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# Tandem amplification of the *vanM* gene cluster drives vancomycin resistance in vancomycin-variable enterococci

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Running title: *vanM* cluster amplification causes vancomycin resistance

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**Background:** Vancomycin-variable enterococci (VVE) are a potential risk factor for vancomycin resistance gene dissemination and clinical treatment failure. *vanM* has emerged as a new prevalent resistance determinant among clinical enterococci in China. A total of 54 vancomycin-susceptible enterococci (VSE) isolates carrying incomplete *vanM* gene clusters were isolated in our previous study.

**Objectives:** To determine the potential of *vanM*-carrying VSE to develop vancomycin resistance and investigate the mechanism of resistance phenotype alteration.

**Methods:** Fifty-four *vanM*-positive VSE strains were induced *in vitro* by culturing in increasing concentrations of vancomycin. Genetic changes between three parent VVE strains and their resistant variants were analysed using Illumina and long-read sequencing technologies, qPCR and Southern blot hybridization. Changes in expression level were determined by qRT-PCR.

**Results:** Twenty-five of the 54 VSE strains carrying *vanM* could become resistant upon vancomycin exposure. A significant increase in *vanM* copy number ranging from 5.28 to 127.64 copies per cell in induced resistant VVE strains was observed. The *vanM* transposon was identified as tandem repeats with IS*1216E* between them and occurred in either the plasmid or chromosome of resistant VVE cells. In addition, an increase in *vanM* expression was observed after resistance conversion in VVE.

**Conclusions:** This study identified tandem amplification of the *vanM* gene cluster as a new mechanism for vancomycin resistance in VVE strains, offering a competitive advantage for VVE under antibiotic pressure.

## Introduction

Glycopeptide resistance in enterococci is mediated by *van* gene clusters, among which *vanA* and *vanB* are the most commonly reported worldwide.[1,2] VanA-type enterococci confer high-level resistance to both vancomycin and teicoplanin, whereas VanB-type enterococci display varying levels of vancomycin resistance but remain susceptible to teicoplanin.[3] In recent years, however, *vanM* has emerged as a new and prevalent resistance determinant in clinical enterococci in China, especially in the cities of Shanghai and Hangzhou.[4,5] The *vanM* gene cluster contains the *vanR, vanS, vanY, vanH, vanM,* and *vanX* genes, along with an IS*1216*-like element, together forming the *vanM* transposon, which usually confers high resistance to vancomycin and teicoplanin.[6]

Vancomycin-variable enterococci (VVE) are a group of enterococci containing *van* genes that exhibit a vancomycin-susceptible phenotype due to a deletion or insertion in the *van* gene cluster but are capable of shifting to a glycopeptide-resistant phenotype under vancomycin therapy *in vivo* or exposure *in vitro*.[7,8] This characteristic of VVE allows them to act as a hidden reservoir for vancomycin resistance genes and pose an underlying clinical risk of treatment failure. Indeed, several outbreaks of VVE have been reported involving *vanA*-containing *Enterococcus* isolates in Canada, Norway, and Denmark.[7, 9, 10] Mutations or the excision of an IS*L3*-family element upstream of *vanHAX* were reported as contributors to switching to a resistant phenotype.[7,10] In a recent study, changes in *ddl* and *vanS* genes or increases in the copy number of *vanA*-carrying plasmids were

shown to lead to vancomycin resistance in *vanA*-type VVE strains containing a deletion in *vanX*.[9] Until now, there have been no reports of VVE carrying *vanM*.

Our previous study showed a high prevalence of silenced *vanM* gene clusters, with 55 *vanM*-carrying strains showing a susceptible phenotype among 1284 clinical isolates.[11] These 55 strains were classified into 5 *vanM* transposon types. It should be noted that the isolate SRR12 of type V, which carried an intact *vanM* gene cluster, was identified to be heteroresistant to vancomycin and therefore was excluded here. In this study, to determine the prevalence of *vanM*-type VVE, 54 vancomycin-susceptible enterococci (VSE) strains carrying incomplete *vanM* gene clusters were investigated to determine whether they had the ability to develop a glycopeptide-resistant phenotype upon vancomycin exposure. Furthermore, we revealed a unique mechanism of vancomycin resistance in *vanM*-type VVE strains.

## Methods

### Bacterial isolates.

The 54 VSE isolates carrying incomplete *vanM* gene clusters included 39 isolates of *vanM* transposon type I, 7 isolates of type II, 3 isolates of type III, 1 isolate of type IV, and 4 isolates of undetermined type, and all were characterized in our previous paper.[11]

### *In vitro* development of vancomycin resistance.

The 54 *vanM*-positive VSE strains were induced *in vitro* by culturing in increasing concentrations of vancomycin to test their ability to convert to a vancomycin-resistant phenotype. A single colony of *vanM*-positive VSE grown on brain heart infusion (BHI) agar was inoculated into 2 mL of BHI broth at 37°C with shaking at 200 rpm for 18 to 20 h. Vancomycin resistance development was initiated by a 1:100 dilution in fresh broth containing 1 mg/L vancomycin until the culture had grown to an $OD_{600}$ of 1, followed by repeating this operation with a two-fold higher concentration of vancomycin. Every culture that underwent this process was transferred to BHI agar with 32 mg/L vancomycin, and the colonies grown up were retained as resistant VVE cells (VVE-R). Their minimal inhibitory concentrations (MICs) of vancomycin and teicoplanin were then determined as described below.

**Antibiotic susceptibility testing.**

The MICs of vancomycin and teicoplanin were measured by the broth microdilution method for the *vanM*-positive VVE strains. *E. faecalis* ATCC 29212 was used as a control. The results were interpreted according to the guidelines of the Clinical Laboratory Standards Institute (CLSI 2017).

**Stability of antibiotic resistance.**

The above-derived VVE-R isolates were cultured in BHI broth in the absence of antibiotic for four continuous passages (1 μL inoculated into 1 mL of BHI broth, ten generations per passage).[12] The MICs of vancomycin and teicoplanin of the population after 40 generations without antibiotic selection were determined. Aliquots

from each culture were subjected to DNA extraction using the QIAamp DNA Mini Kit (Qiagen, Valencia, CA, USA) and then quantitative PCR (qPCR) was used to measure *vanM* copy number, which was individually described in detail.

**Whole genome sequencing (WGS) and analysis.**

The genomic DNA for three pairs of representative VVE strains was extracted using the QIAamp DNA Mini Kit (Qiagen, Valencia, CA, USA) and sequenced using a HiSeq X Ten platform (Illumina, San Diego, CA) with 2 × 150 bp paired-end reads. The raw data were trimmed and assembled using CLC Genomics Workbench 9.5.1 (Qiagen, Aarhus, Denmark).

Genomic DNA for long-read genome sequencing was prepared using the Gentra Puregene Yeast/Bact. Kit (Qiagen, Valencia, CA, USA). Nanopore sequencing using a MinION sequencer (Oxford Nanopore Technologies, Oxford, UK) was performed for the genomic DNA of ZY2, SRR24, ZY11, ZY2-R and ZY11-R. Single-molecule real-time sequencing using an RSII sequencer (Pacific Biosciences, Menlo Park, CA) was performed for the genomic DNA of SRR24-R. Hybrid assembly was achieved by Unicycler using the Illumina reads and Nanopore reads.[13] All the assembled contigs were annotated using the RAST server[14] and manually refined by the NCBI Prokaryotic Genome Annotation Pipeline.[15]

**PCR and local sequencing.**

The *vanM* gene cluster sequences of the VVE isolates were determined by Sanger sequencing with the primers (M1, M2, and M3) described in Table S1.

**Detection of copy number variation and determination of gene copy number by WGS analysis.**

The Illumina reads of each VVE-S and VVE-R strain were mapped against a *vanM*-carrying plasmid/chromosome of VVE-S that was assembled using long-read sequencing, and a read coverage map was generated using CLC Genomics Workbench 9.5.1. The median read coverage for each sequence was calculated as the number of mapped reads × the average length of mapped reads / the length of the target gene.

The copy number for the *vanM* gene was estimated by dividing the median read coverage of *vanM* by that of *purK*. The *purK* gene is involved in housekeeping functions and is present in a single copy within the chromosome. The copy number of *vanM*-carrying plasmids was evaluated by dividing the median read coverage of the plasmid replication gene *rep* by that of *purK*.

**Determination of *vanM* copy number by real-time quantitative PCR.**

The genomic DNA for three pairs of representative VVE strains was extracted using the QIAamp DNA Mini Kit (Qiagen, Valencia, CA, USA) from bacterial cultures grown in the absence or presence of vancomycin. The primer sequences of the *vanM*, *rep* and *purK* genes are listed in Table S1. Quantitative PCRs were carried out in a 10 μL reaction that contained 5 μL SYBR® Premix Ex Taq™ PCR kit (Takara Bio, Kusatsu, Japan), 0.2 μL genomic DNA (10 ng/μL) as template, and 0.2 μL each primer (10 μM). The Ct values of each sample were measured under appropriate PCR

conditions (preheated at 95°C for 5 min; 45 amplification cycles at 95°C for 5 s, 55°C for 15 s and 72°C for 15 s) on a LightCycler® 480 instrument (Roche Molecular Diagnostics, Rotkreuz, Switzerland). Data from three independent experiments were analysed by the $2^{-\Delta\Delta Ct}$ method.[16] The *purK* gene, which is located on the chromosome in a single copy, was chosen as a housekeeping control.

**Expression analysis by quantitative RT-PCR (qRT-PCR)**

RNA for the tested VVE strains was extracted from cultures in the exponential growth phase with an RNeasy® Mini Kit (Qiagen, Valencia, CA, USA). Reverse transcription of the RNA was performed using the PrimeScript® RT reagent kit (Takara Bio, Kusatsu, Japan) according to the manufacturer's instructions. Primers for RT-qPCR of *vanM*, *vanR* and *purK* are listed in Table S1. qPCRs were performed as mentioned above. All qRT-PCR operations were performed in triplicate. Fold changes in expression level in the VVE-R strains were normalized relative to that of the original VVE-S strains, according to the $2^{-\Delta\Delta Ct}$ method.[17]

**Pulsed-field gel electrophoresis (PFGE) and southern blot hybridization.**

Agarose plugs of the VVE strains were prepared to perform PFGE according to a previously described protocol.[18] Restriction fragments of DNA were separated using clamped homogeneous electric fields of 6 V/cm, 120° switch angle at 14°C, and switch time from 3 s to 20 s in 0.5× TBE electrophoresis buffer for 15 h using the CHEF-DR II system (Bio-Rad Laboratories, Hercules, CA, USA). *Salmonella* serotype Braenderup strain H9812 was included as a molecular size marker.

The hybridization probe was designed to bind to *vanM* and was synthesized using the primers *vanM*-probe-F and *vanM*-probe-R (Table S1).

**Statistical analysis.**

A t-test was carried out using GraphPad Prism to analyse the difference in the means between two groups (VVE-S group and VVE-R group). The Jonckheere-Terpstra test was performed by statistics in R to determine if there was a statistically significant trend between the continuous dependent variable (*vanM* copy number) and the ordinal independent variable (vancomycin concentration).

**Accession numbers.**

The Illumina sequencing of ZY2, ZY2-R, SRR24, SRR24-R, ZY11, and ZY11-R were deposited at NCBI under the following accession numbers: SSSK00000000, SRXZ00000000, SIWX00000000, SIWW00000000, SIWV00000000, and SRSK00000000.

The complete sequences of the plasmids pZY2 and pSRR24 were deposited at NCBI under the following accession numbers: CP039730 and CP038997. The chromosome sequence of ZY11 was deposited at NCBI under the accession number CP038995.

The Nanopore/PacBio sequencing long reads carrying the *vanM* transposons of ZY2-R, SRR24-R, and ZY11-R were submitted to the Sequence Read Archive (SRA) database under BioProject PRJNA520784 with the SRA accession numbers

SRR8936547, SRR8599734, and SRR8599755, respectively.

## Results

### The diversity of remnants of the *vanM* gene cluster is related to the potential for resistance development.

In our previous study, 55 VSE strains carrying the *vanM* gene cluster were identified among 1284 clinical enterococcus strains and classified into five *vanM* transposon types.[11] The only strain of type V, SRR12, which carries an intact *vanM* gene cluster, was shown to be heteroresistant to vancomycin and is not discussed in this study. The other 54 VSE strains carrying imperfect *vanM* gene clusters were induced by vancomycin exposure to investigate whether they had the ability to develop resistance. Twenty-nine strains, including 23 strains of type I, one strain of type II, one strain of type IV, and 4 strains of an undetermined type failed to generate resistance (Table 1).

In total, 25 strains were confirmed to be capable of shifting from susceptible to resistant and are thus true VVE strains. The original susceptible VVE strains were named VVE-S, and the induced resistant VVE isolates were termed VVE-R. From Table 1, the strains that were most efficient in becoming VVE were those with partial deletions of *vanRS* (type III *vanM* transposon). Within two days, they could be induced to become VVE-R cells that were high-level resistant to both vancomycin and teicoplanin by exposure to vancomycin at a concentration of 2 mg/L. In comparison, it was difficult for strains with a 60.6% deletion of *vanX* (type II *vanM*

transposon) to convert into VVE-R with a longer incubation time (3 to 6 days) and higher ultimate vancomycin exposure concentrations (4 or 8 mg/L). In addition, five of the type II VVE-R strains remained susceptible to teicoplanin (MIC ≤ 8 mg/L). In contrast, strains with partial deletions of both *vanRS* and *vanX* (type I *vanM* transposon) were the most difficult to evolve into VVE-R isolates. They required a very long time (5 to 14 days) and high ultimate vancomycin exposure concentrations (8 to 32 mg/L) to become VVE-R isolates. Approximately half (7/16) of the obtained VVE-R strains remained susceptible to teicoplanin.

**Increased copy number of the *vanM* gene cluster in VVE-R.**

To further investigate the mechanism of vancomycin resistance development in VVE, one susceptible VVE-S strain and its induced VVE-R isolate from each *vanM*-carrying transposon type (type I: ZY2/ZY2-R, type II: SRR24/SRR24-R, type III: ZY11/ZY11-R) were selected for subsequent studies. To determine whether indels or point mutations were present in the *vanM* transposon after resistance development, we determined the sequences of the *vanM* transposons in each VVE-S and VVE-R strain by Sanger sequencing of PCR products combined with Illumina sequencing data. The sequence of the *vanM* gene cluster was found to be identical between each VVE-S and VVE-R. In addition, no meaningful SNPs or indels such as chromosomal *ddl* gene mutations were identified in other regions of the genome of the resistant strains through short-read WGS comparative analysis. Interestingly, we found changes in DNA patterns by *Sma*I-PFGE as well as alterations in the *vanM*-carrying fragments by Southern blotting with the *vanM* probe, indicating that genomic changes had

occurred during resistance development (Figure S1).

Therefore, we mapped Illumina reads of each VVE strain to a reference sequence (a *vanM*-carrying plasmid/chromosome) in the VVE-S strain. An obvious coverage peak in the region of the *vanM* gene cluster was observed in the coverage map of each VVE-R (Figure 1). Specifically, the average sequence coverage of the *vanM* gene cluster was similar to the surrounding regions in the susceptible ZY2 strain (Figure 1a) but 20.4-fold higher (29,920.0 compared to 1465.0) in ZY2-R (Figure 1b). Similar results were observed in SRR24-R and ZY11-R, with coverage of the *vanM* gene cluster being 20.4-fold higher (21,471.0 compared to 1054.3) and 58.6-fold higher (20,940.0 compared to 357.5) than that of the surrounding region (Figure 1d, f), respectively. The VVE-R cells thus have a remarkably higher copy number of the *vanM* gene cluster than VVE-S cells.

To estimate the copy number of the *vanM* gene and the *vanM*-carrying plasmid per cell, we mapped the Illumina reads from the VVE strains to the *vanM* gene, the *purK* gene (the housekeeping gene in the chromosome), and the *rep* gene (the gene for replication of the *vanM*-carrying plasmid), respectively. The average copy number of the *vanM* gene per cell was found to be 2.38, 2.20 and 1.10 in the susceptible strains ZY2, SRR24 and ZY11 but was significantly higher (93.78, 75.17 and 73.99) in ZY2-R, SRR24-R and ZY11-R, respectively (Table S2). In contrast, the copy number of the *rep* gene was not significantly different between ZY2 and ZY2-R (2.46 versus 2.77) and between SRR24 and SRR24-R (1.74 versus 1.84). The copy number of the *rep* gene in ZY11 or ZY11-R was not determined because the *vanM* gene is located on

252 the chromosome. These findings indicated that the increase in *vanM* gene dosage
253 could not be attributed to the amplification of the *vanM*-carrying plasmid.

254 The above results were also confirmed by q-PCR assay using *purK* as a reference
255 gene. As expected, an increase in *vanM* copy number was observed in the VVE-R
256 strains (Figure 2). There is approximately one copy of the *vanM* gene (0.99, 1.03, and
257 0.49) in each of the three VVE-S strains, but the *vanM* copy numbers increased to
258 79.08, 51.89, and 53.41 in their VVE-R strains, respectively. In comparison, the copy
259 number of the *rep* gene was similar between each VVE-S and its VVE-R (Figure 2).
260 These results further indicated that the increase in *vanM* gene copy number does not
261 result from an increase in plasmid copy number.

262 To investigate whether *vanM* gene amplification was present in all VVE-R
263 strains, we performed qPCR on the other 23 pairs of VVE-S and derived VVE-R.
264 Similarly, there was approximately one copy of *vanM* (0.34 ~ 0.92) in each VVE-S
265 strain, but there was a significantly increased copy number of *vanM* (5.28 ~ 127.64
266 copies) in the VVE-R strains (Figure S2).

267 In addition, we measured the copy number of *vanM* by qPCR of VVE-R after
268 bacterial growth in BHI broth containing a series of sub-lethal concentrations of
269 vancomycin. The Jonckheere-Terpstra test was performed to analyse the trend of
270 *vanM* copy number with respect to vancomycin concentration. We found a significant
271 concentration-dependent gene copy number increase in *vanM* ($p < 0.05$) in ZY2-R and
272 SRR24-R (Figure S3). In contrast, the increasing trend of *vanM* copy number in

ZY11-R with different vancomycin concentrations was not significant, with a p-value of 0.16 (Figure S3c).

**The *vanM* gene clusters are tandemly repeated in the genome.**

To determine how multiple *vanM* copies exist, the VVE isolates were sequenced using long-read sequencing on the PacBio and MinION platforms. Only one single copy of the *vanM* transposon was found to be located in a 97,574-bp plasmid of ZY2 (Figure 3a). However, in the Nanopore raw data of ZY2-R, one MinION read (46,070 bp) containing seven full *vanM* transposons and two partial *vanM* transposons on both sides was identified (indicating at least nine adjoining *vanM* transposons). One *vanM* gene cluster along with an IS*1216E* element was a repeat unit of 5917 bp, and every two adjacent repeats shared a common IS*1216E* between them. Similar results were found in SRR24/SRR24-R and ZY11/ZY11-R (Figure 3b, 3c). One single copy of the *vanM* transposon was located in a 123,020-bp plasmid of SRR24 or the chromosome of ZY11. However, PacBio or MinION reads containing (at least) five adjoining *vanM* transposons were also identified in the genomes of SRR24-R and ZY11-R. Due to the limitations of sequencing read length, we could not obtain the exact copy numbers of *vanM* transposons in ZY2-R, SRR24-R and ZY11-R, but the results revealed the presence of tandem amplification of the *vanM* transposon. The presence of tandem repeats was also verified by a specific PFGE and hybridization method using the restriction enzyme *Pst*I, whose cut sites are located within the *vanY* gene of the *vanM* gene cluster (Figure S4).

**The tandem amplification of the *vanM* gene cluster is unstable.**

The stability of glycopeptide resistance due to tandem amplification of the *vanM* gene cluster was tested. The MICs for vancomycin and teicoplanin and the *vanM* copy numbers of these 25 VVE-R isolates after growth for 40 generations free of antibiotic pressure were measured. The MICs for vancomycin and teicoplanin of all 25 VVE-R isolates were found to be unchanged, except for one isolate, SRR5-R, which had decreased resistance to teicoplanin (Table 1). However, the *vanM* copy numbers for 19 (76%) VVE-R isolates displayed marked decreases (Figure S2). Nevertheless, there were several exceptions. The *vanM* copy number of six VVE-R isolates with low *vanM* copy numbers (<30 copies) displayed no further significant decline after 40 generations without antibiotic pressure (Figure S2).

**Accumulation of the *vanM* gene cluster contributes to an increase in *vanM* gene expression.**

To observe the changes in expression level, we performed qRT-PCR on the *vanM* and *vanR* genes of three pairs of VVE strains in the absence and presence of vancomycin (1/8 MIC). In general, the expression level of *vanM* showed a marked (t-test, $P < 0.001$) increase in each VVE-R isolate compared with that in its VVE-S isolate (Figure 2). For the strains ZY2-R and ZY11-R, both having a deletion in *vanRS*, the expression of *vanR* was undetectable, while the expression level of *vanM* increased by more than 50 times in the VVE-R strains. This observation suggested that despite the lack of *vanRS* genes, the expression of *vanM* increased significantly. With regard to

315 SRR24 harbouring intact *vanRS* genes, the expression of *vanR* increased 9-fold in
316 SRR24-R. Meanwhile, the expression level of *vanM* increased 53.5-fold in SRR24-R.
317 This result suggests that the increase in *vanM* expression appears to be predominantly
318 the result of *vanM* gene accumulation rather than *vanR* regulation. In addition, a
319 sub-lethal concentration (1/8 MIC) of vancomycin had little effect on *vanM*
320 expression in ZY2-R and ZY11-R but could stimulate *vanM* expression to a higher
321 level in SRR24-R.

322

323 **Discussion**

324 Our previous study demonstrated that the vancomycin resistance gene *vanM* had a
325 high prevalence in clinical *Enterococcus* strains in China.[11] In this study, we found
326 that VVE strains capable of converting to vancomycin resistance were present at a
327 high rate (25/54) among *vanM*-carrying VSE strains, suggesting a potential future risk
328 of the prevalence of these VVE strains. In addition, in our study, amplification of the
329 *vanM* gene cluster was first identified as the primary mechanism for vancomycin
330 resistance in *vanM*-type VVE strains. The *vanM* transposon was tandemly repeated
331 with IS*1216E* elements in common in the genomes of VVE-R cells and occurred in
332 either the plasmid or chromosome. It can be inferred that a transposable unit
333 containing the *vanM* gene cluster and a single IS*1216E* might preferentially insert
334 adjacent to an IS*1216E* position in the plasmid or chromosome, similar to that
335 occurring in IS*26*, which also belongs to the IS*6* family.[19] IS*1216E*, as a direct repeat

sequence around the *vanM* gene cluster, might allow frequent spontaneous duplication to accomplish tandem amplification through the "translocatable units" movement mechanism and play a key role in the dissemination of the *vanM* gene cluster.[12,19]

The structural integrity of the *vanM* gene cluster, especially the functional genes *vanH*, *vanM*, and *vanX*, seemed to be related to the capability to develop resistance. VVE strains carrying type III *vanM* transposons with a complete *vanHMX* had a high capability of rapidly developing into high-level resistance to glycopeptides with an induction success rate of 100%. The existence of *vanA*-type VRE carrying Tn*1546*-like transposons missing *vanRS* has previously been reported,[20] and some alternative regulatory mechanisms, such as an alternate promoter in the upstream region of *vanH*, might lead to the expression of the genes that are functionally responsible for vancomycin resistance.[7] In contrast, it was difficult for strains having defects in *vanX* (type I and II) or *vanH* (type IV) to develop vancomycin resistance, so that only a proportion of isolates succeeded in generating resistance, probably owing to the crucial roles of the D,D-dipeptidase encoded by *vanX* and a ketoacid dehydrogenase encoded by *vanH* in vancomycin resistance.[1,21] In addition, derived VVE-R isolates of different *vanM* transposon types differ in teicoplanin susceptibility. The VVE strains carrying a complete *vanHMX* (type III) showed high-level resistance to vancomycin and teicoplanin, similar to VanA-type strains. This result suggested that the accumulation of *vanHMX* leads to an increase in their expression, followed by an increase in the production of VanH, VanM, and VanX, even in the absence of *vanRS*. However, the VVE-R strains of type I and II showed a VanB-like phenotype

with resistance to vancomycin but not to teicoplanin. This phenotype may to some degree be related to *vanX* deficiency. Previous work revealed that a complete elimination of D-Ala-D-Ala-containing precursors was required for teicoplanin resistance.[22] However, for VVEs carrying an incomplete *vanX*, the hydrolysis of D-Ala-D-Ala was limited, followed by synthesis obstruction of UDP-MurNAc-pentadepsipetide, similar to VanB-phenotype enterococci.[22]

The role of tandem gene amplification in the development of antibiotic resistance has been reported in other species, such as *Proteus mirabilis*, *Staphylococcus aureus*, *Acinetobacter baumannii*, and *Pseudomonas aeruginosa*.[23-26] Gene amplification, presumably due to increased gene dosage, might be beneficial for adaptation to environmental stresses, such as drug selection.[27, 28] In our study, the accumulation of the *vanM* gene cluster in VVE-R, probably leading to increased *vanM* transcription, provides VVE with a competitive advantage to survive vancomycin exposure. However, this amplification of the *vanM* gene cluster appeared to increase with higher vancomycin concentration (Figure S3), conversely, the *vanM* gene dosage would decrease after growth in the absence of vancomycin (Figure S2). This result might reflect the instability of gene amplifications, which probably act as an intermediate presence under antibiotic pressure prior to a more stable genetic change.[19] Furthermore, due to its transient nature, the clinical cases and clinical importance of *vanM* gene amplification in vancomycin resistance are probably underestimated.

Guidelines for vancomycin therapeutic drug monitoring recommend that trough serum vancomycin concentrations should be maintained at 10 –15 mg/L in adult

patients.[29, 30] We attempted to expose the *vanM*-type VVE strains directly to 8 mg/L vancomycin, but no growth was observed within 5 days. However, in clinical practice, the serum concentration of vancomycin usually fails to reach 10 mg/L and is likely even lower in other tissues. The treatment of patients carrying these VSE-like VVE strains with vancomycin might thus provide a favourable environment for them to develop into VRE. Therefore, early screening for the presence of VVE and the use of vancomycin according to clinical guidelines is crucial to avoid the risk of therapy failure. Considering that VVE strains are hidden by their susceptible phenotype and can thus easily escape detection, it is essential to perform genotypic screening for the presence of *vanM*. WGS analysis is particularly useful to monitor whether the entire *vanM* gene cluster is present intact and to better judge the risk of evolving into VRE. Because of the rapid emergence and spread of *vanM*-carrying *E. faecium* strains in China, tandem amplification of the *vanM* gene cluster might become the primary resistance mechanism for the emergence of VRE in China in the future.


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## Transparency declarations

None to declare.

## Supplementary data

Tables S1、S2 and Figures S1 to S4 are available as Supplementary data at JAC Online

## References


**1** Ranotkar S, Kumar P, Zutshi S *et al.* Vancomycin-resistant enterococci: Troublemaker of the 21st century. *J Glob Antimicrob Resist* 2014; **2**: 205-12.

**2** Coombs GW, Daley DA, Lee YT *et al.* Australian Group on Antimicrobial Resistance (AGAR) Australian Staphylococcus aureus Sepsis Outcome Programme (ASSOP) Annual Report 2016. *Commun Dis Intell (2018)* 2018; **42**: S2209-6051(18)00021-0.

**3** Baptista M, Depardieu F, Reynolds P *et al.* Mutations leading to increased levels of resistance to glycopeptide antibiotics in VanB-type enterococci. *Mol Microbiol* 1997; **25**: 93-105.

**4** Zhang G, Lin X, Cai J *et al.* Characteristics of clinical and environmental *vanM*-carrying vancomycin-resistant enterococci isolates from an infected patient. *Int J Antimicrob Agents* 2018; **51**: 932-35.

**5** Chen C, Sun J, Guo Y *et al.* High Prevalence of *vanM* in Vancomycin-Resistant *Enterococcus faecium* Isolates from Shanghai, China. *Antimicrob Agents Chemother* 2015; **59**: 7795-8.

**6** Xu X, Lin D, Yan G *et al. vanM*, a new glycopeptide resistance gene cluster found in *Enterococcus faecium*. *Antimicrob Agents Chemother* 2010; **54**: 4643-7.

**7** Thaker MN, Kalan L, Waglechner N *et al.* Vancomycin-variable enterococci can give rise to constitutive resistance during antibiotic therapy. *Antimicrob Agents Chemother* 2015; **59**: 1405-10.

**8** Coburn B, Low DE, Patel SN *et al.* Vancomycin-variable *Enterococcus faecium*: in vivo emergence of vancomycin resistance in a vancomycin-susceptible isolate. *J Clin Microbiol* 2014; **52**: 1766-7.

**9** Hansen TA, Pedersen MS, Nielsen LG *et al.* Emergence of a vancomycin-variable *Enterococcus faecium* ST1421 strain containing a deletion in *vanX*. *J Antimicrob Chemother* 2018; **73**: 2936-40.

**10** Sivertsen A, Pedersen T, Larssen KW *et al.* A Silenced *vanA* Gene Cluster on a Transferable Plasmid Caused an Outbreak of Vancomycin-Variable Enterococci. *Antimicrob Agents Chemother* 2016; **60**: 4119-27.

**11** Sun L, Qu T, Wang D *et al.* Characterization of *vanM* carrying clinical *Enterococcus* isolates and diversity of the suppressed *vanM* gene cluster. *Infect Genet Evol* 2019; **68**: 145-52.

**12** Nicoloff H, Hjort K, Levin BR *et al*. The high prevalence of antibiotic heteroresistance in pathogenic bacteria is mainly caused by gene amplification. *Nat Microbiol* 2019; **4**: 504-14.

**13** 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.

**14** Aziz RK, Bartels D, Best AA *et al.* The RAST Server: rapid annotations using subsystems technology. *BMC Genomics* 2008; **9**: 75.

**15** Tatusova T, DiCuccio M, Badretdin A *et al.* NCBI prokaryotic genome annotation pipeline.

*Nucleic Acids Res* 2016; **44**: 6614-24.

**16** Bubner B, Baldwin IT. Use of real-time PCR for determining copy number and zygosity in transgenic plants. *Plant Cell Rep* 2004; **23**: 263-71.

**17** Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 2001; **25**: 402-8.

**18** Saeedi B, Hallgren A, Jonasson J *et al.* Modified pulsed-field gel electrophoresis protocol for typing of enterococci. *APMIS* 2002; **110**: 869-74.

**19** Harmer CJ, Moran RA, Hall RM. Movement of IS*26*-associated antibiotic resistance genes occurs via a translocatable unit that includes a single IS*26* and preferentially inserts adjacent to another IS*26*. *MBio* 2014; **5**: e01801-14.

**20** Sung K, Khan SA, Nawaz MS. Genetic diversity of Tn*1546*-like elements in clinical isolates of vancomycin-resistant enterococci. *Int J Antimicrob Agents* 2008; **31**: 549-54.

**21** Reynolds PE, Depardieu F, Dutka-Malen S *et al.* Glycopeptide resistance mediated by enterococcal transposon Tn*1546* requires production of VanX for hydrolysis of D-alanyl-D-alanine. *Mol Microbiol* 1994; **13**: 1065-70.

**22** Arthur M, Depardieu F, Reynolds P *et al.* Quantitative analysis of the metabolism of soluble cytoplasmic peptidoglycan precursors of glycopeptide-resistant enterococci. *Mol Microbiol* 1996; **21**: 33-44.

**23** Girlich D, Bonnin RA, Bogaerts P *et al.* Chromosomal Amplification of the $bla_{\text{OXA-58}}$ Carbapenemase Gene in a *Proteus mirabilis* Clinical Isolate. *Antimicrob Agents Chemother* 2017;

**61**: e01697-16.

**24** Gallagher LA, Coughlan S, Black NS *et al.* Tandem Amplification of the Staphylococcal Cassette Chromosome *mec* Element Can Drive High-Level Methicillin Resistance in Methicillin-Resistant *Staphylococcus aureus*. *Antimicrob Agents Chemother* 2017; **61**: e00869-17.

**25** McGann P, Courvalin P, Snesrud E *et al.* Amplification of aminoglycoside resistance gene *aphA1* in *Acinetobacter baumannii* results in tobramycin therapy failure. *MBio* 2014; **5**: e00915.

**26** San Millan A, Toll-Riera M, Escudero JA *et al.* Sequencing of plasmids pAMBL1 and pAMBL2 from *Pseudomonas aeruginosa* reveals a $bla_{\text{VIM-1}}$ amplification causing high-level carbapenem resistance. *J Antimicrob Chemother* 2015; **70**: 3000-3.

**27** Iantorno SA, Durrant C, Khan A *et al.* Gene Expression in *Leishmania* Is Regulated Predominantly by Gene Dosage. *MBio* 2017; **8**: e01393-17.

**28** Dhar R, Bergmiller T, Wagner A. Increased gene dosage plays a predominant role in the initial stages of evolution of duplicate TEM-1 beta lactamase genes. *Evolution* 2014; **68**: 1775-91.

**29** Ye ZK, Chen YL, Chen K *et al.* Therapeutic drug monitoring of vancomycin: a guideline of the Division of Therapeutic Drug Monitoring, Chinese Pharmacological Society. *J Antimicrob Chemother* 2016; **71**: 3020-25.

**30** Matsumoto K, Takesue Y, Ohmagari N *et al.* Practice guidelines for therapeutic drug monitoring of vancomycin: a consensus review of the Japanese Society of Chemotherapy and the Japanese Society of Therapeutic Drug Monitoring. *J Infect Chemother* 2013; **19**: 365-80.

480 **Table 1** Summary of *in vitro* vancomycin induction tests and characterization of 25 VVE-R strains

| *vanM* transposon type | Number of isolates [b] succeeding / failing | Successfully induced strain | MIC (mg/L) | | | | The vancomycin concentration to acquire VVE-R cells (mg/L) | Time to acquire VVE-R cells (d) |
|---|---|---|---|---|---|---|---|---|
| | | | | | After 40 generations [c] | | | |
| | | | Van | Tec | Van | Tec | | |
| Type I (missing partial *vanRS* and *vanX*) | 16 / 23 | **ZY2-R** [a] | 256 | 8 | 256 | 8 | 8 | 5 |
| | | SRR5-R | 256 | 128 | 256 | 2 | 8 | 5 |
| | | SRR26-R | 256 | 8 | 256 | 8 | 16 | 6 |
| | | ZY17-R | 256 | 32 | 256 | 32 | 16 | 6 |
| | | SRR29-R | 256 | 32 | 256 | 32 | 8 | 6 |
| | | SRR13-R | 128 | 8 | 128 | 8 | 8 | 8 |
| | | ZY1-R | 256 | 16 | 256 | 16 | 32 | 8 |
| | | SRR21-R | 128 | 8 | 128 | 8 | 16 | 9 |
| | | ZY5-R | 128 | 1 | 128 | 1 | 16 | 9 |
| | | ZY4-R | 128 | 16 | 128 | 16 | 32 | 10 |
| | | SRR8-R | 256 | 128 | 256 | 128 | 32 | 10 |
| | | SRR27-R | 128 | 16 | 128 | 32 | 32 | 12 |
| | | SRR7-R | 256 | 64 | 256 | 64 | 32 | 12 |
| | | SRR2-R | 256 | 64 | 256 | 64 | 16 | 12 |
| | | SRR17-R | 128 | 4 | 128 | 4 | 16 | 12 |
| | | ZY16-R | 128 | 4 | 128 | 4 | 32 | 14 |
| Type II (missing partial *vanX*) | 6 / 1 | **SRR24-R** [a] | 128 | 1 | 128 | 1 | 4 | 3 |
| | | ZY12-R | 256 | 4 | 256 | 4 | 4 | 3 |
| | | SY3-R | 256 | 4 | 256 | 4 | 4 | 3 |
| | | SRR25-R | 128 | 8 | 128 | 8 | 4 | 3 |
| | | ZY19-R | 256 | 4 | 256 | 4 | 8 | 5 |
| | | ZY6-R | 256 | 16 | 256 | 16 | 8 | 6 |
| Type III (missing partial *vanRS*) | 3 / 0 | **ZY11-R** [a] | 512 | 256 | 512 | 256 | 2 | 2 |
| | | SRR14-R | 512 | 512 | 512 | 512 | 2 | 2 |
| | | SRR15-R | 512 | 512 | 512 | 512 | 2 | 2 |
| Type IV | 0 / 1 | NA | | | | | | |
| Type V | NA | NA | | | | | | |
| Undetermined type | 0 / 4 | NA | | | | | | |

481 Footnote: One isolate of Type IV (having a deletion in *vanRS* and interruption in *vanH*) and four isolates of
482 undetermined type (the sequence of the *vanM* gene cluster was undetermined) failed to develop resistance. One
483 strain, SRR12, of Type V (having an intact *vanM* gene cluster), was shown to be heteroresistant to vancomycin and
484 was excluded from this study. NA, not applicable.

485

a. The strains in bold were selected for mechanism investigation;

b. The number of strains succeeding and failing to generate resistance;

c. MICs for vancomycin and teicoplanin of VVE-R strains grown in the absence of antibiotics for 40 generations;

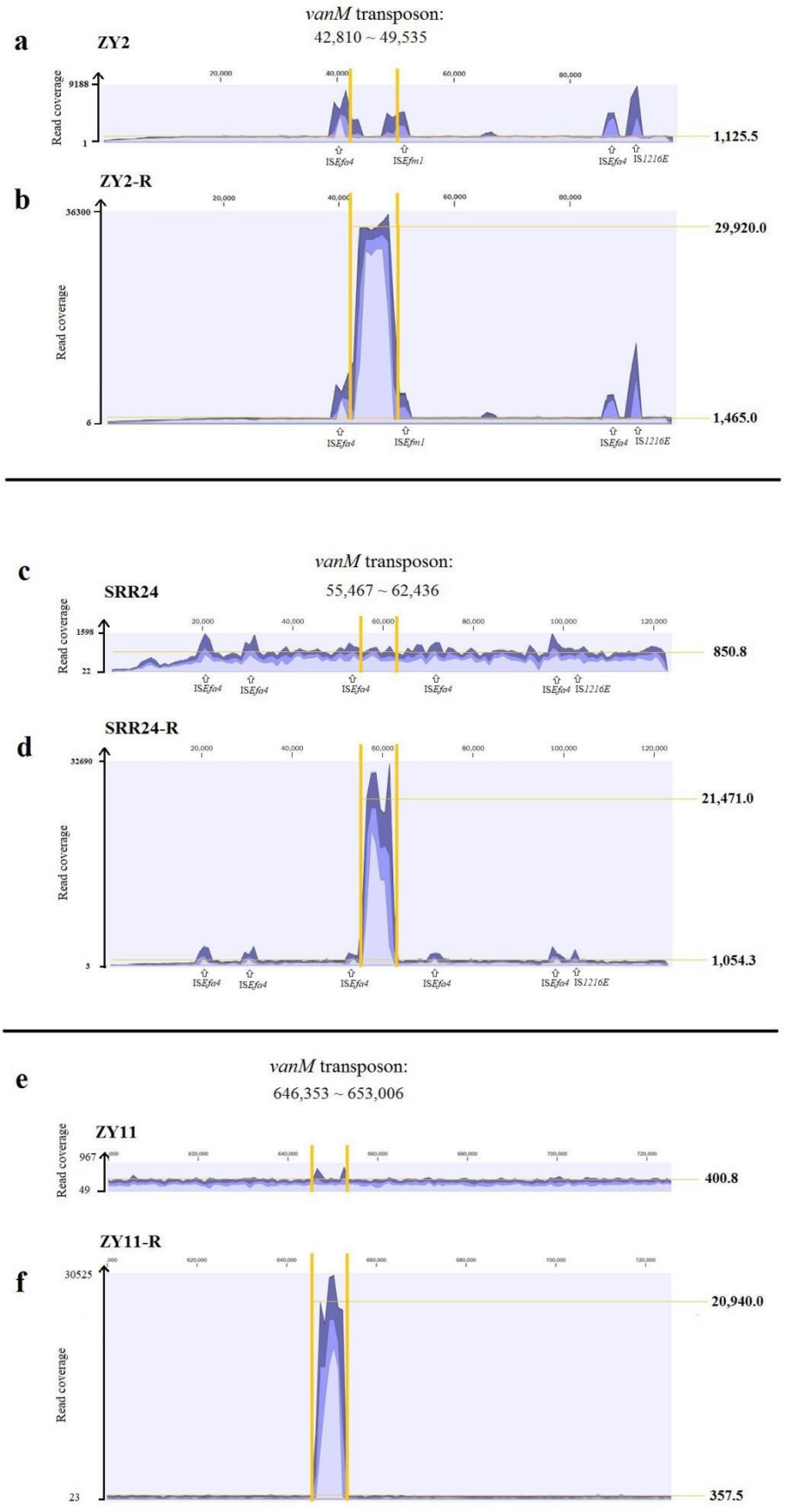
a
ZY2
vanM transposon:
42,810 ~ 49,535
Read coverage
9188
1
1,125.5
ISEfa4
ISEfm1
ISEfa4
IS1216E
b
ZY2-R
36300
6
29,920.0
1,465.0
c
SRR24
55,467 ~ 62,436
1598
22
850.8
d
SRR24-R
32690
3
21,471.0
1,054.3
e
ZY11
967
49
400.8
f
ZY11-R
30525
23
20,940.0
357.5

**Figure 1** Mapping graphs representing read coverage difference (ordinate) between VVE-S and VVE-R in the complete plasmid of ZY2 and SRR24 (the abscissa of a, b, c, d) and in the partial chromosome of ZY11 (the abscissa of e, f). The regions of the *vanM* transposon are framed by two yellow lines. The mean coverages are indicated on the right. The peaks in coverage outside the *vanM* transposon corresponding to IS elements are indicated by the arrows. (This figure appears in colour in the online version of JAC and in black and white in the printed version of JAC.)

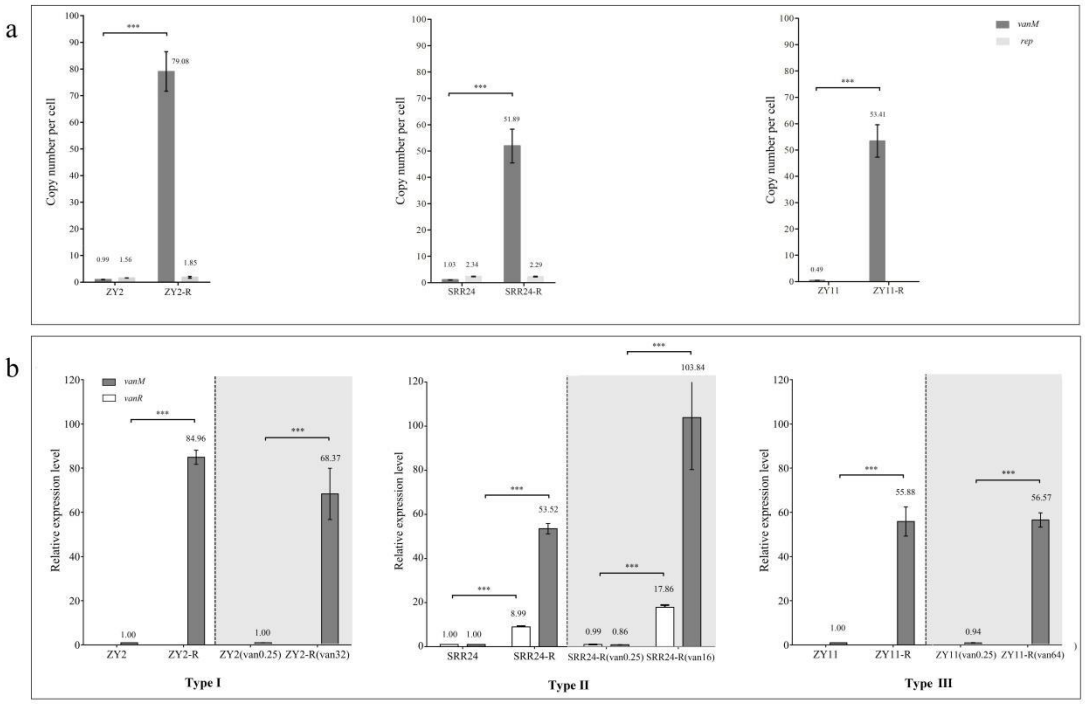


**Figure 2** Change in the copy numbers and transcript levels of *vanM* in each VVE-S and VVE-R pair. (a) The *vanM* copy numbers per cell of three types of VVE strains measured by qPCR. ZY2, SRR24, and ZY11 are susceptible original isolates. The *rep* gene is the gene encoding the replication protein as an indicator of plasmid copy number. ZY2-R, SRR24-R, and ZY11-R are their corresponding VVE-R isolates. (b) Relative expression levels of *vanM* and *vanR* measured by RT-qPCR in the absence (left) and presence of vancomycin (1/8 MIC) (shaded, right). The fold changes of expression level in the VVE-R strains were normalized to that of the original VVE-S strains. All measurements were normalized to the housekeeping gene *purK*. *** Significant difference ($P<0.001$) between the mean values.

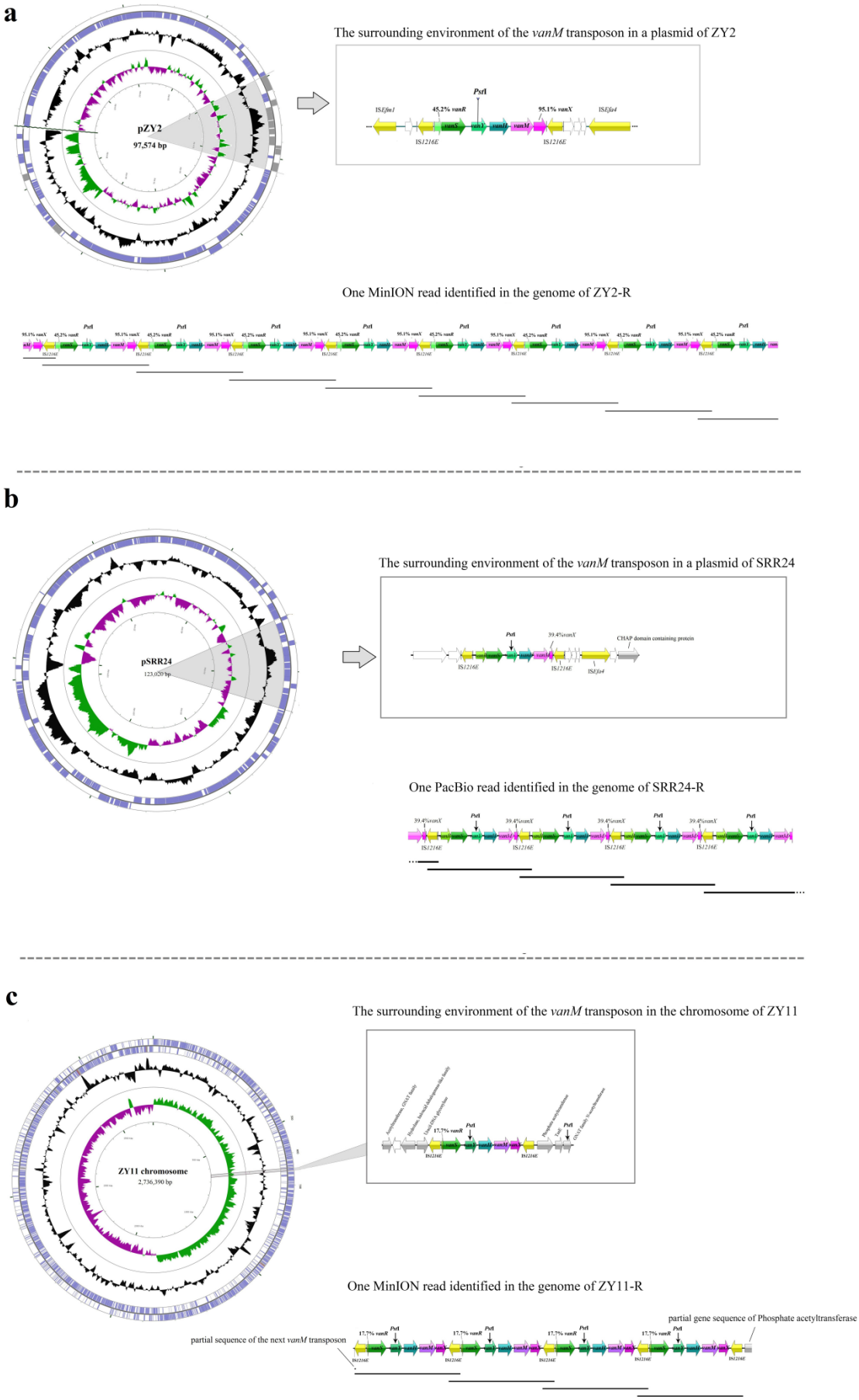
a
pZY2
97,574 bp
The surrounding environment of the vanM transposon in a plasmid of ZY2
PstI
ISEfm1
45.2% vanR
95.1% vanX
ISEfa4
IS1216E
IS1216E
One MinION read identified in the genome of ZY2-R
b
pSRR24
123,020 bp
The surrounding environment of the vanM transposon in a plasmid of SRR24
PstI
39.4%vanX
CHAP domain containing protein
IS1216E
IS1216E
ISEfa4
One PacBio read identified in the genome of SRR24-R
c
ZY11 chromosome
2,736,390 bp
The surrounding environment of the vanM transposon in the chromosome of ZY11
17.7% vanR
PstI
IS1216E
IS1216E
One MinION read identified in the genome of ZY11-R
partial sequence of the next vanM transposon
partial gene sequence of Phosphate acetyltransferase

**Figure 3** Comparison of *vanM* cluster carriers in the genomes of the original strains (ZY2, SRR24, ZY11) and the induced resistant strains (ZY2-R, SRR24-R, ZY11-R). The circular map of the *vanM*-carrying plasmid/chromosome in ZY2, SRR24, and ZY11 is shown at the left of each panel. The *vanM* gene cluster surrounding the region is indicated with grey shading and further displayed in a grey rectangle at the top right of each panel. One MinION read harbouring nine adjoining *vanM* transposons in ZY2-R is shown at the bottom right of panel (a). One PacBio read harbouring at least five tandem repeats of the *vanM* transposon in the genome of SRR24-R is shown at the bottom right of panel (b). One MinION read harbouring at least five tandem repeats of the *vanM* transposon in ZY11-R is at the bottom right of panel (c). (This figure appears in colour in the online version of JAC and in black and white in the printed version of JAC.)

# Supplementary data

**Table S1** Primers designed in this study

| Primers | DNA sequence (5' to 3') | Length of target gene | Reference |
|---|---|---|---|
| M1-F | CAATAATTCCTTCACCATTGATTACG | ~ 2.5 k | Sun *et al.*, 2018[11] |
| M1-R | CTCAGAAGAGCATAATGTATCGGT | | |
| M2-F | CTATATCAATGTTGTTGGCAATCTC | ~ 2.4 k | Sun *et al.*, 2018[11] |
| M2-R | GTTGTTCAAGTTGTGAATTATGGAG | | |
| M3-F | TAGTCCAAGACCAGTGCTATCC | ~ 2 k | Sun *et al.*, 2018[11] |
| M3-R | TCAGTCAAGAAGAAATCATCAGC | | |
| Q-*vanM*-F | CTCAGAAGAGCATAATGTATCGGT | 154 | Sun *et al.*, 2018[11] |
| Q-*vanM*-R | ACAGTGTTCATTATCCCAATCTATAC | | |
| Q-*vanR*-F | GATATGAAGTAACCCGTCAAGTC | 158 | In this study |
| Q-*vanR*-R | CACACGAGCAACCAATTCAATC | | |
| Q-purK-F | GATATCCAAGATGCGATTGACG | 154 | Sun *et al.*, 2018[11] |
| Q-purK-R | CTTCTAAAACACAGGTTCCTTCTC | | |
| Q-*rep*-srr12-F | CGAATCAACTTGTGGATAGCATTG | 157 | In this study |
| Q-*rep*-srr12-R | GATGAACTAGAAGACATCAACGCA | | |
| Q-*rep*-srr24,zy2-F | GTCATCTCACCGCTAGAAATTAC | 156 | In this study |
| Q-*rep*-srr24,zy2-R | CGCATACTTGCTTGTCAAGTTAAC | | |
| *vanM*-probe-F | CAGAGATTGCCAACAACATTGA | 268 | Sun *et al.*, 2018[11] |
| *vanM*-probe-R | AGCCTACATAAGGTATACCAGAC | | |

**Table.S2** Estimation of *vanM* copy number by mapping Illumina reads to reference genes

| Isolates | Mean sequence coverage | | | Estimated gene copy no. | |
|---|---|---|---|---|---|
| | *purK* gene (chromosome) | *rep* gene (plasmid) | *vanM* | *vanM*- carrying plasmid | *vanM* |
| ZY2 | 370.98 | 912.56 | 881.40 | 2.46 | 2.38 |
| ZY2-R | 351.10 | 971.54 | 32925.79 | 2.77 | 93.78 |
| SRR24 | 377.03 | 656.60 | 830.41 | 1.74 | 2.20 |
| SRR24-R | 328.05 | 604.60 | 24658.14 | 1.84 | 75.17 |
| ZY11 | 354.17 | NA | 390.64 | NA | 1.10 |
| ZY11-R | 322.27 | NA | 23844.75 | NA | 73.99 |

Footnote: NA, Not applicable. The *vanM* gene cluster of ZY11 and ZY11-R are located on chromosome.

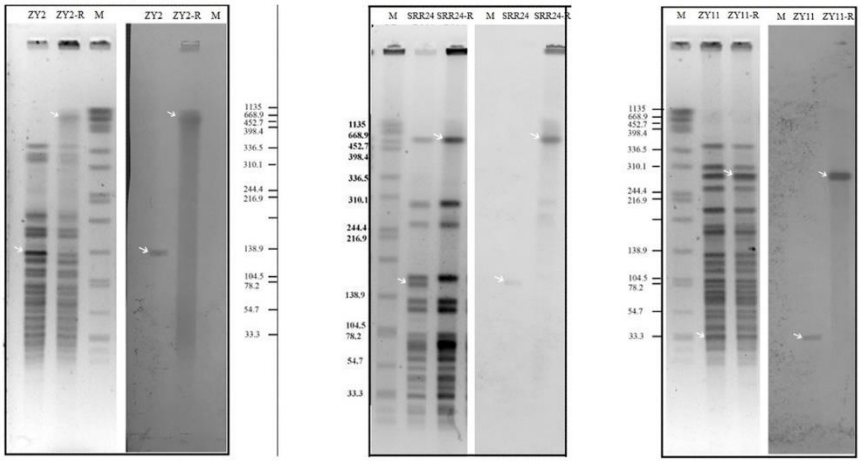


**Figure S1** DNA pattern in each VVE strain by *Sma*I-PFGE and southern blotting hybridization with *vanM*-probes.

The sizes of the molecular marker (M) are indicated.

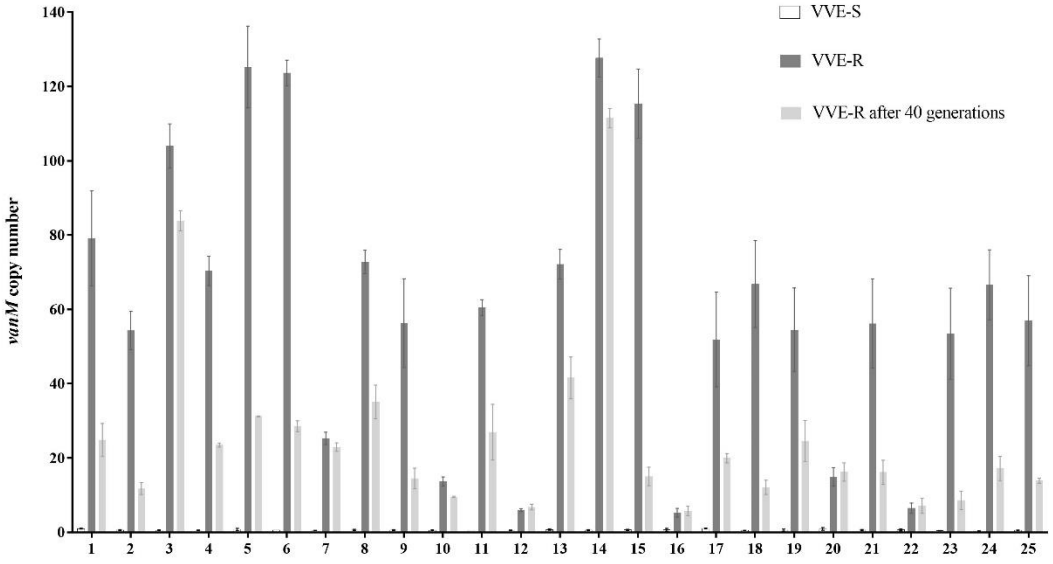


**Figure S2** The *vanM* copy numbers per cell of 25 strains for their VVE-S (□), VVE-R (■) and VVE-R after growth in absence of antibiotic for 40 generations (■) measured by qPCR. All measurements were normalized against the housekeeping gene *purK*. The number 1 ~ 25 represent 25 VVE isolates in the same order as column 3 of Table.1 from ZY2 to SRR15.

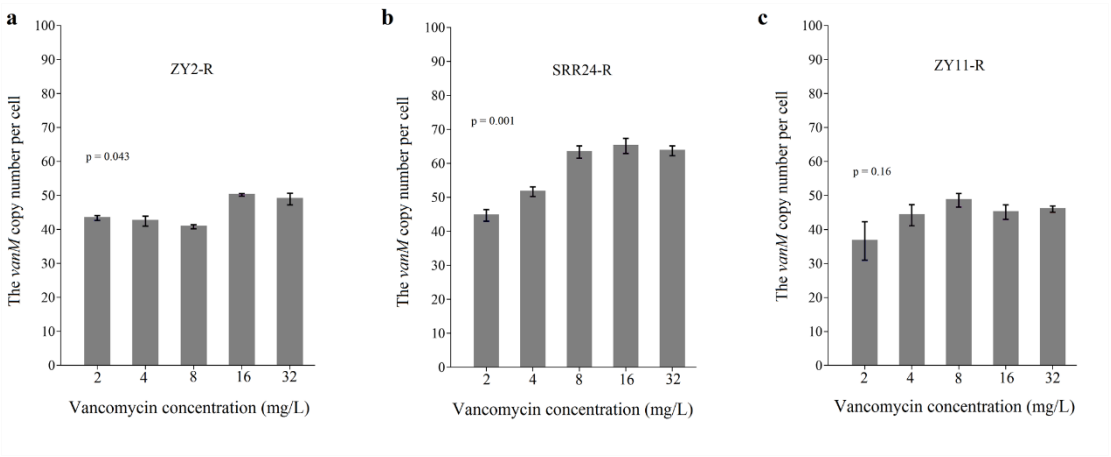


**Figure S3** The *vanM* copy numbers of four types of VVE-R strains after bacterial growth in different concentration of vancomycin. qPCR was performed on the genomic DNA of the sub-cultures in different vancomycin concentrations from the same VVE-R colony culture. Jonckheere-Terpstra test was performed by statistics using R to analysis the trend of *vanM* copy number with vancomycin concentration and the p-value for each group was listed.

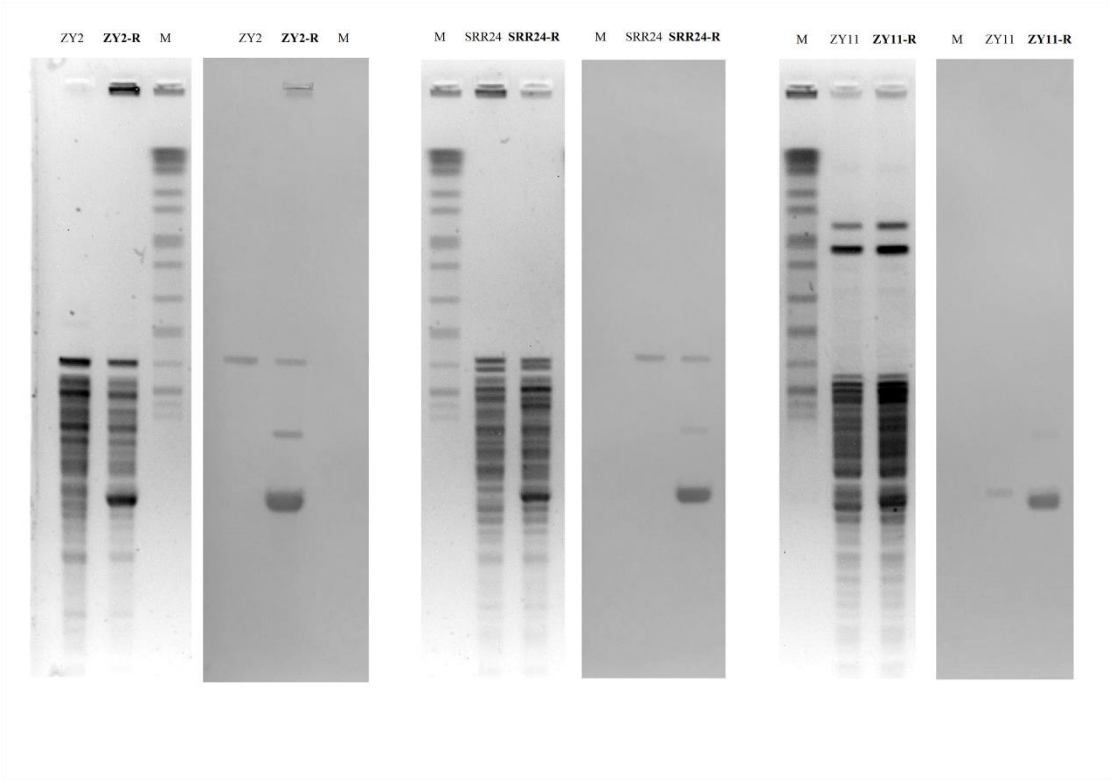


**Figure S4** Determination of the *vanM* gene copy number by *Pst*I-PFGE and *vanM* blotting hybridization. Salmonella H9812 was included as molecular size markers