Molecular analysis of diverse elements mediating VanA glycopeptide resistance in enterococci

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Differences were examined among 24 distinct elements mediating VanA-type glycopeptide resistance in enterococci isolated from hospital patients and non-human sources in the UK. The methods used included long-PCR restriction fragment length polymorphism (L-PCR RFLP) analysis and DNA hybridization. All elements had conserved vanRSHAX genes, but variation occurred upstream of vanR and downstream of vanX. Twenty-one VanA elements had significant alterations upstream of vanR in the transposition genes orf1 and orf2: either parts of these genes were absent or they were disrupted by IS1216V or IS3-like insertion sequences. Among VanA elements with alterations downstream of vanX, seven lacked vanY, one lacked both vanY and vanZ, and ten had copies of insertion sequence IS1216V between vanX and vanY. All VanA elements of group D (from geographically and temporally diverse enterococci) were characterized by the presence of an IS1216V/IS3-like/orf1 complex and a point mutation in vanX, both of which were absent from the other 23 groups of VanA elements. This finding is consistent with the dissemination of a stable resistance element. We conclude that L-PCR RFLP analysis, combined with DNA hybridization, merits further development for studying the evolution and epidemiology of VanA resistance elements in enterococci.

Introduction

The development of resistance to glycopeptide antibiotics poses a therapeutic problem for the treatment of serious enterococcal infections, such as endocarditis. Four phenotypes of glycopeptide resistance are currently recognized in enterococci. The VanA, VanB and VanD phenotypes are examples of acquired resistance, and the VanC phenotype is an intrinsic resistance characteristic of the species Enterococcus gallinarum and Enterococcus casseliflavus. VanA is the phenotype reported most frequently in the UK, and is associated with high-level resistance to vancomycin and cross-resistance to teicoplanin. This is the only form of acquired resistance also confirmed to exist both in the community and in non-human sources, such as the faeces of farm animals, raw meat for human consumption, and sewage. These sources may act as reservoirs of enterococci with VanA elements and so may pose a threat to public health. Several molecular methods, including pulsed-field gel electrophoresis (PFGE), have been used to group isolates into clusters (or strains), but these methods generally fail to show significant relationships between VanA strains from human and non-human sources. Rather, the horizontal spread of VanA elements between strains is proposed to play an important role in the dissemination of resistance.

In the prototype VanA strain of Enterococcus faecium (BM 4147), resistance is conferred by a 10.8 kb transposon, designated Tn1546. However, heterogeneity exists among VanA elements, largely resulting from the presence of insertion sequences (IS) at various positions within Tn1546-like elements. Specifically, an IS1216V/IS3-like complex has been found at the 5′ end of Tn1546, in the intergenic region between orf2 and vanR, IS1251 between vanS and vanH, IS1216V between vanX and vanY, and IS1476 within the reading frame of vanY.
have previously compared the VanA resistance elements of 106 diverse isolates of enterococci from UK hospital patients and non-human sources and divided them into 24 groups, designated A to X. The aim of this study was to characterize and further compare VanA elements representing each of these 24 groups, one of which (group A) corresponded to classical Tn1546.

Materials and methods

Bacteria

The 24 isolates of enterococci included in this study had previously been found to contain VanA elements of groups A to X. Transposon Tn1546, from E. faecium strain B M 4147, was used to represent a VanA element of group A. Investigations for the presence of a known point mutation in the vanX gene (see below) used (i) a previously described collection of 107 diverse enterococci from hospital patients and non-human sources, (ii) 13 additional isolates from animals and hospital patients from Denmark and (iii) Enterococcus faecalis strain BM 4224 (kindly supplied by Michel Arthur, Pasteur Institute, Paris, France).

Long PCR (L-PCR) of Tn1546-like elements and vanRSHAX genes

Genomic DNA was extracted from enterococci, quantitatively spectrophotometrically, and 125 ng amounts were used as templates for L-PCR reactions. Tn1546-like elements were amplified using an Expand Long Template PCR System (Boehringer–Mannheim, Lewes, UK), with the buffer conditions recommended by the manufacturer, except that the reaction volumes were decreased to 25 μL. Primer 1 (5'-GGA AAA ATT GC TTT TCA A C A C T A A G-3') is located in both of the terminal inverted repeats of Tn1546 (Figure 1). Amplification was carried out on Touchdown (Hybaid, Teddington, UK) or Genius (Techne, Cambridge, UK) thermal cyclers with the protocol: (i) 94°C for 2 min; (ii) ten cycles of 94°C for 10 s, 65°C for 30 s, and 68°C for 10 min; (iii) 20 cycles of 94°C for 10 s, 65°C for 30 s, and 68°C for 10 min (with the elongation time increased by 20 s/cycle); and (iv) 68°C for 7 min.

A 4.4 kb fragment of the vanRSHAX genes, corresponding to nucleotides 4101–8549 of Tn1546, was amplified with primer 2 (forward) 5'-AG CAC AGT CAT-3' and primer 3 (reverse) 5'-AT TGA TGC TCA CCA CCA CTC TTG GCC-3'. The amplification conditions were as above, except that the primer annealing temperature in steps (ii) and (iii) was reduced from 65°C to 58.5°C.

The quality of all L-PCR amplicons was checked by electrophoresis through 0.8% agarose gels in 0.5× TBE buffer (1× TBE is 89 mM Tris, 89 mM boric acid, 2 mM EDTA, pH 8), followed by staining with ethidium bromide and visualization under ultraviolet light.

Restriction fragment length polymorphism (RFLP) analysis of L-PCR amplicons

To compare the amplicons derived from different VanA elements, 0.1–0.5 μL amounts (with the precise volumes determined empirically) of the L-PCR products were digested overnight with 30 U of Clal (Tn1546-like elements) or 10 U of Ddel (vanRSHAX genes) (Life Technologies, Paisley, UK). The resulting fragments were separated by electrophoresis through 1% (Tn1546-like elements) or 2% (vanRSHAX genes) agarose gels in 0.5× TBE buffer.

Rapid screening for point mutations in vanX

A 424 bp fragment of vanX (nucleotides 8082–8505 of Tn1546) was amplified by PCR as previously described from 121 enterococci of diverse origins. The products (3 μL) were digested for 4 h with 10 U of Ddel (Life Technologies, Paisley, UK) and analysed by electrophoresis through 2.5% agarose gels. Amplicons with sequences identical to Tn1546 were predicted to generate three Ddel fragments of 185, 149 and 90 bp. However, sequences carrying a G→T mutation at position 8234 should generate only two fragments of 239 and 185 bp.

Amplification of orf1, orf2, vanY and vanZ

The presence of sequences internal to orf1 (750 bp, nucleotides 795–1544 of Tn1546 and 540 bp, nucleotides 1871–2936), orf2 (540 bp, nucleotides 3187–3726), vanY (866 bp, nucleotides 9005–9960) and vanZ (324 bp, nucleotides 10,258–10,581) was investigated (i) by PCR using published primers and amplification conditions and (ii) by hybridization, using PCR products amplified from Tn1546 (BM 4147) and labelled with digoxigenin.

Identification of insertion sequences within VanA elements

Primers described by Jensen et al. were used to amplify internal fragments of IS1216V and IS3-like elements, to investigate the presence of an IS1216V/IS3-like complex and to confirm the association of IS3-like elements with orf1.

Location of VanA elements

Plasmid DNA was extracted by a modified alkaline lysis method and analysed on 0.8% agarose gels. PFGE of
Molecular analysis of VanA elements

SmaI-digested genomic DNA was performed as described. After electrophoresis, plasmid DNA or PFGE-separated genomic DNA was transferred to nylon membranes by vacuum blotting, hybridized overnight with a digoxigenin-labelled probe specific for the vanA gene, and hybrids were detected colorimetrically.

Results

ClaI RFLP analysis of Tn1546-like elements

Tn1546, a VanA element belonging to group A, was amplified from *E. faecium* BM4147 with primer 1 and digested with ClaI. Four restriction fragments were obtained and their sizes were consistent with those predicted from the DNA sequence (Figure 1). When the same method was applied to VanA elements of groups B to X, only 19 were amplified; the exceptions were elements of groups D, K, M and P. The 20 successfully amplified elements ranged in size from c. 8 kb (groups W and X) to c. 15 kb (group H), and ClaI digestion revealed 19 distinct RFLP patterns; elements of groups W and X appeared indistinguishable (Figure 2). All elements included a 2 kb fragment which was confirmed to carry the vanA gene by DNA hybridization (data not shown). This observation suggested that these 20 elements all contained a central conserved block of DNA.

Ddel RFLP analysis of the vanRSHAX genes

To investigate the possibility of a conserved part of VanA elements, a second L-PCR assay was designed to amplify the vanRSHAX genes, with primers 2 and 3. The VanA elements of all 24 groups gave products with the predicted size of 4.4 kb (data not shown).

Recently, a point mutation was reported at position 8234 of Tn1546, lying in the vanX gene. This causes the loss of a Ddel target site. Digestion of the vanRSHAX amplicons with Ddel showed consistency in the banding patterns of 23 of 24 VanA elements (Figure 3), whereas the pattern given by the isolate with the group D element differed in that the mobility of the predicted 874 bp Ddel fragment was reduced. This is consistent with the presence of the point mutation reported in the vanX gene.

Prevalence of the mutation at position 8234

An internal fragment of vanX was amplified from 121 enterococci, comprising 106 isolates from the UK, 13 isolates from Denmark and strains BM4147 and BM4224 from France, and digested with Ddel. The products derived from Tn1546 (in BM4147) and 105 other isolates yielded three Ddel fragments consistent with DNA sequence predictions. However, 15 isolates, including BM4224, gave products yielding only two Ddel fragments (Figure 4). This was consistent with the presence of a

![Figure 1. The structure of VanA transposon Tn1546, indicating the annealing sites for L-PCR primer 1, ClaI target sites (C) and predicted sizes of restriction fragments. The lower panels show the actual fragments obtained for Tn1546 and a schematic representation of the gel.](image-url)
mutation at position 8234. Such ‘mutant’ vanX genes were observed in all enterococci with VanA elements belonging to group D, but in no other groups (data not shown).

Investigation of transposition and accessory genes

Previously, we showed that VanA elements of groups D to X had alterations in the sequences of either or both of the transposition genes, orf1 (encoding a transposase) and orf2 (encoding a resolvase). In this work, large parts of orf1 could not be detected by PCR in enterococci representing VanA elements of groups L, M, N, P, Q, R, S, T, U, V, W and X (Table). The absence of these sequences was confirmed by hybridization. Enterococci of groups R to X also lacked sequences homologous to orf2 (Table). Three elements (groups E, F and G) yielded no product with primers for orf1 nucleotides 795–1544. Nevertheless, hybridization with a probe derived from this fragment suggested limited, but significant changes were present at the annealing site of one or other of the primers rather than absence of the entire sequence.

A vanY amplicon was not yielded by enterococci representing eight VanA groups (Table) and the absence of this sequence was confirmed by hybridization. The vanZ gene was detected by PCR and hybridization in all enterococci except the isolate representing group K.
Molecular analysis of VanA elements

Identification of insertion sequences within VanA elements

IS1216V was detected by PCR in the genomes of enterococci carrying VanA elements representative of all 24 groups, and DNA hybridization indicated the groups in which these elements were located within the actual VanA elements (Table). IS1216V was located on PCR amplicons corresponding to Tn1546 nucleotide positions 6979–8920 in groups B, C, E, H, Q, R, S, T, U and V, nucleotide positions 8889–10473 in group C, and within the reading frame of orf2 in group L. Based on the sizes of amplicons obtained with primers specific for vanX, vanY and vanZ, the IS1216V elements between positions 6956 and 8920 and between positions 8890 and 10473 were probably located at variable positions within the intergenic region between vanX and vanY.

IS3-like sequences were detected in the genomes of enterococci representing 21 groups of VanA elements, excluding groups C, O and Q (Table). An IS1216V/IS3-like complex was detected only in the isolate containing a group D element, and most probably was located immediately upstream of orf1, as suggested by detection of an IS3-like/orf1 complex. Further examination of 15 enterococci with group D elements (including BM 4224) indicated that all had both IS1216V/IS3-like and IS3-like/orf1 complexes. The IS3-like/orf1 complex was also detected in isolates representing VanA elements of groups H, I, J and K (Table), which lacked the IS1216V/IS3-like complex.

Location of VanA elements

Southern blots hybridized with a vanA-specific probe indicated that 17 groups of VanA elements were located on plasmids (Table), and PFGE-separated genomic DNA from the isolates representing these groups failed to hybridize with the probe. The VanA elements of six further groups were probably located on the chromosome, as suggested by hybridization only with PFGE-separated genomic DNA. Hybridization data suggested that the isolate with a group C element contained intact or partial VanA elements on both plasmids and its chromosome.

Discussion

We have previously described 24 groups of VanA resistance elements among enterococci from UK hospital patients and non-human sources. In the present study, we further investigated the differences among these elements by a variety of PCR and hybridization assays. Using L-PCR RFLP analysis, we showed that the vanRSHAAX gene cluster was conserved in all 24 groups and, hence, that the variation in their ClaI RFLPs resulted from sequence alterations upstream of vanR or downstream of vanX.

Previously, we showed that 21 VanA groups (D to X) had alterations upstream of vanR in orf1 and orf2 and seven of these groups had copies of IS1542 in the intergenic region between orf2 and vanR. This study confirmed that elements of groups D to X each have various changes in the sequences of orf1 and orf2. DNA hybridization confirmed that several groups lacked parts of these genes, both of which are associated with the transposition of Tn1546. In other groups, orf1 and orf2 were disrupted by IS3-like and IS1216V elements, respectively. The extent to which these events affect the ability of the elements to transpose requires further investigation, as it may affect the dissemination of VanA resistance.

The groups of VanA elements with alterations downstream of vanX included seven that lacked vanY and one that lacked both vanY and vanZ. These two genes are not essential for the expression of VanA resistance. Disruption of vanY by an IS element has been observed previously, but the absence of vanY from clinical isolates...
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<sup>a</sup>Fragments correspond to specified nucleotide positions on Tn546 (GenBank M97297).
<sup>b</sup>P, plasmid; C, chromosome.
<sup>c</sup>No amplicon was detected by PCR, but elements hybridized with a probe derived from this fragment of Tn546.
<sup>d</sup>Amplicon was larger than that obtained from Tn546 and other elements and hybridized with an IS1216V probe.
of VanA enterococci is novel and only one strain has been described previously that lacks vanZ. Ten groups of VanA elements carried copies of IS1216V, most probably within the intergenic region between vanX and vanY, as reported previously.

The epidemiological value of assigning VanA elements to groups will be limited if the elements prove unstable. Transposition of an IS element into, out of, or to a different position within a VanA element would cause assignment to a different group. Isolates of enterococci that belong to the same strain, as defined by PFGE, may contain VanA elements of distinct groups, which suggests that the resistance elements may change more rapidly than other molecular markers, such as the distribution of Smal target sites (for PFGE). On the other hand, this study suggested that some other VanA elements are both temporally and geographically stable.

Group D elements from UK isolates correspond to those designated ‘type 2’ in Danish isolates by Jensen et al. E. faecalis strain BM 4224, which was derived from a French clinical isolate, was shown here also to contain a group D element. This group of elements has been associated with VanA enterococci isolated from pig faeces and from some hospital patients, but not with enterococci from the faeces of other animals; the epidemiological reasons for this association are unknown. Group D elements were unique in several molecular characteristics. These included the presence of an IS1216V/IS3-like/orf1 complex at the 5’ end of the element and a nucleotide change (from G→T) at position 8234, which lies in the vanX gene. Although the IS1216V/IS3-like/orf1 complex, which was associated with loss of the first 120 nucleotides of Tn1546, was originally identified from a VanA element from the USA, the sequence of the group D element in strain BM 4224 is identical (M. Arthur, personal communication). The original US element would not have been assigned to group D because it had an IS1251 element between vanS and vanH, nevertheless it was located on the chromosome, as was a group D element investigated here. Recognition of VanA resistance in Europe pre-dates its recognition in the USA and IS1251 elements have not been observed in Europe. A group D element appears to be stable over time, the VanA element described by Handwerger & Skoble might have arisen following the transposition of IS1251 into a group D VanA element. Further study of VanA elements might provide evidence to indicate whether VanA resistance was imported into the USA from Europe. This absence of nucleotides 1–120 of Tn1546, which include the left terminal inverted repeat, would prevent annealing of primer 1, and this potentially explains the failure to amplify any group D elements by L-PCR with this primer. The VanA elements of groups K, M and P were also not amplified with primer 1 for reasons that remain undefined: each must lack one, other, or both of the terminal inverted repeats of Tn1546.

L-PCR RFLP analysis may prove to be a useful tool for determining the relatedness of VanA resistance elements in enterococci. It can show point mutations if an appropriate restriction enzyme is used, and can be used, in combination with DNA hybridization, to evaluate the diversity and stability of VanA elements. Amplification and digestion of vanRSAX can be used to screen for major changes in the main resistance genes, e.g. it would indicate the presence of IS1251 or other insertions within the vanS–vanH intergenic region. Currently, we are developing further L-PCR assays that amplify regions upstream of vanR and downstream of vanX, so as to overcome the problems of negative results observed for groups D, K, M and P with primer 1. This approach could be developed to study the evolution of VanA elements and to monitor their spread in local, national, and international epidemiological studies.

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**References**


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