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Vancomycin-Variable Enterococci Can Give Rise to Constitutive Resistance during Antibiotic Therapy

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Vancomycin-resistant enterococci (VRE) are notorious clinical pathogens restricting the use of glycopeptide antibiotics in the clinic setting. Routine surveillance to detect VRE isolated from patients relies on PCR bioassays and chromogenic agar-based test methods. In recent years, we and others have reported the emergence of enterococcal strains harboring a "silent" copy of vancomycin resistance genes that confer a vancomycin-susceptible phenotype (vancomycin-susceptible enterococci [VSE]) and thus escape detection using drug sensitivity screening tests. Alarmingly, these strains are able to convert to a resistance phenotype (VSE→VRE) during antibiotic treatment, severely compromising the success of therapy. Such strains have been termed vancomycin-variable enterococci (VVE). We have investigated the molecular mechanisms leading to the restoration of resistance in VVE isolates through the whole-genome sequencing of resistant isolates, measurement of resistance gene expression, and quantification of the accumulation of drug-resistant peptidoglycan precursors. The results demonstrate that VVE strains can revert to a VRE phenotype through the constitutive expression of the vancomycin resistance cassette. This is accomplished through a variety of changes in the DNA region upstream of the resistance genes that includes both a deletion of a likely transcription inhibitory secondary structure and the introduction of a new unregulated promoter. The VSE→VRE transition of VVE can occur in patients during the course of antibiotic therapy, resulting in treatment failure. These VVE strains therefore pose a new challenge to the current regimen of diagnostic tests used for VRE detection in the clinic setting.

Resistance to the antibiotic vancomycin is a global clinical challenge for the treatment of infections caused by enterococci (1). The first reports of vancomycin-resistant enterococci (VRE) >25 years ago described the VanA phenotype, whose signature is drug-inducible resistance to vancomycin and the lipoglycopeptide antibiotic teicoplanin (2). The biochemical manifestation of resistance is the incorporation of cell wall peptidoglycan terminating in the depsipeptide (ester) D-alanyl–D-lactate (D-Ala-D-Lac) in place of the canonical D-alanyl–D-alanine (D-Ala-D-Ala) dipeptide (3). Subsequently, VanB phenotype VRE was described, which is characterized by strains that are inducible by vancomycin only but retain the D-Ala-D-Lac depsipeptide (4). The synthesis of D-Ala-D-Lac cell wall-terminating depsipeptides requires the action of three enzymes, VanH, VanA, and VanX (Fig. 1) (5). VanX is a dipeptidase that removes the D-Ala-D-Ala that continues to be generated by the constitutively expressed chromosomal cell wall biosynthesis machinery. VanH supplies D-Lac by the biochemical reduction of pyruvate, while VanA is an ATP-dependent ligase that synthesizes the ester D-Ala-D-Lac. This depsipeptide is then incorporated into intracellular cell wall precursor biosynthesis, which supplies the reagents required for extracellular peptidoglycan synthesis, which is necessary for cell division and growth.

The expression of the *vanHAX* three-gene cassette is controlled by the two-component regulatory system VanRS, in which VanR is a response regulator and VanS is an integral membrane histidine kinase that recognizes the presence of vancomycin (by direct binding in the case of $VanS_B$ [3, 6]). The autophosphorylation of VanS and transfer of the phosphate to a VanR aspartyl side chain generates the induction-competent version of VanR that binds to elements upstream of *vanH*, inducing the expression of *vanHAX*, which results in resistance (Fig. 1). The *vanRS* and *vanHAX* genes have been found on a number of mobile genetic elements, including plasmids and transposons, such as Tn*1546*. As a result, they have spread globally, and VRE remains an important drug-resistant pathogen in many clinical settings (1). Consequently, VRE colonization in patients is monitored in clinical microbiology laboratories by screening rectal swab specimens on selective chromogenic medium and by PCR detection of *vanA*/*vanB* (7).

Because VanA- and VanB-type VRE are linked to the presence of the *vanHAX* genes, we were intrigued by the recent identification of strains of vancomycin-susceptible enterococci (VSE) determined by susceptibility testing from Ontario, Canada, which nevertheless were positive for the *vanHAX* genes, as assessed by PCR (8, 9). A similar phenomenon has been described in which genetic alterations in plasmid pS177 give rise to *vanA*-positive enterococcal isolates that are susceptible to vancomycin (VSE)

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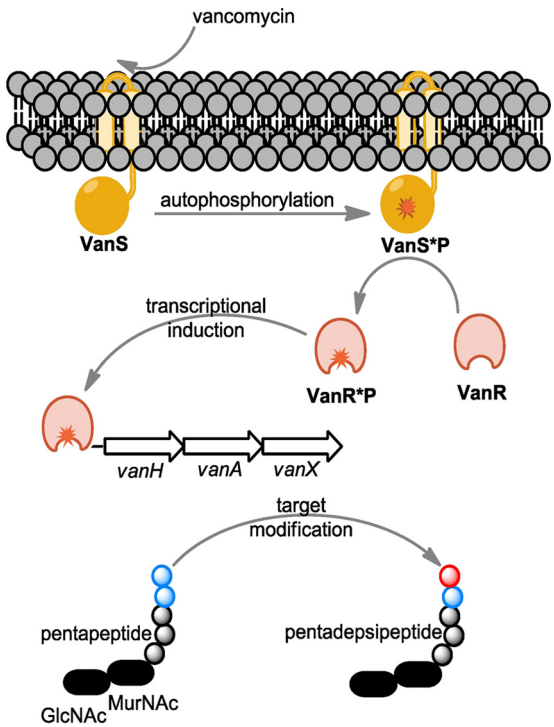

FIG 1 Mechanism of vancomycin resistance. VanS is a membrane-bound histidine kinase that senses the presence of vancomycin, resulting in ATP-dependent autophosphorylation. The phospho-VanS then transfers phosphate to the response regulator VanR in the cytoplasm. Phospho-VanR binds to the intergenic region upstream of the *vanHAX* operon and facilitates transcription, resulting in drug resistance. Among the resistance cassette proteins, VanX is a dipeptidase that removes D-Ala-D-Ala that continues to be generated by the constitutively expressed chromosomal cell wall biosynthesis machinery. VanH supplies D-Lac by the biochemical reduction of pyruvate, while VanA is an ATP-dependent ligase that synthesizes the ester D-Ala-D-Lac. This depsipeptide is then incorporated into intracellular cell wall precursor biosynthesis, which supplies the reagents required for extracellular peptidoglycan synthesis for cell division and growth. This subtle alteration of the target reduces the affinity of the vancomycin by 1,000-fold, rendering the drug ineffective.

(10). We recently reported that these VSE isolates with a "silent" *vanHAX* operon are able to revert to the VRE phenotype during treatment, and these were consequently termed vancomycin-variable enterococci (VVE) (9). If *vanA*-positive strains that are phenotypically drug susceptible yet harbor a *vanHAX* cassette are treated as susceptible, reversion to a resistant phenotype can directly impact the success of therapy. Furthermore, if strains of the same clone might be either vancomycin susceptible or resistant, VRE transmission control programs may be difficult or impossible to manage by phenotypic screening alone. Here, we report the underlying molecular mechanism for the variable resistance phenotype in VVE strains and show that various changes to the region upstream of *vanHAX* can result in the constitutive expression of the gene cassette, conferring resistance even in the absence of *vanRS*.

MATERIALS AND METHODS

Bacterial strains. The enterococcal clinical isolates used in this study were obtained from Mount Sinai Hospital, Toronto, Canada. The *Enterococcus faecium* strains VVE833, VVE844, VVE856, and K845 were isolated from patients before antibiotic therapy. The vancomycin-resistant *E. faecium* strains K860, M658, M223, and M224 were isolated from patients previously colonized with VVE strains, following treatment with vancomycin. VRE844 and VRE856 were selected in the laboratory under increasing concentrations of vancomycin in brain heart infusion (BHI) medium. The standard strains *Enterococcus faecalis* ATCC 29212 (VSE) and *E. faecalis* ATCC 51299 (VRE, VanB phenotype) were procured from the American Type Culture Collection.

MIC determination and growth curves. The MICs were determined by a microbroth dilution method, according to CLSI guidelines. For the growth curve assays, the strains were grown overnight with or without vancomycin (4 μg/ml), and 5 μl of overnight inoculum was used to inoculate each well of a 96-well microtiter plate containing 100 μl of brain heart infusion (BHI) medium supplemented with or without vancomycin (4 μg/ml). Each strain was inoculated in quadruplicate with *E. faecalis* ATCC 29212 (VSE) or *E. faecalis* ATCC 51299 (VRE, VanB phenotype) as negative and positive controls, respectively. The plate was incubated at 37°C with shaking at 250 rpm in a microplate absorbance reader (Sunrise; Tecan), and the optical density at 600 nm (OD_{600}) was measured at 15-min intervals for 20 h. The average of four readings was used to plot graphs of optical density (growth) versus time for each condition and strain.

Development of adaptive mutants. In order to assess whether VSE strains could revert to a VRE phenotype under laboratory conditions, we tested strains 0833, 0844, and 0856 by serial challenge with increasing concentrations of vancomycin. The strains were grown on BHI agar without vancomycin at 37°C for 16 to 18 h. For each strain, a single colony was inoculated into BHI agar containing 1 μg/ml vancomycin. The cultures were incubated at 37°C with shaking at 250 rpm for 18 to 20 h. A 1:100 dilution was made to a fresh broth containing 2 μg/ml vancomycin. This was repeated with 1-μg/ml increments to a final concentration of 10 μg/ml. The cultures were retained and renamed as VRE, and MIC testing was performed as described above.

Diagnostic PCR assay. The sequence-specific primers (Fwd, 5′-CATCCACCAACATCAAAAAGTATAGAGCC, and Rev, 5′-TTCGGGTAGAAATATATTTCACACCGG) were designed to cover the region spanning a small stretch of conserved DNA between the transposon (IS*1251*-like) and the variable *vanHAX* promoter region, extending into the *vanH* gene (see Fig. S1 in the supplemental material). Diagnostic PCR was carried out in a 50-μl reaction mixture containing 100 ng of genomic DNA (gDNA), 200 μM deoxynucleoside triphosphates (dNTPs), 1 U of *Taq* polymerase, 5 μl of 10× buffer with $MgCl_2$, 2.5 μl of dimethyl sulfoxide (DMSO), 0.5 μM each primer, and water to make the volume. The amplification was carried out according to the PCR program: denaturation of 2 min at 95°C, followed by 30 cycles each with 95°C for 30 s, 54°C for 10 s, and 72°C for 1 min, and a final extension step of 7 min at 72°C. The PCR products were run on a 1% agarose gel, and the amplicon bands were eluted and sequenced.

Peptidoglycan precursor analysis. One percent inoculum from overnight cultures of each strain was added to 8 wells of a 96-well plate containing 100 μl of BHI agar per well. The plate was incubated at 37°C with shaking at 250 rpm in a microplate absorbance reader (Sunrise; Tecan), with the OD_{600} measured at 15-min intervals. The cells were harvested at early log phase (OD_{600}, 0.2 to 0.25), and the cultures from the same strain were pooled in a microcentrifuge tube. The cultures were treated with bacitracin (200 μg/ml) for 20 min at 37°C and the cells collected by centrifugation at 13,000 rpm for 30 min at 4°C. The medium was discarded, and the cell pellet was resuspended in 100 μl of ice-cold formic acid and incubated for 30 min on ice. The cell debris was removed by centrifugation, and the supernatant was collected and neutralized by adding an equal volume of 1% NH_4OH. The samples were frozen with liquid N_2 and freeze-dried. For liquid chromatography-mass spectrometry (LC-MS) analysis, the dried precursor samples were resuspended in 100 μl of H_2O and centrifuged at 13,000 rpm for 5 min.

An Agilent 1100 high-performance liquid chromatography system equipped with an autosampler, degasser, and diode array detector was used for solvent delivery and sample introduction. The samples (50 μl) were injected onto a reverse-phase C_{18} Nova-Pak column (3.9 by 150 mm; 4 μm). The column was eluted at a flow rate of 0.5 ml/min with the

following gradient: 0 to 1 min, 0% B; 1.0 to 9.0 min, up to 2% B; 10.0 to 18.0 min, up to 10% B; 18 to 20 min, 10% B; 20 to 25 min, up to 100% B; 25 to 30 min, 100% B; and 31 to 35 min, back to 0% B (solvent A, 0.05% [vol/wt] formic acid in water; solvent B, 0.05% formic acid in acetonitrile).

A QTRAP (Applied Biosystems) mass spectrometer equipped with an electrospray ion source was used for peak detection in positive ion mode. The peptidoglycan precursors UDP-*N*-acetyl-muramyl-L-Ala-D-Glu-L-Lys-D-Ala-D-Ala and UDP-*N*-acetyl-muramyl-L-Ala-D-Glu-L-Lys-D-Ala-D-Lac were detected in the multiple-reaction monitoring mode. The collision energy was set to 25 V. UDP-linked pentapeptides were detected using the following transitions: 1,150.08→746.34 for D-Ala-D-Ala ending precursor and 1,151.16→748.18 for D-Ala-D-Lac (dwell time, 200 ms for both).

Genome sequencing and assembly. We sequenced 2 × 150-bp paired end reads from VRE strains K845, K860, M658, M223, and M224 on an Illumina MiSeq platform at the McMaster Genome Facility. The sequence data were assembled using Velvet 1.2.07, with a *k* value of 31 (11).

Mapping to pS177 and pF856 and coverage plots. The Illumina-generated sequence data for strains K845, K860, M658, M223, and M224 were mapped to plasmid pS177 (GenBank accession no. NC_014959.1) and the assembled plasmid sequence pF856 (GenBank accession no. JQ663598.1) using Bowtie2 version 2.0.0 in local alignment mode (12). Coverage plots were produced using BEDTools version 2.16.1 (13) and Circos (14) after computing the average coverage depth over 1,000 bins of equal width.

***vanA* expression analysis.** The constitutive expression of *vanA* in the clinical isolates was measured using quantitative reverse transcription-PCR (qRT-PCR) in the absence of vancomycin. *E. faecalis* ATCC 51299 (VRE) uninduced and induced with vancomycin (5 μg/ml) served as controls. One milliliter of overnight cultures at an OD_{600} of 0.4 was used to isolate total RNA using the RNeasy mini RNA extraction kit. Isolated RNA was treated with RNase-free DNase to remove residual DNA isolated with the RNA. The reaction was stopped by adding 1 μl of 25 mM EDTA, followed by incubation at 65°C for 10 min. A diagnostic PCR using *vanA*-specific primers was performed on the RNA to confirm the removal of all contaminating DNA. Reverse transcription on the pure RNA was done using the VILO SuperScript cDNA synthesis kit, according to the manufacturer's instructions. The PCR was carried out using *Taq* polymerase and 1× SYBR green I DNA stain in a Bio-Rad C1000 real-time thermocycler using the following cycling conditions: initial denaturation at 95°C for 60 s, followed by 35 cycles of denaturation at 95°C for 20 s, annealing at 59°C for 15 s, amplification at 72°C for 30 s, and one final extension cycle at 72°C for 7 min before termination. The 16S rRNA gene served as an internal reference to quantify the normalized fold expression of *vanA* in different strains.

Nucleotide sequence accession numbers. The raw read sequences were deposited in the Short Read Archive under the study accession no. SRP043196, and for the strains K845, K460, M658, M223, and M224, the accession no. SRR1393715, SRR1393732, SRR1393738, SRR1393809, and SRR1393910 respectively, were used.

RESULTS

Identification of *vanA*-containing VSE and reversion to VRE. We and others have noted the emergence of strains of enterococci in the clinic that may or may not grow on VRE chromogenic agar, are positive for the presence of *vanA* by PCR, and yet paradoxically exhibit a vancomycin-susceptible phenotype. We sequenced the plasmid associated with one of the VVE strains, K845, isolated from a patient treated with ciprofloxacin, piperacillin-tazobactam, and vancomycin. The plasmid sequence identified a variant of the known enterococcal plasmid pS177, which also shows an IS element (IS*1251*) inserted in the region between *vanRS* and *vanHAX* (15). In this new plasmid, an ~8-kb fragment that includes the *vanRS* regulatory system is deleted. The plasmid identified from isolate K845 is an identical match with pF856, associated with a recent VRE outbreak in southern Ontario (8) and a pS177 variant identified earlier in Quebec (10). The plasmid is a chimera formed by a fusion of DNA segments from multiple resistance plasmids. Besides genes conferring resistance to glycopeptides, streptomycin, streptothricin, kanamycin, and erythromycin, the plasmid is also equipped with various transposons and a toxin-antitoxin system.

A subsequent *E. faecium* strain (K460) collected on day 22 from the same patient after 5 days (days 12 to 17) of vancomycin therapy gave a VRE phenotype with an MIC of >256 μg/ml. To our knowledge, such a reversion of the VVE strains to a VRE phenotype during treatment was unprecedented until recently (9). The sequencing of the associated plasmid from the resistant strain identified a variable segment of intergenic DNA upstream of *vanH*, where a 1.5-kb insertion sequence (IS*1167*-like) was inserted at position −21 between the −35 and −10 regions of the *vanH* promoter region (Fig. 2).

Diagnostic PCR-based detection of variable region in VVE strains. A survey of several clinical VSE strains containing *vanA* isolated during the outbreak in Ontario (8) identified an identical plasmid with a loss of the *vanRS* regulatory genes. In a follow-up survey, VRE strains were reisolated from the same patients colonized earlier with VVE. We designed oligonucleotide primers and amplified the region upstream of *vanH* to diagnose any genetic variation in the isolates with restored resistance. Three strains (M658, M223, and M224) that were isolated from patients previously colonized with VVE strains and who were treated with vancomycin showed unexpected results. PCR amplification from the genomic DNA of each of these strains yielded two amplicons, either longer or shorter than the expected 425-bp length (see Fig. S1 in the supplemental material). The subsequent sequencing and analysis of all the amplicons identified multiple genotypes associated with the upstream region (Fig. 2). Shorter amplicons with deletions of 108 bp in M658 and 134 bp in the other two isolates, namely, M223 and M224, resulted in the elimination of the *vanH* promoter region. The results are comparable to the deletion observed in K460. The sequencing of the longer (0.9-kb) amplicons of M223 and M224 identified insertions of an IS*1216*-like sequence in the *vanH* promoter region. Interestingly, the point of insertion and deletion in the longer and shorter amplicons occurred at the same nucleotide in both strains (position +39). The result suggests that the insertion likely represents an intermediate stage leading to the final deleted version observed in the shorter amplicons. Overall, we observed three scenarios in the resistant strains, in which the variable region was altered at three independent locations. The polymorphism reflects independent deletion events rather than a lateral transfer of a single plasmid variant. Regardless of the position of the insertion, all alterations resulted in either a deletion or disruption of the *vanH* promoter region, rendering it nonfunctional.

Genome-wide scan for detection of functional resistance determinants. Although a compromised *vanH* promoter region emerged as a consensus change in all VVE isolates that reverted to the VRE phenotype, this change in sequence might not have a direct role in the reversion of resistance. Given the fact that the vancomycin resistance cassette in enterococci is present on a transposable element (Tn*1546*), a chromosomally encoded canonical copy of the entire resistance machinery or a compensatory *vanRS* capable of imparting resistance cannot be ruled out. We therefore sequenced the genomes of the isolates in this study to explore their complete genomic context. Following curation, the sequence data resulted in average genome-wide coverage with the

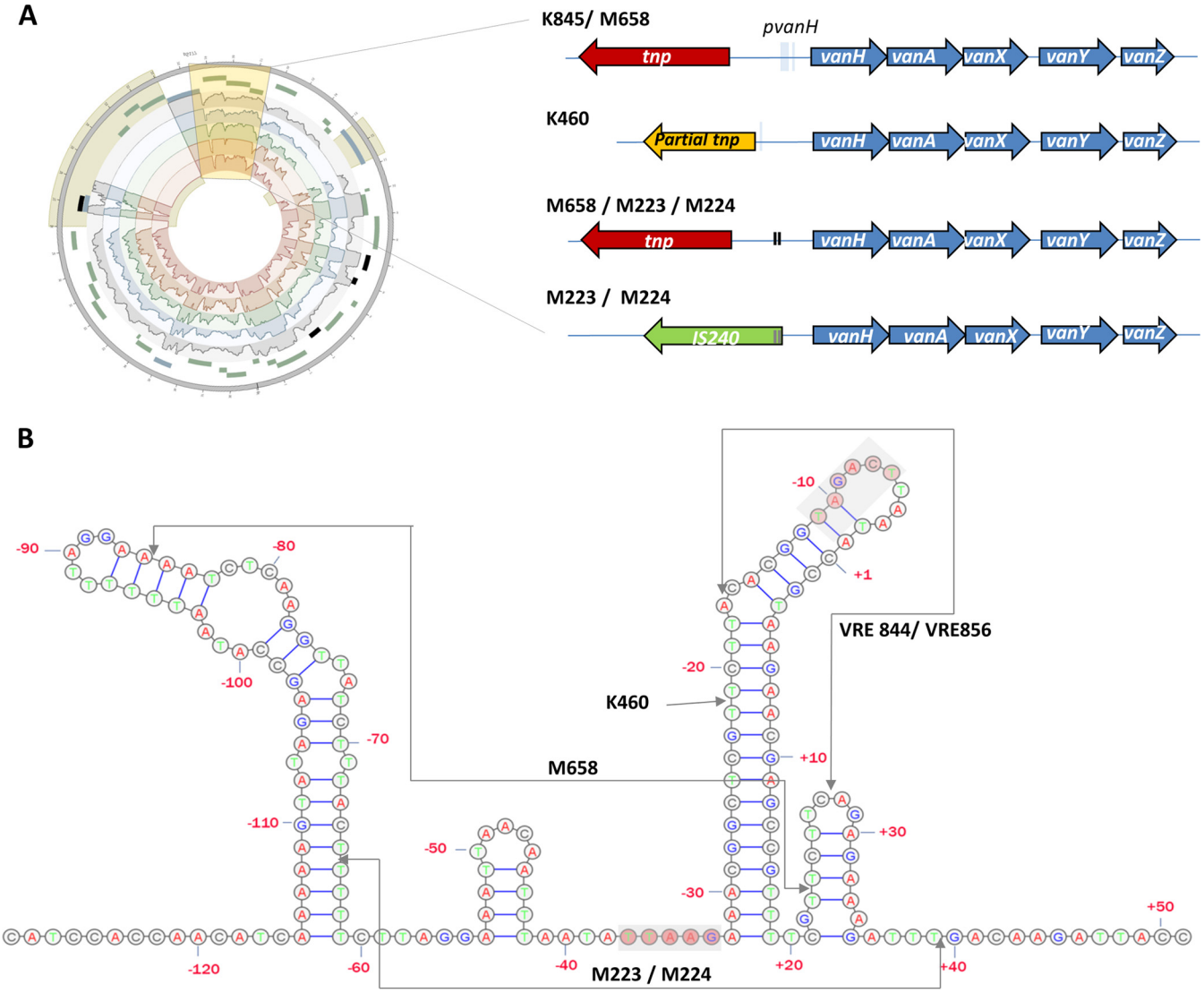

FIG 2 Genotype alterations leading to expression of *vanHAX* resistance genes in the plasmid lacking a *vanRS* regulatory system. (A) A comparative sequence coverage map for the plasmids in different VVE isolates. The outer ring shows the genetic map of pS177 derivatives found in the study. A magnified region containing a vancomycin resistance cassette shows an uninterrupted intergenic region between *tnp* and *vanH* with an intact promoter *pvanH* in the vancomycin-susceptible isolate K845. Three clinical isolates, M658, M223, and M224, showed deletions in the intergenic region, leading to the removal of the *pvanH*. M223 and M224 also had two longer amplicons amplified, showing an insertion of IS*240* in place of the deletion, suggesting that one genotype leads to the other. Strain K460 had the most interesting genotype, in which an ISL3 family transposase partial sequence has disrupted *pvanH*, such that the −10 box is retained. (B) The modeling of DNA for secondary structures using Mfold shows the presence of a hairpin involving *pvanH* in the susceptible isolate K845, without any deletions. Another hairpin structure is formed from −115 to −61 due to the insertion of IS*1251* at position −101. All the deletions or disruptions identified in the vancomycin-resistant isolates mapped around the secondary structure. In K460, a transposon is inserted at position −22, which compromises the second hairpin structure.

following theoretical depths, estimated using a reference strain (NCBI RefSeq accession no. NC_017960.1, NC_017961.1, NC_017962.1, and NC_017963.1): K845, 129× coverage (394 Mbp of raw sequence); K460, 112× coverage (343 Mbp of raw sequence); M658, 131× coverage (398 Mbp of raw sequence); M223, 130× coverage (396 Mbp of raw sequence); and M224, 134× coverage (408 Mbp of raw sequence). Subsequent examination of the assembled genomes did not detect *vanRS* or any additional versions of the vancomycin resistance cassette.

***In silico* analysis of the *vanH* promoter region.** We reasoned that changes in the *vanH* promoter region could relieve the repression of the *vanHAX* gene cassette, resulting in the observed vancomycin resistance phenotype, even when *vanRS* was missing. The deletion of the region upstream of *vanH*, including its promoter, requires an alternate promoter in the upstream region to enable the transcription of the *vanHAX* resistance genes. Using BPROM (16), we identified a putative promoter in the left arm of IS*1251*, present upstream of the variable region, as well as a hybrid promoter arising from the insertion of IS*1167* at the −21 position in K460 (see Fig. S2 in the supplemental material). The presence of hybrid promoters associated with Tn*1546* has been reported in the past (17). These promoters can account for the expression of the *vanHAX* cassette in the absence of *vanRS*.

We also explored the region upstream of *vanH* DNA using the

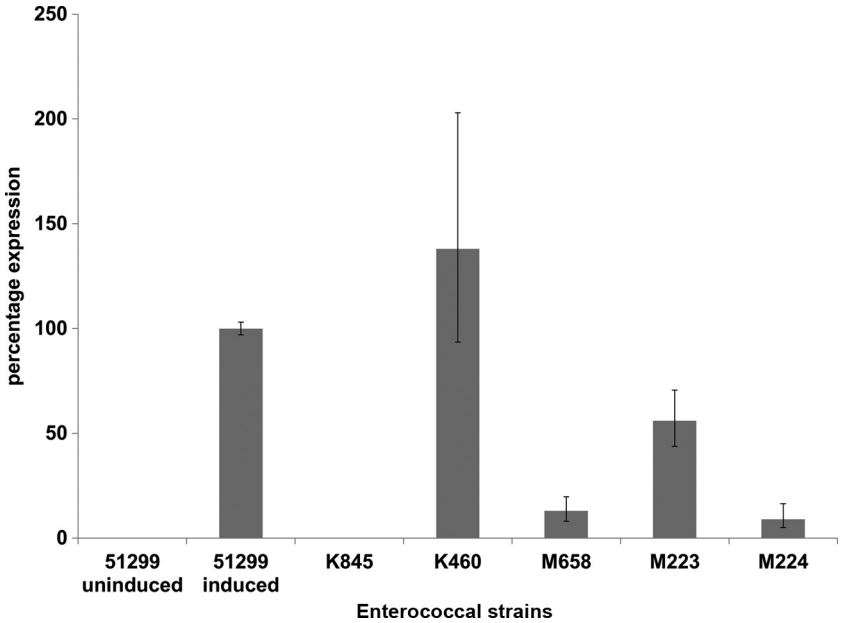

FIG 3 *vanA* expression analysis of the VVE isolates from patients. The results are represented as the percent expression compared to the VRE-positive control, *E. faecalis* ATCC 51299 expression in the presence of 5 μg/ml vancomycin. The vancomycin-susceptible isolate K845 did not show any expression of *vanA*, whereas the other isolates showed various degrees of *vanA* expression, with K460 surpassing the levels of the standard VRE strain. The error bars show 1 standard deviation.

Mfold software (18) and identified a predicted secondary stem-loop structure formed as a result of a 21-bp inverted repeat involving the *vanH* promoter. An additional stem-loop resulting from the IS*1251* insertion, which is absent in the canonical VRE sequence, was identified in the VVE isolates (see Fig. S3 in the supplemental material). The mapping of each VVE deletion with its respective constitutive vancomycin resistance phenotype correlates with a compromised secondary structure in resistant VVE isolates, whereas isolates with an intact stem-loop exhibit a susceptible phenotype, as observed earlier. Thus, the secondary structure upstream of *vanH* probably impairs IS*1251* promoter-mediated transcription in VSE strains, and the deletion of the same restores uninterrupted *vanHAX* expression, imparting a resistance phenotype.

Verification of *vanA* expression and analysis of peptidoglycan precursors in VVE revertants. We analyzed the expression of the resistance cassette by measuring the levels of *vanA* transcripts from the five isolates. Since we expected VanRS-independent constitutive expression, the isolates were cultured without the addition of vancomycin. The qRT-PCR data show the absence of *vanA* transcripts in the VSE strains (Fig. 3). On the other hand, all VVE strains with a resistance phenotype showed constitutive *vanA* expression with various levels of transcripts. The results indicate that the high MICs for the resistant strains are the result of restored *vanHAX* activity. We further verified the results of the qRT-PCR experiment by studying the formation of D-Ala-D-Lac terminating peptidoglycan precursors as a measure of VanHAX activity in all of the isolates. Indeed, we observed an accumulation of significant levels of depsipeptide precursors in the soluble cell wall precursor pool (Table 1). Thus, the alteration of the secondary structure in the *vanH* promoter region of VVE strains correlates with the reversion to the resistant phenotype.

TABLE 1 Microbiological and biochemical analysis of the VVE strains[a]

Strain	MIC (μg/ml) for:		Phenotype	Ratio of D-Ala to D-Lac	% D-Lac precursor
	Vancomycin	Teicoplanin			
ATCC 29212	2	<0.5	Sensitive		0
ATCC 51299	>64	ND	VRE-B	10:1	9
VVE833	<1	<0.5	Sensitive		0
VVE844	<1	<0.5	Sensitive		0
VRE844	16	8	Resistant	4:1	25
VRE856	32	8	Resistant	3:1	33
K845	<1	<0.5	Sensitive		0
K460	>256	>32	Resistant		100
M658	8	8	Resistant		0
M223	8	4	Resistant	4:1	26
M224	4	4	Resistant	3:1	33

[a] MICs of glycopeptide antibiotics and peptidoglycan precursor analysis without induction with vancomycin show a correlation between the resistance phenotype and the presence of D-Ala-D-Lac in the precursor pool.

***In vitro* analysis of reversion in VVE to VRE.** We selected three VVE isolates (VVE833, VVE844, and VVE856) to test if the plasmid modifications required to restore the vancomycin-resistant phenotype in VVE strains could be reproduced under laboratory conditions. We developed adaptive mutants by growing the isolates in the presence of increasing concentrations of vancomycin, starting with subinhibitory doses (1 μg/ml). VVE844 and VVE856 were able to evolve to full vancomycin resistance with increasing drug concentrations, and they withstood 32 μg/ml vancomycin. On the other hand, VVE833 failed to grow beyond the MIC values of vancomycin, even after repeated attempts. PCR amplification and sequencing of the region upstream of the *vanH* gene of both VRE strains identified a deletion in the *vanH* promoter region (Fig. 2).

DISCUSSION

The decoding of this novel molecular mechanism adopted by VVE strains raises concern about the current surveillance techniques used for detecting VRE strains. The use of vancomycin susceptibility testing on chromogenic agar is not sufficient to detect the presence of VVE strains. There are multiple factors that favor the restoration of resistance in these strains. First, because such strains will be missed by VRE control programs, unrestricted transmission of these strains may occur in health care facilities. Infections due to these strains might then be treated with vancomycin and select for resistance. As we observed *in vitro*, as well as in patients, VVE isolates have devised the capacity to recalibrate their ability to express the resistance genes. Second, the pS177-like plasmids carry a toxin-antitoxin system that provides a powerful mechanism for plasmid retention (15). The short-lived antitoxin is continuously synthesized to check the RNase activity of the toxin. The loss of plasmid causes cell death and elimination from the population. The toxin-antitoxin ensures faithful maintenance of the plasmid in the cell, even in the absence of selection pressure, favoring the persistence of these strains. Finally, the transposon-mediated mechanism increases the frequency of deletion compared to that with the gain-of-phenotype point mutations. The presence of a variety of transposons and IS elements identified in the genome occurring in multiple copies creates more opportunities for successful reversion of the susceptible VVE to the resistant form.

Here, we describe the molecular mechanisms leading to constitutive glycopeptide resistance in VVE. Two independent genetic rearrangements are required for the decoupling of VanRS-mediated regulation of the vancomycin resistance cassette and its constitutive expression. First is the intermediate stage in which the deletion of *vanRS* leaves the cell susceptible to the antibiotics vancomycin and teicoplanin (8, 10). The treatment of infections caused by these VSE-like VVE strains with vancomycin provides the selective pressure required for a second genetic alteration that removes an upstream transcription block and inserts a nonregulated promoter, rendering the cell constitutively drug resistant. Regarding the VVE strains as VSE is then dangerous, because therapy can subsequently lead to the propagation of vancomycin-resistant forms of the strain. This in turn translates to an increased risk of treatment failure. We therefore recommend that diagnostic laboratories consider both the genotype and phenotype of enterococcal species for reporting purposes. For the purposes of both infection control and individual patient management, VVE strains, i.e., those positive for *vanA* but lacking *vanRS*, should be reported as VSE, with a warning that they can revert to VRE.

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