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Genetic linkage of the *vanB2* gene cluster to Tn*5382* in vancomycin-resistant enterococci and characterization of two novel insertion sequences

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VanB-type vancomycin resistance is encoded by the *vanB* gene cluster, which disseminates by horizontal gene transfer and clonal spread of vancomycin-resistant enterococci (VRE). Genetic linkage of the *vanB* gene cluster to transposon Tn*5382* and the insertion sequences IS*16* and IS*256*-like has previously been shown. In this study linkage of defined *vanB* gene cluster subtypes to these elements was examined. All the *vanB2* subtype strains studied (*n* = 14) revealed co-hybridization of *vanB* and Tn*5382*, whereas the strains of *vanB1* (*n* = 8) and *vanB3* (*n* = 1) subtypes were Tn*5382* negative. Conjugative cotransfer of the *vanB2* gene cluster and Tn*5382* was demonstrated for two strains. DNA sequencing of the $vanX_B$–ORFC region in *vanB2* strains confirmed that the *vanB2* gene cluster is an integral part of Tn*5382*. No general pattern of linkage was observed with regard to IS*16* and IS*256*-like. Two novel insertion sequences were identified in specific *vanB2* subtype strains. (i) A 1611 bp element (ISEnfa*110*) was detected in the left flank of Tn*5382*. Its insertion site, lack of terminal inverted and direct repeats, and two conserved motifs in its putative transposase all conform to the conventions of the IS*110* family. (ii) A 787 bp element (ISEnfa*200*) was detected in the $vanS_B$–$vanY_B$ intergenic region. Its ORF encoded a putative protein with 60–70% identity to transposases of the IS*200* family. No further copies of ISEnfa*110* were found by colony hybridization of 181 enterococcal isolates, whereas ISEnfa*200* was found in four additional *vanB2* strains from the USA. The five strains had identical ISEnfa*200* element insertion sites, and Tn*5382* was located downstream from a *pbp5* gene conferring high-level ampicillin resistance. These isolates showed related PFGE patterns, suggesting possible clonal spread of a VRE strain harbouring a Tn*5382*–*vanB2*–ISEnfa*200* element linked to a *pbp5* gene conferring ampicillin resistance.

Keywords: *Enterococcus*, insertion element, Tn*5382*, *vanB*

INTRODUCTION

Enterococci are part of the normal faecal flora of humans and animals and have emerged as important nosocomial pathogens. In addition to their many inherent resistance traits, enterococci are able to obtain resistance by acquisition of new DNA (Leclercq, 1997). Insertion sequences (ISs) are small mobile genetic elements that can be involved in acquisition and dissemination of virulence or antimicrobial-resistance genes, as well as genes involved in catabolic pathways. Transposition of ISs has been shown to generate silent mutations, gene inactivation and activation of downstream genes. To

Abbreviations: IS, insertion sequence; VRE, vancomycin-resistant enterococci.

GenBank and GenPept accession numbers are given in Table 3.

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Table 1. VanB-type *Enterococcus* strains used in this study and results from *vanB* gene cluster subtyping and Tn*5382* hybridization

Reference no.	Origin	Species	Subtype of *vanB* gene cluster*	Presence of Tn*5382*	Reference
V583	USA	*E. faecalis*	1	–	Dahl *et al*. (1999); Sahm *et al*. (1989)
TUH1-75	Sweden	*E. faecalis*	2	+	Dahl *et al*. (1999)
TUH1-79	Norway	*E. faecium*	2	+	Dahl *et al*. (1999)
TUH2-18	Norway	*E. faecium*	2	+	Dahl *et al*. (1999)
TUH4-54	UK	*E. faecium*	2	+	Dahl *et al*. (1999)
TUH4-64	USA	*E. faecium*	1	–	Dahl *et al*. (1999)
TUH4-65	USA	*E. faecium*	1	–	Dahl *et al*. (1999)
TUH4-66	USA	*E. faecium*	1	–	Dahl *et al*. (1999)
TUH4-67	USA	*E. faecalis*	2	+	Dahl *et al*. (1999)
TUH4-68	USA	*E. faecium*	1	–	Dahl *et al*. (1999)
TUH7-13	USA	*E. faecalis*	1	–	Dahl *et al*. (1999)
TUH7-14	USA	*E. faecium*	1	–	Dahl *et al*. (1999)
TUH7-15	USA	*E. faecium*	2	+	Dahl *et al*. (1999)
TUH7-16	Norway	*E. gallinarum*	2	+	Dahl *et al*. (1999)
TUH7-53	Germany	*E. faecalis*	1	–	Dahl *et al*. (1999)
TUH7-54	Germany	*E. faecalis*	2	+	Dahl *et al*. (1999)
TUH7-55	Germany	*E. faecium*	2	+	Dahl *et al*. (1999)
VRE 45	USA	*E. faecalis*	3	–	Dahl *et al*. (1999); Patel *et al*. (1998)
C68	Ohio, USA	*E. faecium*	2	+	Carias *et al*. (1998)
D366	France	*E. faecium*	2	+	Carias *et al*. (1998); Williamson *et al*. (1989)
3174	New York, USA	*E. faecium*	2	+	Carias *et al*. (1998)
3332	Ohio, USA	*E. faecium*	2	+	Carias *et al*. (1998)
3568	Missouri, USA	*E. faecium*	2	+	Carias *et al*. (1998)

* Subtypes of the *vanB* gene cluster are indicated by: 1, *vanB1*; 2, *vanB2*; 3, *vanB3*.

limit the potentially detrimental effects of excessive genome rearrangements, transposition is generally kept at low levels (Galas & Chandler, 1989). ISs are grouped into families on the basis of similarities in genetic organization, conservation of domains or motifs in their presumptive transposase sequences, their terminal nucleotide sequences and generation of target duplications (Mahillon & Chandler, 1998). Homologous ISs can mobilize the intervening DNA, creating structures termed composite or compound transposons (Galas & Chandler, 1989; Quintiliani & Courvalin, 1996). The importance of composite transposons in the spread of antimicrobial resistance determinants in enterococci has been emphasized by the description of a large composite chromosomal element Tn*5385* in *Enterococcus faecalis* conferring multiple antimicrobial resistance (Rice & Carias, 1998).

Acquired glycopeptide resistance in enterococci is phenotypically and genotypically heterogeneous, with VanA and VanB phenotypes being the most commonly encountered forms (Arthur *et al*., 1993; Quintiliani & Courvalin, 1996). Their mechanism of resistance involves the synthesis of peptidoglycan precursors that terminate in D-lactate instead of D-alanine, thus reducing the affinity for glycopeptide antibiotics (Evers & Courvalin, 1996). The *vanA* gene cluster is located on the non-conjugative transposon Tn*1546* or related elements, which can be part of large mobile chromosomal elements as well as non-conjugative and conjugative plasmids (Arthur *et al*., 1993; Handwerger & Skoble, 1995; Handwerger *et al*., 1995). Dissemination of the *vanB* gene cluster appears to result from conjugation of plasmids (Woodford *et al*., 1995) or intercellular transfer of chromosomal elements (Carias *et al*., 1998; Quintiliani & Courvalin, 1996) as well as clonal spread. The *vanB* gene cluster has not been universally linked to a specific mobile genetic element. Recently an approximately 27 kb putative conjugative transposon Tn*5382* containing the *vanB* gene cluster was described. Intercellular transfer of Tn*5382* as an integral part of a larger chromosomal element also conferring ampicillin resistance was documented (Carias *et al*., 1998), but transposition of Tn*5382* itself has not been shown. Intracellular transposition of the *vanB* gene cluster as part of the composite transposon

Tn*1547* flanked by insertion sequence elements IS*16* and IS*256*-like to a conjugative plasmid, and interspecies transfer of plasmids or large chromosomal elements containing Tn*1547*, have also been described (Quintiliani & Courvalin, 1996). The epidemic potential of conjugative plasmids containing *vanB* is indicated by the presence of transferable *vanB*-encoding plasmids in unrelated clinical *Enterococcus faecium* and *E. faecalis* strains (Woodford *et al*., 1995).

Based on sequence differences the *vanB* gene cluster can be divided into at least three distinct subtypes: *vanB1*, *vanB2* and *vanB3* (Dahl *et al*., 1999; Patel *et al*., 1998). In this study we report that the *vanB2* subtype gene cluster is an integral part of the putative conjugative transposon Tn*5382*. Two novel ISs, ISEnfa*110* and ISEnfa*200*, were found in specific *vanB2* subtype strains of vancomycin-resistant enterococci (VRE). These IS elements were characterized with respect to their DNA sequence and distribution in enterococci.

METHODS

Bacterial strains. The 23 VanB-type strains (Dahl *et al*., 1999) included in the study are listed in Table 1. *E. faecalis* BM4281 containing Tn*1547* (Quintiliani & Courvalin, 1996) was *vanB* subtyped but not included in the other analyses. When screening for ISEnfa*200*- and ISEnfa*110*-like elements, the following strains were included: (i) 63 *Enterococcus* strains from the European VRE study (Schouten *et al*., 1999) – 29 *E. faecium*, 33 *E. faecalis* and one *Enterococcus gallinarum*, from England, Germany, Hungary, Italy, Poland, Portugal and Spain; (ii) 81 VanA-type VRE strains (Simonsen *et al*., 2000) from diverse ecological and geographical sources – 75 *E. faecium*, three *Enterococcus hirae*, two *Enterococcus durans* and one *E. faecalis*, from England, France, Germany, Ireland, the Netherlands, Norway and the USA; and (iii) 14 reference strains – *E. faecalis* ATCC 51575, ATCC 49757, ATTC 29212 and ATCC 19433, *E. faecium* ATCC 51559 and ATCC 19434, *E. gallinarum* ATCC 49608, *E. casseliflavus* ATCC 25788, *E. saccharolyticus* ATCC 43076, *E. avium* ATCC 14025, *E. durans* ATCC 19432, *E. flavescens* ATCC 49996, *E. hirae* ATCC 8043 and *E. raffinosus* ATCC 49427.

Conjugation experiments. Conjugation was done by filter mating (Trieu-Cuot *et al*., 1988) with minor modifications. A 100 µl sample of mating mixture containing donor strains TUH2-18 or TUH7-15 and the recipient strain *E. faecium* BM4105-RF (Poyart & Trieu-Cuot, 1994) was spread on a 45 µm nitrocellulose membrane filter (Millipore) placed on top of brain heart infusion (BHI) agar. After 18 h incubation at 37 °C, cells were resuspended in 1 ml BHI broth and spread on BHI agar containing 8 µg vancomycin ml^{-1}, 20 µg rifampicin ml^{-1} and 10 µg fucidic acid ml^{-1}.

Preparation of genomic DNA. DNA for genomic digestion was isolated using the QIAGEN Blood & Cell Culture DNA kit. Bacterial DNA for PCR was prepared with a Dynabeads DNA DIRECT Kit (Dynal) as described by Dahl *et al*. (1999) or by isolation of total DNA using the GenomicPrep Cells and Tissue DNA Isolation Kit (Pharmacia Biotech) with modification of the lysis step. The bacterial cells were incubated in fresh lysis solution (10 mM Tris/HCl pH 8·0, 1 mM EDTA, 25%, w/v, sucrose, 10 mg lysozyme ml^{-1} and 0·1 mg RNase A ml^{-1}) at 37 °C for 35 min to achieve optimal lysis.

Table 2. PCR primers used in this study

Amplicon	Primer sequence (5′–3′)	Size of amplicon (bp)	Reference
vanB	CAAAGCTCCGCAGCTTGCATG TGCATCCAAGCACCCGATATAC	484	Dahl *et al*. (1999)
$vanS_B$–$vanY_B$	ATATGCGCTGGAAAACACCTC CCCCAGATTGTTTCATATGCC	309	Dahl *et al*. (1999)
Tn*5382*	GTTCTTATTCCGCAGGTGGTGATT ACGCCATGCTATTTACTTCCGGC	311	Carias *et al*. (1998)
$vanX_B$–ORFC	GATGCCAAGTACGCTACATGGGA TGAGTTGTGGAAGTCGATTAGAG	873	This study
IS*16*	GCCATTGATCTCAGTTAGGAG AAAGTGTTCCAATTATCCGAG	1183	This study
IS*256*	AAAAACATACCCAGGAGGAC GGCTGATGTTTGATTGGGGA	1115	This study
ISEnfa*110*	GGTCTTGACCACTGGCTCACA TATTTGACCGGATTGGTAGGG	642	This study
ISEnfa*200*	GATAAGGATGCTTCAGCTTACA TTTTACCGGCACTGGAGTCTA	647	This study
16S rDNA	TGCATTAGCTAGTTGGTGAGG TCGAATTAAACCACATGCTCC	726	This study
pbp5–Tn*5382**	TCAGCCGATTTGCGACAGGTTATG TGGGGTGGCGGGTATTAGCAGTAT	1079	Carias *et al*. (1998)
pbp5	GCACGGCAAAAATCGAACAGG TGCTCGTTTGGTTGCTGAATCATC	1984	This study

* Primer sequences kindly provided by Louis B. Rice.

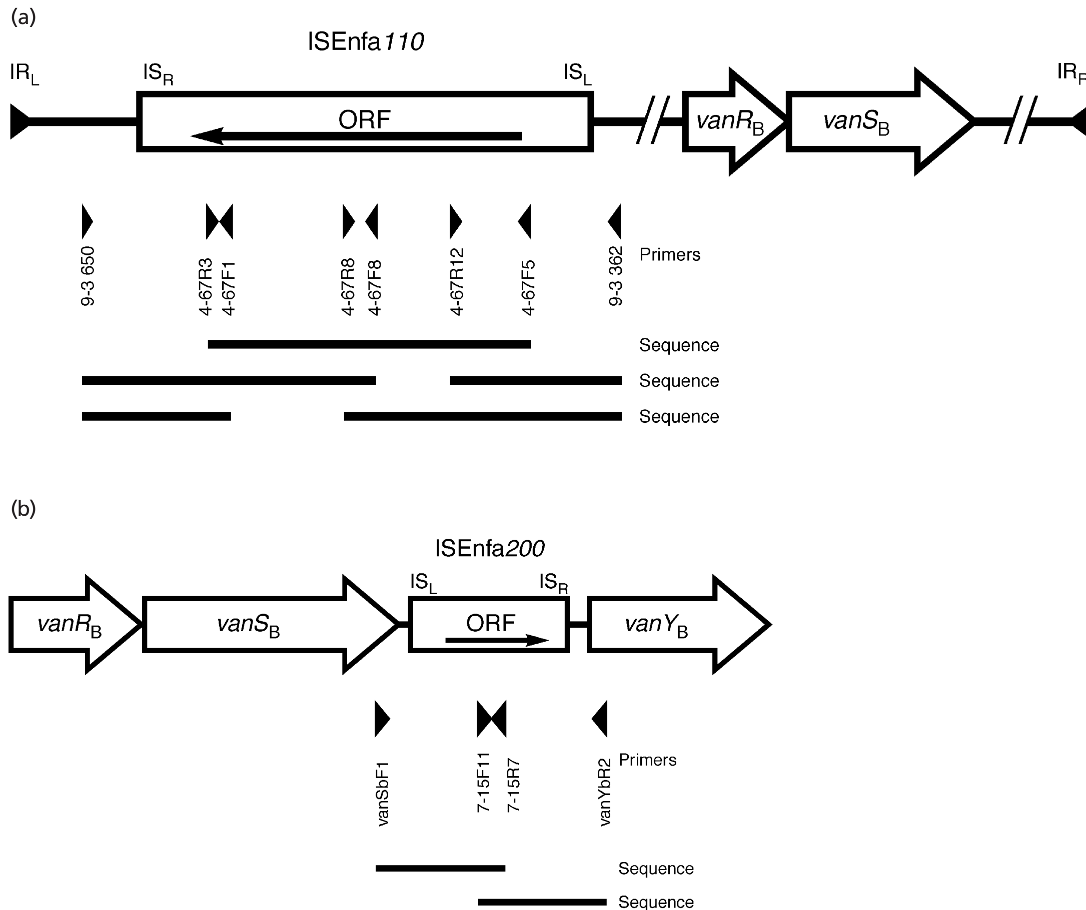

Fig. 1. Schematic representation of (a) Tn*5382* left end of strain TUH4-67 with the ISEnfa*110* element inserted and (b) the *vanS*$_B$–*vanY*$_B$ intergenic region of strain TUH7-15 with insertion ISEnfa*200*. Positions of primers and PCR amplicons used for DNA sequencing of the novel IS elements are indicated in the figure. The open boxes mark ORFs and the arrows indicate the coding strand and direction of transcription. IS ends and terminal inverted repeats of Tn*5382* are indicated. The sequences of the primers are as follows: (a) 9-3650, GTTCTTATTCCGCAGGTGGTGATT; 4-67R3, ACTGGTACACCGGCTCGTCTA; 4-67F1, CTGCAATGTGCTCCAATAGAC; 4-67R8, CACTTCTGGTATTGGGTAGTAAAG; 4-67F8, AGTGGGTGGACTTTGTCGCTA; 4-67R12, ATTGCATTGACTACCGAGACA; 4-67F5, ATCGATGTTTCCAAGGGTAAG; 9-3362, ACGCCATGCTATTTACTTCCGGC; (b) vanSbF1, ATATGCGCTGGAAAACACCTC; 7-15F11, GAAATTCCGCCAAGCATGAGT; 7-15R7, CGCTCGAATATCATTAGTGTACT; vanYbR2, CCCCAGATTGTTTCATATGCC.

DNA transfer and hybridization. Southern transfer of digested genomic DNA to a positively charged nylon membrane (Boehringer Mannheim) was carried out by vacuum blotting (Vacugene XL system, Pharmacia Biotech) according to the manufacturer's instructions. Colony blotting was performed as described by Sambrook *et al.* (1989) with the following modification: the nylon membrane was incubated on a 3 MM paper saturated with fresh lysis solution (10 mM Tris/HCl, 1 mM EDTA, 25%, w/v, sucrose, 10 mg lysozyme ml^{-1}) for 60 min at 37 °C to ensure complete lysis of the enterococci. DNA was fixed to the nylon membrane by UV cross-linking. Probes were labelled using the PCR DIG Probe Synthesis Kit (Boehringer Mannheim) and purified by agarose gel electrophoresis followed by extraction by QIAquick Gel Extraction Kit (QIAGEN). Hybridization was carried out at 68 °C and detection performed using the DIG Luminescent Detection Kit (Boehringer Mannheim). All protocols were performed according to the manufacturers' instructions.

Total DNA from the following bacteria was used as templates for probe synthesis: the *vanB* probe came from *E. faecalis* V583 (Evers *et al.*, 1994), the Tn*5382* and *pbp5* probes from *E. faecium* C68 (Carias *et al.*, 1998), the IS*16* and IS*256* probes from *E. faecalis* BM4281 (Quintiliani & Courvalin, 1996), the ISEnfa*110* probe from TUH4-67, the ISEnfa*200* probe from TUH7-15, and the 16S rDNA probe from *E. faecalis* DS16C2 (Franke & Clewell, 1981). The 16S rRNA genes in *E. faecium* and *E. faecalis* have at least 98% homology.

PCR. PCRs were performed in a GeneAmp PCR System 2400 (Perkin-Elmer) using Perkin-Elmer Standard PCR reaction mix with GeneAmp PCR buffer and *Taq* DNA polymerase. PCR elongation times were adjusted according to the expected size of PCR amplicons and the alignment temperatures were adjusted according to the specific nucleotide sequence of the primers and hence their melting temperatures.

PCR amplicons and primer sequences used in PCR identification, DNA sequencing and probe syntheses are listed in Table 2. Additional primer sequences and amplicons used in the characterization of ISEnfa*110* and ISEnfa*200* are described in Fig. 1(a) and (b), respectively.

DNA sequencing and PFGE. PCR amplicons were directly sequenced on both DNA strands by ABI Prism 377 (Perkin-Elmer), as described by Dahl *et al.* (1999). Agarose plugs containing enterococcal DNA were digested with *Sma*I and analysed by PFGE as described by Dahl *et al.* (1999) with minor modifications. The plugs were incubated with 40 U mutanolysin ml^{-1} in addition to lysozyme to ensure proper lysis of the cells before treatment with 50 µg proteinase K ml^{-1}.

Computer analysis and sequence accession numbers. Editing, initial analysis of the DNA sequences and alignments were performed using the Sequence Navigator (Perkin Elmer) software package. Nucleotide sequences were compared to sequences in the GenBank, EMBL, DDBJ and PDB databases and protein sequences to non-redundant GenBank CDS translations, PDB, SWISS-PROT, SPupdate and PIR by using the BLASTN, BLASTP and BLASTX local alignment search tools (Altschul *et al.*, 1990). Possible ORFs were found using the ORF finder located at the National Center for Biotechnology Information website. Searches for repeats and palindromes were performed using the GCG package. RNAdraw V1.0 was used to determine potential secondary structures. Novel nucleotide accession numbers and previously published GenBank and GenPept sequences mentioned in the text are shown in Table 3.

RESULTS AND DISCUSSION

Characterization of the VanB strain collection and *vanB* typing

The 23 VanB-type strains (Table 1) were characterized by PFGE and DNA sequencing of the *vanB* gene and the $vanS_B$–$vanY_B$ intergenic region as described by Dahl *et al.* (1999). The *vanB* gene cluster of strains C68, D366, 3174, 3332 and 3568 was subtyped as *vanB2* (Table 1). Subtyping of the remaining 18 PFGE unrelated strains (Table 1) has been published previously (Dahl *et al.*, 1999). Altogether, eight strains were of the *vanB1* subtype, 14 of the *vanB2* subtype and one of the *vanB3* subtype. PFGE analyses revealed that TUH7-15, C68, 3174, 3332 and 3568 might be related (see below), whereas the other strains were unrelated according to the criteria for PFGE typing by Tenover *et al.* (1995).

The *vanB2* gene cluster is an integral part of Tn*5382*

A *vanB*-containing 27 kb putative conjugative transposon, Tn*5382*, was recently described in *E. faecium* C68. The presence of Tn*5382* was also documented in 15 strains from the USA as well as in one strain from France (Carias *et al.*, 1998). In this study, genetic linkage of the specific *vanB* subtypes to Tn*5382* was examined. The VanB strain collection was analysed by a Tn*5382*-specific PCR internal to the nonintegrase (left) end of Tn*5382* (Carias *et al.*, 1998), Southern hybridization using Tn*5382* and *vanB* amplicons as probes (Table 2), and DNA sequencing of the $vanX_B$–ORFC intergenic region and flanking coding sequences in Tn*5382* (Fig. 2). Fourteen of the 23 strains (61 %) were Tn*5382* PCR positive (Table 1), which is comparable to the result obtained by Carias *et al.* (1998). All Tn*5382* PCR positive strains were of the *vanB2* subtype (Dahl *et al.*, 1999). *Sma*I-digested total DNA was subjected to PFGE (Fig. 3a) and Southern hybridization (Fig. 3b, c). Co-hybridization of the *vanB* and Tn*5382* probes to the same fragments was detected for all strains ($n = 14$) of the *vanB2* subtype, as illustrated in Fig. 3 for strains TUH2-18 (lane 4) and TUH7-15 (lane 7). Strains of the *vanB1* or *vanB3* subtypes were negative for Tn*5382* by PCR and hybridization (Fig. 3, lanes 2, 3 and 9).

Corresponding analyses were performed on five vancomycin-resistant transconjugants derived from each of TUH2-18 and TUH7-15. All transconjugants were Tn*5382* PCR positive (data not shown). PFGE revealed minor genetic rearrangements in the transconjugants (Fig. 3a), and Southern blot analysis (Fig. 3b, c) showed co-hybridization of *vanB* and Tn*5382* probes to novel fragments consistent with insertion events as illustrated by one transconjugant derived from TUH2-18 (lane 5) and one from TUH7-15 (lane 8). These results indicate cotransfer of the *vanB2* gene cluster and Tn*5382* during conjugation due to genetic linkage. The DNA sequence of the $vanX_B$–ORFC intergenic region and flanking sequences in the *vanB2* strains (Table 3) revealed a 550 bp $vanX_B$–ORFC sequence identical to the corresponding part of the published Tn*5382* sequence (Table 3) except for a 2 bp insertion and a 1 bp substitution. The 301 bp $vanX_B$ sequence upstream of this region revealed sequences almost identical to the corresponding region of the published *vanB1* subtype gene cluster (Table 3) except for 16–18 bp substitutions which probably reflect different $vanX_B$ sequence in *vanB1* and *vanB2* subtypes. Thus, these results confirm that the *vanB2* subtype gene cluster of these strains is an integral part of Tn*5382* as shown in Fig. 2.

The DNA sequence of the *vanB2* ligase gene seems to be less conserved than the *vanB1* ligase gene (Dahl *et al.*, 1999; Patel *et al.*, 1998), indicating that the *vanB2* gene cluster might be older in evolutionary terms or evolve more rapidly due to more efficient mechanisms for horizontal spread. As part of Tn*5382*, the *vanB2* gene cluster may be transferable between cells and within the cell to conjugative chromosomal elements or plasmids, thereby promoting a more efficient spread as indicated by the finding of a wide geographical distribution of the *vanB2* gene cluster. However, molecular epidemiological studies with regard to the relative distribution of *vanB* subtypes have not been performed to test this assumption.

Presence of IS*16* and IS*256*-like in VanB-type VRE strains

The *vanB* gene cluster in *E. faecalis* BM4281 has been reported to be linked to the mobile genetic elements IS*16* and IS*256*-like as a functional composite transposon, Tn*1547*. Hybridization studies of clinical VanB-type VRE isolates revealed that the *vanB* gene cluster was not necessarily linked to IS*16* or IS*256*-like elements (Quintiliani & Courvalin, 1996). The *vanB* gene cluster of Tn*1547* (Quintiliani & Courvalin, 1996) belongs to the *vanB1* subtype (this study, Table 3). In this study the VanB strain collection was examined for prevalence of

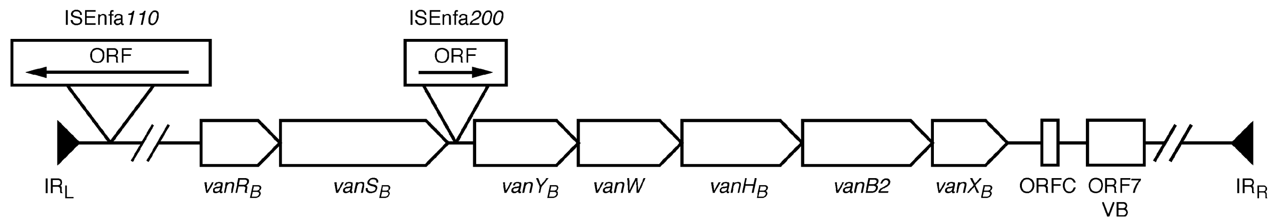

Fig. 2. Map of Tn*5382* showing the localization of the *vanB2* gene cluster, ISEnfa*110* and ISEnfa*200*. The open boxes mark ORFs and the arrows indicate direction of transcription. Terminal inverted repeats of Tn*5382* are indicated.

IS*16*-like and IS*256*-like elements and their genetic linkage to *vanB* gene cluster subtypes.

PCR analyses identified IS*16*-like sequences in 15 of 23 strains (65%) and IS*256*-like sequences in 19 of 23 strains (83%) (data not shown). These observations are consistent with the finding of IS*16*-like sequences in 21 of 32 (66%) and IS*256*-like sequences in 31 of 32 (97%) clinical VanB-type *E. faecium* and *E. faecalis* strains (Quintiliani & Courvalin, 1996), and IS*256*-like sequences in 88 out of 103 (85%) clinical *E. faecium* and *E. faecalis* strains (Rice & Thorisdottir, 1994). PFGE followed by Southern hybridization did not show any universal genetic linkage or co-hybridization of the *vanB* subtypes to IS*16*- and IS*256*-like sequences. Co-hybridization of *vanB* and both IS*16*-like and IS*256*-like sequences was found in only two *vanB2* subtype strains, TUH7-15 and 3174. Co-hybridization of *vanB* and IS*16*-like sequences was found in two different fragments in TUH1-79, and in one fragment in TUH2-18 and 3568. Co-hybridization of *vanB* and IS*256*-like sequences was found in one fragment in V583, TUH7-54 and C68 (data not shown).

Multiple copies of both IS*16*- and IS*256*-like sequences were detected in several strains (data not shown). Our results confirm that IS*16*- and IS*256*-like sequences are common in enterococci, but that these elements are not generally linked to the *vanB1* gene cluster as observed for the composite transposon Tn*1547* in *E. faecalis* BM4281. Multiple copies of these IS elements raise possibilities for IS-based composite transposons and genomic rearrangements. IS256 is capable of forming novel mobile elements in enterococci as illustrated by the detection of Tn*5281* (Hodel-Christian & Murray, 1991), conferring high level resistance to aminoglycosides, and Tn*5384*, encoding macrolide resistance and high level gentamicin resistance (Rice *et al.*, 1995).

Characterization of ISEnfa*110*, a novel member of the IS*110* family

The prevalence of Tn*5382* in the VanB strain collection was determined by a Tn*5382*-specific PCR and hybridization using a 311 bp sequence starting 237 bp downstream of the left inverted repeat of Tn*5382* in a nonintegrase sequence (Carias *et al.*, 1998). Tn*5382* PCR of TUH4-67 DNA showed an enlargement of this region. To identify the exact sequence of this enlargement, the complementary strand of the fragment internal to the nonintegrase (left) end of Tn*5382* (Carias *et al.*, 1998) was sequenced in strain C68 (Table 3). Sequence analysis of the same region in TUH4-67 revealed a 1611 bp insertion with a putative ORF of 1209 bp transcribed in the opposite direction to the integrase gene of Tn*5382* (Figs 1a and 2, Table 3).

No extensive DNA homology was found with known insertion sequences by the BLAST program. However, the deduced 402 amino acid sequence of the ORF within this insert showed low levels (21–24%) of identity to about 380 amino acids from putative transposases of IS*110*, IS*2112*, IS*1110*, IS*902* and IS*901* (Table 3). An alignment of the amino acid sequences of this ORF to transposases of IS elements belonging to the IS*110* family (Mahillon & Chandler, 1998), clearly demonstrated the relatedness between this IS element, hereafter designated ISEnfa*110*, and IS*110* family members. The transposases of the IS*110* family show some regions of identity within the N- and C-terminals. Two regions highly conserved in transposases of the IS*110* family (Hernandez Perez *et al.*, 1994) were present in the ISEnfa*110* transposase.

Like most of the elements in this family, ISEnfa*110* had no terminal inverted repeats. Target sequences of IS*110* elements exhibit similarities to the circle junction of the element. Recent *in vivo* studies of IS*110* elements have shown DNA fragments with abutted IS ends only when the transposase gene is intact. These observations are consistent with the model for transposition of IS*110* elements by circularization and site-specific recombination (Mahillon & Chandler, 1998). A 2 bp sequence (5′-CT-3′) was directly repeated at each end of ISEnfa*110* after insertion into the integration site. According to the IS110 family criteria these elements do not create target duplications, but a match often occurs between one end of the IS and the target site sequence (Hernandez Perez *et al.*, 1994). Thus, the CT sequence is probably present both in the attachment site of the circular form of the element and in the target site. ISEnfa*110* showed additional sequence homology between the putative circle junction and the site of integration (Fig. 4).

The ISEnfa*110* ORF was preceded by a putative RBS (GT$\underline{\text{A}}$TT$\underline{\text{GGAGG}}$N$_7$ATG) that displays some complementarity (underlined) to the 3′ extremity of the *Bacillus subtilis* 16S rRNA. The upstream region also revealed

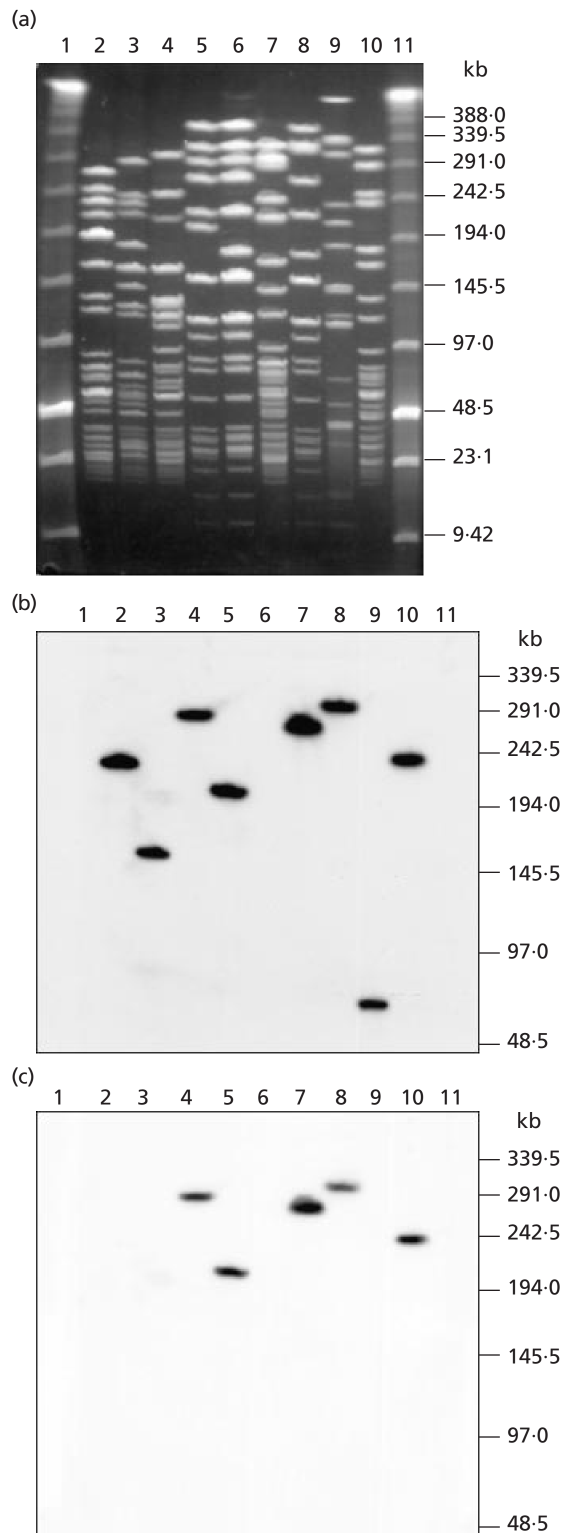

Fig. 3. (a) PFGE of *Sma*I-digested total DNA of VanB-type strains, transconjugants and the recipient strain used in conjugation experiments, and (b, c) corresponding Southern hybridization with a *vanB* probe (b) and Tn*5382* probe (c). Lanes 1 and 11, low-range PFG marker (New England Biolabs); lane 2, TUH4-64 (*vanB1*); lane 3, TUH4-65 (*vanB1*); lane 4, TUH2-18 (*vanB2*) donor; lane 5, BM2-18 transconjugant; lane 6, *E. faecium* BM4105-RF recipient; lane 7, TUH7-15 (*vanB2*) donor; lane 8, BM7-15 transconjugant; lane 9, VRE45 (*vanB3*); lane 10, C68 (positive control *vanB* and Tn*5382*).

two putative promoter regions with some homology to the −35 (TTGACA) and −10 sequences (TATAAT) observed for *Escherichia coli* and *B. subtilis* promoters (Moran *et al.*, 1982). A −35 region (CTGCCA) located in the target sequence was separated by 19 bp from a −10 sequence (TACAAT) starting at nucleotide 5 of the IS element (Fig. 4). The 19 bp spacer region is not optimal for transcription initiation. A stronger promoter might assemble upon circularization of ISEnfa*110* with an optimal spacer region of 17 bp and a putative −35 region with the DNA sequence TTGACT (Fig. 4). The second putative promoter had a −35 (TTCCCT) and a −10 sequence (TACAAT) separated by a spacer region of 17 bp (Fig. 4). This promoter was located between nucleotides 47 to 75 of the IS element.

Characterization of ISEnfa*200*: a novel member of the IS*200* family

The *vanB* subtyping strategy revealed an enlargement of the $vanS_B$–$vanY_B$ intergenic region in strain TUH7-15. Sequence analysis identified a 789 bp insert region (Table 3) with a 471 bp ORF oriented in the same direction as the *vanB* gene cluster (Figs 1b and 2). Searching with the BLAST program in different protein databases revealed sequence similarity to the transposases of the IS*200* family, which was strengthened by the preserved structural features of this family (see below). The IS element was designated ISEnfa*200*.

ISEnfa*200* encoded a putative 156 amino acid protein with 70 % identity to the first 147 amino acids of the putative 151 amino acid transposase of IS*1469* from *Clostridium perfringens* (Brynestad *et al.*, 1997; Table 3). Amino acid identities above 60 % were also detected with putative IS*200* transposases of *Salmonella typhimurium*, *S. typhi*, *Yersinia pestis*, *Escherichia coli* and *Actinobacillus actinomycetemcomitans* (Table 3). The IS*1469* ORF had sequence homology greater than 60 % to ORFs in other IS*200*-like elements (Brynestad *et al.*, 1997). In contrast, the ISEnfa*200* DNA sequence only showed a significant degree of homology to other IS*200* sequences in smaller regions. Thus, ISEnfa*200* had a higher level of conservation at the amino acid level compared to the DNA level, suggesting that the coding capacity of the ORF was selectively maintained.

ISEnfa*200* possessed general features of IS*200* elements. The ISEnfa*200* element (787 bp) was comparable in size to IS*1469* (789 bp) and *Salmonella* spp. IS*200* (708 bp). The element lacked terminal inverted repeats and had one ORF preceded by several sequences capable of forming hairpin structures (see below). The sequence data indicated that the ISEnfa*200* insertion had generated a 2 bp target duplication (5′-TG-3′). However, it is also possible that these 2 bp were part of the

Table 3. GenBank and GenPept sequence accession numbers

Sequence description	GenBank accession no.	Comments
vanX$_B$–ORFC intergenic region	AF203404–AF203417	*vanB2* subtype strains; this study
Tn*5382* right end	AF063010	Strain C68
vanB1 subtype gene cluster	U35369	Strain V583
vanS$_B$–*vanY*$_B$ intergenic region	AF201896	Strain BM4281; this study
Tn*5382* nonintegrase left end	AF175739	Strain C68; this study
ISEnfa*110* and target sequence	AF173641	Strain TUH4-67; this study
ISEnfa*200* and target sequence	AF125554	Strain TUH7-15; this study
pbp5 and Tn*5382* left end	AF117609	
IS designation	**GenPept accession no.**	**Bacterial host**
IS*110*	P19780	*Streptomyces coelicolor*
IS*2112*	AAC15836	*Rhodococcus rhodochrous*
IS*1110*	S49546	*Mycobacterium avium*
IS*902*	S24062	*M. avium*
IS*901*	S16893	*M. avium*
IS*1469*	CAA50690	*C. perfringens*
IS*200*	AAC36995	*S. typhimurium*
IS*200*	CAA71045	*S. typhi*
IS*200*	AAA96353	*Y. pestis*
IS*200*	I41311	*E. coli*
IS*200*	BAA10957	*A. actinomycetemcomitans*

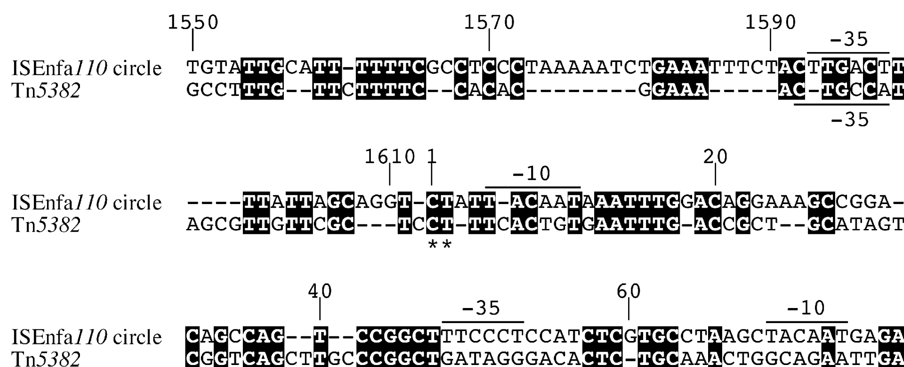

Fig. 4. Sequence similarity between the region surrounding the crossover point of the potential ISEnfa*110* circular form and the integration site in Tn*5382* of strain TUH4-67. Homologies are indicated by black shading. The ISEnfa*110* circle and target sequence have been aligned by the addition of pads (-) to the sequences. Potential promoters (−35 and −10 boxes) upstream of the ISEnfa*110* transposase gene in both the putative circular and the integrated form of the IS element are indicated. Nucleotide numbers corresponding to the linear form of ISEnfa*110* are indicated. The crossover point (CT) is marked by **.

ISEnfa200 element or sequences flanking the donor element at the previous target site. ISEnfa*200* was inserted in an A+T-rich sequence (68% A+T bp in the neighbouring 22 bp) in a non-coding region upstream of the promoter region in the *vanS*$_B$–*vanY*$_B$ intergenic sequence. Thus, the insertion does not seem to affect expression of the proteins encoded by the *vanB* gene cluster.

Sequence analysis showed that the primary transcript may form a potential RNA factor independent transcription terminator at the left end of ISEnfa*200* [nucleotides 2–16, ΔG^0 −10·9 kcal mol^{-1} (−45·6 kJ mol^{-1}), followed by 4 U residues]. A second potential RNA stem–loop structure [nucleotides 119–174, ΔG^0 −22·0 kcal mol^{-1} (−92·0 kJ mol^{-1})] that might occlude a putative RBS was also present. IS*200* in

S. typhimurium, *S. abortusovis* and *Vibrio cholerae* showed high sequence conservation in the stem–loop regions (Beuzón & Casadesús, 1997). These features still seemed to be preserved in ISEnfa*200*, although no significant DNA sequence homology was found to other IS*200* elements in these regions. The RBS (AC-AAAGGAGGN$_7$ATG) upstream of the ISEnfa*200* ORF was located at a standard distance from the start codon and displays a strong complementarity (underlined) to the 3′ extremity of the *B. subtilis* 16S rRNA (UCUUUCCUCC) (Moran *et al.*, 1982). A putative promoter with −35 (CTTACA) and −10 (TATAAT) regions separated by a spacer region of 16 bp was also located upstream of the putative start codon.

Rarity of ISEnfa*110* and ISEnfa*200* in enterococci

The presence of ISEnfa*110*- and ISEnfa*200*-like elements was examined by colony hybridization of 181 *Enterococcus* strains of geographically diverse origins using internal PCR fragments (Table 2) of the IS elements as probes. All colonies hybridized with the positive control 16S rDNA probe (data not shown). Only TUH4-67 was positive for ISEnfa*110*, whereas five strains (TUH7-15, C68, 3174, 3332 and 3568), including the original ISEnfa*200* strain, were positive for ISEnfa*200* (data not shown).

Southern hybridization of total DNA showed hybridization of the ISEnfa*110* probe to an approximately 10 kb *Dra*I restriction fragment of strain TUH4-67 (data not shown), indicating the presence of a single copy of ISEnfa*110*. ISEnfa*110* has a G+C content of 51 mol%, which is higher than the G+C range of enterococci (34–42 mol%) but in the same range as the target site in Tn*5382* (55 mol%). ISEnfa*200* hybridization with total DNA revealed a positive 1·5 kb *Dra*I restriction fragment (data not shown) in the five ISEnfa*200*-positive strains, indicating the presence of a single ISEnfa*200* element. The low prevalence of these novel IS elements might reflect recent acquisition or low transposition frequencies of these elements in enterococci. ISEnfa*200* has a G+C content of 41 mol%, which is typical of that of *Enterococcus* species (34–42 mol%). The low G+C range of ISEnfa*200* reflects to a large extent the preferential use of A and T residues at the third position of codons in the putative ORF.

IS*200* elements are mainly found in Gram-negative bacteria, except for IS*1469* from *C. perfringens* and ISEnfa*200* in *E. faecium*. ISEnfa*200* had the highest degree of homology to IS*1469* (see above). The presence of closely related IS elements in *E. faecium* and *C. perfringens* might indicate interspecies gene flux in a common environmental niche. The ISEnfa*200* element was inserted in the *vanB* gene cluster, which has been shown to be transferable among enterococcal strains (Carias *et al.*, 1998; Quintiliani & Courvalin, 1996; Woodford *et al.*, 1995). Spread of ISEnfa*200* will probably be a result of *vanB* gene cluster transfer rather than transposition of the IS element itself since IS*200* elements have extremely low transposition frequencies.

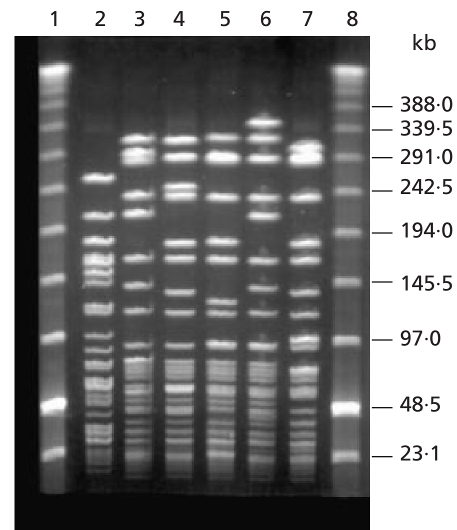

Fig. 5. PFGE of *Sma*I-digested total DNA of a *vanB2* subtype strain from France (D366) and five possibly related *vanB2* subtype strains from the USA. Lanes 1 and 8, low-range PFG marker (New England Biolabs); lane 2, D366; lane 3, TUH7-15; lane 4, C68; lane 5, 3174; lane 6, 3332; lane 7, 3568.

A widespread USA *vanB E. faecium* strain with a chromosomal Tn*5382*–*vanB2*–ISEnfa*200* element genetically linked to *pbp5*?

Recent reports have shown that the putative conjugative transposon Tn*5382* can be located downstream of a *pbp5* gene encoding ampicillin resistance in clonally distinct *E. faecium* strains from different parts of the USA (Carias *et al.*, 1998; Hanrahan *et al.*, 1998). The two resistance determinants were co-transferred during conjugation as parts of a larger genetic element. In this study strains of different geographical origins were analysed for the occurrence of Tn*5382* downstream of *pbp5*, to examine if these resistance elements are linked. The *vanB2* positive strains ($n = 14$) were examined for *pbp5*–Tn*5382* linkage by PCR-amplifying a 1079 bp region between *pbp5* and Tn*5382* (Table 3). Five USA strains (TUH7-15, C68, 3174, 3332 and 3568) revealed a *pbp5*–Tn*5382* amplicon of the expected size (data not shown). PFGE analysis of *Sma*I-digested DNA (Fig. 5) suggests genetic relatedness between these strains. The ISEnfa*200* integration site in the $vanS_B$–$vanY_B$ intergenic region (Table 3) was identical in all five strains. Southern blot analyses showed co-hybridization of the *pbp5* probe, Tn*5382* probe and ISEnfa*200* probe (data not shown), confirming the linkage between *pbp5* and the Tn*5382*–*vanB2*–ISEnfa*200* element.

PFGE has so far been the most accurate method in molecular epidemiological studies of enterococci. Additional molecular markers might be useful. Transposition of IS*200* occurs at extremely low frequency (Casadesus & Roth, 1989), which would make ISEnfa*200* a very stable epidemiological marker. The finding of ISEnfa*200* in the *vanB2* gene cluster of Tn*5382* in these geographically unrelated USA strains could be

due to horizontal transfer of a large chromosomal element among enterococcal strains as suggested by Carias *et al*. (1998). However, the genetic relatedness between strains as shown by PFGE analysis suggests a possible clonal spread in the USA of a VRE strain harbouring a Tn*5382*–*vanB2*–ISEnfa*200* element linked to a *pbp5* gene conferring ampicillin resistance.

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