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Emergence of vancomycin- and teicoplanin-resistant *Enterococcus faecium* via *vanD5*-harbouring large genomic island

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Background: Treatment of VRE is of clinical concern. While certain numbers of *vanD*-type VRE have been isolated, only two *vanD5*-harbouring *Enterococcus faecium* isolates have been reported in Canada and Japan.

Methods: We report the isolation of *vanD5*-type *E. faecium* and the first ever determination of the whole-genome sequence to investigate the possible mechanisms of the acquisition of the *vanD5* gene cluster in *E. faecium*.

Results: Two *vanD5*-harbouring vancomycin-resistant *E. faecium* were isolated from the skin (SMVRE19) and faeces (SMVRE20) of a patient with a skin ulcer in Japan. The isolates exhibited vancomycin and teicoplanin MIC values of 128 mg/L, whilst the previous isolates of *vanD5*-harbouring *E. faecium* were only resistant to vancomycin. SMVRE19 and SMVRE20 were clones related to ST18, which is also seen in *vanA*- and *vanB*-type VRE. These isolates harboured an insertion element, IS*Efm1*, in the *ddl* gene, similar to a previously described teicoplanin-resistant *vanD3*-type *E. faecium*. The *vanD5* gene cluster was integrated into the SMVRE20 chromosome as a part of a large genomic island (approximately 127 kb), similar to other recently spreading *vanD* variants in the Netherlands. The genomic island shared the greatest similarity with a part of the *Blautia coccoides* genome sequence, except for the region surrounding the *vanD* gene cluster.

Conclusions: This study reports that emergence of vancomycin- and teicoplanin-resistant *vanD5*-type *E. faecium* occurred via acquisition of the *vanD5* cluster and IS*Efm1* insertion into *ddl*. Considering the genetic similarity between the various VRE strains, the current study should serve as a warning against the spread of *vanD5*-type VRE.

Introduction

Enterococcus spp. are some of the most important nosocomial pathogens that cause urinary tract infections, surgical wound infection, endocarditis and bacteraemia. VRE are isolated worldwide and their treatment is a serious clinical concern. Vancomycin-resistant *Enterococcus faecium* strains are of particular concern because of the frequently observed MDR phenotype, including resistance to β-lactams, fluoroquinolones and aminoglycosides.[1]

Glycopeptide (vancomycin and teicoplanin) resistance is attributed to the acquisition of vancomycin resistance (*van*) genes. Nine *van* genes that encode a D-Ala:D-Lac or D-Ala:D-Ser ligase (*vanA*, *vanB*, *vanC*, *vanD*, *vanE*, *vanG*, *vanL*, *vanM* and *vanN*) have been identified in *Enterococcus* spp.[1] These resistance genes are usually harboured together with other *van* gene clusters, such as *vanR*, *vanS*, *vanY*, *vanH* and *vanX*, with the different combinations depending on the *van* types.[1] The isolation of *vanD*-type vancomycin-resistant *E. faecium* strains has been reported since the mid-2000s,[2,3] and they have been spreading in clinical fields in recent years.[4–9] In addition to the *vanD* gene cluster, functional disruption of Ddl (D-Ala:D-Ala ligase), which also contributes to vancomycin and teicoplanin resistance,[10] has been commonly observed.

In the current study, we report the isolation of *vanD5*-harbouring *E. faecium* isolates from a patient with a skin ulcer in Japan in 2017. We describe, to the best of our knowledge, the first ever

determination of the whole-genome sequence of a *vanD5*-harbouring *E. faecium* and present the possible pathways of acquisition of the *vanD5* gene cluster.

Materials and methods

Clinical isolates and detection of the vanA–D genes

Two vancomycin-resistant *E. faecium* isolates were isolated from the skin (SMVRE19) and faeces (SMVRE20) of a patient with a skin ulcer in Sapporo Medical University Hospital (Sapporo, Japan) in 2017. The patient had no history of travelling abroad and had received vancomycin treatment 2 months before the isolation. The study was approved by Sapporo Medical University Ethics Committee (No. 302-1031). The *vanA–D* genes were detected by PCR, as previously described.[2,11]

Antimicrobial susceptibility testing

Antimicrobial susceptibility, including susceptibility to vancomycin (Shionogi, Osaka, Japan) and teicoplanin (Sanofi, Tokyo, Japan), was tested using the microbroth dilution method according to the guidelines of CLSI. *E. faecium* ATCC 35667 was used as a reference.

WGS

Genomic DNA of SMVRE19 and SMVRE20 was isolated using the Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA). The complete genome sequence of SMVRE20 was determined using the PacBio RS SMRT Portal (Pacific Biosciences, Menlo Park, CA, USA). The filtered subreads (in fastq format) were assembled with 300 bp paired-end reads obtained by MiSeq sequencing (Illumina, San Diego, CA, USA) using Unicycler (version 0.4.3).[12] To increase accuracy, the sequences were curated by mapping analysis with the paired-end reads using the CLC Genomics Workbench (QIAGEN, Hilden, Germany). The finalized contig lengths were 2 840 573 bp (the SMVRE20 genome), 153 748 bp (pSMVRE20L, the large plasmid of SMVRE20) and 40 229 bp (pSMVRE20S, the small plasmid of SMVRE20). The entire sequences were annotated by DFAST (legacy version) based on PROKKA.[13] Paired-end reads of SMVRE19 were subjected to mapping analysis to compare with SMVRE20, using CLC Genomics Workbench.

Genome analysis

The nucleotide sequences of previously reported *vanD*-positive strains E8043,[6] E9354[6] and NEF1[3] were assembled using the respective short reads available from the European Nucleotide Archive (ENA) database using the CLC Genomics Workbench with default parameters. The sequence of the genomic island of SMVRE20 harbouring the *vanD5* gene cluster was then compared with those of the other *vanD*-positive strains and the corresponding region of *Blautia coccoides* YL58 (accession number CP022713.1), using BLASTn in EasyFig 2.2.2.[14] Putative drug resistance-related genes were identified using CARD version 3.0.1.[15] In addition, core genes were defined using BLASTn, based on the reciprocal best hits between two compared sequences.

Conjugation experiments

Conjugation experiments were performed as previously described.[16] Eight rifampicin- and fusidic acid-resistant *E. faecium* mutants selected from four *E. faecium* isolates were used as the recipients. Brain heart infusion (BHI) agar containing vancomycin (8 mg/L), rifampicin (32 mg/L) and fusidic acid (25 mg/L) was used for the selection of *vanD5*-habouring transconjugants after incubation at 37°C for 3 days. The details of the conjugation experiments are described in Appendix S1 (available as Supplementary data at *JAC* Online).

Natural transformation experiment

Genomic DNA was isolated from *E. faecium* SMVRE19 and SMVRE20 using the Wizard Genomic DNA Purification Kit. Approximately 1 μg, 100 ng or 10 ng of the genomic DNA was added into the mixture of 900 μL of fresh BHI broth and 100 μL of overnight culture of eight rifampicin- and fusidic acid-resistant *E. faecium* mutants in BHI broth (details are in Appendix S1). BHI agar containing vancomycin (8 mg/L), rifampicin (32 mg/L) and fusidic acid (25 mg/L) was used for the selection of *vanD5*-habouring transconjugants after incubation at 37°C for 3 days.

Data availability

The obtained genome sequences were deposited at the DNA Data Bank of Japan (DDBJ)/ENA/GenBank under the accession numbers AP019408 (the SMVRE20 genome; https://www.ncbi.nlm.nih.gov/nuccore/AP019408.1), AP019409 (pSMVRE20L; https://www.ncbi.nlm.nih.gov/nuccore/AP019409) and AP19410 (pSMVRE20S; https://www.ncbi.nlm.nih.gov/nuccore/AP019410).

Results

Antimicrobial susceptibility of vanD-type vancomycin-resistant E. faecium

SMVRE20 and SMVRE19 exhibited identical antimicrobial susceptibility results: resistance to vancomycin and teicoplanin (both MICs were 128 mg/mL), penicillins (penicillin and ampicillin MICs were 128 and 64 mg/L, respectively) and fluoroquinolones (ciprofloxacin and levofloxacin MICs were >64 mg/L); they were susceptible to linezolid (MIC of 1 mg/mL) and daptomycin (MIC of 4 mg/mL). WGS of SMVRE20 and SMVRE19 revealed the presence of only two SNPs among the two strains. This suggested that the two strains originated from an identical clone. Meanwhile, SMVRE19 lacked a small plasmid harboured by SMVRE20 (pSMVRE20S). The two isolates were untypeable by MLST (https://pubmlst.org/efaecium/) because of an IS*Efm1* insertion in *ddl*, one of the seven housekeeping genes used for the determination of ST. However, with the exception of the insertion, ST of the isolates was identical to ST18.

Identification of the vanD variant and sequencing of the vanD cluster and ddl genes

The *vanD* sequence of SMVRE20 was identical to the sequence of a *vanD5* variant (100% identity with *vanD5* of the first ever isolated *E. faecium* strain N03-0072, accession number NG048362). SMVRE20 possessed other *vanD* gene clusters: *vanR*, *vanS*, *vanY*, *vanH* and *vanX*. SMVRE20 had a complete *vanY* sequence, whereas N03-0072 harbours a frameshift, i.e. insertion of thymine between position 77 and 78 in that gene (accession number AY489045.1).[2] In addition, SMVRE20 VanX had two amino acid substitutions (Trp21Cys and Met22Ile) compared with the N03-0072 protein, whereas VanR and VanS were 100% identical (accession numbers, NG_048412.1 and NG_048436.1) with N03-0072. The *ddl* gene of SMVRE20 had been disrupted by a 1050 bp insertion, IS*Efm1*, between nucleotides 762 and 763, inserted via repeated nucleotide sequences, atcaataat (Figures S1 and S2).

Genomic location of the vanD5 gene cluster and the genomic island

The long-read WGS and BLAST analysis revealed that the region surrounding the *vanD5* gene cluster in SMVRE20 harboured

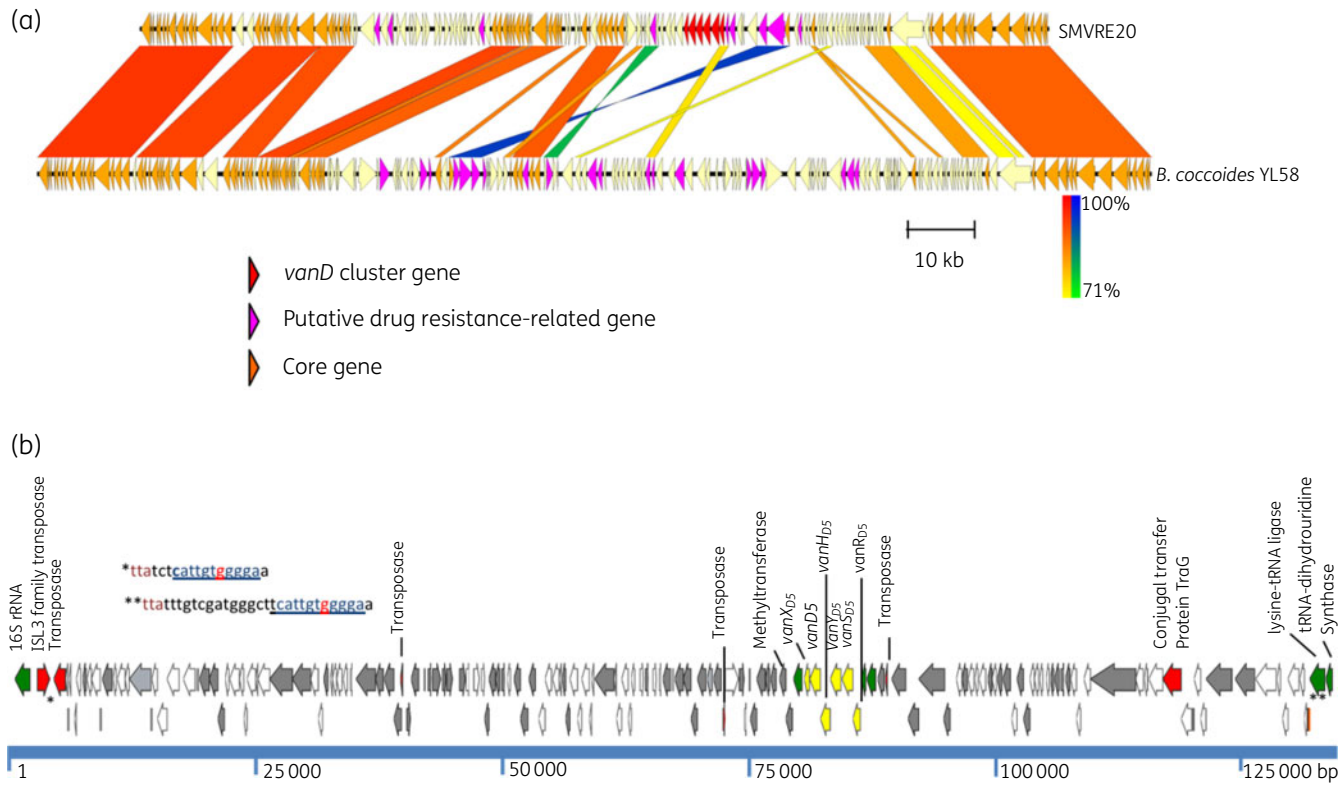

Figure 1. Genomic analysis of the *vanD5* gene cluster and the surrounding genomic island genes in *E. faecium* SMVRE20 (identical with SMRVE19). (a) Genetic comparison of the genomic island harbouring the *vanD5* gene cluster in SMVRE20 and the corresponding region of the *B. coccoides* YL58 genome (accession number CP022713.1). The island was defined by an 11 bp repeat sequence according to Top *et al.*[6] Bi-directional BLAST hits were coloured using a colour gradient corresponding to identity of 71%–100%. (b) Predicted integration site of the *vanD5*-harbouring genomic island in the *E. faecium* SMVRE20 (identical with SMVRE19) chromosome. The arrows correspond to coding sequences (CDSs) originating from *E. faecium* (green), the *vanD5* gene cluster (yellow), transposase genes (red), functional protein genes (grey) and hypothetical protein genes (white). Some features are shown as arrows in the bottom lane for clarity. * and **, the predicted integration site and the associated 11 bp repeated nucleotide sequence, cattgtgggga (underlined). The nucleotide at the seventh position (guanine, marked in red) is different from that (cytosine) reported previously.[5]

sequences of non-*Enterococcus* spp. origin and most of the regions were highly similar to the genome sequence of *B. coccoides* YL58 (Figure 1a). The region containing the *B. coccoides* YL58-like nucleotide sequences consisted of 127 465 bp and was sandwiched in the *E. faecium* chromosome in the vicinity of the 16S rRNA gene and a tRNA-dihydrouridine synthase gene (*lysS*) via an 11 bp repeat nucleotide sequence, cattgtgggga (Figure 1b).

Comparison of the genomic island regions with other *vanD*-type *E. faecium* isolates[8] revealed that SMVRE20 shared greater nucleotide sequence similarity with E9354, which harbours a cluster II *vanD*-containing genomic island, than with E8043 (cluster I) or NEF1 (cluster III) (Figure 2). Further, the *vanD5*-containing genomic island of SMVRE20 was approximately 6.5 kb longer than that of E9354 (approximately 120.8 kb).[6]

Conjugation and natural transformation experiments

In the conjugation experiment, we observed a total of 19 strains that grew on the BHI agar containing vancomycin, rifampicin and fusidic acid. However, no *vanD* gene was observed in the obtained strains by PCR. We also failed to select any *vanD5*-harbouring transconjugants in the natural transformation.

Discussion

The first identification of *vanD5* was reported in an isolate from a rectal/perineal sample of a patient with a nephrostomy in Canada in 2004.[2] A recent study reported the isolation of *vanD5*-harbouring *E. faecium* (ST203) in Japan in 2017.[9] These indicate that the emergence of *vanD5*-type VRE did not exclusively occur in Canada, but independently in another place. The present isolate SMVRE20 represents ST18, when the IS*Efm1* insertion in *ddl* is disregarded. ST18 is a major lineage in which vancomycin resistance acquisition is associated with *vanA*, *vanB* and other *vanD* variants and spread worldwide.[6,17] Therefore, the spread of ST18 strains should be monitored in the clinic because of their ability to acquire various types of *van* genes.

Although the Canadian and recently isolated Japanese *vanD5*-type *E. faecium* strains (N03-0072 and IPHb306, respectively) were resistant to vancomycin (MIC of >16 mg/L) and susceptible to another glycopeptide, teicoplanin (MICs of 1 and 2 mg/L),[2,9] SMVRE20 was resistant to both agents. Teicoplanin resistance is contributed to by imbalance of peptidoglycan precursors (UDP-MurNAc-pentadepsipeptide, UDP-MurNAc-pentapeptide and UDP-MurNAc-tetrapeptide) due to the disruption of Ddl and regulation of VanXY

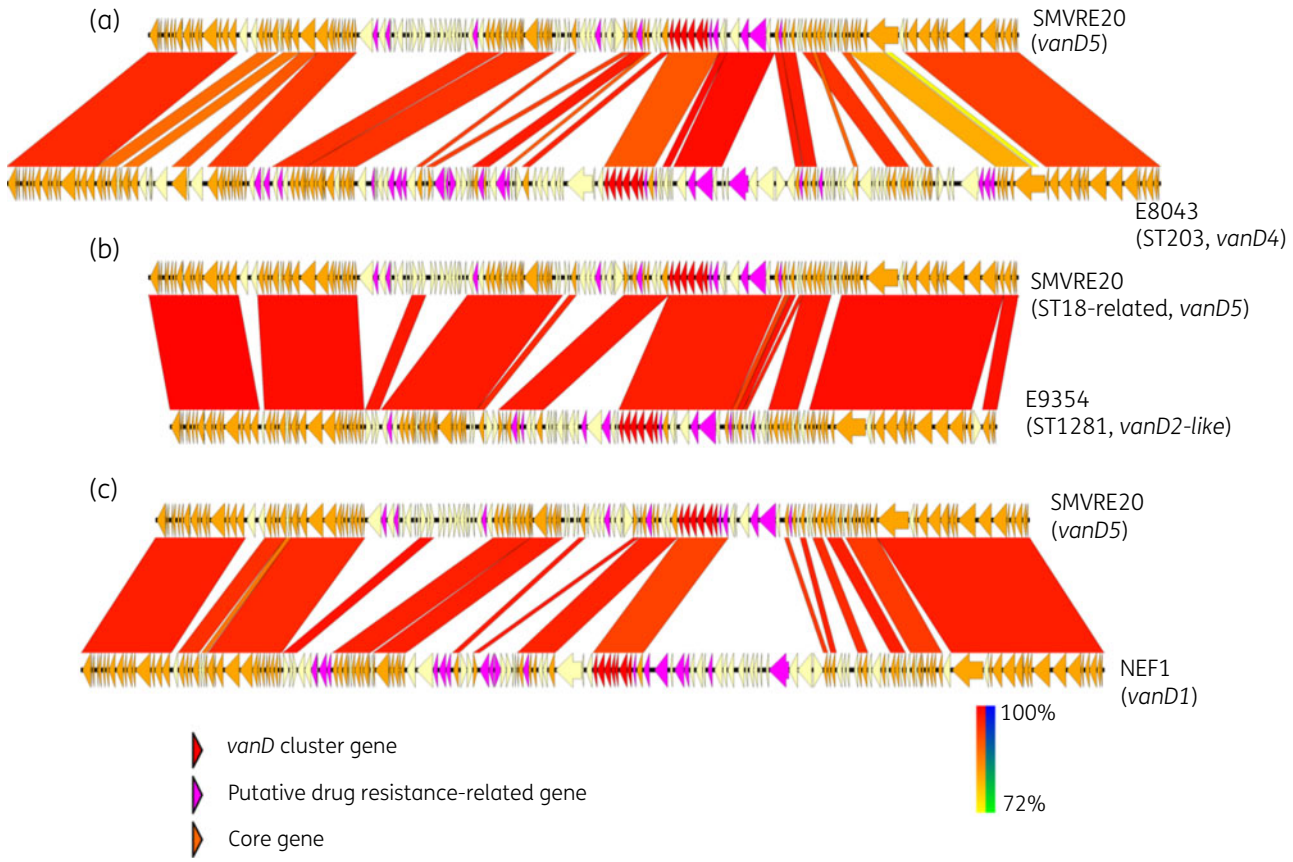

Figure 2. Genetic comparison of genomic islands harbouring the *vanD* gene cluster. Genomic islands harbouring the *vanD* gene cluster in *vanD*-positive strains were compared. Sequences of three strains [E8043[6] (a), E9354[6] (b) and NEF1[3] (c)] were assembled from the respective short reads available from the ENA database using the CLC Genomics Workbench with default parameters. The island was defined by the 11 bp repeat sequence according to Top *et al.*[6] The arrows designate CDSs annotated by DFAST.[13] The sequences were compared using BLASTn in EasyFig 2.2.2.[14] Bi-directional BLAST hits were coloured using a colour gradient corresponding to identity of 72%–100%. The minimum length of a BLAST hit was fixed at 600 bp. Drug resistance-related genes (the descriptor for the pink arrowheads) were searched using CARD version 3.0.1,[15] using BLASTp (filtered at an E-value of 1E-15). The core genes were also defined using BLASTn, based on reciprocal best hits among the four sequences.

and VanSR.[18] Thus, the resistance to teicoplanin of SMVRE20 can be explained by functional VanY and mutated VanX in addition to Ddl disruption from the comparison with N03-0072. The insertion of IS*Efm1* into *ddl* at the same position has been reported in a *vanD3*-type *E. faecium* strain N97-330 in Canada in 2000.[19]

The acquisition mechanism of *vanD5* has not been fully determined, because whole-genome data for the previously reported *vanD5*-type *E. faecium* strains N03-0072 and IPHb306 are not available. We suggested that the *vanD5* of SMVRE20 shared a common pathway for acquisition of the *vanD* gene cluster, the integration of a highly similar large genomic island via 11 bp repeat nucleotide sequence (cattgt**c**ggga, but cattgt**g**ggga in SMVRE20) at the same site of the chromosome with other *vanD*-harbouring *E. faecium* strains (*vanD1-like*, *vanD2-like* and *vanD4*).[3,6] These observations indicate that the acquisition pathway of *vanD* variants occurs via a hotspot in the *E. faecium* chromosome in many lineages.

Several segments of the SMVRE20 genomic island were identical to the nucleotide sequence of *B. coccoides*. *B. coccoides*, a commensal anaerobic Gram-positive bacterium, resides in the human gut, as do *Enterococcus* spp.[20] Therefore, the emergence of *vanD5*-harbouring vancomycin-resistant *E. faecium* could be mediated by horizontal gene transfer of a large part of the genomic island originating from *B. coccoides* to the *E. faecium* chromosome in the human gut.[21] However, transfer of the genomic island from SMVRE20 to *E. faecium* recipients did not occur in our *in vitro* experiments. This suggested that the *vanD5*-harbouring genomic island is stable in the *E. faecium* chromosome after the integration.

In conclusion, we report here the genetic similarity (ST18-related, large genomic island integration and IS*Efm1* insertion) of *vanD5*-harbouring vancomycin- and teicoplanin-resistant *E. faecium* between the various VREs.

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Transparency declarations

None to declare.

Supplementary data

Appendix S1 and Figures S1 and S2 are available as Supplementary data at *JAC* Online.

References

1 Cetinkaya Y, Falk P, Mayhall CG. Vancomycin-resistant enterococci. *Clin Microbiol Rev* 2000; **13**: 686–707.

2 Boyd DA, Kibsey P, Roscoe D *et al*. *Enterococcus faecium* N03-0072 carries a new VanD-type vancomycin resistance determinant: characterization of the VanD5 operon. *J Antimicrob Chemother* 2004; **54**: 680–3.

3 Lavigne JP, Marchandin H, Bouziges N *et al*. First infection with VanD-type glycopeptide-resistant *Enterococcus faecium* in Europe. *J Clin Microbiol* 2005; **43**: 3512–5.

4 Boyd DA, Lalancette C, Levesque S *et al*. Characterization of a genomic island harbouring a new *vanD* allele from *Enterococcus faecium* N15-508 isolated in Canada. *J Antimicrob Chemother* 2016; **71**: 2052–4.

5 Starlander G, Tellgren-Roth C, Melhus Å. Fatal acquisition of *vanD* gene during vancomycin treatment of septicaemia caused by *Enterococcus faecium*. *J Hosp Infect* 2016; **92**: 409–10.

6 Top J, Sinnige JC, Brouwer EC *et al*. Identification of a novel genomic island associated with *vanD*-type vancomycin resistance in six Dutch vancomycin-resistant *Enterococcus faecium* isolates. *Antimicrob Agents Chemother* 2018; **62**: e01793–17.

7 Lam PW, Kozak RA, Eshaghi A *et al*. Nosocomial outbreak of *vanD*-carrying vancomycin-resistant *Enterococcus faecium*. *Infect Control Hosp Epidemiol* 2018; **39**: 1266–8.

8 Flipse J, von Wintersdorff CJH, van Niekerk JM *et al*. Appearance of *vanD*-positive *Enterococcus faecium* in a tertiary hospital in the Netherlands: prevalence of *vanC* and *vanD* in hospitalized patients. *Sci Rep* 2019; **9**: 6949.

9 Terada S, Harada T, Yokota M *et al*. First isolation and characterization of vancomycin-resistant *Enterococcus faecium* harboring *vanD5* gene cluster recovered from a 79-year-old female inpatient in Japan. *Diagn Microbiol Infect Dis* 2019; **95**: 114883.

10 Gholizadeh Y, Prevost M, Van Bambeke F *et al*. Sequencing of the *ddl* gene and modeling of the mutated D-alanine:D-alanine ligase in glycopeptide-dependent strains of *Enterococcus faecium*. *Protein Sci* 2001; **10**: 836–44.

11 Dutka-Malen S, Evers S, Courvalin P. Detection of glycopeptide resistance genotypes and identification to the species level of clinically relevant enterococci by PCR. *J Clin Microbiol* 1995; **33**: 24–7.

12 Wick RR, Judd LM, Gorrie CL *et al*. Unicycler: resolving bacterial genome assemblies from short and long sequencing reads. *PLoS Comput Biol* 2017; **13**: e1005595.

13 Tanizawa Y, Fujisawa T, Kaminuma E *et al*. DFAST and DAGA: web-based integrated genome annotation tools and resources. *Biosci Microbiota Food Health* 2016; **35**: 173–84.

14 Sullivan MJ, Petty NK, Beatson SA. Easyfig: a genome comparison visualizer. *Bioinformatics* 2011; **27**: 1009–10.

15 Jia B, Raphenya AR, Alcock B *et al*. CARD 2017: expansion and model-centric curation of the comprehensive antibiotic resistance database. *Nucleic Acids Res* 2017; **45**: D566–73.

16 García-Solache M, Lebreton F, McLaughlin RE *et al*. Homologous recombination within large chromosomal regions facilitates acquisition of β-lactam and vancomycin resistance in *Enterococcus faecium*. *Antimicrob Agents Chemother* 2016; **60**: 5777–86.

17 Freitas AR, Tedim AP, Francia MV *et al*. Multilevel population genetic analysis of *vanA* and *vanB Enterococcus faecium* causing nosocomial outbreaks in 27 countries (1986–2012). *J Antimicrob Chemother* 2016; **71**: 3351–66.

18 Depardieu F, Foucault ML, Bell J *et al*. New combinations of mutations in VanD-type vancomycin-resistant *Enterococcus faecium*, *Enterococcus faecalis*, and *Enterococcus avium* strains. *Antimicrob Agents Chemother* 2009; **53**: 1952–63.

19 Boyd DA, Conly J, Dedier H *et al*. Molecular characterization of the *vanD* gene cluster and a novel insertion element in a vancomycin-resistant enterococcus isolated in Canada. *J Clin Microbiol* 2000; **38**: 2392–4.

20 Liu C, Finegold SM, Song Y *et al*. Reclassification of *Clostridium coccoides*, *Ruminococcus hansenii*, *Ruminococcus hydrogenotrophicus*, *Ruminococcus luti*, *Ruminococcus productus* and *Ruminococcus schinkii* as *Blautia coccoides* gen. nov., comb. nov., *Blautia hansenii* comb. nov., *Blautia hydrogenotrophica* comb. nov., *Blautia luti* comb. nov., *Blautia producta* comb. nov., *Blautia schinkii* comb. nov. and description of *Blautia wexlerae* sp. nov., isolated from human faeces. *Int J Syst Evol Microbiol* 2008; **58**: 1896–902.

21 Domingo MC, Huletsky A, Giroux R *et al*. High prevalence of glycopeptide resistance genes *vanB, vanD*, and *vanG* not associated with enterococci in human fecal flora. *Antimicrob Agents Chemother* 2005; **49**: 4784–6.