

## Diversity of Tn1546 Elements in Clinical Isolates of Glycopeptide-Resistant Enterococci from Scottish Hospitals

A. R. BROWN, A. C. TOWNSLEY, AND S. G. B. AMYES\*

Department of Medical Microbiology, University of Edinburgh, Edinburgh, United Kingdom

Received 17 July 2000/Returned for modification 3 October 2000/Accepted 10 January 2001

**The Tn1546-related elements of 48 Van glycopeptide-resistant enterococci were compared. Ten distinct Tn1546 types were identified with variation primarily due to IS1542 and IS1216V-like insertions. Clonal isolates frequently differed in their Tn1546 type, indicating instability of Tn1546-related elements. A putative hybrid promoter was identified, generated upstream of *vanR* by the insertion of IS1542. The presence of this hybrid promoter was associated with constitutive expression of the *van* genes and elevated teicoplanin resistance.**

While all VanA phenotype glycopeptide-resistant enterococci (GRE) share the same basic Tn1546 structure, as described for *Enterococcus faecium* BM4147 (2), considerable diversity has now been identified within Tn1546-related elements. This variation, in the form of point mutations, insertion sequence (IS) elements, and deletions, has been exploited in several epidemiological studies (8, 10, 13), often in combination with well-established methods (e.g., pulsed-field gel electrophoresis [PFGE] and ribotyping). However, the possible transient nature of insertion sequences has led to questions about the suitability of this type of analysis in epidemiological studies (4, 15). In this study, we have compared 48 VanA enterococcal isolates by PFGE and by molecular analysis of Tn1546-related elements.

Forty-eight clinical isolates of enterococci, collected from eight hospitals in Scotland over a 5-year period (1995 to 1999) were confirmed as *vanA* positive by PCR (5) and identified with the AP120 Strep system (BioMerieux). Sixty-nine percent of isolates were *E. faecium*, and the remainder were *E. faecalis*. Vancomycin and teicoplanin MICs were determined by incorporation of the antimicrobial agents into Mueller-Hinton agar (Oxoid) and inoculation of plates with approximately 10<sup>4</sup> CFU per inocula. All 48 isolates displayed resistance levels that were typical of the VanA phenotype. Vancomycin and teicoplanin MICs ranged from 64 to 1,024 mg/liter and 8–128 mg/liter, respectively.

PFGE analysis and interpretation were performed as previously described (9, 12). Following digestion with *Sma*I, genomic DNA was separated by electrophoresis for 24 h at 200 V with 5- to 40-s pulse times. Discounting those isolates belonging to a previously described outbreak strain of VanA *E. faecium* (3), the 48 isolates were largely heterogeneous in nature, with only small discrete clusters of related isolates identified. No GRE isolated from different geographic regions of Scotland shared PFGE patterns.

Template DNA for PCRs was prepared by the guanidium

thiocyanate extraction method (11). Long-template PCR (L-PCR; Expand long-template PCR system; Boehringer Mannheim) was used according to the manufacturer's instructions. The inverted repeat (IR)-specific primers described previously (16) enabled amplification of the Tn1546-related elements and subsequent restriction fragment length polymorphism (RFLP) analysis by *Cla*I digestion. Five of the 48 isolates failed to yield an L-PCR product with the Tn1546-IR primer, suggesting that they lacked at least one of the IRs. The loss of the left IR has been previously described and is often associated with the presence of IS1216V-like elements alone or in combination with a truncated IS3-like element (6, 13). The reason for the L-PCR failure in these five isolates was not ascertained. The Tn1546-related elements of the remaining 43 isolates were assigned to 10 distinct types on the basis of *Cla*I RFLP analysis. Fourteen isolates harbored Tn1546 elements that were indistinguishable from the prototype Tn1546 element by *Cla*I RFLP analysis. The remaining 29 isolates harbored nonprototype elements. Different Tn1546 types were evident in clonally related isolates, indicating instability within Tn1546-related elements. Such instability has been previously described (15).

Using the primers listed in Table 1, all nonprototype Tn1546-related elements were further studied by PCR and by hybridization analysis with the ECL (enhanced chemiluminescence) random prime labeling and detection system (Amersham Life Sciences Ltd.). PCR products of interest were sequenced in both directions by the dideoxy method on an ABI Prism automated sequencer. All 29 nonprototype elements harbored IS1542 within the *orf2-vanR* intergenic region (nucleotide position 3932) and an IS1216V-like element within the *vanX-vanY* intergenic region (nucleotide position 8839). Both IS elements have been described previously at the same nucleotide positions (13, 15). In addition, all nonprototype Tn1546-related elements had considerable variation within the *orf1-orf2* region. Hybridization analysis, performed following *Bam*HI digestion of Tn1546-related elements, confirmed that this variation was due at least in part to the insertion of an IS1216V-like element within the *orf1-orf2* region. The precise location of the insertion was not ascertained.

The point mutation at Tn1546 nucleotide position 8234 (G→T) within the *vanX* gene was screened for as described

\* Corresponding author. Mailing address: Department of Medical Microbiology, University of Edinburgh Medical School, Teviot Place, Edinburgh, EH8 9AG, United Kingdom. Phone: 44-131-6503163. Fax: 44-131-6511385, E-mail: s.g.b.amyes@ed.ac.uk.

TABLE 1. Primers used in the analysis of Tn1546-related elements<sup>a</sup>

Primer	Sequence	Coordinates	Reference
<b>Amplification</b>			
Tn1546-IR	5'-GGAAAATGCGGATTTACAACGCTAAG	13-38; 10839-10814	16
<i>orf1</i> -f	5'-GATGGTGGCTCCTTTTCC	906-923	
<i>orf1</i> -r	5'-CCATTGTTGGCAGCAGAC	1597-1580	
<i>vanR</i> -f	5'-GCCGAAAAGCCCTTTGATA	3841-3859	
<i>vanR</i> -r	5'-TCGTTCTAGTTTGGAAATAGTCG	4699-4678	
<i>vanS</i> -f	5'-TAGGGTAGAGCTTCCAGCGA	5752-5771	
<i>vanH</i> -r	5'-ATTATCGTTGCCATAACGCC	6115-6096	
<i>vanX</i> -f	5'-GATGAACGCTCATCATGC	8448-8467	
<i>vanY</i> -r	5'-TTCCTGAGAAAACAGTGCTTCA	9138-9117	
<i>vanX1</i>	5'-ACTTGGGATAATTCACCGG	8082-8101	8
<i>vanX2</i>	5'-TGCATTGTCGCTTCATTG	8505-8486	8
<b>Sequencing</b>			
1216V-A	5'-TTGAAGATGTAAGGCAGAGC	8727-8746	
1216V-B	5'-AGGCTGAACTGCCTGTTGAC	8857-8838	
1216V-C	5'-GATCGCATAGAGGGGTGGTA	8802-8821	
1542-A	5'-TAGCTGTTTCGAGCGAGTTCA	980-961 <sup>b</sup>	
1542-B	5'-TCGGCAATTCATGTTTCATC	4022-4003	
<b>Hybridization</b>			
1216V-D	5'-GTACAGACCGAAAACCCGAA	88-107 <sup>c</sup>	
1216V-E	5'-GCAATTCAGCAGGATGTGA	714-695 <sup>c</sup>	

<sup>a</sup> Coordinates are based on the published Tn1546 sequence (GenBank accession number M97297) unless stated otherwise. Sequencing primers were used in the sequencing of the IS1542 and IS1216V-like elements within the *orf2-vanR* and *vanX-vanY* intergenic regions, respectively. Hybridization primers 1216V-D and 1216V-E were used to obtain IS1216V-like-specific PCR product from which labeled probes were produced. Primers without a reference were designed by Primer3 (<http://www.genome.wi.mit.edu/cgi-bin/primer>).

<sup>b</sup> Corresponds to the published sequence of IS1542 (GenBank accession number AF114715).

<sup>c</sup> Corresponds to the published sequence of the IS1216V-like element (GenBank accession number AF093508).

previously (10). This point mutation, previously associated with porcine isolates of VanA GRE (14), was not evident in any of the isolates studied.

Sequencing of *orf2-vanR* intergenic regions harboring IS1542 identified a putative hybrid promoter. The -10 TATAAT box that forms part of the native *vanR* promoter proposed by Holman et al. (7) is duplicated by the 8-bp target site duplication generated by IS1542 insertion at nucleotide position 3932. This duplicated -10 box forms a putative promoter sequence in conjunction with an outwardly directed -35 box (TTTACA) located within the inverted repeat of IS1542. The impact of the IS1542 insertion and the resulting hybrid promoter on the expression of glycopeptide resistance was assessed by growth curve analysis following glycopeptide challenge and by VanX<sub>D,D</sub>-dipeptidase enzyme assays (1). Growth curves were consistent with induced expression of *van* genes, irrespective of whether encoded by a prototype or nonprototype Tn1546 element. However, VanX enzyme assays revealed significant constitutive expression of the *van* genes of nonprototype Tn1546-related elements. Specific activities in the absence of induction

were, on average, 10-fold greater than the background expression from prototype Tn1546 elements. Constitutive expression, which we propose is mediated by the IS1542-generated hybrid promoter, supplemented rather than replaced the inducible *van* gene expression. In addition, nonprototype Tn1546 elements conferred higher levels of teicoplanin resistance than did prototype Tn1546 elements. While this phenomenon was partly medium dependent (Table 2), the consistency of teicoplanin MICs conferred by prototype Tn1546 elements suggested that the elevated resistance was not solely attributable to the media used. The reason for the medium dependency is unclear.

In conclusion, this study has revealed considerable diversity within the Tn1546-related elements of VanA GRE in Scotland. The types of variation witnessed were consistent with the findings of previous studies. Many IS elements have the potential to form hybrid promoters, owing to the presence of outwardly directed -35 regions within their inverted repeats. This study describes the first such case within the *van* gene cluster. We propose that constitutive expression of the *van* genes from the

TABLE 2. Summary of teicoplanin MIC data for VanA isolates with prototype and nonprototype Tn1546 elements

Medium used for sensitivity testing <sup>a</sup>	Prototype Tn1546			Nonprototype Tn1546 <sup>b</sup>		
	MIC <sub>50</sub> <sup>c</sup>	MIC <sub>90</sub> <sup>c</sup>	Range <sup>c</sup>	MIC <sub>50</sub>	MIC <sub>90</sub>	Range
Iso-Sensitest agar	8	32	4-32	64	128	16-128
Mueller-Hinton agar	16	64	4-64	32	64	16-128
Brain heart infusion agar	16	32	8-32	256	512	32-1024

<sup>a</sup> The control organism, *E. faecalis* NCTC 775, was consistently susceptible on all media tested (MIC, 0.5 mg/liter).

<sup>b</sup> Defined as having IS1542 and IS1216V-like insertions within the *orf2-vanR* and *vanX-vanY* intergenic regions, respectively.

<sup>c</sup> Expressed in milligrams per liter.

hybrid promoter results in the elevated teicoplanin resistance conferred by nonprototype Tn1546 elements. The elevation in teicoplanin resistance and not vancomycin resistance could potentially reflect different abilities of the glycopeptide agents to act against residual D-Ala-D-Ala-terminating precursors.

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