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EPIDEMIOLOGY AND SURVEILLANCE

Identification of a Novel Genomic Island Associated with *vanD*-Type Vancomycin Resistance in Six Dutch Vancomycin-Resistant *Enterococcus faecium* Isolates

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ABSTRACT Genomic comparison of the first six Dutch *vanD*-type vancomycin-resistant *Enterococcus faecium* (VRE) isolates with four *vanD* gene clusters from other enterococcal species and anaerobic gut commensals revealed that the *vanD* gene cluster was located on a genomic island of variable size. Phylogenetic inferences revealed that the Dutch VRE isolates were genetically not closely related and that genetic variation of the *vanD*-containing genomic island was not species specific, suggesting that this island is transferred horizontally between enterococci and anaerobic gut commensals.

KEYWORDS *Enterococcus faecium*, genomic island, *vanD* type, vancomycin resistance

The *vanD*-type gene cluster encoding vancomycin resistance has been identified among different bacterial species, including enterococci and anaerobic gut commensals (1, 2). Analysis of *vanD* vancomycin-resistant enterococci revealed some general characteristics. In all of these strains, (i) the D-Ala-D-Ala ligase housekeeping enzyme is not active due to mutations, insertions, and/or deletions in the *ddl* gene; (ii) vancomycin resistance is constitutively expressed due to mutations, insertions, and/or deletions of the vancomycin sensor and regulator genes *vanS* and *vanR*; (iii) the *vanD* gene cluster is chromosomally located and not shown to be transferable (1, 3, 4).

In the Netherlands, so far, only *vanA* and *vanB* types of resistance have been found in *Enterococcus faecium* isolates, but over the last 4 years, a total of six *vanD*-positive vancomycin-resistant *E. faecium* (VRE) isolates were identified in five different patients from four different hospitals in the Netherlands (Table 1). All patients received antibiotics before detection of the *vanD*-positive VRE isolate, including vancomycin in four of the five patients (Table 1). For patient D, two ampicillin-resistant *E. faecium* (ARE) (E9352 and E9353) were isolated from blood cultures before the isolation of two *vanD*-positive VRE isolates (E8429 and E9354). Whole-genome sequencing (WGS) (Illumina NextSeq and, for 3 strains, MinION nanopore [see Table S1 in the supplemental material for assembly statistics]) was performed to determine the genetic relatedness of the six *vanD*-positive VRE isolates and two ARE strains and the genomic organization of the *vanD* gene clusters. Based on allelic variation in the 1,423 core genome (cg) MLST loci using Ridom SeqSphere + v3.5.0 (5), a phylogenetic neighbor-joining (NJ) tree was

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TABLE 1 Strain characteristics and WGS sequence statistics

Strain	Resistance classification[a]	Hospital	Patient	Age (yrs)	Underlying disease	Isolation date (mo-day-yr)	Isolation site	Antibiotic use	Interval vancomycin treatment and VanD[b]	MICs (mg/liter)			Variation in Ddl[c]	Variation in VanS[c]	Size of genomic island (bp)
										Vancomycin	Teicoplanin	Ampicillin			
E7962	VRE	1	A	73	Acute myeloid leukemia	11-25-2013	Rectum	Vancomycin cotrimoxazole, ciprofloxacin	Unknown	16	<1	>256	$S_{185}F$	$S_{57}R$, $K_{169}E$, $T_{170}P$, $V_{280}L$	185,412
E8043	VRE	2	B	57	Acute lymphoid leukemia	3-24-2014	Rectum	vancomycin	Unknown	16	<1	>256	Insertion 2 aa at pos. 280	$S_{57}R$, $A_{165}V$, $V_{280}L$	168,512
E9242	VRE	2	C	18	Acute lymphoid leukemia	3-31-2016	Rectum	Cotrimoxazole, ciprofloxacin	Unknown	256	4	>256	Δ2 bp at pos. 319	$K_{308}Q$	144,229
E9352[d]	ARE					12-9-2012	Blood	Amoxicillin-clavulanic acid, cefotaxime, ciprofloxacin, metronidazole, vancomycin	NA	<1	<1	>256	Normal	NA	
E9353[d]	ARE	3	D	65	Cardiovascular disease, cholangitis	12-27-2012	Blood		NA	<1	<1	>256	Normal	NA	
E8429[d]	VRE					1-22-2013	Rectum		43 days	256	2	>256	Δ1 bp at pos. 165	$S_{57}R$, $A_{68}V$, $K_{308}Q$, $V_{280}L$	120,918
E9354[d]	VRE					1-27-2013	Rectum		48 days	256	2	>256	Δ4 aa at pos. 280	$S_{57}R$, $A_{68}V$, $K_{308}Q$, $V_{280}L$	120,844
E9641	VRE	4	E	63	Non-Hodgkin lymphoma	2-27-2017	Rectum	Ciprofloxacin, ceftazidime, piperacillin-tazobactam, vancomycin	4 mo	8	<0.5	>256	$S_{185}F$	$S_{57}R$, $V_{280}L$	168,541

[a]VRE, vancomycin-resistant *E. faecium*; ARE, ampicillin-resistant *E. faecium*.

[b]NA, not available.

[c]Indicated positions are based on amino acid (aa) sequence for Ddl (D-Ala-D-Ala ligase) and VanS (vancomycin sensor protein).

[d]These four strains were isolated from the same patient at different time points.

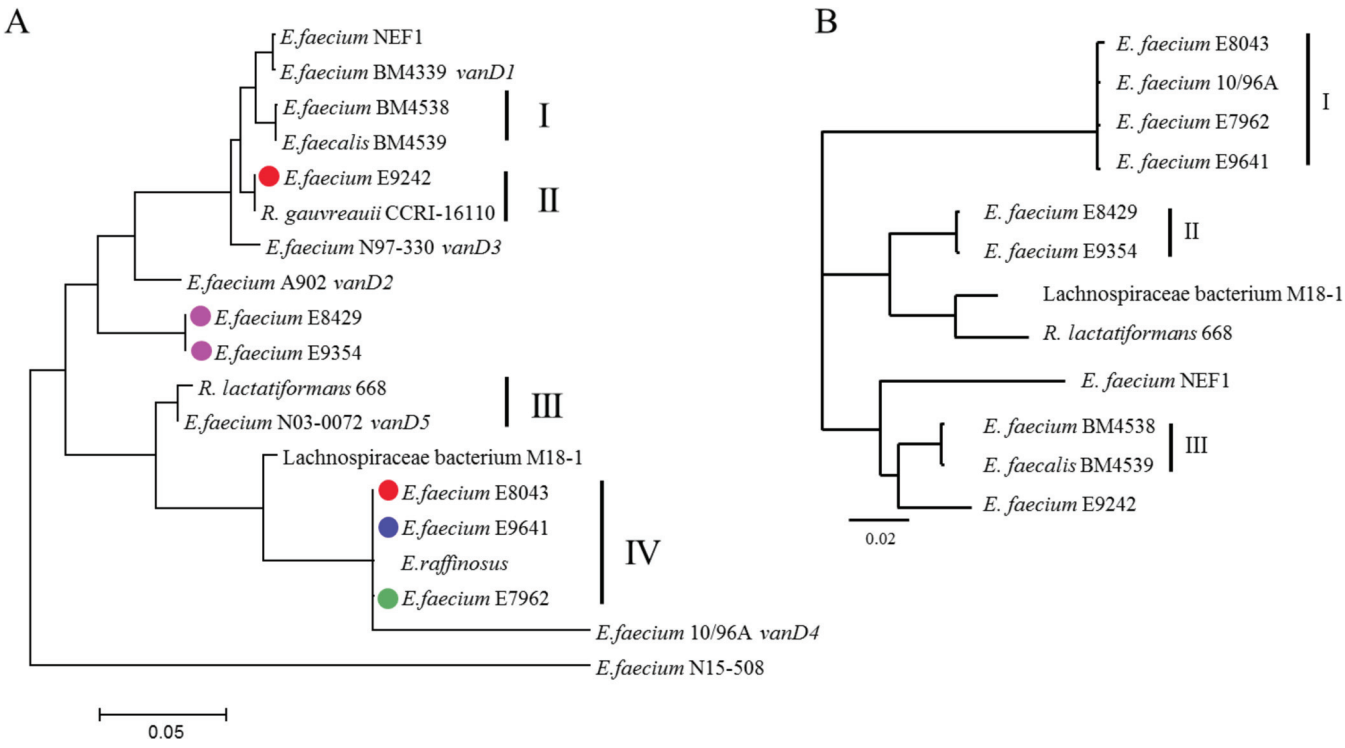

FIG 1 (A) Phylogenetic neighbor-joining (NJ) tree of the *vanD* gene cluster. The phylogenetic NJ tree was generated from a ClustalW multiple alignment of DNA sequences using MegAlign, DNAStar Lasergene v.14 software. The six Dutch isolates are indicated with green, red, purple, and blue dots (hospitals 1 through 4, respectively). Vertical lines and Roman numerals indicate clusters of highly related (>90% identity) *vanD* gene clusters, including multiple species. (B) Phylogeny of the 120- 190-kb genomic island. The phylogenetic NJ tree was generated from a ClustalW multiple alignment of the *vanD*-containing genomic island using Geneious 8.1.2 software. Vertical lines and Roman numerals indicate clusters of highly related genomic islands (>90% identity).

generated that revealed that the *vanD*-positive VRE isolates from the four different hospitals had different cgMLST profiles, indicating that they were not clonally related (see Fig. S1 in the supplemental material) and belonged to the phylogenetic group clade A1 (data not shown; 6–8). In contrast, the four strains from patient D (hospital 3), two ARE and two VRE, appeared very similar, e.g., the two *vanD*-positive VRE strains differed in no more than 3 or 4 of 1,423 loci from ARE E9352, indicating that they are genetically very closely related. All six *vanD*-positive VRE isolates contained a mutated or truncated *ddl* gene, presumably resulting in a nonfunctional housekeeping ligase (Table 1). Of note, the two VRE isolates from patient D appeared to have two different mutations in *ddl*. The $vanS_D$ vancomycin sensor gene of the six *vanD*-positive VRE isolates contained several of the previously described nonsynonymous mutations (Table 1). It has been shown that these amino acid substitutions in $VanS_D$ may lead to constitutive expression of the *vanD* gene clusters, thereby rescuing *vanD*-positive VRE with a nonfunctional housekeeping *ddl*, which would have otherwise been lethal (1, 9, 10).

Phylogenetic analysis of the complete *vanD* gene clusters from the 6 Dutch isolates and 13 *vanD* gene clusters retrieved from GenBank, including representative sequences of the 5 previously described different types of *vanD* gene clusters (*vanD1* to *vanD5*) (9, 11–14); 3 *vanD* gene clusters from other *E. faecium* strains (1, 15, 16); *vanD* gene clusters from *Enterococcus faecalis* (16), *Enterococcus raffinosus* (17), anaerobic gut commensals *Ruminococcus gauvreauii* (2), *Ruthenibacterium lactatiformans* 668 (18), and *Lachnospiraceae* bacterium M18-1 (see Table S2 in the supplemental material), revealed that the *vanD* gene clusters of the Dutch VRE isolates did not cluster together in a single branch and that *vanD* gene clusters did not cluster according to the species in which they were contained (Fig. 1A). In contrast, highly similar clusters, including multiple species, were observed. This may suggest genetic exchange of the *vanD* gene cluster

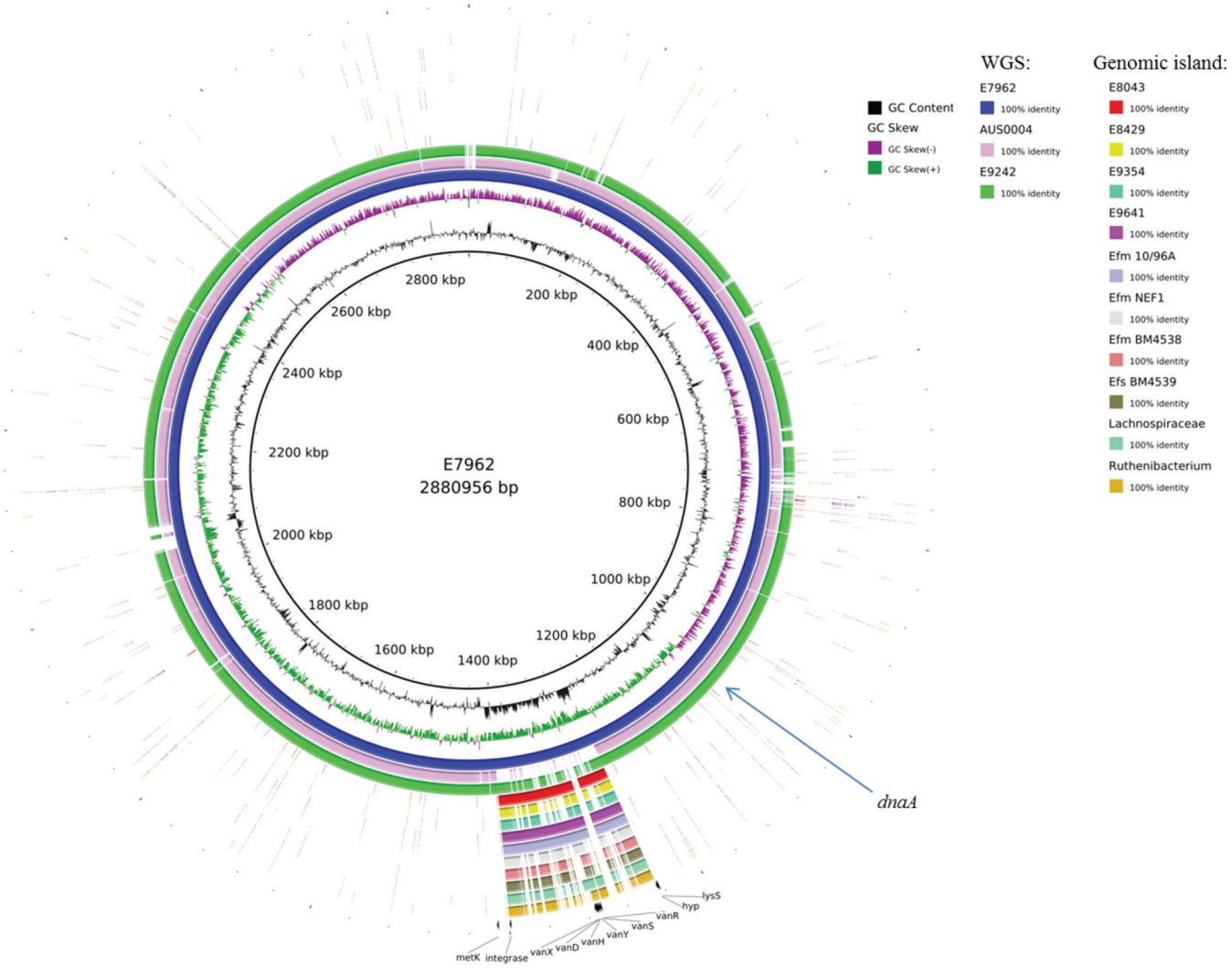

FIG 2 Circular representation (BLAST Ring Image Generator [24]) of complete genomes and genomic islands using *E. faecium* E7962 as reference.

between species and genera. Recently, Boyd et al. (15) described a *vanD* gene cluster-containing element, designated IME*Efm*15508 (integrative mobilizable element in *E. faecium* strain N15-508). Based on our analysis, this *vanD* gene cluster clustered completely separately from all other *vanD* gene clusters. A phylogenetic NJ tree based on aligned protein sequences of all ligases conferring vancomycin resistance indicated that the *E. faecium* N15-508 VanD belonged to a separate lineage between the VanB and VanD ligases (see Fig. S2 in the supplemental material).

To investigate whether the *vanD* gene cluster in the Dutch VRE isolates was located on a plasmid or other mobile genetic element, we performed long-read nanopore sequencing for strains E7962, E8429, and E9242 and, in combination with short-read Illumina reads, generated a hybrid assembly using SPAdes 3.8.0. This resulted in completely assembled chromosomes for strains E7962 and E9242 of 2,880,956 and 2,831,933 nucleotides, respectively. A comparative circular alignment, using E7962 as reference and including the *vanD*-negative complete genome sequence of *E. faecium* AUS0004 (accession no. NC_017022) revealed that the *vanD* gene cluster was part of a large genomic island of 185 kb with a divergent GC content compared to the rest of the genome of strain E7962 and that this island in E9242 is slightly smaller (144 kb) (Fig. 2 and Table 1). In the four other Dutch VRE isolates and the six *vanD*-positive strains from GenBank for which WGSs were available, the *vanD* gene cluster was also part of a

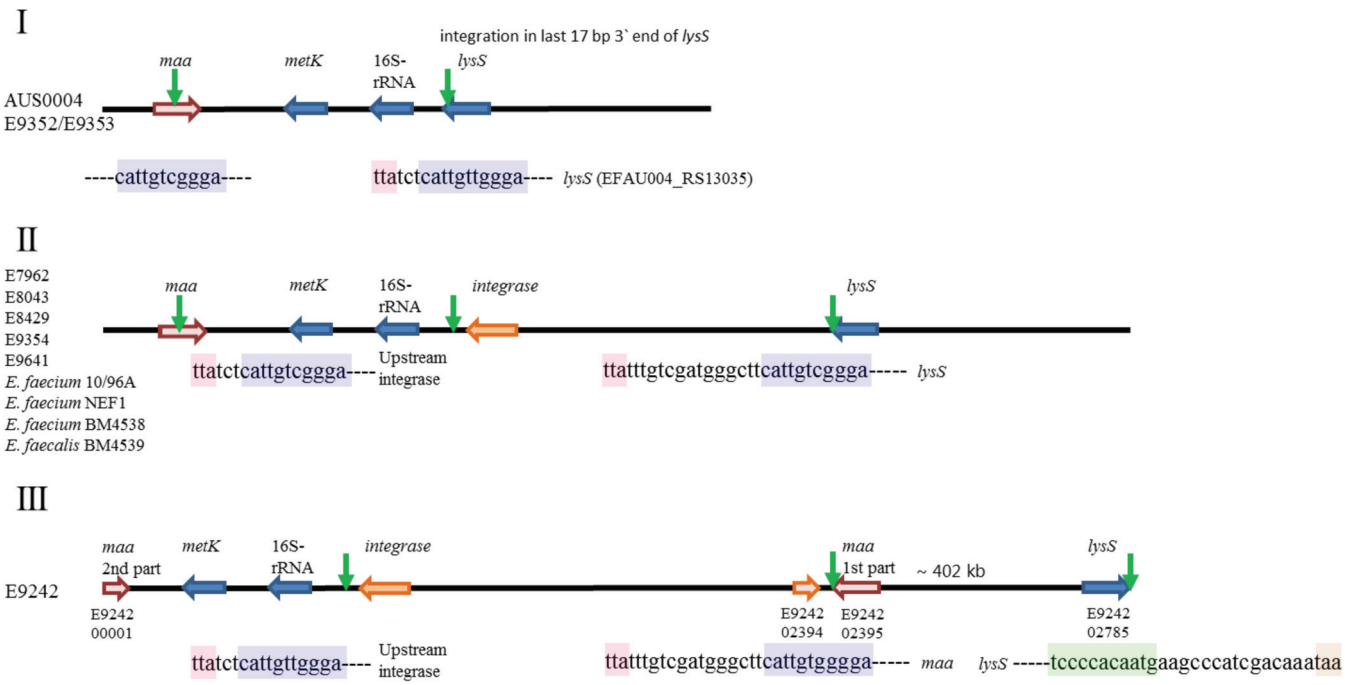

FIG 3 Genomic organization (not drawn to scale) indicating the integration of the genomic island. (I) *vanD*-negative isolates AUS0004, E9352, and E9353. Green arrows indicate the presence of an 11-bp repeat unit (blue box) in the 3′ end of *lysS* (red box, stop codon) and *maa* genes. (II) *vanD*-positive isolates *E. faecium* E7962, E8043, E8429, E9354, E9641, 10/96A, NEF1, and BM4538 and *E. faecalis* BM4539. In these strains, integration always occurred by the 11-bp repeat sequence present in *lysS*, resulting in a duplication at the 11-bp repeat unit upstream to the integrase and an alternative stop codon for *lysS*. (III) Genomic rearrangement in *vanD*-positive strain E9242. In this strain, after integration of the genomic island, a large genomic rearrangement had occurred between the 11-bp repeat present in the *lysS* and *maa* genes, resulting in a split of the *maa* gene and an inverted repeat (green box) in the *lysS* gene.

similar large genomic island with a variable size of 120 to 168 kb (Fig. 2 and Table 1; see also Table S2). The previously mentioned IME*Efm*15508 element, however, is different in gene content and size (66.7 kb) from the genomic island we described in this study and was therefore excluded from further analysis (15).

Based on comparison with the *E. faecium* AUS0004 genome sequence, the insertion site of the *vanD*-containing genomic island was located between the 16S ribosomal rRNA gene (EFAU004_RS13030) and *lysS* (EFAU004_RS13035) (Fig. 3, I). In both *E. faecium* and *E. faecalis* strains, insertion had occurred in the last 11 bp of the 3′ end of *lysS* (Fig. 3, I), resulting in a duplication of this 11-bp insertion site at both ends of the island, thereby generating an alternative stop codon for *lysS* (Fig. 3, II; Table S2). In strain E9242, the *lysS* gene (E9242_02785) was at the end of the contig in the forward orientation (Fig. 3, III). PCR analysis confirmed a large genomic rearrangement of ~402 kb (data not shown), which should have occurred after integration of the island via a 29-bp inversed repeat region in the *maa* and *lysS* genes (Fig. 3, III). The presence of an integrase gene and duplication of the insertion site suggest exchange via a circular intermediate, which is a general characteristic for an integrative and conjugative element (19–21).

A phylogenetic tree based on a multiple alignment of the *vanD*-containing genomic islands revealed three clusters of closely related islands (Fig. 1B). Cluster I in Fig. 1B contained Dutch isolates E7962, E8043, and E9641 and strain *E. faecium* 10/96A from Brazil; cluster II contained the two *vanD*-positive VRE isolates from patient D; and cluster III contained *E. faecium* BM4538 and *E. faecalis* BM4539. The genomic island of *vanD*-positive VRE E9242 clustered separately from the other Dutch isolates. A multiple alignment of the *vanD*-containing genomic islands revealed five conserved blocks of genes (see regions A through E in Fig. S3A and B in the supplemental material), including the *vanD* gene cluster (region D, Fig. S3B and C) interspersed with variable regions (Fig. S3A and B). A nonsupervised

orthologous group (eggNOG v4.5 [22]) analysis revealed that the overall distribution of Clusters of Orthologous Groups (COG) categories was very similar for all the strains (see Fig. S4 and Tables S3 to S14 in the supplemental material), including a high number of transcriptional regulators and putative two-component systems (category K, T and depicted as blue and purple genes, respectively, in Fig. S3A and B) and genes involved in replication, recombination, and repair (category L, green genes in Fig. S3A and B). In addition to the vancomycin resistance genes (category M, orange genes in Fig. S3A and B), at least 12 other putative antibiotic resistance genes were identified (yellow genes in Fig. S3A and B).

Based on the diversity in cgMLST allelic profiles, sequence variation in the *vanD* gene clusters and in the genomic islands carrying the *vanD* gene clusters, we conclude that the six Dutch *vanD*-positive VRE isolates are not epidemiologically linked and thus have not emerged through either clonal spread or horizontal transmission of the vancomycin resistance genes. In contrast, our results point toward independent acquisition of a large genomic island containing the *vanD* gene cluster, possibly from the patient's own anaerobic microbiota, which might have occurred in patient D. Domingo et al. (23) described high prevalences of *vanB*-, *vanG*-, and *vanD*-type resistance genes not associated with enterococci present in the human fecal flora. The level of similarity among the genomic islands containing the *vanD* gene clusters between the anaerobic bacteria and *E. faecium* described in this study support the hypothesis that anaerobic gut commensals may represent a reservoir for the *vanD* type of vancomycin resistance; however, so far, there is no experimental evidence for genetic exchange between gut commensals and enterococci.

The fact that we did not find indications for clonal spread of *vanD*-positive VRE suggests that these VREs do not transmit easily between patients, in contrast to *vanA*- or *vanB*-positive VRE. However, because the genomic island described in this study contains a high number of additional antibiotic resistance genes, acquisition of the island and subsequent infection with *E. faecium* strains containing the island may lead to particularly difficult or even nontreatable infections.

Accession number(s). The raw reads obtained for the eight *E. faecium* strains used in this study have been deposited at the European Nucleotide Archive under the following project accession numbers: PRJEB21556 and PRJEB21647.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AAC.01793-17.

SUPPLEMENTAL FILE 1, XLSX file, 0.1 MB.
SUPPLEMENTAL FILE 2, PDF file, 2.8 MB.

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