J Antimicrob Chemother 2021; **76**: 876–882
doi:10.1093/jac/dkaa541 Advance Access publication 26 December 2020

Journal of Antimicrobial Chemotherapy

Alternative *vanHAX* promoters and increased *vanA*-plasmid copy number resurrect silenced glycopeptide resistance in *Enterococcus faecium*

Theresa Maria Wagner [1], Jessin Janice [1,2], Audun Sivertsen[2]†, Ingegerd Sjögren[3], Arnfinn Sundsfjord[1,2] and Kristin Hegstad [1,2]*

[1]*Research group for Host-Microbe Interactions, Department of Medical Biology, Faculty of Health Sciences, UiT The Arctic University of Norway, Tromsø, Norway;* [2]*Norwegian National Advisory Unit on Detection of Antimicrobial Resistance, Department of Microbiology and Infection Control, University Hospital of North-Norway, Tromsø, Norway;* [3]*Department of Clinical Microbiology and Infection Control, Hospital of Halland, Halmstad, Sweden*

*Corresponding author. E-mail: Kristin.Hegstad@uit.no
†Present address: Department of Microbiology, Haukeland University Hospital, 5021 Bergen, Norway.

Received 17 June 2020; accepted 4 December 2020

Background: Vancomycin variable enterococci (VVE) are *van*-positive isolates with a susceptible phenotype that can convert to a resistant phenotype during vancomycin selection.

Objectives: To describe a vancomycin-susceptible *vanA*-PCR positive ST203 VVE *Enterococcus faecium* isolate (VVE*Swe*-S) from a liver transplantation patient in Sweden which reverted to resistant (VVE*Swe*-R) during *in vitro* vancomycin exposure.

Methods: WGS analysis revealed the genetic differences between the isolates. Expression of the *van*-operon was investigated by qPCR. Fitness and stability of the revertant were investigated by growth measurements, competition and serial transfer.

Results: The VVE*Swe*-R isolate gained high-level vancomycin (MIC >256 mg/L) and teicoplanin resistance (MIC = 8 mg/L). VVE*Swe*-S has a 5′-truncated *vanR* activator sequence and the VVE*Swe*-R has in addition acquired a 44 bp deletion upstream of *vanHAX* in a region containing alternative putative constitutive promoters. In VVE*Swe*-R the *vanHAX*-operon is constitutively expressed at a level comparable to the non-induced prototype *E. faecium* BM4147 strain. The *vanHAX* operon of VVE*Swe* is located on an Inc18-like plasmid, which has a 3–4-fold higher copy number in VVE*Swe*-R compared with VVE*Swe*-S. Resistance has a low fitness cost and the vancomycin MIC of VVE*Swe*-R decreased during *in vitro* serial culture without selection. The reduction in MIC was associated with a decreased *vanA*-plasmid copy number.

Conclusions: Our data support a mechanism by which vancomycin-susceptible VVE strains may revert to a resistant phenotype through the use of an alternative, constitutive, *vanR*-activator-independent promoter and a *vanA*-plasmid copy number increase.

Introduction

Enterococcus faecium is an important opportunistic pathogen causing severe MDR infections in hospitalized patients. The increase in VRE causes concerns due to severely limited treatment options. Vancomycin resistance occurs by the acquisition of one of several *van*-gene clusters—*vanA, B, C, D, E, G, L, M*, and *N*—of which *vanA* and *vanB* are the most significant clinically.[1]

Vancomycin-variable enterococci (VVE) is a term used for VRE where expression of the *van* genes is phenotypically silenced by genetic rearrangements, which may be reversed under vancomycin selection.[2,3] A complex of seven genes (*vanRSHAXYZ*) support the expression of the prototype VanA-type high-level vancomycin and teicoplanin resistance. Upon exposure to glycopeptides the two-component regulators, sensor VanS and

activator VanR, up-regulate the expression of the enzymes (VanHAXY) involved in changing the peptidoglycan sidechain terminus from D-Ala-D-Ala to D-Ala-D-Lac. However, only expression of *vanHAX* is essential in gaining resistance in strains with a functional host D-alanine:D-alanine ligase (Ddl).[4] In 2009, a Canadian ST18 VVE. *faecium* was able to convert to a resistant phenotype by the introduction of IS elements providing novel promoters for constitutive expression of *vanHAX* and deletions in the promoter region.[5,6] By 2015–16, a regional spread of VVE in the Ontario region had occurred and 47% of the *vanA*-positive isolates were VVE.[7] In 2016, a Danish ST1421 VVE. *faecium* strain with a 252 bp *vanX*-truncation was described. The resistant phenotype was associated with an increased *vanA*-plasmid copy number or by disruption of the host *ddl* ligase gene.[8] By 2019 this clone had spread from the capital region to all five Danish regions, and the Faroe Islands.[9] Early identification of VVEs and their reversion mechanism is important for therapeutic and infection control measures.

We have previously described a VVE. *faecium* ST203 outbreak strain in Norway, where excision of a *vanA*-operon-inserted IS*L3* restored the resistant phenotype.[3] The occurrence of VVE will likely increase corresponding to the increase in VanA-type VRE as the *vanA*-gene cluster will be affected by random genetic alterations.

In this study, we show yet another different variant of variable resistance in a Swedish VVE strain.

Materials and methods

Isolation of VVE

The susceptible parental faecal VVE strain (VVE*Swe*-S) was isolated from a liver-transplanted patient at Halmstad Hospital, Sweden after faecal VRE-screening, but the patient never had an *E. faecium* infection. The VRE-screening was performed by enrichment in Bile aesculin azide (BEA) broth supplemented with aztreonam (60 mg/L) and vancomycin (4 mg/L) at 36°C for 20–24 h and subsequent *vanA/B* PCR using Rotor-Gene and TaqMan probes.[10]

Vancomycin resistance phenotype reversion and frequency

Conversion to vancomycin resistance was initiated as described previously[3] by incubating a single susceptible VVE colony in 5 mL of Brain Heart Infusion (BHI) broth (Oxoid) overnight, followed by a 1:100 dilution into 5 mL of BHI broth containing 2 or 8 mg/L vancomycin.

Resistance reversion frequency determination was based on Sivertsen *et al.*[3] Ten-fold serial dilution samples of an overnight VVE*Swe*-S strain BHI-broth culture in biological triplicates and technical triplicates were plated on BHI agar with and without 6 mg/L vancomycin. The plates were incubated at 35°C and cfu counted after 24 h for plates without vancomycin and after 48 h and 72 h for plates with vancomycin. Vancomycin-resistant revertant (VVE*Swe*-R) colonies from vancomycin-containing plates were verified by MALDI-TOF (Bruker), antimicrobial susceptibility testing (AST), and Sanger sequencing of the *vanSH* PCR product using primers as described before[11] and BigDye 3.1 technology (Applied Biosystems). AST was performed with vancomycin MIC test strips (Liofilchem) and/or Sensititre EUENCF plate (Thermo Fisher Scientific) according to the manufacturers' instructions with ATCC 29212 as control. JumpStart REDTaq ReadyMix (Merck KGaA) was used for PCRs. DNA extractions for PCRs were performed using the NucliSens EasyMAG instrument and reagents (BioMeriéux) according to the manufacturer's instructions.

WGS and bioinformatics

Bacterial genomic DNA was isolated with the Qiagen MagAttract HMW DNA isolation kit (Qiagen) 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). Sequence reads were trimmed using Trimmomatic v.0.36,[12] assembled with Spades v.3.9.0,[13] and annotated with Prokka v.1.11.[14] MLST profiles were determined using MLST software[15] and core genome (cg)MLST cluster types (CTs) were determined using SeqSphere+.[16]

In order to confirm the location of the *vanA*-gene cluster, the genomes were closed by nanopore sequencing technology. Nanopore reads were error-corrected and assembled along with Illumina reads by the hybrid assembler Unicycler v0.4.7.[17] Resistance genes and the replicon type of *van*-operon-containing plasmid sequences were identified by scanning the genomes in Abricate v.8.5 using the NCBI resistance database and PlasmidFinder database, respectively. Illumina reads were mapped on the nanopore assemblies using bwa-mem.[18] Genome coverage was calculated by bedtools genomcov option,[19] which permitted quantifiable coverage ratios between the chromosome and the *vanA*-containing plasmid. VVE*Swe*-S and VVE*Swe*-R sequences were compared with MUMmer v3.23[20] and the SNPs were called using GATK. Genome syntenies of the VVE*Swe*-S and VVE*Swe*-R genomes and between the VVE*Swe*-S/-R *vanA* gene cluster and prototypic Tn*1546* (GenBank Acc. No. M97297) were visualized with ACT,[21] and alignment figures were produced with EasyFig v.2.2.2.[22] Sanger sequencing of the *vanSH* region was performed on the prototypic Tn*1546* as described above to confirm that the sequence has not changed in reference strain BM4147.

Promoters were predicted in the intergenic region between *vanS* and *vanH* using Softberry.[23]

Accession numbers

The sequences have been posted to NCBI and can be found under the BioProject number PRJNA551094 (CP041261-8 and CP041270-8).

Reverse transcription-quantitative PCR (RT-qPCR) on cDNA and qPCR on gDNA

Quantification of mRNA levels of *vanRS* and *vanHAX* was done as described previously. Primer sequences for qPCR were also described previously.[3]

For quantification of gDNA levels of *vanRS* and *vanHAX*, DNA was extracted from VVE*Swe*-S, VVE*Swe*-R and BM4147 grown to mid-log-phase in BHI broth without and with 8 mg/L vancomycin, using the GenElute Bacterial Genomic DNA Kit (Sigma Aldrich). qPCR was performed using probes with 5′FAM and a 3′BHQ-1 quencher (Eurogentec), qPCR Master Mix Plus Low ROX (Eurogentec) and run on a 7300 Fast Real-Time PCR System (Applied Biosystems). Cycle threshold (Ct) values were normalized to the housekeeping gene *gdh* and ΔCt was calculated as $\Delta Ct_{vanRS} = Ct_{vanRS} - Ct_{gdh}$ and $\Delta Ct_{vanHAX} = Ct_{vanHAX} - Ct_{gdh}$. The plasmid copy number was calculated as $2^{\Delta Ct(vanHAX)}$ since only one copy of *gdh* is found in all strains, and *vanHAX* only localizes to the plasmid of interest (based on Lee *et al.*[24]).

Statistical data analysis of qPCR data was performed in GraphPad Prism 7 using an unpaired two-tailed *t*-test.

Fitness measurements

The relative fitness was assessed through growth rate measurements and head-to-head competition as described previously.[25]

Overnight cultures were diluted 1:100 in BHI and growth was measured in an Epoch 2 Spectrophotometer with Gen5 Software (BioTek Instruments Inc.) at 37°C, shaking at 425 rpm, with OD_{600} measurement every tenth minute for 24 h. Growth rates were calculated in the logarithmic growth phase with the program GrowthRates.[26] The relative fitness was calculated by

comparing the growth rates of VVE*Swe*-S and VVE*Swe*-R using the equation $w = \text{GrowthRate}_{\text{VVESwe-R}}/\text{GrowthRate}_{\text{VVESwe-S}}$.

For pairwise competition, overnight cultures of VVE*Swe*-S and VVE*Swe*-R were OD-adjusted (OD_{600}), mixed at a ratio of 1:1 and diluted 1:100 in 5 mL BHI and incubated for 24 h at 37°C with shaking. The initial and final cfu counts of the competitors were determined on BHI agar without and with 8 mg/L vancomycin. The relative fitness w of the revertant VVE*Swe*-R was estimated as a ratio of ln(cfu t_{24}/cfu t_0) of the revertant to the susceptible VVE*Swe*-S using the equation $w = \ln(\text{cfu}_{\text{VVESwe-R}}\ t_{24}/\text{cfu}_{\text{VVESwe-R}}\ t_0)/\ln(\text{cfu}_{\text{VVESwe-S}}\ t_{24}/\text{cfu}_{\text{VVESwe-S}}\ t_0)$.[27]

Statistical data analysis for fitness cost experiments was performed in GraphPad Prism 7 using an unpaired two-tailed *t*-test to calculate whether the value significantly differs from 1.

Resistance stability in VVESwe-R

The revertant was cultured continuously in absence of antibiotic with serial transfer every 24 h in biological triplicates (30 μL inoculated into 3 mL BHI). Colonies were counted on BHI plates with and without 8 mg/L vancomycin and the ratio of total cfu to resistant cfu was determined. Colonies were counted after 24 h and re-checked after 48 h, since the colonies were growing slowly on the selective plates. One hundred colonies were exposed to differential plating on BHI plates without and with 8 mg/L vancomycin.

Ten colonies were selected for qPCR analysis to determine *vanA*-plasmid copy, subjected to Sanger sequencing of the *vanSH* PCR product as described above and vancomycin MIC test strip (MTS-Liofilchem) with *E. faecalis* ATCC 29212 and *E. faecium* BM4147 as control strains.

Results and discussion

VVESwe resistance phenotype reversion and frequency

The original VVE faecal sample gave a positive *vanA*-PCR result from the BEA broth culture which supported growth of single colonies of enterococci on chromogenic and blood agar. Disc diffusion showed an inhibition zone diameter of 15 mm with a sharp edge and Etest gradient strips a vancomycin MIC of 2 mg/L. The isolate was named VVE*Swe*-S. In broth microdilution VVE*Swe*-S expressed susceptibility to vancomycin (MIC 1 mg/L) and teicoplanin (MIC <0.5 mg/L) (Tables S1 and S2, available as Supplementary data at *JAC* Online).

The VVE*Swe*-S strain reverted to a vancomycin-resistant phenotype (VVE*Swe*-R) during exposure to vancomycin 6 mg/L or 8 mg/L for 48–72 h. VVE*Swe*-R had gained high-level vancomycin resistance (MIC >256 mg/L) and teicoplanin resistance (MIC 8 mg/L) (Table S2). The frequency of vancomycin resistance reversion was 2×10^{-8} resistant colonies per parent cell *in vitro* after 48 h and 5×10^{-8} after 72 h. A similar reversion frequency (3×10^{-8} after 24 h, 7×10^{-8} after 48 h) was also detected for the previously described VV*E. faecium* ST203 outbreak strain from Norway, although the reversion mechanism was different.[3] A bacterial load of above 10^8 could be reached in certain infection sites (e.g. infected peritoneal fluids) in a patient[28] and thus reversion is clinically relevant and may occur during vancomycin treatment as previously observed for other VVE strains.[2,3] The VVE isolates reverted at a frequency that is too low and over a time that is too long to be detected by standard antimicrobial susceptibility testing methods, which explains why VVE resistant revertants are difficult to detect by standard AST methods using a much lower inoculum (10^5 bacteria) and reading after 24 h.

Genetic differences between VVESwe-S and VVESwe-R

VVE*Swe* belongs to ST203/CT20 and is unrelated to any global or local surveillance isolates or the Norwegian ST203/CT465 VVE.[3] Early detection of the VVE*Swe* by PCR may have hindered the spread of this clone before it became prevalent. However, *vanA*-positive ST203/CT20 strains were prevalent in Germany among VRE blood culture isolates (2015–18), but expressed a normal VanA phenotype (G. Werner, personal communication).

High-quality assemblies were achieved for VVE*Swe*-S and VVE*Swe*-R genomes with pertinent genome size, GC content, and coverage of 305× and 252×, respectively. The genomes of the parental VVE*Swe*-S and the resistant revertant VVE*Swe*-R were compared and aligned with the prototypical *vanA* cluster of BM4147. Both VVE*Swe*-S and VVE*Swe*-R have a 5′-truncated *vanR* activator gene (Figure 1). The difference between the VVE*Swe*-S and VVE*Swe*-R strain was a 44 bp deletion covering the inducible *vanHAX* promoter region in the resistant strain. Alternative promoters in the *vanHAX* promoter region were predicted *in silico* (P1 to P5 in Figure 1), but only promoters P1 to P3 changed their proximity to *vanH*, thus we predict these promoters to be responsible for the phenotypic reversion. The additional alternative promoters were also found in the prototypical *vanA* cluster of BM4147 (Figure S1). Promoter prediction solely *in silico* is a limitation of this study. However, we were unable to perform an experimental approach giving high enough resolution to distinguish putative promoters at the single nucleotide level, which would be required since the predicted promoters are overlapping.

The deletion occurred in a region with an IR and a DR and precisely removes one of the DR sequences as well as the nucleotides between the DR, which suggests that the deletion occurs by illegitimate recombination.[29] Genome comparisons and read mapping with subsequent SNP and variation calling revealed no other obvious relevant genomic alterations that could be linked to the phenotypic differences between these isogenic strains, as shown in Table S3. The whole genome sequence of an additional independent revertant VVE*Swe*-R (VVE*Swe*-R2), was also compared with VVE*Swe*-S and the same 44 bp deletion in the *vanHAX* promoter region was found (Table S3). Additionally, Sanger sequencing of the *vanSH* PCR product of two additional independent revertants showed the sequence to be identical to that in VVE*Swe*-R.

To elucidate the observed difference in vancomycin susceptibility, we explored the localization and expression of *vanHAX* in the VVE*Swe*-R isolate compared with VVE*Swe*-S.

The vanA cluster in VVESwe is located on a plasmid

Genome sequence analysis revealed that the *vanA* cluster of VVE*Swe* is located on a 35 kb non-conjugative plasmid (GenBank Acc. No. CP041279) with a *rep* previously described as a CDS1 putative replicon of the plasmid pRE25 belonging to Inc18 theta replicating plasmids of *rep* class 2.[30] In other VVEs the *vanA* gene cluster localized on a transferable plasmid, which thus has a potential to spread vancomycin resistance.[3,6,8] However, we were unable to show transfer of the *vanA* VVE*Swe*-R in mating experiments.

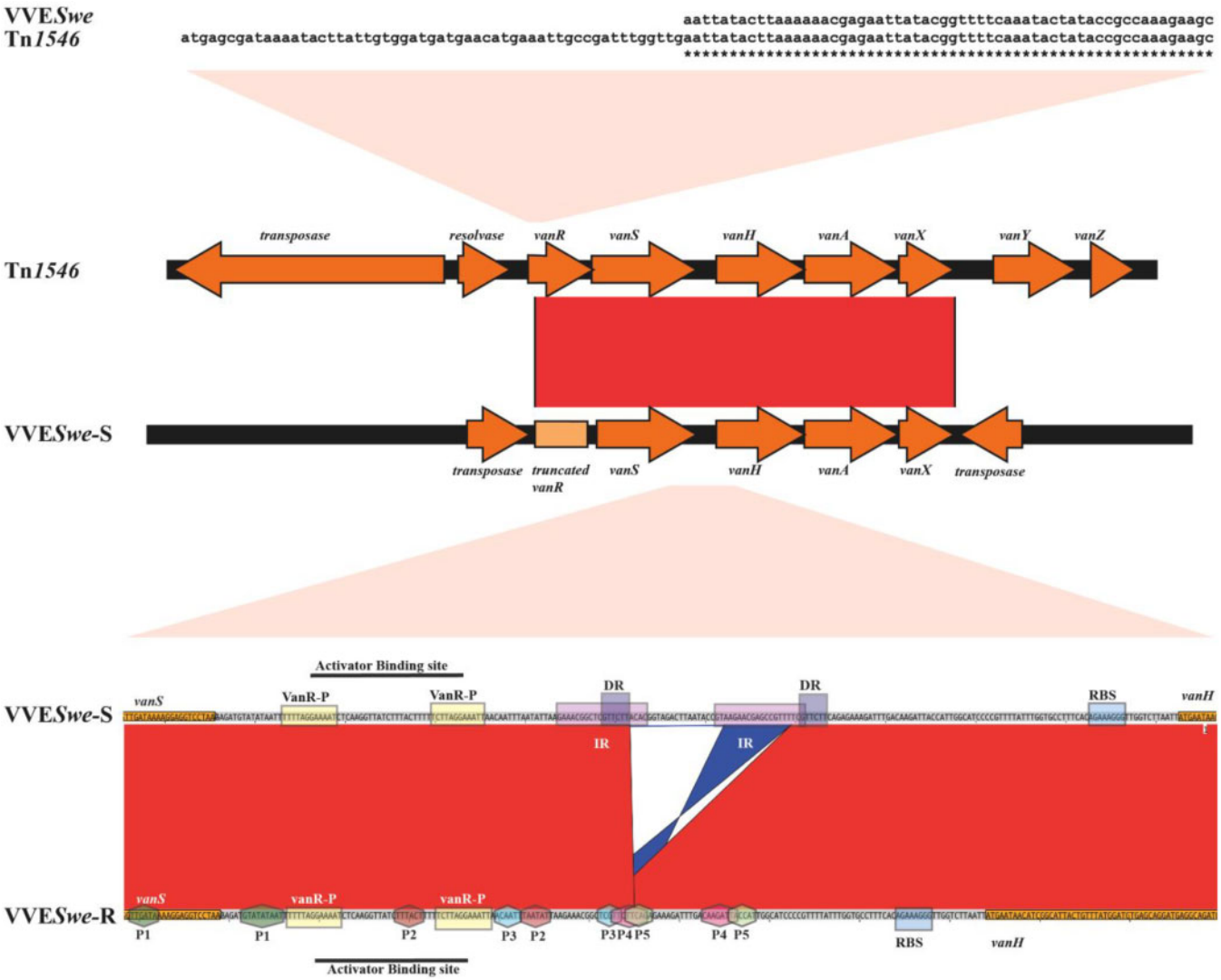

Figure 1. Comparison of the *van* cluster of VVE*Swe* with the prototype. (Top) Pairwise alignment starting with the start codon up to bp 113 of the *vanR* gene of Tn*1546* of BM4147 and the 5′-truncated *vanR* of VVE*Swe*. Asterisks represent identity. (Middle) Pairwise alignment of the *vanA* cluster of Tn*1546* of BM4147 and VVE*Swe*-S. Both VVE*Swe* isolates lack a functional *vanR* activator gene (light orange box). (Bottom) Pairwise alignment of the *vanSH* intergenic region of VVE*Swe*-S and VVE*Swe*-R. VVE*Swe*-S and VVE*Swe*-R comparisons revealed a 44 bp deletion in VVE*Swe*-R, which is connected to direct repeat (DR) and inverted repeat (IR) sequences covering the inducible promoter region. Alternative promoters in VVE*Swe* are marked with hexagons and numbered P1 to P5. Activator binding sites are indicated as boxes (VanR-P, VanR-phosphate binding site; RBS, ribosomal binding site). 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.

The vanHAX-operon was constitutively expressed in VVESwe-R

We examined the functionality of the alternative promoter in absence of the inducible promoter (Figure 1) by RT-qPCR on BM4147, VVE*Swe*-S and VVE*Swe*-R.

First, the transcription profile of the VanA-prototype strain BM4147 was analysed. Sanger sequencing of the *vanSH* PCR product confirmed that no genetic changes had occurred in Tn*1546* of BM4147 compared with the reference sequence (GenBank Acc. No. M97297). Without vancomycin induction, BM4147 expressed *vanRS* and *vanHAX* (Figure 2a), in line with early studies at the protein level.[31,32] This observation was also confirmed in MH broth, the standard medium for MIC testing (Figure S2). We therefore assume that in the absence of vancomycin induction, the above-described alternative constitutive promoters (Figure 1 and Figure S1) may be used for expression of *vanHAX* in BM4147, or low-level activation of the prototype promoter occurs.

Vancomycin exposure significantly increased the expression of both *vanRS* and *vanHAX* in BM4147, in line with the observed requirement of an intact *vanRS* for vancomycin induction of *vanHAX* (Figure 2a).[32–34] The presence of vancomycin triggers phosphorylation of VanS, phospho-VanS then phosphorylates the transcriptional activator VanR and Phospho-VanR induces transcription of the *vanHAX* operon.[35]

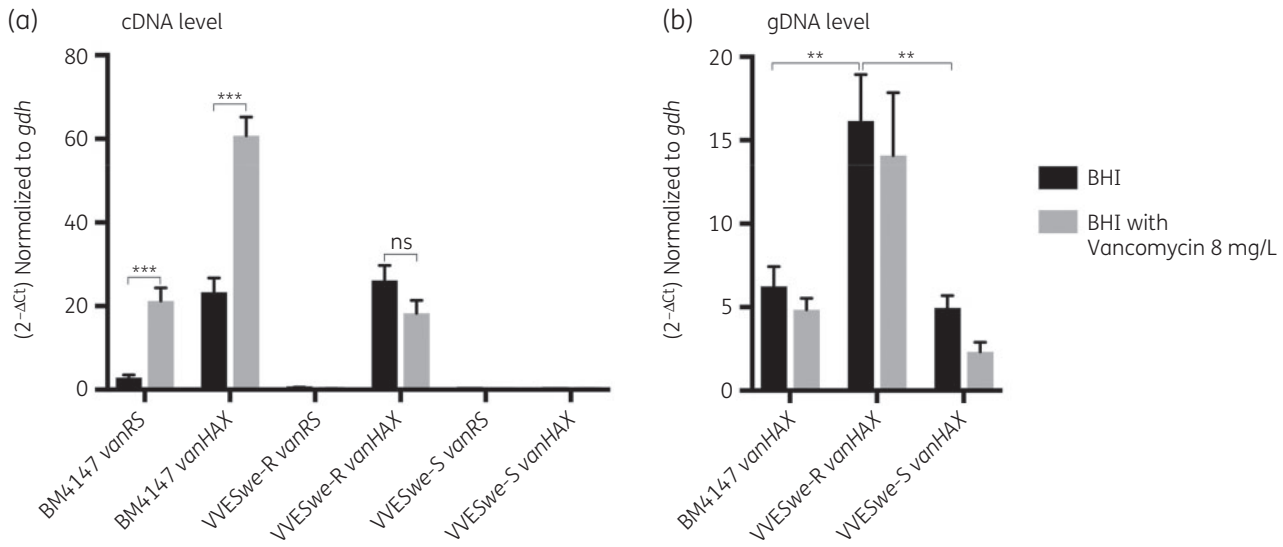

Figure 2. mRNA and gDNA levels of *vanRS* and *vanHAX* operons relative to the housekeeping gene *gdh* in BM4147, the susceptible (VVE*Swe*-S) and resistant (VVE*Swe*-R) isogenic VVE isolates grown in BHI broth without antimicrobials or in BHI broth with vancomycin 8 mg/L until mid-log phase. (a) Expression level as measured by RT-qPCR (*t*-test, two-tailed, $P_{BM4147\ vanRS} < 0.0001$, $P_{BM4147\ vanHAX} < 0.0001$, $P_{VVESwe\text{-}R\ vanHAX} = 0.141$). (b) gDNA level as measured by qPCR (*t*-test, two-tailed, $P_{BM4147\ versus\ VVESwe\text{-}R} = 0.0026$, $P_{VVESwe\text{-}R\ versus\ VVESwe\text{-}S} = 0.0030$). Bars are averages with SEM of three biological replicates including four technical repeats each.

The *van*-operon is considered as a textbook example of inducible resistance to glycopeptide antibiotics. Former studies observed that the regulatory expression of the *vanHAX*-operon is not tight, since VanA was detected in BM4147 membrane extracts even in the absence of vancomycin induction.[31] Our data are consistent with previous studies, supporting the notion of an inducible prototypic *vanA* operon of Tn*1546* and constitutive low-level expression of *vanHAX* in the absence of vancomycin induction.

The transcription profile of VVE*Swe* showed very low expression of *vanRS* in both VVE*Swe*-S and VVE*Swe*-R, confirming non-functionality of *vanRS* in VVE*Swe*, as predicted by sequence analysis. VVE*Swe*-S does not express *vanHAX*, whereas VVE*Swe*-R expresses *vanHAX*. The *vanHAX*-transcription level of VVE*Swe*-R was similar with and without vancomycin exposure and comparable to the non-induced prototype BM4147-level (Figure 2a), supporting the notion of an alternative constitutive promoter. Constitutive expression of *vanHAX* has been described in Canadian VVE-R strains, but was due to a different set of mutations in the promoter region.[5,6]

Furthermore, the copy number of *vanHAX* at the gDNA level was measured in BM4147, VVE*Swe*-S and VVE*Swe*-R, in BHI both with and without vancomycin. Vancomycin exposure did not significantly alter the copy number of *vanHAX* in any strains. However, VVE*Swe*-R harboured a higher copy number of the *vanHAX*-operon (16 ± 3), compared with both BM4147 (6 ± 1) and VVE*Swe*-S (5 ± 1) (Figure 2b). A higher *vanA*-plasmid copy number may support higher expression levels of *vanHAX* from an alternative constitutive promoter, and thus in the absence of a functional *vanRS* may be responsible for the resistant phenotype. Moreover, both Illumina and Nanopore WGS analyses confirmed the plasmid copy number of the *vanA*-plasmids in VVE*Swe*-R ($n = 14$) and VVE*Swe*-S ($n = 2$). Similarly, *vanA*-plasmid copy number was described to confer reversion in an ST1421 strain.[8] Recently, increased *vanM* gene cluster copy number by tandem amplification and increased expression was also described to confer resistance reversion[36,37] but for the VVE*Swe*-S and VVE*Swe*-R nanopore assembly data showed that the *vanA* cluster appears as a single copy in the Inc18-plasmid.

In conclusion, an alternative promoter conveys *vanHAX* expression independent of the *vanR* activator and is thus not inducible by vancomycin. In addition, increased plasmid copy numbers add to the resistant phenotype.

The novel resistance phenotype posed a low fitness cost and was replaced by a susceptible phenotype over time

Acquisition of vancomycin resistance has been described to reduce the fitness of the resistant strain compared with its susceptible competitor in the absence of selective pressure.[25,38,39] The relative fitness cost for the revertant VVE*Swe*-R was low: 6% as measured by growth rate measurements and 9% in 24 h head-to-head competition experiments (Figure 3a). A comparable fitness cost of 4%–9% was previously described for strains possessing *vanA*-plasmids when compared with their plasmid-free counterpart.[25,39]

We further investigated the stability of the resistance phenotype under non-selective conditions where the *vanA*-plasmid would not be selected for. Over a period of 5 days the ratio of resistant colonies was reduced significantly to 50% (Figure 3b). Of note, the colonies were smaller on the vancomycin-containing plates compared with the plates without vancomycin after 24 h, and the plates were therefore re-checked after 48 h.

After serial transfer over 5 days, single colonies ($n = 100$) were collected and subjected to differential plating on BHI plates without and with vancomycin 8 mg/L. However, all 100 colonies were able to grow on vancomycin plates after 72 h, suggesting

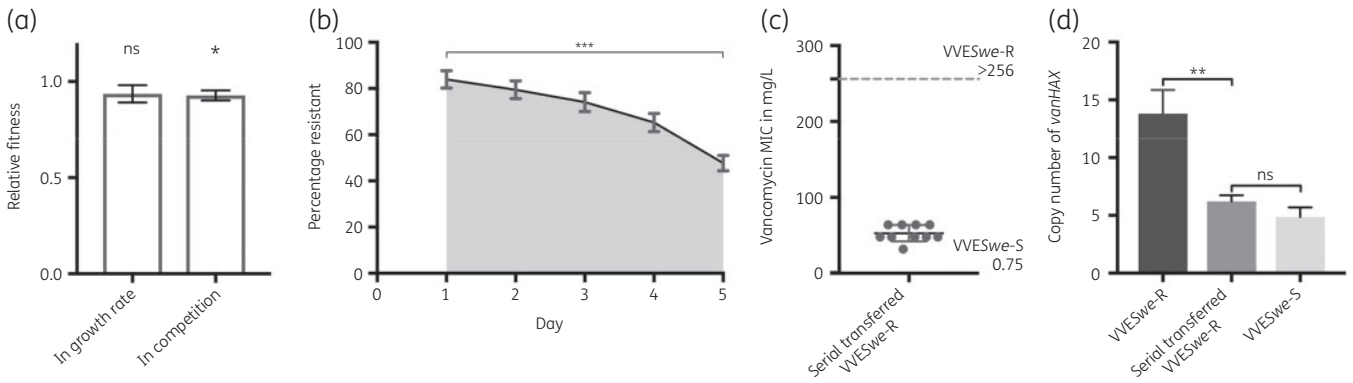

Figure 3. Fitness and stability of the resistant revertant. (a) Relative fitness as assessed by growth rate and in head-to-head competition of VVE*Swe*-S and VVE*Swe*-R. Bars show mean with SEM of three biological replicates in three technical repeats each (*t*-test, two-tailed; ns, $P = 0.1746$; *$P = 0.0134$). (b) Stability of the VVE*Swe*-R by serial transfer over 5 days. The plot shows mean with SEM of five biological replicates in three technical repeats each (*t*-test, two-tailed, ***$P < 0.0001$). (c) Vancomycin MIC (mg/L) of serial transferred VVE*Swe*-R-colonies compared with VVE*Swe*-R and VVE*Swe*-S as measured with a MIC test strip. (d) Copy number of *vanHAX* normalized to *gdh* in VVE*Swe*-R, VVE*Swe*-S and serial transferred VVE*Swe*-R-colonies measured by qPCR on gDNA isolated from the strains grown in BHI broth without vancomycin until mid-log phase. Bars show mean with SEM (*t*-test, two-tailed, **$P = 0.0022$; ns, $P = 0.1766$).

that even though the number of susceptible colonies read after 24 h had increased, these colonies had not lost *vanHAX*. Sanger sequencing of the *vanSH* PCR product of ten selected serial transferred colonies showed that no genetic changes had occurred compared with VVE*Swe*-R. Illumina WGS analysis of two selected serial transferred colonies confirmed this. The vancomycin MIC of the ten selected serial transferred colonies ranged from 32–64 mg/L (Figure 3c).

The copy number of *vanHAX* under non-selective conditions was significantly reduced in serial transferred VVE*Swe*-R compared with non-serial transferred VVE*Swe*-R and at a similar level to the original VVE*Swe*-S strain (Figure 3d). Thereby, the *vanHAX*-plasmid copy number combined with the promoter region deletion are likely responsible for the resistant phenotype and MIC variation of VVE*Swe*-R. We hypothesize that this decrease of *vanHAX*-copy number also reduces the fitness cost of resistance and is therefore evolutionarily advantageous. Further experimental studies are needed to elucidate the mechanism of plasmid copy number regulation.

In summary, we describe a VVE strain that can convert from a vancomycin-susceptible to a vancomycin-resistant phenotype and further to reduced resistance in response to differential vancomycin exposure. During vancomycin exposure the parental VVE*Swe*-S strain converts to a VVE*Swe*-R by a 44 bp deletion in the van*HAX*-promoter region and an increased *vanA*-plasmid copy number (Figures 1 and 2). In the absence of vancomycin, the VVE*Swe*-R population acquired a phenotype of a lower vancomycin MIC, which correlated to a decrease in *vanA*-plasmid copy number (Figure 3d).

In line with previous publications,[5,6] we observed the ability of VVE to convert to a resistant phenotype during vancomycin selection. In a patient with a susceptible VVE infection, vancomycin would provide the selective pressure needed for selection and amplification of a resistant phenotype. Vancomycin is therefore not a treatment option for VVE. Years after the discovery of VVEs, a regional spread of VVE in Canada[7] and a national spread of VVE in Denmark was reported,[9] which highlights the importance of characterizing VVE clones and screening for their presence.

Acknowledgements

We thank Ellen H. Josefsen and Bjørg C. Haldorsen at the Norwegian National Advisory Unit on Detection of Antimicrobial Resistance for excellent technical assistance, Seila Pandur at the Institute for Chemistry, UiT The Arctic University of Norway, for genome sequencing using MiSeq, Hagar Taman at the Genomics Support Center Tromsø[TM], for genome sequencing using NextSeq500, Julia M. Kloos and Vidar Sørum at the Institute for Pharmacy, UiT The Arctic University of Norway, for advise on fitness experiments, and Petra Edquist, at The Public Health Agency of Sweden, for assistance with surveillance data.

Funding

This project was conducted at the Norwegian National Advisory Unit on Detection of Antimicrobial Resistance, Department of Microbiology and Infection Control, University Hospital of North-Norway, Tromsø, Norway and no other specific funding has been received for this study. This work was supported by travel grants from the National Graduate School in Infection Biology and Antimicrobials (grant number 249062). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Transparency declarations

None to declare.

Supplementary data

Tables S1 to S3 and Figures S1 and S2 are available as Supplementary data at *JAC* Online.

References

1 Bender JK, Cattoir V, Hegstad K *et al*. Update on prevalence and mechanisms of resistance to linezolid, tigecycline and daptomycin in enterococci in Europe: towards a common nomenclature. *Drug Resist Updat* 2018; **40**: 25–39.

2 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.

3 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.

4 Arthur M, Depardieu F, Cabanié L *et al*. Requirement of the VanY and VanX D, D-peptidases for glycopeptide resistance in enterococci. *Mol Microbiol* 1998; **30**: 819–30.

5 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.

6 Szakacs TA, Kalan L, McConnell MJ *et al*. Outbreak of vancomycin-susceptible *Enterococcus faecium* containing the wild-type *vanA* gene. *J Clin Microbiol* 2014; **52**: 1682–6.

7 Kohler P, Eshaghi A, Kim HC *et al*. Prevalence of vancomycin-variable *Enterococcus faecium* (VVE) among *vanA*-positive sterile site isolates and patient factors associated with VVE bacteremia. *PLoS One* 2018; **13**: 1–11.

8 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.

9 Hammerum AM, Justesen US, Pinholt M *et al*. Surveillance of vancomycin-resistant enterococci reveals shift in dominating clones and national spread of a vancomycin-variable *vanA Enterococcus faecium* ST1421-CT1134 clone, Denmark, 2015 to March 2019. *Euro Surveill* 2019; **24**: 1900503.

10 Folkhälsomyndigheten. Bilagor till VRE-diagnostiken - Referensmetodik för laboratoriediagnostik. http://referensmetodik.folkhalsomyndigheten.se/w/Bilagor_till_VRE-diagnostiken#Bilaga_3.

11 Simonsen GS, Myhre MR, Dahl KH *et al*. Typeability of Tn*1546*-like elements in vancomycin-resistant enterococci using long-range PCRs and specific analysis of polymorphic regions. *Microb Drug Resist* 2000; **6**: 49–58.

12 Bolger AM, Lohse M, Usadel B. Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics* 2014; **30**: 2114–20.

13 Bankevich A, Nurk S, Antipov D *et al*. SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. *J Comput Biol* 2012; **19**: 455–77.

14 Seemann T. Prokka: rapid prokaryotic genome annotation. *Bioinformatics* 2014; **30**: 2068–9.

15 Seemann T. Scan contig files against traditional PubMLST typing schemes. https://github.com/tseemann/mlst.

16 Jünemann S, Sedlazeck FJ, Prior K *et al*. Updating benchtop sequencing performance comparison. *Nat Biotechnol* 2013; **31**: 294–6.

17 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.

18 Li H, Durbin R. Fast and accurate long-read alignment with Burrows-Wheeler transform. *Bioinformatics* 2010; **26**: 589–95.

19 Quinlan AR, Hall IM. BEDTools: a flexible suite of utilities for comparing genomic features. *Bioinformatics* 2010; **26**: 841–2.

20 Kurtz S, Phillippy A, Delcher AL *et al*. Versatile and open software for comparing large genomes. *Genome Biol* 2004; **5**: R12.

21 Carver TJ, Rutherford KM, Berriman M *et al*. ACT: the Artemis comparison tool. *Bioinformatics* 2005; **21**: 3422–3.

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

23 Solovyev V, Salamov A. Automatic annotation of microbial genomes and metagenomic sequences. In: Li RW, ed. *Metagenomics and Its Applications in Agriculture, Biomedicine and Environmental Studies*. Nova Science Publishers, 2011; 61–78.

24 Lee C, Kim J, Shin SG, Hwang S. Absolute and relative QPCR quantification of plasmid copy number in *Escherichia coli*. *J Biotechnol* 2006; **123**: 273–80.

25 Starikova I, Al-Haroni M, Werner G *et al*. Fitness costs of various mobile genetic elements in *Enterococcus faecium* and *Enterococcus faecalis*. *J Antimicrob Chemother* 2013; **68**: 2755–65.

26 Hall BG, Acar H, Nandipati A, Barlow M. Growth rates made easy. *Mol Biol Evol* 2014; **31**: 232–8.

27 Wiser MJ, Lenski RE. A comparison of methods to measure fitness in *Escherichia coli*. *PLoS One* 2015; **10**: 1–11.

28 König C, Simmen HP, Blaser J. Bacterial concentrations in pus and infected peritoneal fluid–implications for bactericidal activity of antibiotics. *J Antimicrob Chemother* 1998; **42**: 227–32.

29 Rocha EPC. An appraisal of the potential for illegitimate recombination in bacterial genomes and its consequences: from duplications to genome reduction. *Genome Res* 2003; **13**: 1123–32.

30 Mikalsen T, Pedersen T, Willems R *et al*. Investigating the mobilome in clinically important lineages of *Enterococcus faecium* and *Enterococcus faecalis*. *BMC Genomics* 2015; **16**: 282.

31 Dutka-Malen S, Mohnas C, Arthur M, Courvalin P. The VANA glycopeptide resistance protein is related to d-alanyl-d-alanine ligase cell wall biosynthesis enzymes. Mol Gen Genet 1990; **224**: 364–72. 10.1007/BF00262430

32 Arthur M, Molinas C, Courvalin P. The VanS-VanR two-component regulatory system controls synthesis of depsipeptide peptidoglycan precursors in *Enterococcus faecium* BM4147. *J Bacteriol* 1992; **174**: 2582–91.

33 Arthur M, Reynolds PE, Depardieu F *et al*. Mechanisms of glycopeptide resistance in enterococci. *J Infect* 1996; **32**: 11–6.

34 Arthur M, Depardieu F, Gerbaud G *et al*. The VanS sensor negatively controls VanR-mediated transcriptional activation of glycopeptide resistance genes of Tn*1546* and related elements in the absence of induction. *J Bacteriol* 1997; **179**: 97–106.

35 Courvalin P. Vancomycin resistance in Gram-positive cocci. *Clin Infect Dis* 2006; **42**: S25–34.

36 Zhou Y, Yang Y, Ding L *et al*. Vancomycin heteroresistance in *vanM*-type *Enterococcus faecium*. *Microb Drug Resist* 2020; **26**: 776–82.

37 Sun L, Chen Y, Hua X *et al*. Tandem amplification of the *vanM* gene cluster drives vancomycin resistance in vancomycin-variable enterococci. *J Antimicrob Chemother* 2020; **75**: 283–91.

38 Foucault ML, Courvalin P, Grillot-Courvalin C. Fitness cost of VanA-type vancomycin resistance in methicillin-resistant *Staphylococcus aureus*. *Antimicrob Agents Chemother* 2009; **53**: 2354–9.

39 Johnsen PJ, Simonsen GS, Olsvik Ø *et al*. Stability, persistence, and evolution of plasmid-encoded VanA glycopeptide resistance in enterococci in the absence of antibiotic selection *in vitro* and in gnotobiotic mice. *Microb Drug Resist* 2002; **8**: 161–70.