

## Tn1546 is part of a larger plasmid-encoded genetic unit horizontally disseminated among clonal *Enterococcus faecium* lineages

H. Sletvold<sup>1</sup>, P. J. Johnsen<sup>1</sup>, O.-G. Wikmark<sup>1</sup>, G. S. Simonsen<sup>2-4</sup>, A. Sundsfjord<sup>2,3</sup> and K. M. Nielsen<sup>1,5\*</sup>

<sup>1</sup>Department of Pharmacy, Faculty of Medicine, University of Tromsø, 9037 Tromsø, Norway; <sup>2</sup>Department of Medical Biology, Faculty of Medicine, University of Tromsø, 9037 Tromsø, Norway; <sup>3</sup>Department of Microbiology and Infection Control, University Hospital of North Norway, 9038 Tromsø, Norway; <sup>4</sup>Norwegian Institute of Public Health, Nydalen, 0403 Oslo, Norway; <sup>5</sup>Genøk-Center for Biosafety, Research Park, 9294 Tromsø, Norway

\*Corresponding author. Tel: +47-77-646165; Fax: +47-77-646151; E-mail: kaare.nielsen@uit.no

Received 3 March 2010; returned 23 March 2010; revised 4 May 2010; accepted 21 May 2010

**Objectives:** To determine the genetic composition of the first VanA-type plasmid (pIP816) reported, which was isolated from a clinical *Enterococcus faecium* (BM4147) strain in France in 1986, and to reveal the genetic units responsible for the dissemination of the *vanA* gene cluster by comparisons with current, published and additionally generated *vanA*-spanning plasmid sequences obtained from a heterogeneous *E. faecium* strain collection ( $n=28$ ).

**Methods:** Plasmid sequences were produced by shotgun sequencing using ABI dye chemistry and primer walking, and were subsequently annotated. Comparative sequence analysis of the *vanA* region was done with published plasmids, with a partial *vanA* plasmid (pVEF4) reported here and to >140 kb of sequence obtained from a collection of *vanA*-harbouring plasmid fragments.

**Results:** Bioinformatic analyses revealed that pIP816 from 1986 and contemporary *vanA* plasmids shared a conserved genetic fragment of 25 kb, spanning the 10.85 kb *vanA* cluster encoded by Tn1546, and that the larger unit is present in both clinical and animal complexes of *E. faecium*. A new group II intron in pVEF4 was characterized.

**Conclusions:** Comparative DNA analyses suggest that Tn1546 disseminates in and between clonal complexes of *E. faecium* as part of a larger genetic unit, possibly as a composite transposon flanked by IS1216 elements.

**Keywords:** glycopeptide drug resistance, mobile elements, horizontal gene transfer, *vanA* plasmids, poultry

### Introduction

The mammalian gut-dwelling genus *Enterococcus*, particularly *Enterococcus faecium* and *Enterococcus faecalis*, have rapidly emerged as troublesome nosocomial pathogens. This has primarily occurred due to a combination of favourable antibiotic selective pressures with respect to intrinsic resistances,<sup>1</sup> as well as an inherent propensity for inter- and intraspecies gene transfer<sup>2</sup> (for an excellent review, see Willems and Bonten<sup>3</sup>). Plasmids and transposons have been shown to play key roles in the acquisition and dissemination of drug resistance in the genus *Enterococcus*.<sup>4-12</sup> The emergence and rapid spread of glycopeptide-resistant *E. faecium* (GREF) has been a particular challenge, as there are few remaining options for antimicrobial treatment.<sup>1</sup> The first clinical GREF was discovered in France in 1986 and harboured the VanA-type plasmid pIP816.<sup>13</sup> Subsequently, nosocomial GREF has been reported worldwide, with

an increasing prevalence in the USA in particular,<sup>14</sup> but also in European countries in recent years (<http://www.rivm.nl/earss/>). In addition to the limited options for GREF treatment, there is also a concern for further horizontal transfer of glycopeptide resistance determinants into more pathogenic Gram-positive species, such as *Staphylococcus aureus* and *Clostridium difficile*.<sup>15</sup> This is underscored by nine cases of VanA-type vancomycin-resistant *S. aureus* isolated in the USA since 2002, and the evidence for an enterococcal origin as well as plasmid-mediated transfer is compelling.<sup>16</sup> More sequence information on VanA-type plasmids from different reservoirs is necessary to clarify their role and function in the maintenance and dissemination of glycopeptide resistance determinants in *Enterococcus* spp.

The use of the glycopeptide avoparcin as an animal growth promoter in European countries provided the opportunity for a build-up of a community reservoir of GREF.<sup>17,18</sup> Avoparcin resistance mediates cross-resistance to vancomycin, a clinically

important antibiotic,<sup>19</sup> and avoparcin was thus prohibited for further use in animal husbandry. However, diverse GREF strains have persisted on farms several years after the ban.<sup>20–22</sup> The persistent GREF populations carry *vanA* plasmids harbouring the non-conjugative transposon Tn1546.<sup>21,23</sup> Tn1546 is also frequently located on plasmids in GREF strains isolated from hospitalized patients and healthy volunteers in the community.<sup>24–26</sup> The presence of conserved Tn1546 elements in genomically heterogeneous *E. faecium* isolates from various environments suggests the spread of resistance by horizontal gene transfer.<sup>27</sup> We have previously reported the presence of a common 372 bp DNA stretch immediately flanking Tn1546 in a polyclonal *E. faecium* population.<sup>21,22</sup> It was hypothesized that the *vanA* gene cluster (Tn1546) disseminates as a larger genetic unit than the transposon, but smaller than a plasmid, due to the heterogeneous plasmid sizes and restriction patterns observed in the strain collection examined.

The aim of this study was to increase the knowledge on enterococcal plasmid population genetics and dynamics, with an emphasis on *E. faecium vanA* plasmids. We present: (i) the complete sequence of the Tn1546-containing pIP816,<sup>13</sup> first isolated in France 1986; (ii) a partial sequence of the Tn1546-containing *E. faecium* pVEF4, isolated from a Norwegian poultry farm previously exposed to avoparcin; and (iii) 28 Tn1546-containing genetic fragments amplified and sequenced from diverse *E. faecium* strains. Comparative analyses suggest that a genetic unit larger than the 10.85 kbp Tn1546 has facilitated the horizontal spread of plasmid-encoded glycopeptide resistance between different *E. faecium* clonal lineages. Sequence data indicate horizontal dissemination as a composite transposon. Lastly, a novel enterococcal group II intron was identified within the composite transposon of several of the *vanA* plasmids and is functionally analysed here. Group II introns are ribozymes that catalyse their own excision and ligation of flanking exon sequences.<sup>28</sup>

## Materials and methods

### Bacterial strains and plasmids

The bacterial strains and plasmids, and their relevant characteristics are given in Table 1. All strains were grown at 37°C using brain heart infusion (BHI) agar or broth (Fluka BioChemika). The VanA-positive strains of *E. faecium* were grown in media supplemented with 10 mg/L vancomycin (Sigma).

### DNA sequencing and analyses of pIP816 and pVEF4

Plasmid DNA was isolated by alkaline lysis, as previously described.<sup>21,29</sup> The fragmentation of plasmid pIP816 and subsequent cloning in *Escherichia coli* was done with the TOPO shotgun subcloning kit, as described by the manufacturer (Invitrogen). Plasmid DNA was purified prior to sequencing with the Perfectprep Plasmid 96 Vac system (Eppendorf). Custom primers (Sigma–Genosys) were used in PCRs for gap closure. The sequencing was done using ABI BigDye terminator chemistry (Perkin–Elmer Applied Biosystems Inc.) with ABI3130XL automated sequencers. Assembly of the sequence data was done using the Staden package<sup>30</sup> and Phrap (<http://www.phrap.org/>). The initial plasmid sequence of pIP816 was presented as a poster at the International Symposium on Plasmid Biology, 2006.<sup>31</sup> The draft contig sequences of pVEF4 were provided by Macrogen, Korea, using BigDye chemistry and with a sequence depth of >14× coverage. Further extensive primer walk and gap closure experiments were done; however, we did not succeed in plasmid

closure. Artemis was used to annotate the nucleotide sequence of pIP816 and pVEF4.<sup>32</sup> GLIMMER was used to predict coding sequences (CDSs)<sup>33</sup> that were checked manually by correlation scores of the open reading frames with ≥50 amino acids. Sequence similarity searches by FASTA and BLASTP refined the predictions.<sup>34,35</sup>

Five complete *E. faecium vanA* plasmids were published in the nucleotide databases EMBL, GenBank and DDBJ as of May 2010 (Table 2). These plasmids as well as the partial pVEF4 sequence were analysed according to genetic composition and synteny using the Artemis Comparison Tool.<sup>32</sup>

### PCR amplification and DNA sequencing of fragments of *vanA* plasmids

The DNA regions flanking the *vanA* gene cluster were also determined for 28 different *vanA* plasmids isolated from genomically diverse *E. faecium* strains of human or animal origin (Table 1). Isolated plasmid DNA was treated with PlasmidSafe DNase (Epicentre), and subsequently digested separately with PstI, EcoRI and BamHI according to the manufacturer's protocol (New England BioLabs), and separated by agarose gel electrophoresis. Undigested plasmid DNA was used in PCR assays, where *rTth* DNA polymerase (GeneAmp XL PCR Kit, Applied Biosystems) and JumpStart *Taq* DNA Polymerase (JumpStart ReadyMix *Taq*, Sigma) were used according to the manufacturers' protocols to amplify DNA fragments up- and downstream of Tn1546 using the primers specified in Table 3. Four PCR assays with the following primer combinations were used: PCF1 and PCF2 (7.1 kb amplicon); PCF1 and PCF3 (7.6 kb amplicon); PCF1 and PCF4 (6.0 kb amplicon); and PCF5 and PCF6 (1.2 kb amplicon). The PCR cycling parameters used in the amplifications of long fragments were initial denaturation at 94°C for 3 min followed by 25 cycles of 94°C for 1 min and 64°C for 11 min, and a final extension at 72°C for 10 min. For the JumpStart *Taq* PCRs, the cycling parameters were denaturation at 94°C for 2 min followed by 30 cycles of denaturation (94°C, 30 s), annealing (58°C, 30 s) and extension (72°C, 1 min 30 s), and a final extension at 72°C for 5 min. PCR products were analysed by agarose gel electrophoresis. Positive PCR products were confirmed by sequencing, as described above. Custom sequencing primers were used for primer walking when necessary (primers not given).

### Group II intron characterization

Intron RNA secondary structure predictions were performed by the mfold server v. 3.2<sup>36,37</sup> and classification was based on group II intron consensus structures.<sup>38</sup> The broader distribution of the group II intron, and its presence in the topoisomerase I gene, was investigated among the 28 *vanA* plasmids isolated from the *E. faecium* strain collection (Table 1) by PCR with the primers giiF/giiR3 and ip3F/giiR7 (Table 3) and JumpStart *Taq* DNA Polymerase, as described above. RNA extraction and subsequent RT-PCR were used to confirm intron splicing. From overnight cultures of *E. faecium* 399/S99/A7 and *E. faecium* 399/F98/A4, 10 µL of each culture was transferred to 10 mL fresh BHI broth and further cultured to a density of ~1 × 10<sup>8</sup> cells. The cell cultures were treated with RNAlater (Ambion), according to the supplier's protocol, followed by total RNA extraction by QIAGEN RNeasy Mini kit and DNaseI treatment with QIAGEN RNase-Free DNase (QIAGEN). RNA extracts were verified free of DNA contamination by using 1 µg of total RNA as template in a PCR with the DyNAzyme II DNA polymerase (Finnzymes). cDNA syntheses were done using the SuperScript III enzyme (Invitrogen) on ~1 µg of total RNA as template and, in other respects, according to protocol. RT-PCRs were carried out with the primers given in Table 3. The RT-PCR cycling parameters used were denaturation at 94°C for 2 min followed by 30 PCR cycles of 94°C for 45 s, 58°C for 30 s and 72°C for 30 s to 1.5 min (depending on the length of the product), and a final extension at 72°C for 5 min. The products were analysed by agarose gel electrophoresis

**Table 1.** The *vanA*-containing *E. faecium* strains used in this study and their characteristics

Strain	Geographical origin	Sample source	Epidemiology <sup>a</sup>	MLST/CC <sup>b</sup>	PFGE	Reference/source <sup>c</sup>
399/F99/H8	Norway	human	cs	195/CC9	7	21
399/F99/A9	Norway	animal	cs	241/CC9	10A	21
399/S99/A7	Norway	animal	cs	ND	11	21
BM4147	France	human	ci	95/CC22	ND	13
399/F98/A4	Norway	animal	cs	ND	5	21
BM4147-1	France	—	—	95/CC22	—	13
399/F98/A1	Norway	animal	cs	ND	9	21
399/F99/A8	Norway	animal	cs	9/CC9	11	21
64/F98/H2	Norway	human	cs	242/ND	14	21
356/98/H	Norway	human	cs	ND	ND	22
31/F01/H	Norway	human	cs	ND	ND	22
399/F99/A10	Norway	animal	cs	310/CC9	5A	21
64/F99/A6	Norway	animal	cs	146/ND	15	21
64/F99/H6	Norway	human	cs	246/CC9	17	21
58/F01/H	Norway	human	cs	ND	ND	22
399/F98/H2	Norway	human	cs	8/CC9	2	21
399/S99/H6	Norway	human	cs	2/CC1	6	21
64/F98/H1	Norway	human	cs	48/CC9	13	21
64/F98/A3	Norway	animal	cs	8/CC9	20	21
K8-61	Norway	human	ci	ND	ND	K-res <sup>c</sup>
64/F98/A2	Norway	animal	cs	195/CC9	19	21
TUH32-76	Portugal	human	ci	132/CC17	ND	57
32/F02/H	Norway	human	cs	ND	ND	22
K9-72	Norway	human	ci	ND	ND	K-res <sup>c</sup>
64/S99/A5	Norway	animal	cs	ND	21	21
TUH2-8	Sweden	human	ci	ND	ND	K-res <sup>c</sup>
TUH32-64	UK	human	ho	18/CC17	ND	57
TUH32-71	Netherlands	human	ho	16/CC17	ND	57
TUH32-72	USA	human	ho	17/CC17	ND	57
TUH32-74	USA	human	ho	20/ND	ND	57
TUH32-77	Portugal	human	ci	125/ND	ND	57
TUH32-78	Netherlands	human	ci	21/CC9	ND	57
TUH32-79	Netherlands	human	hs	5/CC5	ND	57
TUH43-7	Norway	human	cs	ND	ND	22

MLST, multilocus sequence type; PFGE, pulsed-field gel electrophoresis; CC, clonal complex; ND, not determined.

<sup>a</sup>ci, clinical isolate; cs, community survey; ho, hospital outbreak; hs, hospital survey.

<sup>b</sup>CC, clonal complex, extracted from references.<sup>22,45,48,49,56</sup>

<sup>c</sup>K-res, Reference Centre for Detection of Antimicrobial Resistance, Department of Microbiology and Infection Control, University Hospital of North Norway.

and stained with ethidium bromide. To confirm the splicing boundaries, the PCR fragments of ligated exons were sequenced.

## Results

### General features of the *VanA*-type plasmids pIP816 and pVEF4

The complete sequence of the plasmid pIP816 was determined and a partial sequence of pVEF4 is presented. pIP816 was extracted from the first glycopeptide-resistant *E. faecium* reported (strain BM4147) and was sampled from a patient with leukaemia in France in 1986.<sup>13</sup> pVEF4 was isolated from an *E. faecium* strain (399/F98/A4) sampled from poultry in 1998

on a Norwegian poultry farm where avoparcin was previously used as a growth promoter.<sup>21</sup> pIP816 (EMBL accession no. AM932524) is 34 616 bp in size, and has 36 CDSs and an average G+C content of 36.9% (Figure 1 and Table 4). The partial sequence of pVEF4 (EMBL accession no. FN424376) is 44 443 bp in length, and has a G+C content of 36.2% and 46 CDSs (Figure 1 and Table 5). Repeated attempts were made to achieve gap closure, but we did not succeed. We do, however, include the 44.4 kb partial, single sequence in this study, because it provides valuable additional information for the analysis of Tn1546 junctions (see below) as well as on the composition of the overall *E. faecium* plasmid pool. Similarity searches identified 31 and 39 CDSs with known or predicted functions in pIP816 and in pVEF4, respectively. pIP816 and

**Table 2.** The completely sequenced *vanA* plasmids of enterococci as of May 2010

Plasmid	Host strain	Size (kbp)	Reference	Accession number
pIP816	<i>E. faecium</i> BM4147	34.6	This study	AM296544
pVEF1	<i>E. faecium</i> 399/F99/H8	39.6	11	AM410096
pVEF2	<i>E. faecium</i> 399/F99/A9	39.6	11	AM410096
pVEF3	<i>E. faecium</i> 399/S99/A7	63.1	58	AM931300
pHT $\beta^a$	<i>E. faecium</i> FH	63.7	59	AB183714

<sup>a</sup>The *vanA* plasmid (pHT $\beta$ ) isolated from an *E. faecium* strain in Japan was included in the presented analysis. However, no DNA sequence identity was found, except from the presence of Tn1546.

**Table 3.** PCR and RT-PCR primers used in the study

Primer	Sequence (5'–3')	Binding site or reference
PCF1	AGGGATTCGTCAGGAAAATAGG	pVEF1 nt 19439–19460
PCF2	AGCGTGATGGTTTCAATCC	pVEF1 nt 20705–20685
PCF3	TCTCTACGATTTTCTCATCCACA	pVEF1 nt 26932–26909
PCF4	TCGTGACAATCGGAACAAAAC	pIP816 nt 27553–27575
PCF5	AGTAACAAAGAAAGCCCAATTATCA	pVEF1 nt 8748–8724
PCF6	ACTTTTAGTTGGCTTGACTGAAC	pVEF1 nt 2742–2765
giiF	TGGAATGATAGGGTAACG	Intron (5' end, forward)
giiF5	TGGTTGCGAGACTTAGGAAAAC	Intron (3' end, forward)
giiR3	AYACGGCGTCCATCAA	Intron (3' end, reverse)
giiR7	TAAGGTATAAGGTGGGCGTTTG	<i>topo</i> (3' end, reverse)
giiR8	TGTTCTACCCGACACATTCTCG	Intron (5' end, reverse)
ip3F	AGACCCACTATTACAGATG	<i>topo</i> (5' end, forward)
Ent1	TACTGACAAACCATTATGATG	60
Ent2	AACTTCGTACCAACGCGAAC	60

pVEF4 both encode a pseudo-streptomycin resistance gene (*str*) that the Tn1546 transposon has transposed into, as well as genes involved in plasmid partitioning, a resolvase, truncated transposases and a hypothetical protein (Figure 1). A topoisomerase is also present on both plasmids, but in pVEF4 a group II intron with an intron-encoding protein (CDS19) was identified inserted into the *topo* gene (see below). The entire Tn1546 transposon, with the 38 bp inverted repeats together with the 5 bp direct repeats (5'-GTCCT-3') of the Tn1546 target site in *str*, is conserved in both plasmids.

The plasmids pIP816 and pVEF4 contain putative CDSs involved in their own replication and maintenance. The replication of pIP816 is probably ensured through the replication protein RepE (CDS17) described in the Inc18 plasmid pAM $\beta$ 1 from *E. faecalis*.<sup>39</sup> The origin of replication (*oriR*), copy number repressor (CopF), and hypothetical proteins ORFC and ORFD of pAM $\beta$ 1 are also 100% conserved in pIP816 (Table 4). Thus, pIP816 is predicted to replicate by a DNA polymerase I-dependent  $\theta$  mechanism, such as pAM $\beta$ 1.<sup>40,41</sup> pVEF4 encodes a replication protein (CDS24) previously reported in pVEF1 and pVEF2,<sup>11</sup> and an *oriR* identical to *oriR* of pAM $\beta$ 1 is found

downstream of the Rep protein. Recently, Jensen *et al.*<sup>42</sup> presented a classification system for plasmids from enterococci and other Gram-positive bacteria based on the conserved areas of the replication initiation genes (*rep*). According to this classification system, pIP816 groups into *rep* families 1 and 2, whereas pVEF4 is a member of group 1 (but also harbours an additional *rep* gene with limited sequence similarity to group 11). The presence of more than one *rep* gene may indicate increased host range, and groups 1 and 2 contain strains from *Enterococcus*, *Staphylococcus* and *Streptococcus*.

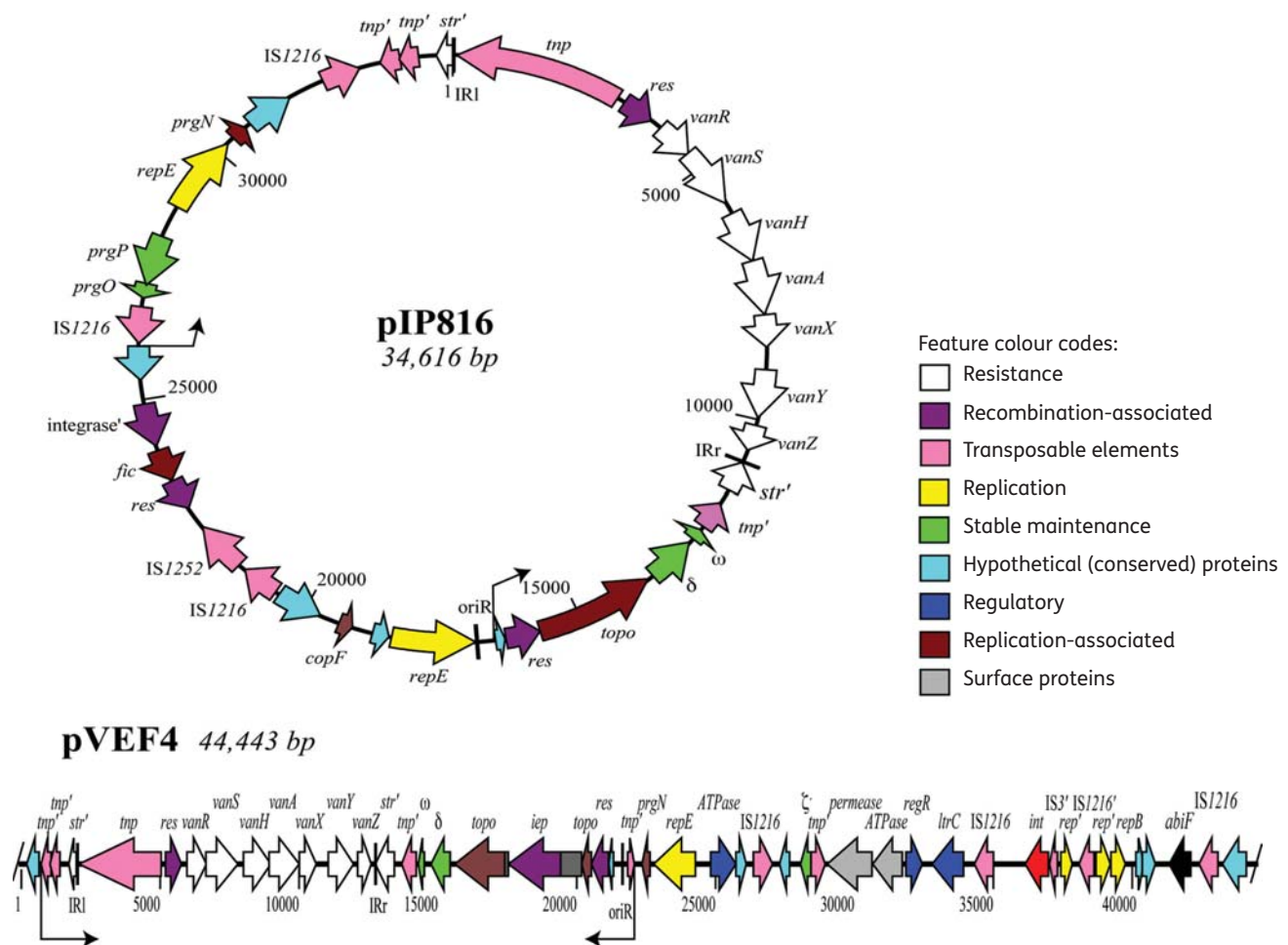
Genes involved in site-specific recombination (resolvases) and active partition processes of plasmids are found on pIP816 and pVEF4, putatively contributing to stable inheritance. Thus, the segregational stability of pIP816 is putatively under the control of the active partitioning systems delta-omega ( $\delta$ - $\omega$ ), as described for plasmid pSM19035 from *Streptococcus pyogenes*,<sup>43</sup> and/or the *prgP-prgO* gene products as described for pCF10 from *E. faecalis*.<sup>44</sup> Complete and fragmented insertion sequences (ISs) and transposases constitute 21% ( $n=8$ ) and 18% ( $n=11$ ) of the DNA sequence of pIP816 and the partial pVEF4, respectively. Inverted repeats of the IS6 (5'-ggttctgtgcaagtttaactactactcaaa-3') or IS30 families (5'-cgccgattgtaaaattaagctagacaata-3') are found along with most of the IS elements.

### Comparative analysis of *vanA* plasmids

A genetic element (~18 kb) including the non-conjugative Tn1546 was found conserved between the *vanA* plasmids pIP816, pVEF1, pVEF2, pVEF3 and partial pVEF4. An additional 7 kb element, flanked by two IS1216, was present immediately upstream in pIP816 (inverted), pVEF1, pVEF2 and pVEF3 (Figure 2). Thus, a common conserved genetic fragment of ~25 kb was present in three *vanA* plasmids with a separate evolutionary history over >13 years.

The presence of the conserved 25 kb *vanA*-containing fragment was further examined among a collection of 28 heterogeneous *vanA* plasmids, as determined by restriction fragment length polymorphism analyses (data not shown), from genomically different *E. faecium* strains of diverse epidemiology and geographical origin (Table 1). The complete 25 kb fragment was only present in the completely sequenced pVEF1, pVEF2 and pVEF3. These three plasmids are present in genomically diverse strains, as previously shown by PFGE.<sup>21</sup> In pVEF1 and pVEF2, the 25 kb fragment is flanked by IS1216. Parts of the elements flanking the *vanA* gene cluster were also found on 15 additional plasmids. Of these, 12 were extracted from *E. faecium* strains sampled from Norwegian poultry farms (four poultry strains and eight poultry farmer strains at six different timepoints from 1998–2002) and three extracted from clinical strains (two from Norway and one from Portugal). Available multilocus sequence typing (MLST) data revealed that the different strains with intact or parts of the common Tn1546-containing fragment group into clonal complexes 1, 9, 17 and 22 (Table 1).

DNA sequence alignment of the amplified plasmid fragments demonstrates high sequence identity, but a deletion of the  $\delta$  gene was found for eight plasmids (Figure 2). The 5' end of Tn1546 is inverted in pIP816 compared with pVEF1, pVEF2 and pVEF3, and both gene order patterns are found on other plasmids (Figure 2).



**Figure 1.** Genetic map of pIP816 and pVEF4. Coding regions are represented by arrows indicating the direction of transcription and are coloured according to their predicted functions. The inverted repeats (IR) of the Tn1546 transposon and the predicted origin of replication (*oriR*) of the plasmids are given as black boxes. The group II intron *En.fm.I2* of pVEF4 is shown as dark grey boxes flanking the intron-encoding protein. Thin arrows indicate the 25 kb larger genetic unit. Truncated CDSs are indicated with a prime symbol (e.g. *tnp'*).

### pVEF4 encodes the group II intron *En.fm.I2*

The annotation of the partial pVEF4 from *E. faecium* 399/F98/A4 identified a 2770 bp group II intron inserted into the topoisomerase I (*topo*) gene and it was named *En.fm.I2* according to the mobile group II intron database nomenclature.<sup>45</sup> The predicted secondary RNA structure (Figure 3a) shows that *En.fm.I2* displays structural features similar to introns belonging to the bacterial class B of group II introns. *En.fm.I2* carries a putative intron-encoded protein (IEP) (CDS19, Table 5) located in domain IV, with domains responsible for the maturase function, reverse transcriptase function and endonuclease function (Figure 3b).

To test if the *En.fm.I2* is a functional ribozyme and splices *in vivo*, total RNA was extracted, and RT-PCR was run with exon-exon-specific primers on DNA of pVEF4 and on pVEF3 as a negative control. *En.fm.I2* intron splicing was established (Figure 4, lane 1) and the ligated exons were confirmed by sequencing (data not shown). Specific primers targeting the intron-exon junctions (both 5' and 3') were used in RT-PCR reactions and confirmed the presence of unspliced intron in pVEF4, but not in

the negative control (pVEF3) (Figure 4). Primers amplifying the 2'-5' junction allowed the identification of the splicing pathway of *En.fm.I2* (Figure 4, lanes 7 and 8). DNA sequencing of the generated PCR product showed that the *En.fm.I2* forms a lariat structure *in vivo* with the 5' end docking into the bulged A in domain VI.

### Distribution of the intron *En.fm.I2* in *vanA* plasmids

Because the *topo* gene and *En.fm.I2* of pVEF4 were found adjacent to Tn1546, the wider presence of *En.fm.I2* was determined among the 28 heterogeneous *vanA* plasmids (Figure 2). In total, 15 *En.fm.I2*-encoding plasmids were identified, for which 14 of the *En.fm.I2* introns were found localized in the *topo* gene. The intron-positive PCR products were confirmed by sequencing (data not shown). A highly conserved DNA sequence identity over the entire intron, including the IEP, was found. Only two of the *En.fm.I2* introns (from *E. faecium* strains 31/F01/H and TUH32-79) displayed non-synonymous substitutions in the IEP protein (Figure 3b).

**Table 4.** Coding sequences (CDSs) of the circular *vanA* plasmid pIP816

CDS <sup>a</sup>	Protein length (amino acids)	Database match	Amino acid identity (%)
1	<i>tnp</i> Tn1546	pIP816 Tn1546 transposase (Q06238)	100
2	<i>res</i> Tn1546	pIP816 resolvase (Q06237)	100
3	<i>vanR</i>	pIP816 VanR protein (Q06239)	100
4	<i>vanS</i>	pIP816 VanS protein (Q06240)	100
5	<i>vanH</i>	pIP816 VanH protein (Q05709)	100
6	<i>vanA</i>	pIP816 VanA protein (P25051)	100
7	<i>vanX</i>	pIP816 VanX protein (Q06241)	100
8	<i>vanY</i>	pIP816 VanY protein (P37711)	100
9	<i>vanZ</i>	pIP816 VanZ protein (Q06242)	100
10	<i>str'</i>	N-terminal part of pS194 streptomycin resistance protein, <i>Staphylococcus aureus</i> (P12055)	95
11	<i>tnp'</i>	pLI100 transposase, <i>Listeria innocua</i> (CAC42047)	98
12	$\omega$	pSM19035 transcriptional repressor $\omega$ , <i>Streptococcus pyogenes</i> (YP_232757)	100
13	$\delta$	pIlo8 $\delta$ protein, <i>Oenococcus oeni</i> (CAD70616) / pSM19035 active partitioning $\delta$ protein, <i>S. pyogenes</i> (YP_232765)	100/98
14	<i>topo</i>	pAM $\beta$ 1 type 1 topoisomerase, <i>Enterococcus faecalis</i> (AAC38606)	97
15	<i>resIP</i>	pGB354 resolvase, <i>Streptococcus agalactiae</i> (AAB48454)	100
16	<i>hcp</i>	pRE25 <i>orf7</i> hypothetical conserved protein, <i>E. faecalis</i> (CAC29163)	100
17	<i>repE</i>	pAM $\beta$ 1 RepE replication protein, <i>E. faecalis</i> (Q52249)	100
18	<i>hcp</i>	pAM $\beta$ 1 <i>orfD</i> , <i>E. faecalis</i> (Q52248)	100
19	<i>copF</i>	pAM $\beta$ 1 copy number repressor, CopF, <i>E. faecalis</i> (Q52247)	100
20	<i>hcp</i>	pAM $\beta$ 1 <i>orfC</i> , <i>E. faecalis</i> (Q52246)	100
21	IS1216	IS1216 on pRE25, pRUM, pTEF1, pTEF3, pUW786 and pE418 (e.g. CAC29206)	100
22	IS1252	pHKK701 IS1252 transposase (AAB42161)	99
23	<i>res</i>	pRE25 <i>orf53</i> resolvase, <i>E. faecalis</i> (Q9AKZ9)	100
24	<i>fic</i>	pRE25 <i>orf52</i> cell filament protein, Fic, <i>E. faecalis</i> (Q9AL00)	100
25	<i>Integrase'</i>	Integrase, <i>Enterococcus faecium</i> DO (Q3Y1H6)	81
26	<i>hcp'</i>	Plasmid2 hypothetical protein, <i>Nitrosomonas eutropha</i> C71 (Q3N6Z6)	39
27	IS1216	IS1216, <i>E. faecium</i> (Q9KI43) / IS1216 on pRE25, pRUM, pTEF1, pTEF3, pUW786, pUW1965 and pE418 (e.g. CAC29206)	100/99
28	<i>prgO</i>	pRE25 PrgO protein, <i>E. faecalis</i> (Q9AKZ5)	100
29	<i>prgP</i>	pRE25 PrgP protein, <i>E. faecalis</i> (Q9AKZ4)	100
30	<i>repE</i>	pRE25 ORF1, putative replication protein, <i>E. faecalis</i> (Q9AL28)	100
31	<i>prgN</i>	pRE25 PrgN protein, <i>E. faecalis</i> (Q9AL27)	100
32	<i>hcp</i>	Hypothetical protein of <i>E. faecium</i> DO (EAN10371) / pRE25 hypothetical protein ORF4, <i>E. faecalis</i> (Q9AL25)	100/99
33	IS1216	IS1216 on pRE25, pRUM, pTEF1, pTEF3, pUW786 and pE418 (e.g. CAC29206)	100
34	<i>tnp'</i>	pLI100 pLI0071 protein, putative transposase of <i>L. innocua</i> (Q925W6)	85
35	<i>tnp'</i>	pLI100 pLI0020 protein, putative transposase of <i>L. innocua</i> (Q926N5)	87
36	<i>str'</i>	C-terminal part of pS194 streptomycin resistance protein, <i>S. aureus</i> (P12055)	95

<sup>a</sup>Prime indicates truncated CDS; *hcp*, hypothetical conserved protein.

## Discussion

We present the complete sequence of pIP816, the first *vanA* plasmid isolated in France 1986, as well as a partial, annotated sequence of the fourth plasmid isolated from a single Norwegian poultry farm, pVEF4. The comparative analyses with other completely sequenced *vanA* plasmids reveal that pVEF1–pVEF4 share a common genetic element of ~18 kb that spans the entire Tn1546 transposon, a topoisomerase I (*topo*) gene and the genes encoding the active partitioning system  $\delta$ - $\omega$  of plasmid pSM19035<sup>43</sup> upstream of the *vanA* gene cluster. Three shared CDSs are located downstream of Tn1546.

Surprisingly, an identical (>99% identity at the nucleotide level) 18 kb fragment is present in pIP816. When the inverted fragment located upstream is included, the size of the common fragment present in pIP816, pVEF1, pVEF2 and pVEF3 is ~25 kb. The observation that the same plasmid-borne DNA fragment (harbouring Tn1546) was found on a Norwegian poultry farm 13 years after the original isolation of pIP816 in a clinical isolate in France<sup>13</sup> suggests either successful clonal spread or horizontal transfer between *E. faecium* strains of different origins. As determined by Werner and colleagues,<sup>46</sup> the pIP816 host BM4147 belongs to the MLST clonal complex (CC) 22, one of three CCs considered to be host-specific for humans

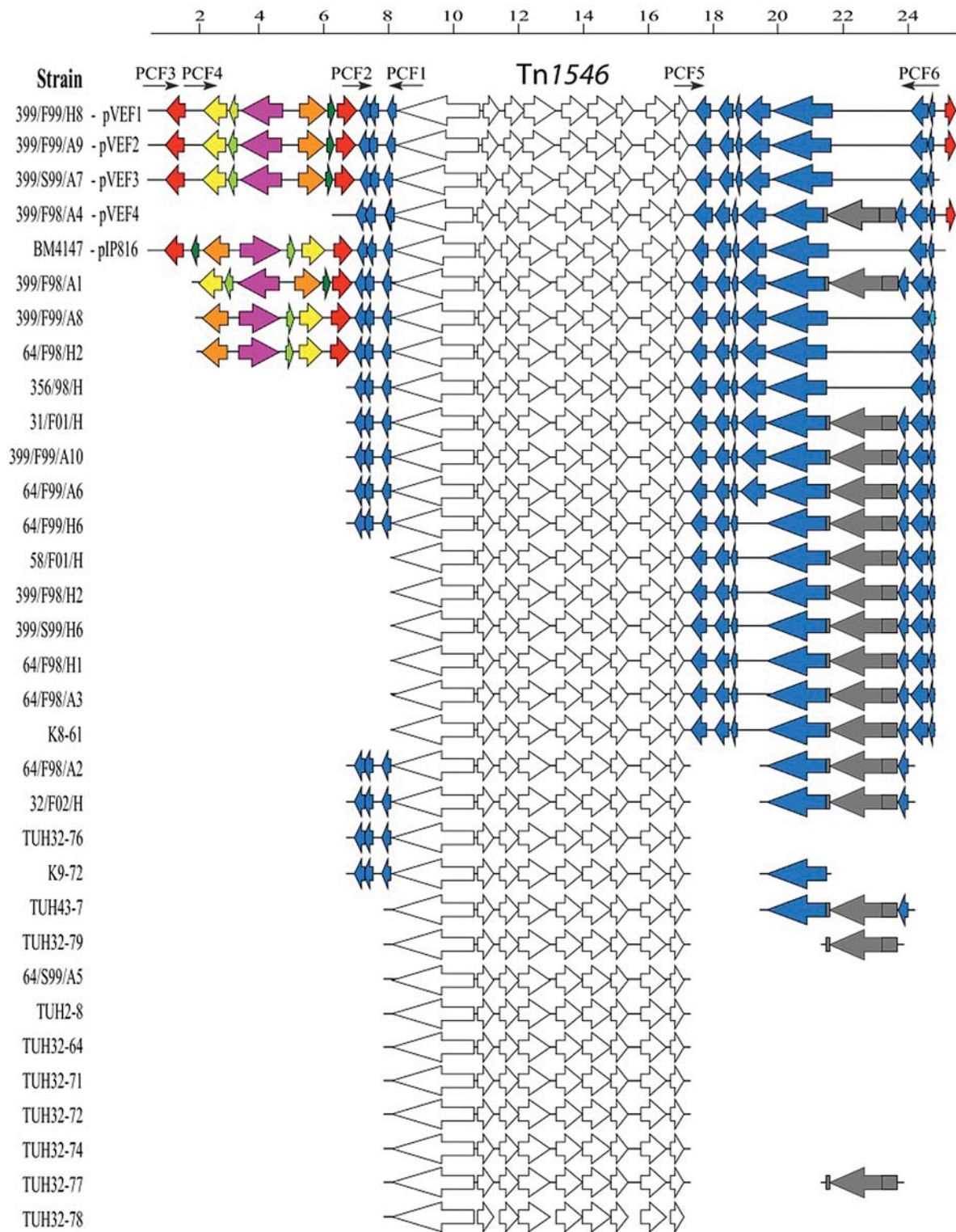
**Table 5.** Coding sequences (CDSs) of the *vanA* plasmid pVEF4 (partial)

CDS <sup>a</sup>	Protein length (amino acids)	Database match (accession number)	Amino acid identity (%)	
1	<i>hp</i>	135	—	
2	<i>tnp'</i>	118	pVEF2 truncated transposase, C-terminal part (CAL90948)	100
3	<i>tnp'</i>	114	pVEF1/pVEF2 truncated transposase, N-terminal part (CAL36541/CAL90947)	100
4	<i>str'</i>	94	pS194 streptomycin resistance protein, <i>Staphylococcus aureus</i> (P12055)	95
5	<i>tnp</i> Tn1546	988	pIP816 Tn1546 transposase (Q06238)	100
6	<i>res</i> Tn1546	191	pIP816 resolvase (Q06237)	100
7	<i>vanR</i>	231	pIP816 VanR protein (Q06239)	100
8	<i>vans</i>	384	pIP816 VanS protein (Q06240)	100
9	<i>vanH</i>	322	pIP816 VanH protein (Q05709)	100
10	<i>vanA</i>	343	pIP816 VanA protein (P25051)	100
11	<i>vanX</i>	202	pIP816 VanX protein (Q06241)	100
12	<i>vanY</i>	303	pIP816 VanY protein (P37711)	100
13	<i>vanZ</i>	161	pIP816 VanZ protein (Q06242)	100
14	<i>str'</i>	188	pS194 streptomycin resistance protein, <i>S. aureus</i> (P12055)	95
15	<i>tnp'</i>	176	pVEF1/pVEF2 truncated transposase, C-terminal part (CAL36529/CAL90935)	100
16	$\omega$	71	pVEF1/pVEF2 putative transcriptional repressor $\omega$ (CAL36528/CAL90934)	100
17	$\delta$	298	pVEF1/pVEF2 putative $\delta$ protein (CAL36527/CAL90933)	100
18	<i>topo</i>	715	pVEF1/pVEF2 putative topoisomerase I (CAL36526/CAL90932)	100
19	<i>iep</i>	628	pBc10987 group II reverse transcriptase, <i>Bacillus cereus</i> ATCC 10987	59
20	<i>res</i>	205	pVEF1/pVEF2 putative resolvase (CAL36525/CAL90931)	100
21	<i>hcp</i>	56	pRE25 and pVEF1/pVEF2 hypothetical conserved protein (Q9AL24, CAL36524/CAL90930)	100
22	IS1216'	82	IS1216 on pRE25, pRUM, pTEF1, pTEF3, pUW786 and pE418 (e.g. CAC29206)	97
23	<i>prgN</i>	95	pVEF1/pVEF2 putative PrgN protein (CAL36522/CAL90928)	100
24	<i>repE</i>	499	pVEF1/pVEF2 putative replication protein (CAL36521/CAL90927)	100
25	<i>ATPase</i>	303	pVEF1/pVEF2 putative ATPase (CAL36563/CAL90969)	100
26	<i>hcp</i>	123	pVEF1/pVEF2 hypothetical protein (CAL36562/CAL09068)	100
27	IS1216	228	IS1216 on pRE25, pRUM, pTEF1, pTEF3, pUW786 and pE418 (e.g. CAC29206)	100
28	<i>hcp</i>	121	pIP501 <i>orf7</i> , <i>Streptococcus agalactiae</i> (Q7AYQ0)	100
29	$\zeta'$	84	pVEF1/pVEF2 $\zeta$ toxin (CAL36553/CAL90959)	95
30	<i>hcp</i>	196	pVEF1/pVEF2 hypothetical protein (CAL36558/CAL909064)	99
31	<i>permease</i>	537	Putative tetracycline resistance transmembrane protein, <i>Streptococcus pyogenes</i> MGAS10750 (YP_603196)	69
32	<i>ATPase</i>	293	Putative tetracycline resistance ATP-binding protein, <i>S. pyogenes</i> MGAS10750 (YP_603195)	85
33	<i>regR</i>	198	pVEF1/pVEF2 putative regulatory protein, TetR family (CAL36555/CAL90961)	97
34	<i>ltrC</i>	374	pMRC01 low temperature requirement C protein LtrC, <i>Lactococcus lactis</i> (AAC56005)	58
35	IS1216	228	IS1216 on pRE25, pRUM, pTEF1, pTEF3, pUW786 and pE418 (e.g. CAC29206)	100
36	<i>int</i>	278	Putative integrase, catalytic region, <i>Enterococcus faecium</i> DO (EAN09812)	99
37	IS3/IS911	96	Putative IS3/IS911, <i>E. faecium</i> DO (EAN09811)	100
38	<i>repA'</i>	137	pEF418 putative replication protein, <i>Enterococcus faecalis</i> (AAL05545)	98
39	IS1216'	179	IS1216 on pRE25, pRUM, pTEF1, pTEF3, pUW786 and pE418 (e.g. CAC29206)	100
40	<i>rep'</i>	172	pEFR putative replication protein, <i>E. faecium</i> (Q8KSS2)	66
41	<i>repB</i>	174	pB82 replication protein RepB, <i>E. faecium</i> (A0JBS1)	49
42	<i>hcp</i>	93	Hypothetical conserved protein, <i>E. faecium</i> DO (EAN10162)	60
43	<i>hcp</i>	138	pEF1 <i>orf33</i> hypothetical conserved protein, <i>E. faecium</i> 6T1a (A3QN12)	55
44	<i>abiF</i>	258	pNP40 abortive infection bacteriophage resistance protein, <i>L. lactis</i> DRC3 (AAB52386)	53
45	IS1216	228	IS1216 on pRE25, pRUM, pTEF1, pTEF3, pUW786 and pE418 (e.g. CAC29206)	100
46	<i>hp</i>	293	Hypothetical protein	—

<sup>a</sup>Prime indicates truncated CDS; *hcp*, hypothetical conserved protein; *hp*, hypothetical protein.

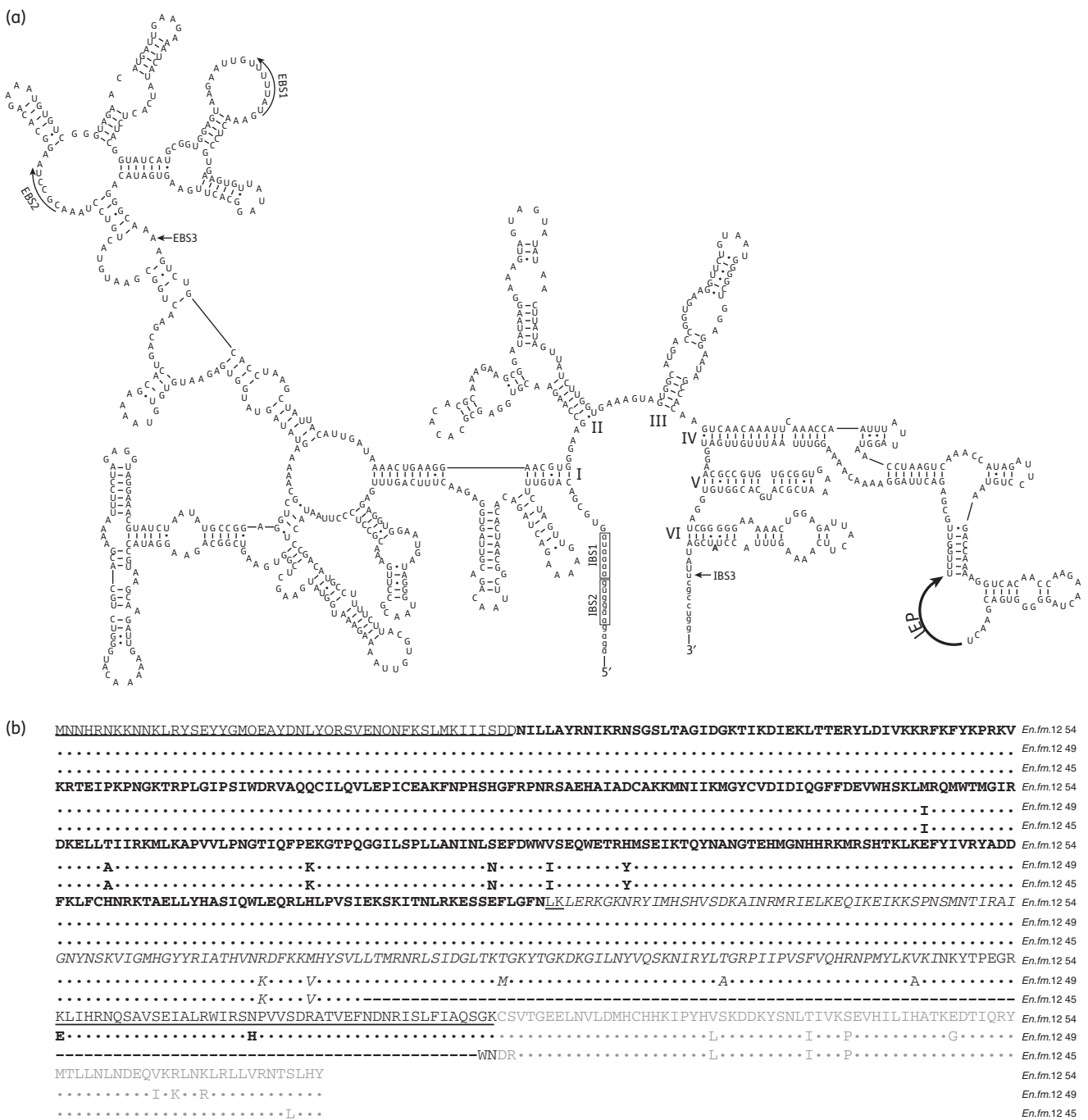
(CC17, CC22 and CC94).<sup>47–49</sup> In contrast, most of the strains isolated from the Norwegian poultry farms (of both poultry and farmer origin) belong to CC9, considered to be host-specific for poultry (including the hosts of pVEF1 and pVEF2).<sup>47,48</sup> This

observation is consistent with the suggested horizontal transfer of a mobile genetic unit larger than Tn1546 between different clonal complexes of *E. faecium*, although the direction and frequency of transfer is uncertain.



**Figure 2.** Gene organization of Tn1546 and flanking areas in *vanA* plasmids from genomically different *E. faecium* strains of human or animal origin. Identical coding regions are colour-coded to highlight similarities in the Tn1546 flanking regions. White, Tn1546; red, IS1216; dark grey, intron *En.fm.12* and *iep*. Note that the ~7 kbp region flanked by IS1216 (red arrows) in pVEF1–pVEF3 is inverted in pIP816, and that similar organization was found in plasmids from two GREF from two Norwegian poultry farms (strains 399/F99/A8 and 64/F98/A1). The top line indicates the size of the aligned Tn1546 flanking regions, with positional marks in kbp.

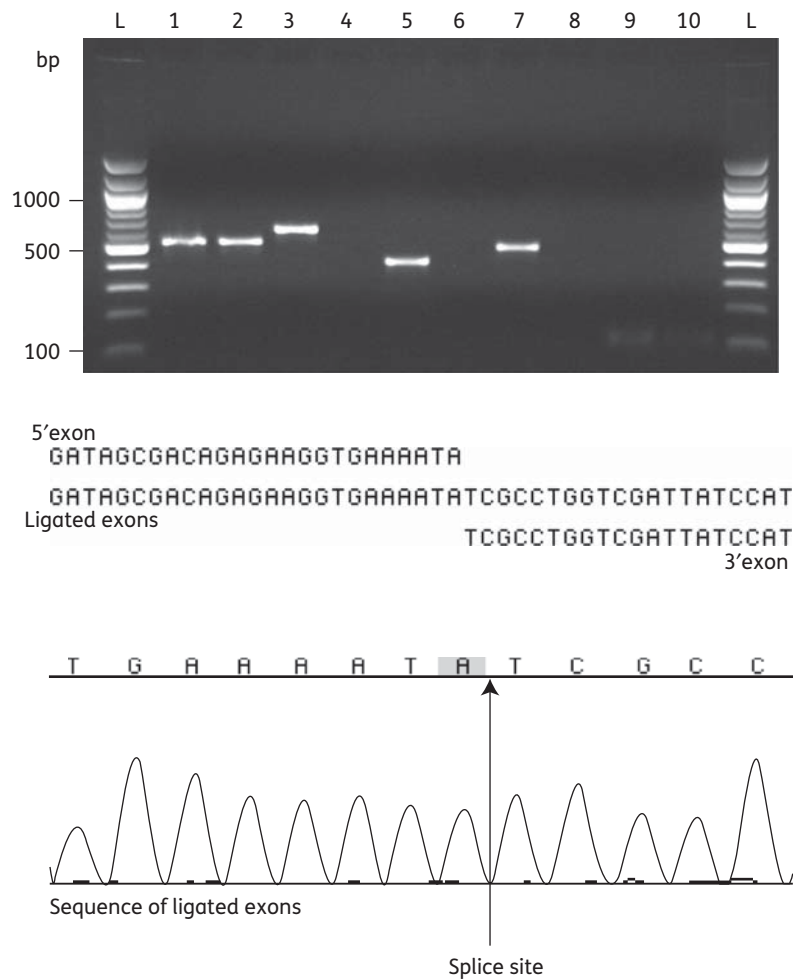




**Figure 3.** Structural features of *En.fm.12* and its intron-encoded protein (IEP). (a) Predicted secondary RNA structure of *En.fm.12*. Intron nucleotides are written in capital letters and exon sequences are written in lowercase letters. Roman numerals denote the domains I–VI. The IEP is found in domain IV. Intron-binding sites (IBSs) 1 and 2 along with the exon-binding sites (EBSs) 1 and 2 are marked by arrows and boxes, respectively. IBS/EBS3 is a single nucleotide interaction and denoted by pointing arrows. The bulged A (branch site) is located in domain VI and shown in bold. (b) The putative IEP displays a reverse transcriptase (RT) domain (bold letters), a maturase (X) domain (italics) and an endonuclease (En) domain (grey). All introns analysed, except two, had identical amino acid composition to *En.fm.12* of pVEF4 (no. 54, top). The non-synonymous substitutions in *En.fm.12* from *E. faecium* strains 31/F01/H (no. 49) and TUH32-79 (no. 45) are shown in the alignment (identical amino acids are represented by a dot; a dash indicates gaps or substitutions).

Tn1546 was found inserted into a streptomycin resistance gene and conserved direct repeats were found in all *vanA* plasmids studied. The majority of these host strains were from

Norwegian poultry farms previously exposed to avoparcin. Available MLST typing data<sup>22,46,47,50</sup> showed that the different MLST types clustered into CC9. These data further extend previous



**Figure 4.** RT-PCR analyses of the topoisomerase, intron, intron splicing products and enterococcal elongation factor. RT-PCR products from pVEF4 and pVEF3 are given on alternate lanes 1–10. PCR products are shown as follows: *topo* mRNA without intron (lanes 1 and 2, primer pair ip3F/giiR7); 5' intron–exon junction (lanes 3 and 4, primer pair ip3F/giiR8); 3' intron–exon junction (lanes 5 and 6, primer pair giiF5/giiR7); intron lariat structure (lanes 7 and 8, primer pair giiF5/giiR8); and positive RT-PCR control (lanes 9 and 10, primer pair Ent1/Ent2). Ladder (lanes L), 100 bp DNA molecular size marker from New England Biolabs. The sequence data of the ligated exon with the indicated splice site is shown in the lower half of the figure.

reports of the successful spread of a common plasmid-mediated genetic element surrounding Tn1546 on Norwegian poultry farms.<sup>21,22</sup> Our observation is also consistent with the original pIP816 characterization with respect to insertion junctions,<sup>23</sup> and a recent Tn1546 junction fragment analysis of *E. faecium* strains from the UK, Denmark, the Netherlands and Norway,<sup>51</sup> presenting evidence of the geographical distribution of two different Tn1546–plasmid insertion junctions. Garcia-Migura *et al.*<sup>51</sup> hypothesize that a common genetic element has spread across different clonal lines within the European broiler sector. The data presented here shed light on the composition and size of this element. Moreover, our data also suggest genetic interactions between *E. faecium* in animal reservoirs and CC17, the only *E. faecium* clinical high-risk CC.<sup>47,49</sup> Strain TUH32-76, a clinical CC17 GREF isolate from Portugal, displays Tn1546 insertion in the streptomycin resistance gene and two immediately adjacent CDSs are linked to the 5' end of the *vanA* gene cluster.

Tn1546 belongs to the non-conjugative class II transposable elements of the Tn3 family<sup>52</sup> and transfers intracellularly by replicative transposition to diverse sites.<sup>23</sup> Thus, the expected transfer by replicative transposition is inconsistent with the genetic patterns observed flanking the Tn1546 transposon reported here and elsewhere.<sup>51</sup> Our data, as well as previous experimental results,<sup>23</sup> do not support the hypothesis that the streptomycin resistance gene represents a hotspot for Tn1546 integration.

A detailed look at the plasmid sequences analysed here reveals that IS1216 is present in one or both flanking regions of the larger glycopeptide resistance-encoding region (of 18–25 kb), suggesting movement of Tn1546 as part of a composite transposon. Composite transposons are well known to mobilize antibiotic resistance genes in enterococci, e.g. Tn5281 flanked by IS256, Tn1547 flanked by IS16- and IS256-like elements, and Tn5385 flanked by IS1216.<sup>8,9,53</sup> Composite transposition enabled by the IS1216 elements flanking the *vanA* operon has

also previously been described.<sup>54</sup> The authors showed that IS1216 elements facilitated the transfer of Tn1546 from a non-conjugative plasmid to a pheromone-responsive conjugative plasmid present in the same enterococcal cell, with subsequent intercellular transfer of the *vanA* gene cluster.

The broader comparative analysis of the genetic composition and gene order (synteny) of pIP816 and the pVEF-type *vanA* plasmids reveals multiple copies of plasmid replication and maintenance genes interspersed with several copies of IS elements. The mosaic plasmid structures indicate frequent formations of plasmid hybrids, recombination and transposition events. Interestingly, no known conjugal transfer genes have been found in any of the completely sequenced *vanA* plasmids from Norwegian poultry farms. This, however, does not mean that the plasmids or parts of them are constrained to a single strain. Plasmid co-transfer by the conjugative transposon Tn916 has been reported in enterococci.<sup>55</sup> Conjugative mobilization of enterococcal plasmids has also been reported.<sup>5,10</sup>

The sequence comparison of the *E. faecium vanA* plasmids enabled the discovery of the novel group II intron *En.fm.I2* inserted into the *topo* gene of pVEF4. Mobile genetic group II intron elements are commonly found in bacteria, although they are not well characterized in enterococci.<sup>45</sup> The intron *En.fm.I2* belongs to the group II bacterial class B introns, has an IEP that displays features of a multifunctional protein that might enable its mobility and follows the standard group II intron splicing pathway.<sup>28</sup> Bacterial mobile group II introns are generally site-specific on insertion and rapidly spread horizontally.<sup>56</sup> However, *vanA* plasmids encoding intron-free *topo* genes were detected in *E. faecium* strains (64/F98/H2 and 64/F98/H1) of human origin (one farmer) sampled at one poultry farm on the same occasion, where both plasmids are *topo*-encoding but only one is invaded by *En.fm.I2*.

In conclusion, comparative analysis of the original *vanA* plasmid pIP816 with the recently sequenced pVEF-type plasmids revealed a conserved genetic fragment (including Tn1546) of 18–25 kb. Taken together, the data show that VanA-type glycopeptide resistance is present in different clonal complexes of *E. faecium* and suggests that glycopeptide resistance can be disseminated through IS1216-facilitated composite transposition.

## Funding

This work was in part supported by the Research Council of Norway, the European Commission (QLK2-CT-2002-00843 'ARTRADI' and LSHE-CT-2007-037410 'ACE') and the Medical Research Foundation, North-Norway.

## Transparency declarations

None to declare.

## References

- Murray BE. Vancomycin-resistant enterococcal infections. *N Engl J Med* 2000; **342**: 710–21.
- Paulsen IT, Banerjee L, Myers GS et al. Role of mobile DNA in the evolution of vancomycin-resistant *Enterococcus faecalis*. *Science* 2003; **299**: 2071–4.
- Willems RJ, Bonten MJ. Glycopeptide-resistant enterococci: deciphering virulence, resistance and epidemicity. *Curr Opin Infect Dis* 2007; **20**: 384–90.
- De Boever EH, Clewell DB, Fraser CM. *Enterococcus faecalis* conjugative plasmid pAM373: complete nucleotide sequence and genetic analyses of sex pheromone response. *Mol Microbiol* 2000; **37**: 1327–41.
- Francia MV, Clewell DB. Transfer origins in the conjugative *Enterococcus faecalis* plasmids pAD1 and pAM373: identification of the pAD1 *nic* site, a specific relaxase and a possible TraG-like protein. *Mol Microbiol* 2002; **45**: 375–95.
- Francia MV, Haas W, Wirth R et al. Completion of the nucleotide sequence of the *Enterococcus faecalis* conjugative virulence plasmid pAD1 and identification of a second transfer origin. *Plasmid* 2001; **46**: 117–27.
- Hirt H, Manias DA, Bryan EM et al. Characterization of the pheromone response of the *Enterococcus faecalis* conjugative plasmid pCF10: complete sequence and comparative analysis of the transcriptional and phenotypic responses of pCF10-containing cells to pheromone induction. *J Bacteriol* 2005; **187**: 1044–54.
- Quintiliani R Jr, Courvalin P. Characterization of Tn1547, a composite transposon flanked by the IS16 and IS256-like elements, that confers vancomycin resistance in *Enterococcus faecalis* BM4281. *Gene* 1996; **172**: 1–8.
- Rice LB, Carias LL. Transfer of Tn5385, a composite, multiresistance chromosomal element from *Enterococcus faecalis*. *J Bacteriol* 1998; **180**: 714–21.
- Schwarz FV, Perreten V, Teuber M. Sequence of the 50-kb conjugative multiresistance plasmid pRE25 from *Enterococcus faecalis* RE25. *Plasmid* 2001; **46**: 170–87.
- Sletvold H, Johnsen PJ, Simonsen GS et al. Comparative DNA analysis of two *vanA* plasmids from *Enterococcus faecium* strains isolated from poultry and a poultry farmer in Norway. *Antimicrob Agents Chemother* 2007; **51**: 736–9.
- Tomita H, Tanimoto K, Hayakawa S et al. Highly conjugative pMG1-like plasmids carrying Tn1546-like transposons that encode vancomycin resistance in *Enterococcus faecium*. *J Bacteriol* 2003; **185**: 7024–8.
- Leclercq R, Derlot E, Duval J et al. Plasmid-mediated resistance to vancomycin and teicoplanin in *Enterococcus faecium*. *N Engl J Med* 1988; **319**: 157–61.
- Hidron AI, Edwards JR, Patel J et al. NHSN annual update: antimicrobial-resistant pathogens associated with healthcare-associated infections: annual summary of data reported to the National Healthcare Safety Network at the Centers for Disease Control and Prevention, 2006–2007. *Infect Control Hosp Epidemiol* 2008; **29**: 996–1011.
- Weigel LM, Donlan RM, Shin DH et al. High-level vancomycin-resistant *Staphylococcus aureus* isolates associated with a polymicrobial biofilm. *Antimicrob Agents Chemother* 2007; **51**: 231–8.
- Perichon B, Courvalin P. VanA-type vancomycin-resistant *Staphylococcus aureus*. *Antimicrob Agents Chemother* 2009; **53**: 4580–7.
- Aarestrup FM. Occurrence of glycopeptide resistance among *Enterococcus faecium* isolates from conventional and ecological poultry farms. *Microb Drug Resist* 1995; **1**: 255–7.
- Klare I, Heier H, Claus H et al. *vanA*-mediated high-level glycopeptide resistance in *Enterococcus faecium* from animal husbandry. *FEMS Microbiol Lett* 1995; **125**: 165–71.
- Aarestrup FM, Ahrens P, Madsen M et al. Glycopeptide susceptibility among Danish *Enterococcus faecium* and *Enterococcus faecalis* isolates of animal and human origin and PCR identification of genes within the VanA cluster. *Antimicrob Agents Chemother* 1996; **40**: 1938–40.

- 20 Borgen K, Simonsen GS, Sundsfjord A *et al.* Continuing high prevalence of VanA-type vancomycin-resistant enterococci on Norwegian poultry farms three years after avoparcin was banned. *J Appl Microbiol* 2000; **89**: 478–85.
- 21 Johnsen PJ, Østerhus JI, Sletvold H *et al.* Persistence of animal and human glycopeptide-resistant enterococci on two Norwegian poultry farms formerly exposed to avoparcin is associated with a widespread plasmid-mediated *vanA* element within a polyclonal *Enterococcus faecium* population. *Appl Environ Microbiol* 2005; **71**: 159–68.
- 22 Sørum M, Johnsen PJ, Aasnes B *et al.* Prevalence, persistence, and molecular characterization of glycopeptide-resistant enterococci in Norwegian poultry and poultry farmers 3 to 8 years after the ban on avoparcin. *Appl Environ Microbiol* 2006; **72**: 516–21.
- 23 Arthur M, Molinas C, Depardieu F *et al.* Characterization of Tn1546, a Tn3-related transposon conferring glycopeptide resistance by synthesis of depsipeptide peptidoglycan precursors in *Enterococcus faecium* BM4147. *J Bacteriol* 1993; **175**: 117–27.
- 24 Biavasco F, Foglia G, Paoletti C *et al.* VanA-type enterococci from humans, animals, and food: species distribution, population structure, Tn1546 typing and location, and virulence determinants. *Appl Environ Microbiol* 2007; **73**: 3307–19.
- 25 Kawalec M, Gniadkowski M, Hryniewicz W. Outbreak of vancomycin-resistant enterococci in a hospital in Gdask, Poland, due to horizontal transfer of different Tn1546-like transposon variants and clonal spread of several strains. *J Clin Microbiol* 2000; **38**: 3317–22.
- 26 Werner G, Klare I, Fleige C *et al.* Increasing rates of vancomycin resistance among *Enterococcus faecium* isolated from German hospitals between 2004 and 2006 are due to wide clonal dissemination of vancomycin-resistant enterococci and horizontal spread of *vanA* clusters. *Int J Med Microbiol* 2008; **298**: 515–27.
- 27 Willems RJ, Top J, van den Braak N *et al.* Molecular diversity and evolutionary relationships of Tn1546-like elements in enterococci from humans and animals. *Antimicrob Agents Chemother* 1999; **43**: 483–91.
- 28 Robart AR, Zimmerly S. Group II intron retroelements: function and diversity. *Cytogenet Genome Res* 2005; **110**: 589–97.
- 29 Sambrook J, Russel DW. *Preparation of Plasmid DNA by Alkaline Lysis with SDS*. Cold Spring Harbor: Cold Spring Harbor Laboratory Press, 2001.
- 30 Staden R, Beal KF, Bonfield JK. The Staden package, 1998. *Methods Mol Biol* 2000; **132**: 115–30.
- 31 Sletvold H, Johnsen PJ, Simonsen GS *et al.* Complete DNA sequence of the *Enterococcus faecium vanA* plasmid pIP816, and comparative analysis to the *vanA* plasmids pVEF1/pVEF2. *Plasmid* 2007; **57**: 239.
- 32 Carver TJ, Rutherford KM, Berriman M *et al.* ACT: the Artemis Comparison Tool. *Bioinformatics* 2005; **16**: 3422–3.
- 33 Delcher AL, Harmon D, Kasif S *et al.* Improved microbial gene identification with GLIMMER. *Nucleic Acids Res* 1999; **27**: 4636–41.
- 34 Altschul SF, Gish W, Miller W *et al.* Basic local alignment search tool. *J Mol Biol* 1990; **215**: 403–10.
- 35 Pearson WR, Lipman DJ. Improved tools for biological sequence comparison. *Proc Natl Acad Sci USA* 1988; **85**: 2444–8.
- 36 Walter AE, Turner DH, Kim J *et al.* Coaxial stacking of helices enhances binding of oligoribonucleotides and improves predictions of RNA folding. *Proc Natl Acad Sci USA* 1994; **91**: 9218–22.
- 37 Zuker M. Mfold web server for nucleic acid folding and hybridization prediction. *Nucleic Acids Res* 2003; **31**: 3406–15.
- 38 Toor N, Hausner G, Zimmerly S. Coevolution of group II intron RNA structures with their intron-encoded reverse transcriptases. *RNA* 2001; **7**: 1142–52.
- 39 Swinfield TJ, Oultram JD, Thompson DE *et al.* Physical characterisation of the replication region of the *Streptococcus faecalis* plasmid pAM beta 1. *Gene* 1990; **87**: 79–90.
- 40 Bidnenko V, Ehrlich SD, Janniere L. *In vivo* relations between pAM $\beta$ -encoded type I topoisomerase and plasmid replication. *Mol Microbiol* 1998; **28**: 1005–16.
- 41 Bruand C, Ehrlich SD. Transcription-driven DNA replication of plasmid pAM $\beta$ 1 in *Bacillus subtilis*. *Mol Microbiol* 1998; **30**: 135–45.
- 42 Jensen LB, Garcia-Migura L, Valenzuela AJ *et al.* A classification system for plasmids from enterococci and other Gram-positive bacteria. *J Microbiol Methods* 2010; **80**: 25–43.
- 43 Dmowski M, Sitkiewicz I, Ceglowski P. Characterization of a novel partition system encoded by the  $\delta$  and  $\omega$  genes from the streptococcal plasmid pSM19035. *J Bacteriol* 2006; **188**: 4362–72.
- 44 Hedberg PJ, Leonard BA, Ruhfel RE *et al.* Identification and characterization of the genes of *Enterococcus faecalis* plasmid pCF10 involved in replication and in negative control of pheromone-inducible conjugation. *Plasmid* 1996; **35**: 46–57.
- 45 Dai L, Toor N, Olson R *et al.* Database for mobile group II introns. *Nucleic Acids Res* 2003; **31**: 424–6.
- 46 Werner G, Klare I, Witte W. The current MLVA typing scheme for *Enterococcus faecium* is less discriminatory than MLST and PFGE for epidemic-virulent, hospital-adapted clonal types. *BMC Microbiol* 2007; **7**: 28.
- 47 Freitas AR, Novais C, Ruiz-Garbajosa P *et al.* Dispersion of multidrug-resistant *Enterococcus faecium* isolates belonging to major clonal complexes in different Portuguese settings. *Appl Environ Microbiol* 2009; **75**: 4904–8.
- 48 Homan WL, Tribe D, Poznanski S *et al.* Multilocus sequence typing scheme for *Enterococcus faecium*. *J Clin Microbiol* 2002; **40**: 1963–71.
- 49 Leavis HL, Bonten MJ, Willems RJ. Identification of high-risk enterococcal clonal complexes: global dispersion and antibiotic resistance. *Curr Opin Microbiol* 2006; **5**: 454–60.
- 50 Rosvoll TC, Pedersen T, Sletvold H *et al.* PCR-based plasmid typing in *Enterococcus faecium* strains reveals widely distributed pRE25-, pRUM-, pIP501- and pHT $\beta$ -related replicons associated with glycopeptide resistance and stabilizing toxin-antitoxin systems. *FEMS Immunol Med Microbiol* 2010; **58**: 254–68.
- 51 Garcia-Migura L, Hasman H, Svendsen C *et al.* Relevance of hot spots in the evolution and transmission of Tn1546 in glycopeptide-resistant *Enterococcus faecium* (GREF) from broiler origin. *J Antimicrob Chemother* 2008; **62**: 681–7.
- 52 Merlin C, Mahillon J, Nesvera J *et al.* Gene recruiters and transporters: the modular structure of bacterial mobile elements. In: Thomas M, ed. *The Horizontal Gene Pool*. Amsterdam: Harwood Academic Publishers, 2000; 363–409.
- 53 Hodel-Christian SL, Murray BE. Characterization of the gentamicin resistance transposon Tn5281 from *Enterococcus faecalis* and comparison to staphylococcal transposons Tn4001 and Tn4031. *Antimicrob Agents Chemother* 1991; **35**: 1147–52.
- 54 Heaton MP, Discotto LF, Pucci MJ *et al.* Mobilization of vancomycin resistance by transposon-mediated fusion of a VanA plasmid with an *Enterococcus faecium* sex pheromone-responsive plasmid. *Gene* 1996; **171**: 9–17.
- 55 Flannagan SE, Clewell DB. Conjugative transfer of Tn916 in *Enterococcus faecalis*: trans activation of homologous transposons. *J Bacteriol* 1991; **173**: 7136–41.
- 56 Lambowitz AM, Zimmerly S. Mobile group II introns. *Annu Rev Genet* 2004; **38**: 1–35.

- 57** Willems RJ, Top J, van Santen M *et al.* Global spread of vancomycin-resistant *Enterococcus faecium* from distinct nosocomial genetic complex. *Emerg Infect Dis* 2005; **11**: 821–8.
- 58** Sletvold H, Johnsen PJ, Hamre I *et al.* Complete sequence of *Enterococcus faecium* pVEF3 and the detection of an omega-epsilon-zeta toxin–antitoxin module and an ABC transporter. *Plasmid* 2008; **60**: 75–85.
- 59** Tomita H, Ike Y. Genetic analysis of transfer-related regions of the vancomycin resistance *Enterococcus* conjugative plasmid pHT $\beta$ : identification of *oriT* and a putative relaxase gene. *J Bacteriol* 2005; **187**: 7727–37.
- 60** Ke D, Picard FJ, Martineau F *et al.* Development of a PCR assay for rapid detection of enterococci. *J Clin Microbiol* 1999; **37**: 3497–503.