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Reversible vancomycin susceptibility within emerging ST1421 *Enterococcus faecium* strains is associated with rearranged *vanA*-gene clusters and increased *vanA* plasmid copy number

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ABSTRACT

Vancomycin variable enterococci (VVE) are *van*-positive enterococci with a vancomycin-susceptible phenotype (VVE-S) that can convert to a resistant phenotype (VVE-R) and be selected for during vancomycin exposure. VVE-R outbreaks have been reported in Canada and Scandinavian countries. The aim of this study was to examine the presence of VVE in whole genome sequenced (WGS) Australian bacteremia *Enterococcus faecium* (*Efm*) isolates collected through the Australian Group on Antimicrobial resistance (AGAR) network. Eight potential VVE*Aus* isolates, all identified as *Efm* ST1421, were selected based on the presence of *vanA* and a vancomycin-susceptible phenotype. During vancomycin selection, two potential VVE-S harboring intact *vanHAX* genes, but lacking the prototypic *vanRS* and *vanZ* genes, reverted to a resistant phenotype (VVE*Aus*-R). Spontaneous VVE*Aus*-R reversion occurred at a frequency of 4-6 $\times$ 10^{-8} resistant colonies per parent cell in vitro after 48 h and led to high-level vancomycin and teicoplanin resistance. The S to R reversion was associated with a 44-bp deletion in the *vanHAX* promoter region and an increased *vanA* plasmid copy number. The deletion in the *vanHAX* promoter region enables an alternative constitutive promoter for the expression of *vanHAX*. Acquisition of vancomycin resistance was associated with a low fitness cost compared with the corresponding VVE*Aus*-S isolate. The relative proportion of VVE*Aus*-R vs. VVE*Aus*-S decreased over time in serial passages without vancomycin selection. *Efm* ST1421 is one of the predominant VanA-*Efm* multilocus sequence types found across most regions of Australia, and has also been associated with a major prolonged VVE outbreak in Danish hospitals.

1. Introduction

Acquired vancomycin resistance in enterococci can be conferred by different *van* gene clusters, of which *vanA* and *vanB* genotypes are the most prevalent [1]. The VanA phenotype is characterized by high-level resistance to vancomycin and teicoplanin [2]. The *vanA* gene cluster typically consists of seven genes (*vanRSHAXYZ*), but only the expression of *vanHAX* is essential for resistance in iso-

Abbreviations: AGAR, Australian Group on Antimicrobial resistance; AST, antimicrobial susceptibility testing; bp, base pair; BMD, broth microdilution; CFU, colony forming unit; Efm, *Enterococcus faecium*; MIC, minimum inhibitory concentration; ST, sequence type; VVE, vancomycin variable enterococci; WGS, whole genome sequencing.

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lates with a functional host D-alanine:D-alanine ligase (Ddl). Exposure to glycopeptides activates the two-component regulators, sensor VanS and activator VanR, which upregulate the expression of the enzymes (VanH, VanA, VanX, VanY) to alter the peptidoglycan sidechain terminus from D-Ala-D-Ala to D-Ala-D-Lac [3]. Vancomycin variable enterococci (VVE) are vancomycin-susceptible enterococci (VVE-S) containing a silenced *vanA* gene cluster that reverts to a resistant phenotype (VVE-R) through genetic rearrangements occurring at low frequencies [4]. The presence of VVE has clinical implications as the VVE-R subpopulation may be selected for during antibiotic exposure, causing therapeutic failure [5,6].

The molecular mechanisms involved in transition to a VVE-R phenotype include insertion sequence (IS) excision and restoration of the promoter [5] or IS-elements providing a functional promoter [4]. Alternative molecular mechanisms comprise changes in the host *ddl* gene to counteract loss of *vanX*, deletions in the promoter region, creation of novel alternative constitutive *vanHAX* promoters or support of their use, and increase in *vanA* plasmid copy number [4,7,8]. VV*Efm* associated with hospital outbreaks have been described in Canada, the Republic of Korea, and Scandinavia [5,9–12]. The occurrence of VVE may go undetected as many clinical laboratories routinely support only phenotypic antimicrobial susceptibility testing of enterococci [13–17].

The prevalence of vancomycin-resistant enterococci (VRE) is increasing worldwide, enlarging the population of *van* gene clusters exposed to genetic alterations [18]. In the USA, 80% of clinical *E. faecium* isolates were reported resistant to vancomycin by 2008 [19]. The prevalence of vancomycin resistance in blood stream *E. faecium* isolates in Australia has been decreasing slightly: 47.7% in 2016 [20], 47.0% in 2017 [21], 45.0% in 2018 [22], 41.8% in 2019 [23], 32.6% in 2020 [24] and 37.9% in 2021 [Coombs, G. W. et al., Australian Group on Antimicrobial Resistance, personal communication]. In 2019, 41.8% of Australian bloodstream *E. faecium* isolates expressed vancomycin resistance, but 45.4% carried a *vanA* and/or *vanB* gene cluster, indicating that 3.6% carried silenced resistance [23]. In Europe, the prevalence of vancomycin resistance in invasive *E. faecium* isolates varies substantially between countries, from <1% to >50%, with a mean of 11.6% in 2016 that increased to 16.8% in 2020 [25].

Until recently, the majority of Australian VRE has been of the *vanB*-type [26], in contrast to North and South America, where the *vanA*-type dominates [27–30], and Europe, where both *vanA*- and *vanB*-type are prevalent [31,32]. However, the prevalence of *vanA* in Australia increased from <1% in 2011 [33] to 17.8% in 2015 [34] and 22% in 2016 [35]. Within the *vanA*-VRE, the emerging *pstS*(-) clade ST1421 *E. faecium* has been predominant [34]. The polyclonal Australian *vanA E. faecium* isolates indicate repeated cross-continental introduction [34,36].

The aim of this study was to examine the potential presence of susceptible VVE (VVE*aus*-S) within Australian bacteremia *E. faecium* collected by the Australian Group on Antimicrobial resistance (AGAR) network [37] and explore their potential to revert to a resistant phenotype (VVE*aus*-R) in vitro.

2. Materials and methods

2.1. Bacterial strains

The bacterial isolates (n=9) covered all potential VVE isolates from the AGAR 2015–2016 bloodstream collection based on the selection criteria: (i) *vanA* gene presence as shown by WGS, and (ii) a vancomycin-susceptible phenotype in routine antimicrobial susceptibility testing (AST), as performed by semi-automated methods (Vitek® 2 and BD Phoenix™) in the AGAR-associated laboratories [37]. The selection was done in February 2018, before the AGAR 2017 collection was available.

2.2. Antimicrobial susceptibility testing (AST)

Vancomycin minimum inhibitory concentration (MIC) determinations were performed for the selected strains before and after vancomycin selection by broth microdilution (BMD) according to ISO-standards, by an in-house method or using a commercial test (EUENCF plate, Sensititre, Trek diagnostic systems, Cleveland, USA). *Enterococcus faecalis* ATCC® 29212 (wild type) and *E. faecalis* ATCC® 51299 (*vanB* VRE) were used as control strains. The European Committee on Antimicrobial Susceptibility Testing (EUCAST) clinical breakpoints were utilized for interpretation [38].

2.3. Selection of potential VVEAus-R

Single colonies of potential VVE*Aus*-S were cultured overnight in 10 mL brain heart infusion (BHI) broth. A 100 µL aliquot of the overnight culture was inoculated in two 10 mL volumes of BHI broth with vancomycin selection (4 and 8 mg/L) and incubated for 48 h. All incubations were performed at 35°C with shaking at 200 rpm, including blank negative controls (without vancomycin) and *E. faecalis* ATCC® 29212 and *E. faecalis* ATCC® 51299 as negative and positive controls (with vancomycin selection), respectively. Vancomycin-selected VVE*Aus*-S positive cultures were plated on horse blood agar (HBA) with a vancomycin 5 µg disc (Oxoid) and on CHROMID VRE agar (bioMérieux, Marcy l'Étoile, France). A single VRE Chrom agar colony from each positive culture was spread for AST. Potential VVE*Aus*-R colonies with BMD vancomycin MIC ≥8 mg/L were selected for further characterization by MALDI-TOF species identification, extended AST-analysis and WGS.

2.4. Spontaneous VVEAus-R frequency

The spontaneous VVE*Aus*-S to VVE*Aus*-R reversion phenotype was examined as previously described [8]. Ten-fold serial dilution samples of an overnight VVE*Aus*-S BHI broth culture were plated on BHI agar with and without 6 mg/L vancomycin, in biological triplicates and technical triplicates. The plates were incubated at 37°C and colony-forming units (CFU) counted after 24 h for plates without vancomycin and after 48 h and 72 h for plates with vancomycin. DNA was extracted using the Wizard Genomic DNA Purification Kit with lysozyme (20 mg/mL) from eight colonies from each strain picked from vancomycin-containing agar, and subjected to PCR using the Phusion proofreading polymerase and primers specific to the promoter region (VVEAus_promoter_Fw 5'- GCTCGTTCTTCCGATACGGG -3'; VVEAus_promoter_Rv 5'-TTCACACCGGCTCTCTTCAG-3'). The PCR amplicon was sequenced with BigDye™ 3.1 Sanger sequencing technology. For molecular biology analysis, two pairs of VVE*Aus*-S and –R were chosen. The first pair was AUSMDU00023981(S) and AUSMDU00015095 (R), called VVE*Aus*-S1 and VVE*Aus*-R1, and the second pair was AUSMDU00023980 (S) and AUSMDU00015187 (R), called VVE*Aus*-S2 and VVE*Aus*-R2.

2.5. Reverse transcription-quantitative PCR (RT-qPCR) and qPCR

Quantitation of mRNA levels of *vanRS* and *vanHAX* transcripts were measured using RT-qPCR, as previously described [8]. In short, VVE*Aus*-S to VVE*Aus*-R, as well as *E. faecium* BM4147, were grown in BHI broth with and without 8 mg/L vancomycin. Total RNA was extracted using the RNeasy Mini Kit (Qiagen, Hilden, Germany) with 50 U mutanolysin and 1 mg lysozyme added to the lysis step, DNA was removed using the Heat and Run Kit (ArcticZymes, Tromsø, Norway) and cDNA was produced using the high-capacity RNA-to-cDNA kit (Thermo Fisher Scientific, Waltham, Massachusetts US). gDNA was extracted with a Wizard Genomic DNA Purification Kit (Promega, Madison, Wisconsin USA). qPCR

Table 1
Characteristics of *vanA*-positive *E. faecium* strains used in this study.

Strain	ST	Plasmids	Vancomycin MIC (mg/L)	Teicoplanin MIC (mg/L)
VVE*Aus*-S1	ST1421	Three plasmids, one *vanA rep2*	1	<0,5
VVE*Aus*-S2	ST1421	Three plasmids, one *vanA rep2*	1	<0,5
VVE*Aus*-R1	ST1421	Seven plasmids, three *vanA*	256	64
VVE*Aus*-R2	ST1421	Four plasmids, one *vanA rep2*	256	64
BM4147 [56]	ST95	pIP816 (*repE*) [59]	>16	>8

MIC, minimum inhibitory concentration; ST, sequence type

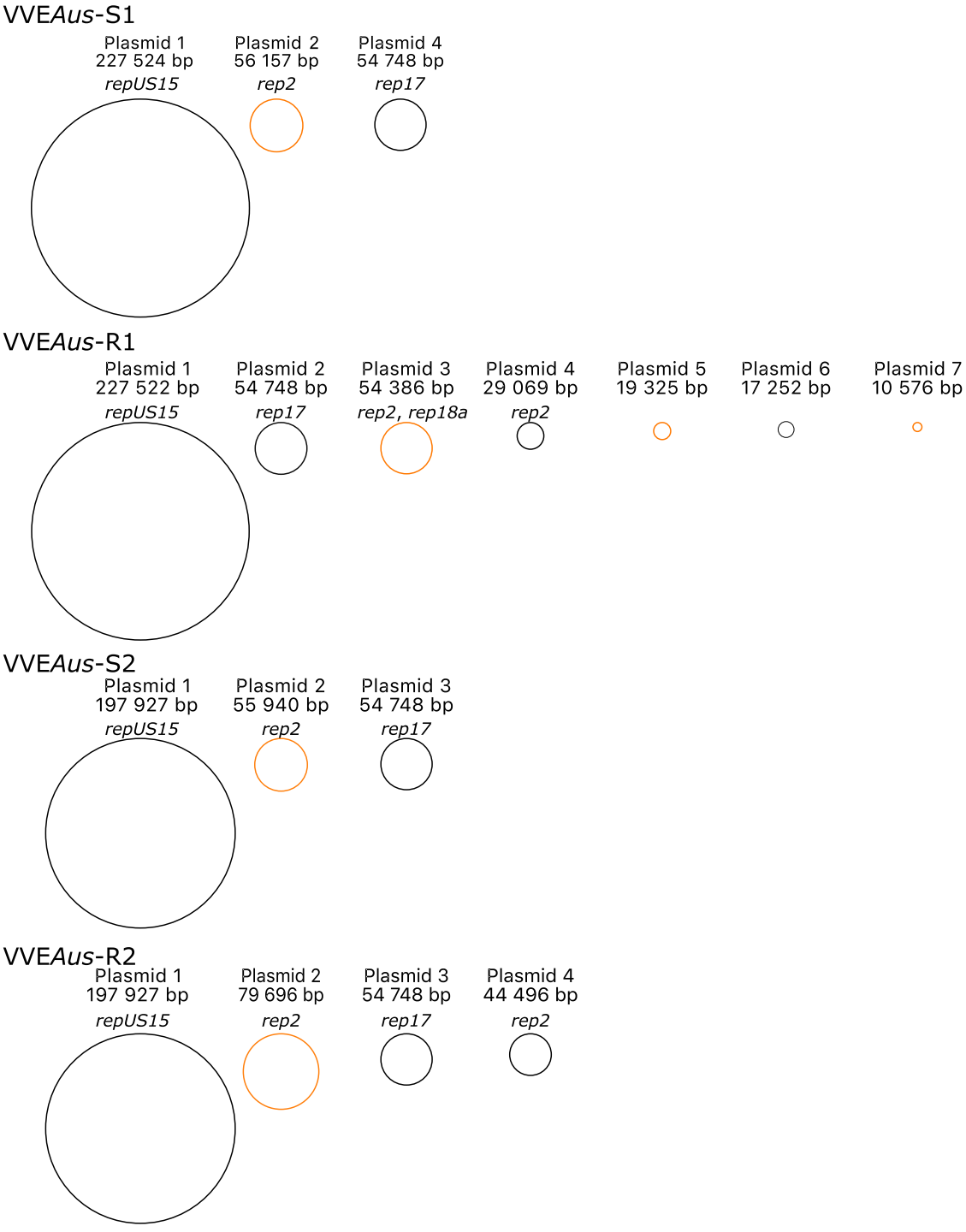

Figure 1. Plasmid configuration in VVE*Aus* strains. Plasmids are drawn as circles to the scale of their size. Plasmids containing *vanA* are colored in orange. Plasmid size is given in bp, and the replicon type is indicated if typeable by PlasmidFinder.

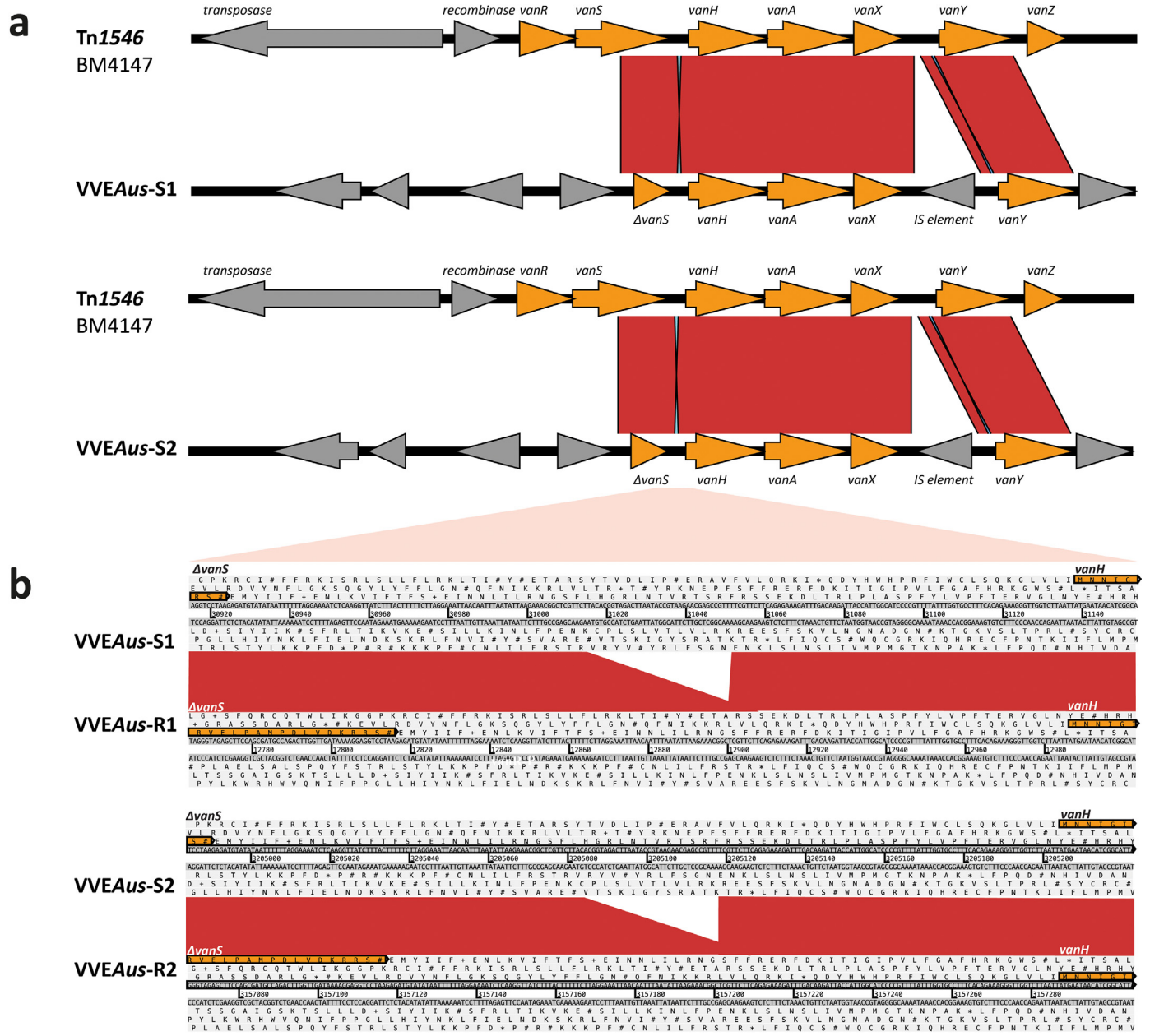

Figure 2. Comparison of the VVE*Aus van*-cluster to the prototype Tn*1546* (a) and between VVE*Aus*-S and VVE*Aus*-R (b). Compared with the prototype Tn*1546* of BM4147, both VVE*Aus*-S1 and VVE*Aus*-S2 lack *vanRS* and *vanZ*. The alignment of VVE*Aus*-S and VVE*Aus*-R revealed a 44-bp deletion in the *vanH* promoter region in VVE*Aus*-R1 and -R2. The *van*-cluster is shown in dark orange and the truncated *vanS* is indicated as Δ*vanS*. Light red shapes indicate which region of the *van*-cluster is zoomed in to. Red and blue bands between sequences represent forward and reverse complement matches, respectively.

was run with probes with 5'FAM and 3'BHQ-1 quencher (Eurogentec, Seraing, Belgium) in qPCR Master Mix Plus Low ROX (Eurogentec, Seraing, Belgium) on a 7300 Fast Real-Time PCR System (Applied Biosystems Waltham, Massachusetts US). Statistical data analysis of qPCR data was performed in GraphPad Prism 7 using an unpaired two-tailed t-test.

2.6. Stability, growth, and fitness of *VVE*Aus

Stability of resistance, growth curve measurements and calculation of relative fitness were conducted as previously described [8]. Briefly, the VVE*Aus*-R were subjected to serial transfer in the absence of vancomycin to monitor the vancomycin resistance phenotype stability. Cultures (30 µL overnight culture in 3 mL BHI) were transferred every 24 h, i.e., every twelfth generation, in biological triplicates for 5 days (60 generations). For every transfer, the ratio of total CFU to resistant CFU was determined using differential plating using BHI agar without and with 6 mg/L vancomycin. For stability over 25 days (300 generations), cultures were also transferred every 24 h but the ratio of total CFU to resistant CFU was determined every 5 days. After 25 days, VVE*Aus*-R_{25d} was obtained. The relative fitness of VVE*Aus*-R and -S was assessed through growth rate measurements, as previously described [39]. Overnight cultures were diluted 1:100 in BHI and growth was measured in an Epoch 2 Spectrophotometer with Gen5 Software (BioTek Instruments Inc., Winooski, Vermont US) at 37°C, shaking at 425 rpm, with OD600 measurement every tenth min for 24 h. The relative fitness was calculated by comparing the growth rates of the susceptible and the resistant isolate and statistical data analysis was performed in GraphPad Prism 7.

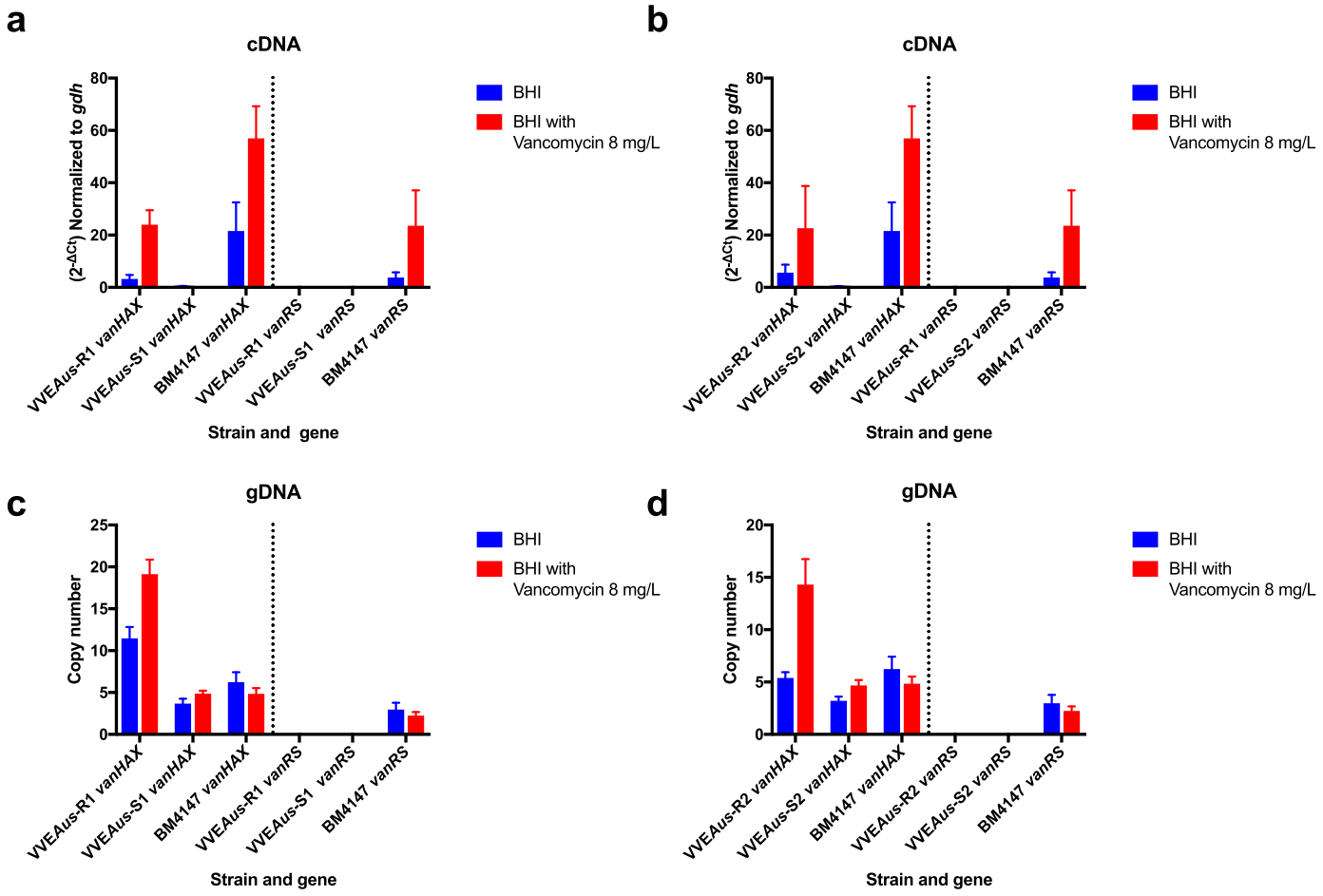

Figure 3. Expression and copy number of the *van*-cluster in VVE*Aus*. mRNA and gDNA levels of *vanRS* and *vanHAX* operons relative to the housekeeping gene *gdh* in BM4147, VVE*Aus*-S and resistant VVE*Aus*-R grown in BHI broth or in BHI broth with vancomycin 8 mg/L until mid-log phase. a) Expression level as measured by RT-qPCR for the pair VVE*Aus*-S1 and -R1, b) Expression level as measured by RT-qPCR for the pair VVE*Aus*-S2 and -R2, c) gDNA level as measured by qPCR for the pair VVE*Aus*-S1 and -R1, d) gDNA level as measured by qPCR for the pair VVE*Aus*-S2 and -R2. Bars are averages with SEM of three biological replicates including three technical repeats each (n=9).

2.7. WGS and bioinformatics analyses

The isolates, VVE*Aus*-S1 and VVE*Aus*-S2, as well as their resistant revertants, VVE*Aus*-R1 and VVE*Aus*-R2, were PacBio sequenced before serial passage. Therefore, bacterial genomic DNA was isolated with the Qiagen MagAttract HMW DNA isolation kit (Qiagen, Hilden, Germany) and sequenced by MiSeq using Nextera library construction on 250-bp paired-end runs or by NextSeq500 using the Nextera XT DNA library preparation kit and the Mid Output 300 cycles cell according to standard protocols (Illumina, San Diego, California US). Bioinformatic analysis was performed as previously described [8]. Sequence reads were trimmed using Trimmomatic v.0.36 [40], assembled with Spades v.3.9.0 [41], and annotated with Prokka v.1.11 [42]. MLST profiles were determined using the mlst software [43]. To confirm the location of the *vanA* gene cluster, the genomes were closed by PacBio sequencing technology. PacBio reads were assembled on their own and also along with Illumina reads by the assembler Unicycler v0.4.7 [44]. Resistance genes and the replicon type of *van*- plasmid sequences were identified by scanning the genomes in AMRFinderPlus [45] and PlasmidFinder [46], respectively. Illumina reads were mapped on the PacBio assemblies using bwa-mem [47]. Genome coverage was calculated by SAMtools [48], which permitted quantifiable coverage ratios between the chromosome and the *vanA*-containing plasmid. VVE*Aus*-S and -R sequences were compared using MUMmer v3.23 [49] and the SNPs were called using Snippy [50]. Genome synteknies of the VVE*Aus*-S and -R genomes and between the VVE*Aus*-S and -R *vanA* gene cluster and prototypic Tn*1546* (GenBank Acc. No. M97297.1) were visualized with ACT [51] and Bandage [52], and alignment figures were produced with EasyFig v.2.2.2 [53]. Sanger sequencing of the *vanSH* region PCR amplicons from vancomycin-resistant colonies from the spontaneous VVE*Aus*-R frequency assay was also performed.

2.8. Accession numbers

The sequences have been posted to NCBI and can be found under BioProject number PRJNA830442.

3. Results and discussion

*3.1. VVE*Aus*-R selection*

Eight of the nine selected AGAR 2015-2016 *E. faecium* isolates were vancomycin-susceptible by BMD (vancomycin MIC ≤4 mg/L) and were subsequently used for VVE*Aus*-R selection studies. The eight strains were identified as *vanA* ST1421, which has been associated with a major prolonged outbreak of VVE in Danish hospitals [12] and has emerged as a predominant clone in Australia [34]. Vancomycin selection (4 and 8 mg/L) did not support the growth of one isolate. Two of the vancomycin selection culture-positive isolates supported only slow-growing, small *E. faecium* colonies

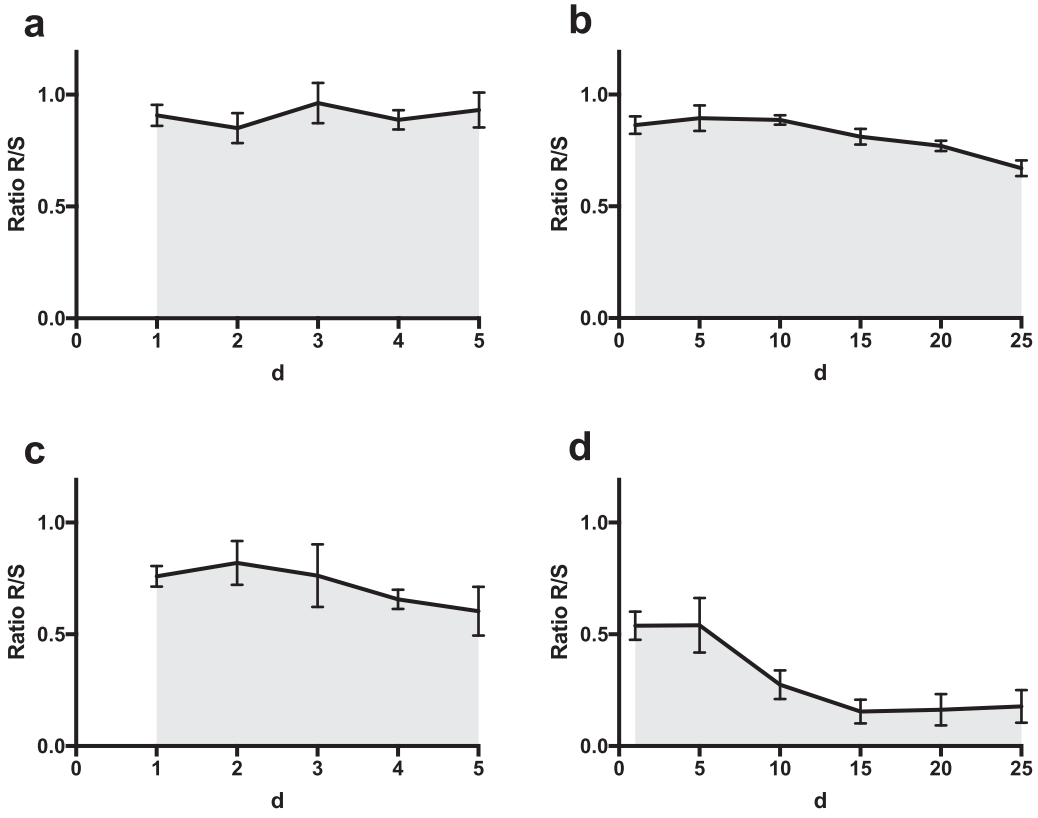

Figure 4. Stability of the two VVE*Aus*-R isolates. a) Ratio of resistant to susceptible colonies of VVE*Aus*-R1 for 5 days, b) Ratio of resistant to susceptible colonies of VVE*Aus*-R1 for 25 days c) Ratio of resistant to susceptible colonies of VVE*Aus*-R2 for 5 days d) Ratio of resistant to susceptible colonies of VVE*Aus*-R2 for 25 days. Bars are averages with SEM of three biological replicates with three technical repeats (n=9).

with BMD-susceptible vancomycin MICs (≤4 mg/L). Vancomycin selected *E. faecium* colonies from the other five parental isolates showed BMD vancomycin MIC ≥8 mg/L (range 8–256 mg/L) and were chosen for extended AST analyses and WGS. Two of those five isolates (VVE*Aus*-S1 and VVE*Aus*-S2) with an intact *vanHAX* cluster and consistent expression of high-level vancomycin and teicoplanin resistance were selected for further analysis (Table 1 and S1). A complete *vanHAX* region is considered necessary for the expression of a vancomycin-resistant phenotype in isolates with an intact host *ddl*-gene [3]. The corresponding revertant isolates, VVE*Aus*-R1 and VVE*Aus*-R2, expressed high-level vancomycin (MIC = 256 mg/L) and teicoplanin (MIC = 64 mg/L) resistance. The resistance reversion frequencies of VVE*Aus*1 and VVE*Aus*2 in vitro were $4 \pm 0.9 \times 10^{-8}$ and $6 \pm 2.4 \times 10^{-8}$, respectively after 48 h. This is comparable to the reversion frequency of Scandinavian VVE ($2–7 \times 10^{-8}$ within 48 h) [5,8]. The reversion rate is considered clinically relevant and below the detection limit of standard AST methods.

3.2. WGS of VVEAus-R revertants and comparisons with their parent VVEAus-S strains

WGS analysis of assembled PacBio data showed that the *vanA* cluster was located on *rep2*-plasmids of similar size in both VVE*Aus*-S1 and VVE*Aus*-S2 (56.157 kb and 55.940 kb, respectively). Chromosomal *van* clusters were not detected. In VVE*Aus*-R1, four different plasmids carried the *vanA* cluster, one combined *rep2-rep18a* and three *rep*-non-typeable derivatives of plasmid 3. In VVE*Aus*-R2, a single enlarged *rep2* plasmid carried the *vanA* cluster. The plasmid data are summarized in Figure 1 and a detailed alignment is shown in supplementary Figure 1.

Sequence comparison between VVE*Aus*-S1/-S2 and the *vanA* cluster prototype Tn*1546* (Acc. No. M97297) showed alterations in *vanRS* in both isolates, whereas *vanHAX* was intact and *vanZ* was lacking. Comparison of the susceptible parental isolates and their resistant revertant revealed an identical 44-bp deletion in the promoter region of *vanHAX* in both VVE*Aus*-R1 and -R2, and no other alterations occurred, as shown in Figure 2. The deletion is identical to the deletion recently described in a Swedish ST203 VVE, called VVE*Swe*-R [8], although the *vanA*-plasmid backbone was unrelated. The PCR amplicon of the promoter region of eight additional resistant revertant colonies of both VVE*Aus*-S1 and VVE*Aus*-S2 from the spontaneous resistance assay showed the same 44-bp deletion in Sanger sequencing, supporting the significance.

As calculated from the coverage of Illumina sequencing data, the plasmid copy number (PCN) of the *vanHAX*-bearing plasmid increased 5-fold in VVE*Aus*-R compared with VVE*Aus*-S. VVE*Aus*-S1 had a *vanHAX*-PCN of 4, whereas *vanHAX* was present in three contigs of VVE*Aus*-R1 with a PCN of 6, 5, and 4 (plasmid 3, 5, and 7, respectively). VVE*Aus*-S2 had a *vanHAX*-PCN of 2.5, and VVE*Aus*-R2 had a *vanHAX*-PCN of 11. A PCN increase was also associated with the resistance reversion of the Swedish VVE*Swe*-R, where the *vanHAX*-PCN increased 3-fold [8]. An alternative or additional mechanism for an increased *vanHAX* copy number could be tandem repeats. However, the detailed mapping of *vanA*-positive plasmids and the plasmid alignment, as shown in the supplementary Figure 1, did not disclose any tandem repeats affecting the *van*-genes.

Similar to VVE*Swe*-R [8], VVE*Aus*-R have an intact *vanHAX* but lack *vanZ* and are resistant to vancomycin and teicoplanin (Table 1). *vanZ* mediates resistance to teicoplanin when *vanHAX* is expressed at low levels [54] and can increase teicoplanin MICs but is not considered essential for the VanA phenotype [2].

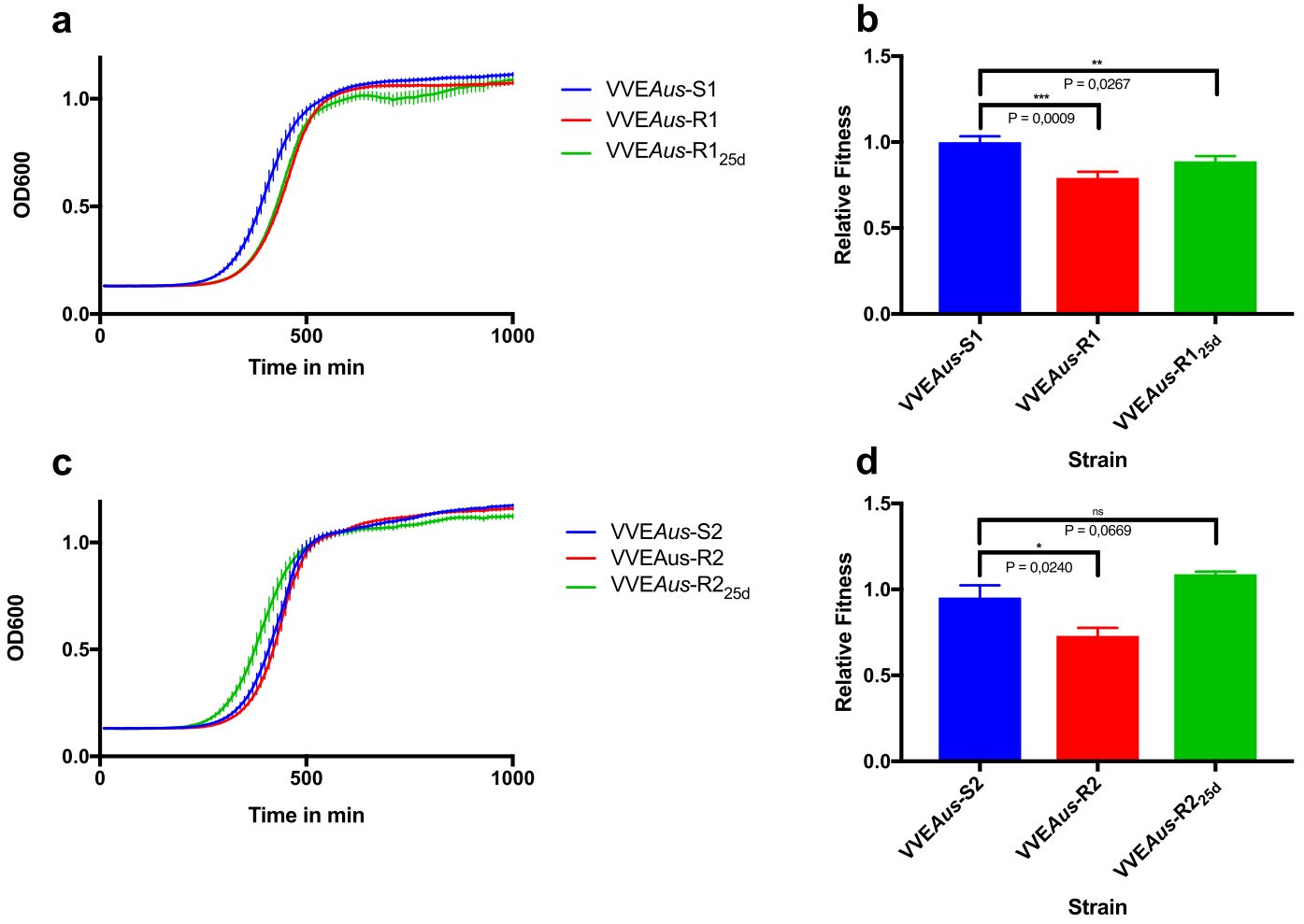

Figure 5. Growth and fitness of VVE*Aus*. a) Growth curve of VVE*Aus*-S1, VVE*Aus*-R1 and VVE*Aus*-R1$_{25d}$, b) Relative fitness of VVE*Aus*-S1, VVE*Aus*-R1 and VVE*Aus*-R1$_{25d}$, c) Growth curve of VVE*Aus*-S2, VVE*Aus*-R2 and VVE*Aus*-R2$_{25d}$, d) Relative fitness of VVE*Aus*-S2, VVE*Aus*-R2 and VVE*Aus*-R2$_{25d}$. Bars are averages with SEM of three biological replicates with three technical replicates (n=9, t-test, two-tailed).

A potential Australian VVE isolate with a partial deletion in *vanA* and absence of *vanXYZ* has recently been described [55]. However, the ability to revert to vancomycin resistance was not examined and the combined *vanA* deletion and lack of *vanX* makes it less likely. VanX encode a d-Ala-d-Ala dipeptidase, which is critical in VanA-type vancomycin resistance involving depletion of d-Ala–terminating precursors to prevent interaction of vancomycin with its target [3]. Moreover, Australian *vanA*-positive VREfm ST80 strains have been shown to carry a *vanS* deletion, indicating the potential development of VVE, but the strains were not specifically discussed with regard to their glycopeptide resistance phenotype [34]. The ST80 strains had a 48-bp deletion within *vanS* only. The VVE*Aus*-S1-2/R1-2 isolates carried a truncated *vanR* (Δ*vanR*) downstream of a prototypic *vanHAX* (supplementary Figure 1) and a truncated *vanS* (Δ*vanS*) upstream of *vanHAX* (supplementary Figure 1, Figure 2). The VVE*Aus*R1-2 strains carried a 44-bp deletion between *vanS* and *vanH* compared with their parental VVE*Aus*S1-2. ST1421 was the dominant potential VVE in the AGAR 2015-16 collection. The predominance of ST1421 or related lineages among potential *vanA* VVE was also observed in subsequent AGAR surveys, underlining the clinical epidemiological relevance of our observations [23].

3.3. *VVE*Aus-*R harbors a functional alternative promoter*

Expression of the *vanA* operon in VVE*Aus* was determined by RT-qPCR and compared to the *E. faecium* BM4147 reference strain [56]. Without induction, BM4147 expressed low levels of *vanHAX* and *vanRS*, which were increased upon exposure to vancomycin (Figure 3 a, b). This is in line with previous observations [8,57]. In the two VVE*Aus*-R isolates, *vanHAX* was inducible by vancomycin at a level that is comparable to the non-induced expression of *vanHAX* in BM4147. In the two VVE*Aus*-S isolates, *vanHAX* expression was not detectable. Expression of *vanRS* was also not detected in VVE*Aus* S1-2/R1-2 isolates (Figure 3 a, b).

At the gDNA level, the BM4147 reference strain showed similar copy numbers of *vanHAX* and *vanRS* with and without vancomycin exposure (Figure 3 c, d). The gDNA level of BM4147 *vanHAX* was comparable to the two susceptible VVE*Aus*-S isolates. The two VVE*Aus*-R isolates, however, showed an elevated *vanHAX* copy number in the presence of vancomycin. Furthermore, the *vanHAX* copy number is increased in VVE*Aus*-R compared with VVE*Aus*-S, consistent with the previous PCN calculations from the Illumina sequencing overage data.

Similar observations have been reported for the Swedish VVE*Swe*-R [8].

3.4. *Stability of VVE*Aus-*R over time and fitness cost of resistance*

The two resistant VVE isolates, VVE*Aus*-R1 and VVE*Aus*-R2, were subjected to serial passage without vancomycin selection, initially for 5 days (60 generations), and then for a prolonged period of 25 days (300 generations). The VVE*Aus*-R1 population retained its resistant phenotype proportion to 93% over 5 days and to 67% over

25 days (Figure 4 a, b), whereas the VVE*Aus*-R2 resistant proportion remained only at 60% over 5 days and was reduced to 18% at Day 25 (Figure 4 c, d). The differences in resistance phenotype stability might be attributed to the differences in *vanA*-plasmid composition as resolved by PacBio sequencing (Figure 1). Before serial passage, VVE*Aus*-R1 contained multiple small *vanA*-plasmids, whereas VVE*Aus*-R2 carried only one large *vanA*-plasmid. It could be beneficial for the host to propagate smaller plasmids, which might pose less fitness cost.

The fitness cost of vancomycin resistance was assessed by measuring the growth rates of the VVE*Aus*-S compared to VVE*Aus*-R and the evolved VVE*Aus*-R_{25d}. The fitness cost for VVE*Aus*-R1 was 21% and was reduced to 12% after 25 days without vancomycin exposure compared to VVE*Aus*-S1. The fitness cost for VVE*Aus*-R2 was 18% and was completely removed after 25 days without antibiotic selection, supporting the development of adaptive compensatory mutations (Figure 5). This fitness cost is higher than the cost reported for a Swedish VVE (6%) [8] and other strains possessing *vanA* plasmids when compared with their plasmid-free counterpart (4–9%) [39,58].

4. Conclusions

To our knowledge, this is the first comprehensive report of Australian VVE. The VVE were identified as *vanA E. faecium* ST1421 lacking *vanRS* and *vanZ*. The reverted VVE-R phenotype was associated with an increased *vanA* plasmid copy number and a 44-bp deletion in the promoter region of *vanHAX* enabling the expression of *vanHAX*. ST1421 is a predominant *vanA E. faecium* multilocus sequence type across most Australian regions and has been related to a prolonged major VVE outbreak in Danish hospitals. The reversion to vancomycin resistance was linked to an increased fitness cost, which was reduced or completely removed after serial passages without vancomycin selection. The VVE-R phenotype was unstable, but the majority of the VVE*Aus*-R1 population expressed resistance after 25 serial passages without antibiotic selection.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ijantimicag.2023.106849.

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