin	> 1024	> 128		HLGR	*aac(6')-aph(2")*
Streptomycin	2048	> 512		HLSR	*ant(6)*-Ia, *ant(6')*-Ii
Ciprofloxacin	> 256	> 4		R	S80I in ParC, S83I in GyrA
Levofloxacin	> 256	> 4		R	S80I in ParC, S83I in GyrA
Moxifloxacin	> 256	n/a	> 1[a]	R	S80I in ParC, S83I in GyrA
Rifampicin	> 256	n/a	> 8[a,f]	R	S491F in RpoB
Quinupristin/dalfopristin	2	> 4		S	n/p
Daptomycin	4	n/a	> 8[a]	S	See text
Chloramphenicol	64	n/a	> 32[a]	R	*fexB*
Erythromycin	1.5	n/a	> 4[a]	S	n/p
Fosfomycin	> 256	n/a	> 128[a,f]	R	Unknown

Table 1 continued

Compound	MIC (mg/l)	EUCAST breakpoint (R)	ECOFF[a]/ TECOFF[b]/PK/ PD[c]	Interpretation[d]	Determinant
Nitrofurantoin (100 µg disc)	10 mm inhibition zone	< 15 mm[f]	< 15 mm[a,f]	R	See text

[a]ECOFF, ecological cut-off (used when the EUCAST breakpoint was not available)
[b]TECOFF, tentative ECOFF
[c]PK/PD, pharmacokinetic/pharmacodynamic breakpoint; n/p, not applicable; n/a, not available; n/d, not determined; R, resistant; S, susceptible
[d]HLGR, high-level gentamicin resistance; HLSR, high-level streptomycin resistance
[e]Increased copy number
[f]Available for *E. faecalis* only

counterpart of the M23 family-peptidase gene EFAU004_02748 present in the *E. faecium* Aus0004 genome [44]. The 6-bp coupling sequence 5′-ATTATG was present at the right transposon terminus. The same Tn*1549* integration site was observed in two other *E. faecium* isolates, UW13781 and UW13763, from Germany (GCA_015365525.1 and GCA_015363845.1, respectively). These isolates represented ST564, a triple-locus variant of ST18, and belonged to a different SNP group (PDS000063135.1), thus being only distantly related to 4995/20.

Linezolid and Tigecycline Resistance and the *poxtA-fexB-tet*(M)-*tet*(L) Plasmid

The LRE Finder analysis of 4995/20 sequencing reads yielded the wild-type genes of 23S rRNA and revealed the presence of *poxtA*_1 [45] on the p4995_2 plasmid. The comparison of ribosomal protein genes *rplC*, *rplD* and *rplV* with their counterparts in the DO strain of *E. faecium* (CP003583) revealed no mutations. The only detected linezolid resistance determinant, *poxtA*, was located between two direct copies of IS*1216* (Fig. 2 and Supplementary Fig. 4). The *fexB* gene located upstream from the *poxtA*-PCT was responsible for the observed chloramphenicol resistance [46]. The whole 8.9-kb segment, consisting of *poxtA, fexB* and three copies of IS*1216*, formed a potential PCT. Very similar structures occurred also in plasmids of *E. faecium* (21.4–52.5 kb) and *Enterococcus hirae* (24.8–53.0 kb) and in chromosomes of *E. faecalis* and *S. aureus* from various sources and countries (Supplementary Table 2). These PCTs differed from 4995/20 and, among each other, by the length of sequences immediately adjacent to terminal inverted repeats of two of IS*1216* elements as well as by the presence/absence of a 904-nt deletion affecting an ORF of unknown function, located in the *fexB*-IS*1216* region (Supplementary Fig. 4).

No mutations were observed in the *rpsJ* gene and 5′ untranslated region (UTR) of *tet*(M), described as determinants of tigecycline resistance in enterococci [18–20, 47]. The *tet*(M) and *tet*(L) tetracycline-resistance genes, together with the MOB_V gene, were included in a 9.5-kb structure flanked by direct copies of IS*1216*, also a potential PCT (Fig. 2 and Supplementary Fig. 4), commonly found on enterococcal plasmids, 18.5–149.5 kb in size, reported to GenBank (Supplementary Table 3). The most similar PCT in the pAT456-d plasmid of *E. faecium* differed from its counterpart in 4995/20 by four nucleotide substitutions and a 3-nt indel in *tet*(M); PCTs in other enterococcal plasmids demonstrated differences in length of the sequences adjacent to terminal repeats of one of IS*1216* (Supplementary Fig. 4), similarly to the situation described above for the *fexB-poxtA-*

Table 2 Genome of 4995/20 isolate of *E. faecium*

Contig	Size (bp)	Copy number[a]	%GC	CDSs[b]	AMR gene(s)[c]	Virulence-associated genes[d]	Plasmid *rep* type[e]	ISs[f]
Chromosome	2 726 121	1.0	38.3	2522	*aac(6′)-Ia, vanB* operon, *msr(C)*	*acm* $efaA_{Efm}$ esp_{Efm}	n/a	n/d
Plasmids								
p4995_1	282 637	3.4	35.8	298	*ant(6')-Ia, aac(6')-aph(2'')*	hyl_{Efm}	$repUS15_{DO3}$	IS*16* IS*256* (3) IS*1062* (2) IS*1216* (2) IS*1251* IS*1252* (5) IS*1297* IS*1476* (3) IS*1485* (2) IS*Ef1* (3) IS*Efa4* (2) IS*Efa5* (2) IS*Efa7* IS*Efa8* IS*Efa11* (2) IS*Efa13* IS*fm1* IS*Rob1* (4) IS*Spn10* (2)
p4995_2	42 382	10.5	34.7	52	*poxtA, fexB, tet*(M), *tet*(L)	None	$rep2_{pRE25}$ $rep29_{AUS0085p4}$ $rep14a_{AUS0085p5}$	IS*1062* IS*1216* (7)
p4995_3	39 491	1.9	35.9	52	*vanA* operon	None	$rep2_{pRE25}$ $repUS1_{pVEF1}$ $repUS12_{pUB110}$	IS*1062* IS*1216* (7) IS*1251* IS*1485*
p4995_4	11 625	11.9	32.6	13	None	None	$rep18a_{p200B}$	IS*Efa4*
p4995_5	4 461	12.1	31.9	4	None	None	Unknown (Rep_3)	None
p4995_6	2 056	13.4	37.7	4	None	None	Unknown (Rep_2)	None

n/a not applicable, *n/d* not determined
[a]Based on the sequencing depth
[b]CDSs, coding DNA sequences
[c]Established using ResFinder
[d]Established using VirulenceFinder
[e]Established using PlasmidFinder
[f]Number of copies, if different from one, given within the brackets

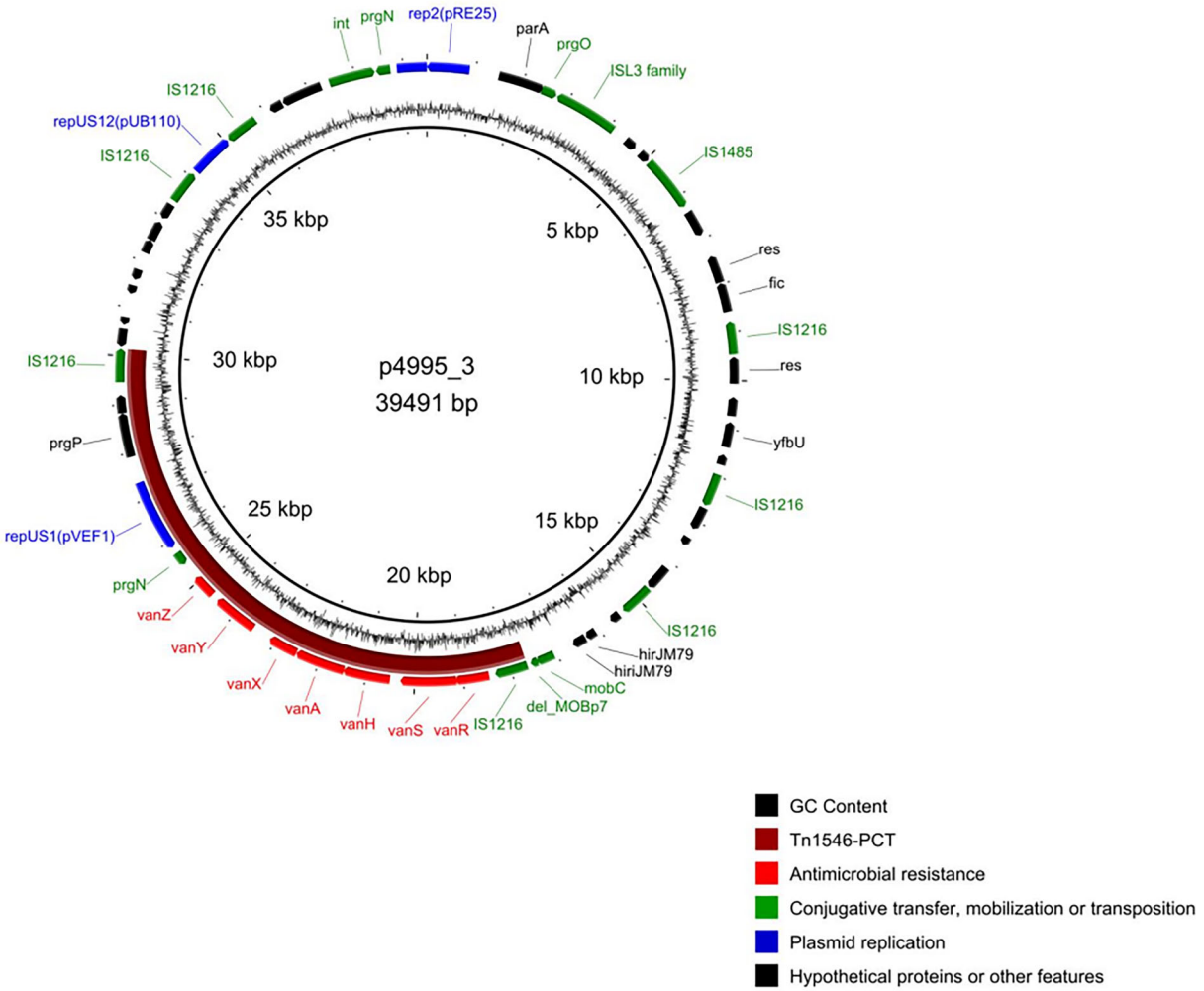

Fig. 1 The p4995_3 plasmid map. Inner circle, backbone of plasmid; outer circle, CDS with predicted functions marked with colors as in the legend; middle circle, CG skew. The putative PCT containing Tn*1546* marked in purple

PCTs. Based on sequencing depth, the p4995_2 plasmid had approximately 10 copies/cell (Table 2), resulting in tigecycline resistance [20].

The p4995_2 plasmid (42.4 kb; Fig. 2), carrying *poxtA, fexB, tet*(M) and *tet*(L), had 52 probable protein-coding genes, including three *rep* genes, $rep2_{pRE25}$ (Inc18), $rep29_{Aus0085p4}$ (Rep_3) and a 5′-truncated $rep14a_{AUS0085p5}$ (Rep_trans). p4995_2 also carried two mobilization genes belonging to MOBv family, with *oriT* sequence adjacent to one of these genes, and a single gene of MOB_{P7} family. Similarly to p4995_3, p4995_2 represented a unique mosaic structure, composed of segments separated by IS*1216*. The 5.8-kb part of p4995_2 surrounding the $rep2_{pRE25}$ gene shared an extensive similarity with the 6.9-kb region in p4995_3, differing by the presence of IS*1062* in p4995_3 (Supplementary Fig. 2). As described above, most of this region in p4995_3 was almost identical to the region *prgP*-$rep2_{pRE25}$-*prgN*-IS*1062* in pRE25.

Conjugative Transfer of Vancomycin, Linezolid and Tigecycline Resistance

Transconjugants were obtained only with the *E. faecium* 64/3 as a recipient and selection for TGC, TET or VAN; selection on LIN regardless of its concentration repeatedly yielded no transconjugants. Obtained transconjugants

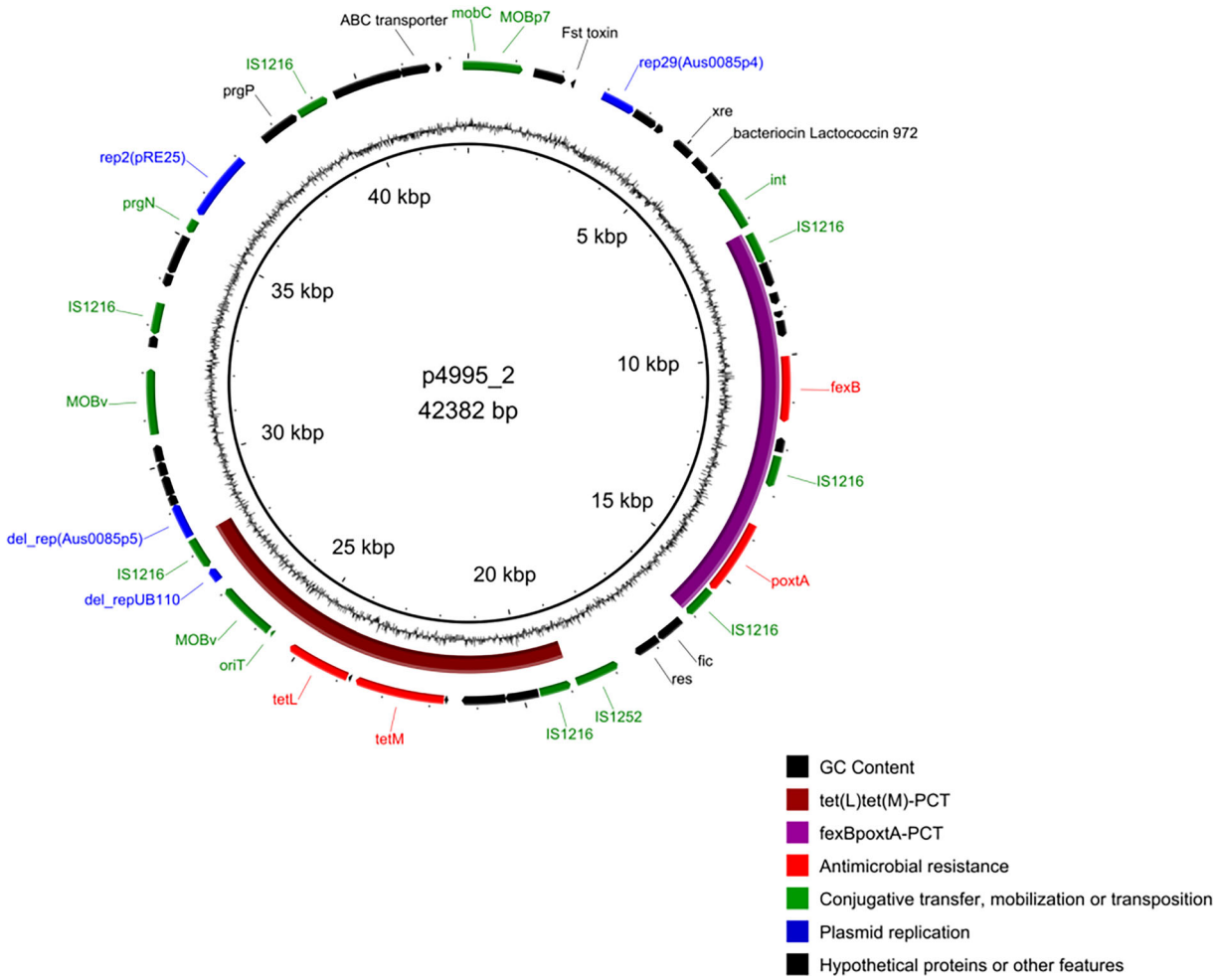

Fig. 2 The p4995_2 plasmid map. Inner circle, backbone of plasmid; outer circle, CDS with predicted functions marked with colors as in the legend; middle circle, CG skew. The putative PCTs containing the *tet*(L) and *tet*(M) and the *fexB* and *poxtA* genes marked in purple and brown, respectively

were characterized by PCR detection of *vanA*, *vanB*, *poxtA*, *tet*(M), *tet*(L) and *aac(6′)-aph(2″)* and all nine plasmid *rep* genes found in 4995/20 as well as by WGS and antimicrobial susceptibility testing for selected representatives (Table 3). Selection on VAN yielded transconjugants TC64_3 × 4995_20_VAN with an efficiency of 2.5×10^{-4}, harboring genes characteristic for the p4995_3 plasmid, namely *vanA*, $repUS1_{pVEF1}$, $repUS12_{pUB110}$ and, with an exception of a single transconjugant, $rep2_{pRE25}$. All transconjugants selected on TGC (TC64_3 × 4995_20_TGC; efficiency of 1.6×10^{-7}) and on TET (TC64_3 × 4995_20_TET; efficiency of 1.5×10^{-4}) carried the *tet*(M), *tet*(L) and *vanA* genes. Moreover, most of them (19 of 28) contained *poxtA*, and five showed the presence of *aac(6′)-aph(2″)*. All TGC/TET transconjugants were positive for $rep2_{pRE25}$ and $repUS1_{pVEF1}$. All *poxtA*-positive transconjugants had $rep29_{AUS0085p4,}$ and 16 of them harbored $repUS12_{pUB110}$. In addition, $repUS15_{DO3}$ was detected in 15 transconjugants, all of which were positive for *aac(6′)-aph(2″)* in the original p4995_1 located 86.1 kb apart. All 42 analyzed VAN and TGC/TET transconjugants, except for two selected on VAN, harbored $rep_{p4995\_5,}$ and five transconjugants selected on TIG/TET

Table 3 Antimicrobial resistance genes, plasmid *rep* genes, STs and antimicrobial resistance profiles of the transconjugants of the 4995/20 isolate

Strain/isolate/transconjugants	*aac(6')-aph(2")*	*ant(6)*-Ia	*aac(6')*-Ii	*msr*(C)	*fexB*	*poxtA*	*tet*(M)	*tet*(L)	*vanA*	*vanB*
Antimicrobial resistance genes[A]										
4995/20 (localization)^	+ (p1)	+ (p1)	+ (ch)	+ (ch)	+ (p2)	+ (p2)	+ (p2)	+ (p2)	+ (p3)	+ (ch)
64/3 (CP012522.1)^	–	–	+	+	–	–	–	–	–	–
TC64_3 × 4995_20_VAN (14)	–	ND	ND	ND	ND	–	–	–	14	–
TC64_3 × 4995_20_V3	–	–	+	+	–	–	–	–	+	–
TC64_3 × 4995_20_TIG (4)	–	ND	ND	ND	ND	4	4	4	4	–
TC64_3 × 4995_20_LTV6	–	–	+	+	+	+	+	+	+	–
TC64_3 × 4995_20_LTV7	–	–	+	+	+	+	+	+	+	–
TC64_3 × 4995_20_TET (24)	5	ND	ND	ND	ND	15	24	24	24	–
TC64_3 × 4995_20_TV8 (localization)^	–	–	+ (ch)	+ (ch)	–	–	+ (p1)	+ (p1)	+ (p1)	–

Strain/isolate/transconjugants	RepA_N	Inc18		Rep_3			Rep1	Rep_2	Rep_trans
	repUS15$_{DO3}$	*rep2*$_{pRE25}$	*repUS1*$_{pVEF1}$	*rep18a*$_{p200B}$	*rep29*$_{AUS0085p4}$	*rep*$_{p4995\_5}$	*repUS12*$_{pUB110}$	*rep*$_{p4995\_6}$	*rep14a*$_{AUS0085p5}$
Plasmid *rep* genes[B]									
4995/20 (localization)^	+ (p1)	+ (p2, p3)	+ (p3)	+ (p4)	+ (p2)	+ (p5)	+ (p3)	+ (p6)	+ (p2)
64/3 (CP012522.1)^	–	–	–	–	–	–	–	–	–
TC64_3 × 4995_20_VAN (14)	–	13	14	–	–	12	14	–	1
TC64_3 × 4995_20_V3	–	+	+	–	–	+	+	–	+
TC64_3 × 4995_20_TIG (4)	–	4	4	3	4	4	1	–	2
TC64_3 × 4995_20_LTV6	–	+	+	–	+	+	–	–	–
TC64_3 × 4995_20_LTV7	–	+	+	+	+	+	–	–	–
TC64_3 × 4995_20_TET (24)	15	24	24	2	15	24	15	–	22
TC64_3 × 4995_20_TV8 (localization)^	–	+ (p1)	+ (p1)	–	–	+ (p2)	–	–	+ (p1)

Table 3 continued

Strain/isolate	ST	Streptomycin	Gentamicin	Chloramphenicol	Linezolid	Tigecycline	Tetracycline	Vancomycin	Teicoplanin
STs and MIC values (mg/l) for selected transconjugants submitted for WGS[C]									
4995/20	18	2048 (HLSR)	> 1024 (HLGR)	64 (R)	8 (R)	2 (R)	48 (R)	> 256 (R)	256 (R)
64/3	21	96 (S)	≤ 8 (S)	8 (S)	4 (S)	0.023 (S)	≤ 1 (S)	≤ 1 (S)	0,5 (S)
TC64_3 × 4995_20_V3	21	32 (S)	4 (S)	8 (S)	4 (S)	0.03 (S)	0.25 (S)	256 (R)	32 (R)
TC64_3 × 4995_20_LTV6	21	32 (S)	2 (S)	32 (S)	8 (R)	8 (R)	64 (R)	32 (R)	16 (R)
TC64_3 × 4995_20_LTV7	21	32 (S)	2 (S)	32 (S)	8 (R)	8 (R)	64 (R)	32 (R)	32 (R)
TC64_3 × 4995_20_TV8^	21	32 (S)	8 (S)	8 (S)	4 (S)	2 (R)	64 (R)	256 (R)	16 (R)

[A]+ positive; −, negative; ch, chromosome; p1, p4995_1; p2, p4995_2; p3, p4995_3; ^, the complete genome sequence available; TC_TIG, TC_TET and TC_VAN, transconjugants obtained with TIG, TET and VAN selection, respectively (the number of representatives within brackets); ND, not determined

[B]+, positive; −, negative; p1, p4995_1; p2, p4995_2; p3, p4995_3; p4, p4995_4; p5, p4995_5; p6, p4995_6; ^, the complete genome sequence available; TC_TIG, TC_TET and TC_VAN, transconjugants obtained with TIG, TET and VAN selection, respectively (the number of representatives within brackets)

[C]Interpretation provided within the brackets. HLSR, high-level streptomycin resistance; HLGR, high-level gentamicin resistance; R, resistant; S, susceptible; ^, complete genome sequence available

contained $rep18a_{p200B}$ characteristic for p4995_4. The rep_{p4995_6} gene was not detected in any transconjugant.

The results of WGS analysis of a representative transconjugant TC64_3 × 4995_20_V3 selected on VAN were consistent with an acquisition of p4995_3 and p4995_5. This transconjugant demonstrated vancomycin and teicoplanin resistance but remained susceptible to tetracycline, tigecycline and linezolid. The WGS and antimicrobial susceptibility testing of two representative TGC transconjugants (TC64_3 × 4995_20_LTV6 and TC64_3 × 4995_20_LTV7) indicated an acquisition of both p4995_2 and p4995_3. A complete genomic sequence of the TC64_3 × 4995_20_TV8 transconjugant, which was selected on TET and carried *tet*(M), *tet*(L) and *vanA* but lacked *poxtA*, revealed a 50.2-kb recombinant plasmid, harboring all three resistance genes as well as $rep2_{pRE25}$, $repUS1_{pVEF1}$ and $rep14a_{AUS0085p5}$. This plasmid was composed of parts of p4995_2 and p4995_3 (Supplementary Fig. 6). This isolate also contained the intact p4995_5.

Decreased Susceptibility to Daptomycin

Susceptibility testing of 4995/20 resulted in MIC of 4 mg/l for daptomycin, which represented a relatively high value, considering that the EUCAST epidemiological cut-off value for *E. faecium* equals 8 mg/l. The genome of 4995/20 was searched for known and potentially novel resistance mutations in genes of 43 proteins associated with daptomycin resistance in *E. faecium* [48]. Two amino acid substitutions were detected, including the T120A substitution in LiaS, a putative histidine kinase of the LiaFSR regulatory system, and the S340L substitution in SulP, a putative sulfate transporter. Moreover, the isolate had the S491F mutation in RpoB (see also below) and the T641K mutation in RpoC, also proposed to be involved in daptomycin resistance [49].

Resistance to Other Antimicrobials

The isolate demonstrated resistance to penicillin, ampicillin and fluoroquinolones

(Table 1), a phenotype typical for hospital *E. faecium*, and carried *aac(6′)-aph(2″), ant(6)-Ia* and *ant(6′)-Ii,* responsible for high-level resistance to aminoglycosides. Analysis of *rpoB* in the search for rifampicin resistance mutations revealed the S491F change in the deduced amino acid RpoB sequence. The same mutation was seen in rifampicin-resistant isolates of *E. faecium* in Australia and New Zealand [49]. The studied isolate was also resistant to fosfomycin but lacked any *fos* genes, including *fosB3* and *fosX*, described as fosfomycin resistance determinants in enterococci [50, 51], and had the wild-type *murA* gene, whose mutation resulted in fosfomycin resistance in *E. faecium* [52]. Analysis of two nitroreductase genes present in the 4995/20 genome, the counterparts of the RS06170 and RS12585 loci in the DO genome, revealed mutations Q48K and T207M in the deduced amino acid sequence of the RS12585 counterpart as potential determinants of nitrofurantoin resistance [53], specific for 4995/20.

Other Plasmids

Besides two resistance plasmids p4995_2 and p4995_3 described above, four other plasmids were detected in 4995/20 (Table 2). The p4995_1 plasmid was a 282.6-kb megaplasmid carrying *repUS15*$_{DO3}$ (RepA_N) typical for pDO3 and pLG1 [54, 55]. p4995_1 also had mobilization gene *mobL* [56] belonging to the MOB$_{P2}$ family and genes of type IV coupling proteins (T4CP). The p4995_1 plasmid also harbored *hyl*$_{Efm}$, aminoglycoside resistance determinants *aac(6′)-aph(2″)* and *ant(6)-Ia*, regions encoding heavy-metal resistance and carbohydrate metabolism enzymes, as well as genes of four plasmid addiction systems (Phd/Doc, MazE/F, PemK/PemI and RelB/RelE). The structure of p4995_1 was unique but this plasmid shared the 149.6-kb part, located from 14.3 to 163.9 kb, with the 215.9-kb plasmid unnamed1 from the *E. faecium* VRE3382 strain isolated in Australia (CP065529.1). The 11.6-kb plasmid p4995_4 represented the *rep18a*$_{p200B}$ (Rep3_theta) replicon. Nearly identical plasmids (99.6–99.9% identity) were recently detected in *E. faecium* (Supplementary Table 4). Two remaining small plasmids, p4995_5 and p4995_6, had *rep* genes unclassified so far, and both of these genes exhibited high similarity (> 99%) to *rep* characteristic for several plasmids from *E. faecium* in GenBank (Supplementary Tables 5 and 6, respectively). Moreover, *rep*$_{p4995\_6}$ was also observed in Poland in *E. faecalis* [27].

DISCUSSION

The isolate of *E. faecium* analyzed in the current study demonstrated resistance to several drugs used in anti-enterococcal therapy, including (amino)penicillins, imipenem, aminoglycosides (high level), almost all glycopeptides, fluoroquinolones and other compounds as well as "last-resort" drugs linezolid and tigecycline. This isolate also had reduced susceptibility to daptomycin and erythromycin. Thus, therapeutic options were limited to oritavancin, a glycopeptide not affected by the presence of *vanA* and *vanB* [57], but available on the market in Poland since May 2022, and quinupristin/dalfopristin, which, because of severe side effects, is not used for treatment. The patient, hospitalized for an extensive period of time, received several various antimicrobials, including linezolid and tigecycline, prior to isolation of 4995/20. Isolations of linezolid- and tigecycline-resistant enterococci in several cases followed the therapy with these drugs [58–61]. Moreover, the use of cephalosporins and carbapenems, which were also received by the patient, represents a significant risk factor for colonization and infection of hospital patients by VRE [62–66]. The genome size of 4995/20 isolate, high load of various antimicrobial resistance genes and presence of several plasmids, including a megaplasmid, are typical features of hospital *E. faecium* distributed worldwide [67]. The isolate, as deduced from genomic data, represented ST18. Enterococci of this ST showed a wide distribution and were observed in 22 countries over a 30-year period (https://pubmlst.org/organisms/enterococcus-faecium; date last accessed 26 September 2022).

The *vanA* and *vanB* gene clusters are the most common vancomycin resistance determinants among clinical isolates of *E. faecium*. Both

genotypes were reported for Polish VRE [39, 67]. Plasmid localization of *vanA* and chromosomal localization of *vanB* are typical for these determinants [10, 68]. Although *E. faecium* carrying concomitantly *vanA* and *vanB* was observed already in 1993 in the UK [69], such strains are still reported very rarely, either as single isolates or in small clusters in various countries, including Finland, France, Greece, Saudi Arabia, Vietnam and Australia, and in different STs, such as 17, 117 and 796 [70–76], all belonging to the hospital meroclone of *E. faecium*. The *vanA* gene in 4995/20 was located in B5-type Tn*1546,* commonly observed in both *E. faecium* and *E. faecalis* VanA isolates in Poland. This variant of transposon lacks ORF1 and ORF2, which are replaced by IS*1216*. Moreover, the B5-type harbors a characteristic single-nucleotide deletion at the 9064-bp position, resulting in a frameshift in *vanY* and truncated VanY [27, 39, 40]. Tn*1546* in 4995/20 was located within a potential 12.6-kb PCT, almost identical to the one present in previously characterized *E. faecalis* [40], in agreement with proposed genetic exchange of *vanA* among hospital strains of these two species [27]. The acquisition of *vanA* by *E. faecium* results in resistance to all glycopeptides except oritavancin [57] as indeed observed for 4995/20. The *vanB* gene was associated with Tn*1549*, the most typical carrier of this determinant [68]. The localization site of Tn*1549* constitutes an important feature of a particular disseminating *vanB* clone [77, 78]. The observed Tn*1549* integration site in 4995/20 indicated that this isolate did not belong to any previously characterized Polish clones [77]. Instead, the identical localization of Tn*1549* was observed in two unrelated isolates from Germany, consistent with an acquisition of the *vanB* determinant by a chromosomal recombination event [79].

Linezolid-resistant enterococci, mostly *E. faecium*, were observed in Poland previously, demonstrating the presence of such determinants as mutations in the 23S rRNA genes and plasmid-borne *optrA* [80]. The *poxtA* gene was observed for the first time in clinical settings in Poland in the current study; however, it represented the most frequent linezolid resistance determinant in enterococci from food of animal origin in our country, constituting a potential source of such genes for clinical strains [81]. The *poxtA* gene was first described in *E. faecalis* and *S. aureus* [45], and this gene was reported then in clinical isolates of *E. faecium* in Portugal, Germany, France, Switzerland and Pakistan [59, 82–85].

Tigecycline resistance remains sporadic among *E. faecium*, and so far invasive enterococci in Polish hospitals have showed full susceptibility to this drug [86]. The 4995/20 isolate lacked typical tigecycline resistance determinants in enterococci such as mutations in the *rpsJ* gene or deletions in the 5′UTR of *tet*(M), resulting in higher expression of *tet*(M) and *tet*(L), located downstream [18–20, 47]. Observed resistance was most likely caused by an increased copy number of plasmid harboring these two genes, reaching approximately 10 copies per cell. Such a mechanism was proposed previously [20]. Moreover, our study demonstrated that the conjugative transfer of *tet*(M)/*tet*(L)-plasmid yielded transconjugants resistant to tigecycline.

The *vanA, fexB-poxtA* and *tet*(M)-*tet*(L) resistance genes were located in structures corresponding to potential PCTs [41]. Although structures very similar to both *fexB-poxtA* and *tet*(M)-*tet*(L) PCTs of the 4995/20 isolate were observed in several other isolates of enterococci and *S. aureus*, the differences in sequence length immediately adjacent to the IS*1216* termini (Supplementary Figs. 3 and 4) would be consistent with an independent formation of such PCTs rather than with a single origin of these structures.

Vancomycin, linezolid and tigecycline resistance was transferable from 4995/20 to a susceptible *E. faecium* by conjugation. Both plasmids carrying the respective resistance determinants were devoid of their own conjugation genes, and only p4995_3 carried mobilization genes and *oriT*. Transfer of such plasmids requires the presence of a helper plasmid and is frequently accompanied by such events as plasmid recombination, plasmid fusion and transfer of PCT [87, 88]. In the case of 4995/20, the p4995_1 megaplasmid might provide transfer functions for other co-resident plasmids; genes specific for p4995_1, such as

aac(6′)-aph(2″) and *repUS15*$_{DO3}$ were indeed detected in some transconjugants but only in these selected with TET. All tested transconjugants selected with VAN lacked *poxtA* and the *tet* genes; however, all transconjugants selected on TET or TGC also harbored *vanA* despite the lack of VAN selection. In addition, only some transconjugants selected on TET or TGC had *poxtA*, residing on the same plasmid as the *tet* genes. Obtaining a complete sequence of one of the tranconjugants positive for *vanA, tet*(M) and *tet*(L) and negative for *poxtA* revealed that a 50.2-kb plasmid in one of transconjugants was composed of parts of p4995_2 and p4995_3. Regions shared by these two plasmids, namely the approximately 3.5-kb part with *rep2* and one of IS*1216*, might have facilitated formation of a fusion plasmid via recombination. However, further investigations, involving establishing the complete sequences of plasmids in other transconjugants would be required to follow events during the transfer of resistance plasmids from 4995/20.

The isolate showed a borderline MIC value for daptomycin (4 mg/l), and it might be in fact clinically non-susceptible to daptomycin. A failure of high-dose daptomycin patient treatment against *E. faecium* with the MIC equal to 3 mg/l was described [89]. Recently, an association of daptomycin resistance with the *vanA* genotype was noticed among *E. faecium* isolates from Australia and New Zealand [49]. Mutations in LiaFSR, a regulatory system involved in cell envelope homeostasis, are the most common changes in daptomycin-resistant *E. faecium* [48, 90]; however, the T120A substitution in LiaS present in the 4995/20 isolate was also commonly found in unrelated *E. faecium* isolates with low daptomycin MIC values [48] and may probably occur even without daptomycin selective pressure [91]. Several substitutions in SulP were observed in *E. faecium* isolates with high daptomycin MICs [48, 90]. The S340L substitution in SulP in 4995/20, located three amino acids before the beginning of the eighth predicted transmembrane domain, is a novel mutation as yet with no obvious association with daptomycin resistance. Mutations in RpoB, RpoC and DltC were proposed as novel determinants of daptomycin resistance among *vanA-E. faecium* from Australia and New Zealand [49], and an independent appearance of the same changes in RpoB and RpoC in 4995/20 strongly supports the potential role of these determinants. Moreover, a replacement of the wild-type *rpoB* by its mutated variant in *S. aureus* leads to heteroresistance to daptomycin and vancomycin mediated by an increased expression of the *dlt* operon and thickening of the cell wall [92]. Thus, selection of resistant *rpoB* mutants by rifampicin might contribute to reducing susceptibility to daptomycin in *E. faecium*.

Our study had certain limitations. No other *E. faecium* isolates from the same center and the same period were obtained; thus, it was not possible to find a potential direct ancestor, retaining susceptibility to some of the antimicrobial compounds, which by an acquisition of resistance yielded the 4995/20 strain. This study also raises further questions about the events occurring during the conjugal transfer of enterococcal plasmids. This issue was, however, beyond the scope of the current work.

CONCLUSIONS

The 4995/20 isolate of *E. faecium* is, to our knowledge, the first reported clinical representative of this species combining resistance or decreased susceptibility to such wide range antimicrobial compounds, including drugs crucial for successful therapy of infections caused by *E. faecium*. Our genomic analyses demonstrated the presence of several resistance determinants responsible for this phenotype in the genetic background of a strain belonging to a hospital-adapted meroclone of *E. faecium*. The acquisition of resistance was associated with mutations of chromosomal genes, plasmids and transposons harboring resistance genes, increased copy number of resistance genes and yet unknown determinants. Moreover, further concomitant dissemination of vancomycin-, tigecycline- and linezolid resistance is possible because of a localization of the respective

determinants on mobilizable plasmids of mosaic structure within potential PCTs. The appearance of such a strain with extremely limited treatment options in the hospital settings is a particularly worrisome phenomenon.

Author Contribution. Ewa Wardal was involved in the conception of the study, designed the experiments, performed the experiments, analyzed and interpreted the data, and drafted the manuscript; Dorota Żabicka was involved in the conception of the study, designed the experiments, analyzed and interpreted the data; Tomasz Skalski obtained the studied isolate, performed its initial characterization, collected the relevant epidemiological data and analyzed and interpreted the data; Joanna Kubiak-Pulkowska obtained the studied isolate, performed its initial characterization, collected the relevant epidemiological data, and analyzed and interpreted the data; Waleria Hryniewicz was involved in the conception of the study and analyzed and interpreted the data; Ewa Sadowy was involved in the conception of the study, designed the experiments, performed the experiments, analyzed and interpreted the data, and drafted the manuscript. All authors critically revised and approved the final manuscript.

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Data Availability. The datasets generated during the current study are available in the GenBank repository in the BioProjects PRJNA1002867 and PRJNA1002867.

Declarations

Conflict of interest. Ewa Wardal, Dorota Żabicka, Tomasz Skalski, Joanna Kubiak-Pulkowska, Waleria Hryniewicz and Ewa Sadowy have no relevant financial or non-financial interests to disclose.

Ethical approval. The isolate used in the current study was obtained during a routine national surveillance activity of the National Reference Centre for Susceptibility Testing, under the mandate of the Ministry of Health. The study was performed in a retrospective manner with an anonymization of the patient's data; thus, ethical approval and informed consent were not required.

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