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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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Yunsong Yu and Tingting Qu made an equal contribution to the article.
**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 IS1216E 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 \textit{van} gene clusters, among which \textit{vanA} and \textit{vanB} are the most commonly reported worldwide.\textsuperscript{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.\textsuperscript{3} In recent years, however, \textit{vanM} has emerged as a new and prevalent resistance determinant in clinical enterococci in China, especially in the cities of Shanghai and Hangzhou.\textsuperscript{4,5} The \textit{vanM} gene cluster contains the \textit{vanR}, \textit{vanS}, \textit{vanY}, \textit{vanH}, \textit{vanM}, and \textit{vanX} genes, along with an IS\textit{1216}-like element, together forming the \textit{vanM} transposon, which usually confers high resistance to vancomycin and teicoplanin.\textsuperscript{6}

Vancomycin-variable enterococci (VVE) are a group of enterococci containing \textit{van} genes that exhibit a vancomycin-susceptible phenotype due to a deletion or insertion in the \textit{van} gene cluster but are capable of shifting to a glycopeptide-resistant phenotype under vancomycin therapy \textit{in vivo} or exposure \textit{in vitro}.\textsuperscript{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 \textit{vanA}-containing \textit{Enterococcus} isolates in Canada, Norway, and Denmark.\textsuperscript{7,9,10} Mutations or the excision of an IS\textit{L3}-family element upstream of \textit{vanHAX} were reported as contributors to switching to a resistant phenotype.\textsuperscript{7,10} In a recent study, changes in \textit{ddl} and \textit{vanS} genes or increases in the copy number of \textit{vanA}-carrying plasmids were
shown to lead to vancomycin resistance in vanA-type VVE strains containing a
deletion in vanX. 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. 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.

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). 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 \textit{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.\textsuperscript{13} All the assembled contigs were annotated using the RAST server\textsuperscript{14} and manually refined by the NCBI Prokaryotic Genome Annotation Pipeline.\textsuperscript{15}

**PCR and local sequencing.**

The \textit{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 \textit{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 \times the average length of mapped reads / the length of the target gene.

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

Determination of \textit{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 \textit{vanM}, \textit{rep} and \textit{purK} genes are listed in Table S1. Quantitative PCRs were carried out in a 10 \textmu L reaction that contained 5 \textmu L SYBR\textsuperscript{®} Premix Ex Taq\textsuperscript{TM} PCR kit (Takara Bio, Kusatsu, Japan), 0.2 \textmu L genomic DNA (10 ng/\textmu L) as template, and 0.2 \textmu L each primer (10 \textmu M). 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. 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.

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. 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 \textit{vanM} and was synthesized using the primers \textit{vanM-probe-F} and \textit{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 (\textit{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: SSSK0000000, SRXZ0000000, SIWX0000000, SIWW0000000, SIWV0000000, and SRSK0000000.

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 \textit{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. 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 \(\leq 8\) 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 \(SmaI\)-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
the chromosome. These findings indicated that the increase in \textit{vanM} gene dosage could not be attributed to the amplification of the \textit{vanM}-carrying plasmid.

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

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

In addition, we measured the copy number of \textit{vanM} by qPCR of VVE-R after bacterial growth in BHI broth containing a series of sub-lethal concentrations of vancomycin. The Jonckheere-Terpstra test was performed to analyse the trend of \textit{vanM} copy number with respect to vancomycin concentration. We found a significant concentration-dependent gene copy number increase in \textit{vanM} (p<0.05) in ZY2-R and SRR24-R (Figure S3). In contrast, the increasing trend of \textit{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 IS1216E element was a repeat unit of 5917 bp, and every two adjacent repeats shared a common IS1216E 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 *PstI*, 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
SRR24 harbouring intact vanRS genes, the expression of vanR increased 9-fold in SRR24-R. Meanwhile, the expression level of vanM increased 53.5-fold in SRR24-R. This result suggests that the increase in vanM expression appears to be predominantly the result of vanM gene accumulation rather than vanR regulation. In addition, a sub-lethal concentration (1/8 MIC) of vancomycin had little effect on vanM expression in ZY2-R and ZY11-R but could stimulate vanM expression to a higher level in SRR24-R.

Discussion

Our previous study demonstrated that the vancomycin resistance gene vanM had a high prevalence in clinical Enterococcus strains in China. In this study, we found that VVE strains capable of converting to vancomycin resistance were present at a high rate (25/54) among vanM-carrying VSE strains, suggesting a potential future risk of the prevalence of these VVE strains. In addition, in our study, amplification of the vanM gene cluster was first identified as the primary mechanism for vancomycin resistance in vanM-type VVE strains. The vanM transposon was tandemly repeated with IS1216E elements in common in the genomes of VVE-R cells and occurred in either the plasmid or chromosome. It can be inferred that a transposable unit containing the vanM gene cluster and a single IS1216E might preferentially insert adjacent to an IS1216E position in the plasmid or chromosome, similar to that occurring in IS26, which also belongs to the IS6 family. IS1216E, 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 Tn1546-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 \textit{vanX} deficiency. Previous work revealed that a complete elimination of D-Ala-D-Ala-containing precursors was required for teicoplanin resistance.\textsuperscript{22} However, for VVEs carrying an incomplete \textit{vanX}, the hydrolysis of D-Ala-D-Ala was limited, followed by synthesis obstruction of UDP-MurNAc-pentadepsipeptide, similar to VanB-phenotype enterococci.\textsuperscript{22}

The role of tandem gene amplification in the development of antibiotic resistance has been reported in other species, such as \textit{Proteus mirabilis}, \textit{Staphylococcus aureus}, \textit{Acinetobacter baumannii}, and \textit{Pseudomonas aeruginosa}.\textsuperscript{23-26} Gene amplification, presumably due to increased gene dosage, might be beneficial for adaptation to environmental stresses, such as drug selection.\textsuperscript{27, 28} In our study, the accumulation of the \textit{vanM} gene cluster in VVE-R, probably leading to increased \textit{vanM} transcription, provides VVE with a competitive advantage to survive vancomycin exposure. However, this amplification of the \textit{vanM} gene cluster appeared to increase with higher vancomycin concentration (Figure S3), conversely, the \textit{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.\textsuperscript{19} Furthermore, due to its transient nature, the clinical cases and clinical importance of \textit{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.\textsuperscript{29,30} We attempted to expose the \textit{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 \textit{vanM}. WGS analysis is particularly useful to monitor whether the entire \textit{vanM} gene cluster is present intact and to better judge the risk of evolving into VRE. Because of the rapid emergence and spread of \textit{vanM}-carrying \textit{E. faecium} strains in China, tandem amplification of the \textit{vanM} gene cluster might become the primary resistance mechanism for the emergence of VRE in China in the future.

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\textbf{Transparency declarations}

None to declare.
Supplementary data

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

References


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


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

<table>
<thead>
<tr>
<th>vanM transposon type</th>
<th>Number of isolates</th>
<th>Successfully induced strain</th>
<th>MIC (mg/L)</th>
<th>The vancomycin concentration to acquire VVE-R cells (mg/L)</th>
<th>Time to acquire VVE-R cells (d)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Van Tec</td>
<td>Van Tec</td>
<td></td>
</tr>
<tr>
<td>Type I</td>
<td>16 / 23</td>
<td>ZY2-R *</td>
<td>256 8</td>
<td>256 8</td>
<td>8 5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>SRR5-R</td>
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<td>256 2</td>
<td>8 5</td>
</tr>
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<td>ZY17-R</td>
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<td>256 32</td>
<td>16 6</td>
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<td>SRR29-R</td>
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<td>8 6</td>
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<td>SRR13-R</td>
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<td>128 8</td>
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<td></td>
<td></td>
<td>ZY1-R</td>
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<td>256 16</td>
<td>32 8</td>
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<td></td>
<td>SRR21-R</td>
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<td>128 8</td>
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<td>ZY5-R</td>
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<td>128 1</td>
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<td>128 16</td>
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<td>SRR27-R</td>
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<td>32 12</td>
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<td>SRR7-R</td>
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<td>256 64</td>
<td>32 12</td>
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<td></td>
<td>SRR2-R</td>
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<td>256 64</td>
<td>16 12</td>
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<td>128 4</td>
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<td></td>
<td>ZY16-R</td>
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<td>128 4</td>
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<td>128 1</td>
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<td>256 4</td>
<td>4 3</td>
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<td>SY3-R</td>
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<td>256 4</td>
<td>4 3</td>
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<td>SRR25-R</td>
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<td>128 8</td>
<td>4 3</td>
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<td>ZY19-R</td>
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<td>256 4</td>
<td>8 5</td>
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<td></td>
<td>ZY6-R</td>
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<td>256 16</td>
<td>8 6</td>
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<td>ZY11-R *</td>
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<td>512 256</td>
<td>2 2</td>
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<tr>
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<td>SRR14-R</td>
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<td>512 512</td>
<td>2 2</td>
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<td>SRR15-R</td>
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<td>512 512</td>
<td>2 2</td>
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<td>Type IV</td>
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<td>Type V</td>
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<td>Undetermined type</td>
<td>0 / 4</td>
<td>NA</td>
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</table>

Footnote: One isolate of Type IV (having a deletion in *vanRS* and interruption in *vanH*) and four isolates of undetermined type (the sequence of the *vanM* gene cluster was undetermined) failed to develop resistance. One strain, SRR12, of Type V (having an intact *vanM* gene cluster), was shown to be heteroresistant to vancomycin and was excluded from this study. NA, not applicable.
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;
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) The surrounding environment of the vanM transposon in a plasmid of ZY2

One MinION read identified in the genome of ZY2-R

b) The surrounding environment of the vanM transposon in a plasmid of SRR24

One PacBio read identified in the genome of SRR24-R

c) The surrounding environment of the vanM transposon in the chromosome of ZY11

One MinION read identified in the genome of ZY11-R
**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.)
Table S1 Primers designed in this study

<table>
<thead>
<tr>
<th>Primers</th>
<th>DNA sequence (5’ to 3’)</th>
<th>Length of target gene</th>
<th>Reference</th>
</tr>
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<tr>
<td>M1-F</td>
<td>CAATAATTCCTTCACCATGATTACG</td>
<td>~ 2.5 k</td>
<td>Sun et al., 2018</td>
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<td>M1-R</td>
<td>CTCAGAAGACATAATGTATCGGT</td>
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<tr>
<td>M2-F</td>
<td>CTATATCAATGGTGTTGGCAATCT</td>
<td>~ 2.4 k</td>
<td>Sun et al., 2018</td>
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<td>M2-R</td>
<td>GGTGTTCAAGTTGAATTAAGG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>M3-F</td>
<td>TAGTCAGAGCAGTGTATCC</td>
<td>~ 2 k</td>
<td>Sun et al., 2018</td>
</tr>
<tr>
<td>M3-R</td>
<td>TCAGTCAAGAGAAATCATCAG</td>
<td></td>
<td></td>
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<tr>
<td>Q-vanM-F</td>
<td>CTCAGAAGACATAATGTATCGGT</td>
<td>154</td>
<td>Sun et al., 2018</td>
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<td>Q-vanM-R</td>
<td>ACAGTGTTCATTATCCCAATCATAC</td>
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<td>Q-vanR-F</td>
<td>GATATGAAGTAACCGTCAAGTC</td>
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<td>In this study</td>
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<td>Q-purK-F</td>
<td>GATATCCAGATGCGATGACG</td>
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<td>Sun et al., 2018</td>
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<td>Q-purK-R</td>
<td>CTCTAAAAACACAGTCTTCTCT</td>
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<td>Q-rep-srr12-F</td>
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<td>GATGAACCTAGAACATCAACGCA</td>
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<td>Q-rep-srr24,zy2-F</td>
<td>GTCATCTCACCCTAGAAAATTAC</td>
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<td>Q-rep-srr24,zy2-R</td>
<td>CGCATATGGCTTGTAAGTTAAC</td>
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<tr>
<td>vanM-probe-F</td>
<td>CAGAGATGCCAACACATATTG</td>
<td>268</td>
<td>Sun et al., 2018</td>
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<td>vanM-probe-R</td>
<td>AGCCTACATAAGGTATACCAGAC</td>
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</table>
Table S2 Estimation of \textit{vanM} copy number by mapping Illumina reads to reference genes

<table>
<thead>
<tr>
<th>Isolates</th>
<th>Mean sequence coverage</th>
<th>Estimated gene copy no.</th>
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<tbody>
<tr>
<td></td>
<td>\textit{purK} gene</td>
<td>\textit{rep} gene</td>
</tr>
<tr>
<td></td>
<td>(chromosome)</td>
<td>(plasmid)</td>
</tr>
<tr>
<td>ZY2</td>
<td>370.98</td>
<td>912.56</td>
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<tr>
<td>ZY2-R</td>
<td>351.10</td>
<td>971.54</td>
</tr>
<tr>
<td>SRR24</td>
<td>377.03</td>
<td>656.60</td>
</tr>
<tr>
<td>SRR24-R</td>
<td>328.05</td>
<td>604.60</td>
</tr>
<tr>
<td>ZY11</td>
<td>354.17</td>
<td>NA</td>
</tr>
<tr>
<td>ZY11-R</td>
<td>322.27</td>
<td>NA</td>
</tr>
</tbody>
</table>

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

Figure S1 DNA pattern in each VVE strain by \textit{SmaI}-PFGE and southern blotting hybridization with \textit{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 PstI-PFGE and vanM blotting hybridization. Salmonella H9812 was included as molecular size markers.