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Molecular Analysis of Tn*1546* in *Enterococcus faecium* Isolated from Animals and Humans

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The internal areas and the position of integration of the glycopeptide resistance element Tn*1546* were characterized by using PCR fragment length polymorphism, sequencing, and DNA hybridization techniques with 38 high-level vancomycin-resistant *Enterococcus faecium* isolates of human and animal origins from Europe and the United States. Only minor variations in the coding regions within Tn*1546* were found, suggesting high genetic stability. The isolates originated from broilers ($n = 5$), a chicken ($n = 1$), a duck ($n = 1$), a turkey ($n = 1$), pigs ($n = 8$), a pony ($n = 1$), and humans ($n = 23$). A total of 13 different types were defined based on a single-nucleotide difference in the *vanX* gene, the presence of insertion sequences, and hybridization patterns. For some types more than one isolate were found. For type 1, 10 isolates of both human and animal origins were 1found. All were indistinguishable from the reference strain, BM4147. For type 2, 11 isolates of human and animal origins were found. Six human isolates from England were all of type 3. Two human isolates from the United States, indistinguishable from each other, were type 9. These results showed that vancomycin-resistant *E. faecium* of animal and human origins can contain indistinguishable genetic elements coding for vancomycin resistance, indicating either horizontal gene transfer between *E. faecium* organisms of human and animal origins or the existence of a common reservoir for glycopeptide resistance.

The enterococci are a dominant bacterial group in the intestinal flora of human and animals (14). Over the last two decades, the number of infections in hospitalized patients due to enterococci has increased (26–29). Until recently most of these infections have been successfully treated by combinations of vancomycin and other antibiotics, but since the emergence of vancomycin-resistant *Enterococcus faecium* in 1986 (23), isolates resistant to all known antibiotics can be found. Vancomycin-resistant *E. faecium* has been found increasingly not only in hospitalized patients (10, 13, 15, 18), but also in the healthy human population (20, 34), in animals (1, 8, 22), and in sewage plants (8, 21, 32). In Europe a glycopeptide, avoparcin, has been used as a growth promoter in animal feed, and its use has been shown to create a reservoir for vancomycin-resistant *E. faecium* in animals (7). Avoparcin is not used as a growth promoter in the United States, and no VanA-positive isolates have been found in animals (31) or in healthy volunteers (11) in the United States.

Studies of vancomycin-resistant enterococci have shown high clonal diversity, indicating that horizontal gene transfer to some extent plays a part in the dissemination of vancomycin resistance (13, 15, 25). The VanA gene cluster, encoding high-level glycopeptide resistance, is located on the mobile DNA element Tn*1546* (3, 6). By investigation of selected vancomycin-resistant *E. faecium* isolates, variations in this element have been found (16, 25). Molecular characterization of the VanA gene cluster could therefore provide additional information regarding the variation or identities of isolates of different origins and could allow for epidemiological studies of the dissemination of vancomycin resistance due to horizontal gene transfer.

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In this study we have characterized isolates of human and animal origins in order to determine if identical Tn*1546* could be found in nonidentical strains, and results obtained from 38 vancomycin-resistant *E. faecium* isolates of human and animal origins in Europe and the United States are described.

MATERIALS AND METHODS

Strains. A total of 40 vancomycin-resistant *E. faecium* isolates were included (Table 1). The Danish isolates were selected from a previously described collection (1). The English isolates have been described previously (8, 19). Seven clinical isolates of vancomycin-resistant *E. faecium* from the United States, collected from patients in Columbus, Ohio (EFM 10-601), Philadelphia, Pa. (EFM 11-803), Bronx, N.Y. (EFM 12-901), New York, N.Y. (EFM 15-1201), Richmond, Va. (EFM 17-2302), New Haven, Conn. (EFM 19-2501), and Chicago, Ill. (EFM 26-4507), were included (Table 1).

Phenotypic test. A phenotypic test for vancomycin and teicoplanin resistance was performed with the Sensititre system (Accumed International Limited, East Grinstead, West Sussex, England). All isolates were tested for resistance to concentrations of vancomycin and teicoplanin in twofold dilutions from 64 to 0.5 μg/ml. Avoparcin resistance was determined on Mueller-Hinton II agar (Becton Dickinson Microbiology Systems, Cockeysville, Md.) according to the description provided by Aarestrup et al. (1). All isolates were tested for resistance to concentrations of avoparcin in twofold dilutions from 256 to 4 μg/ml.

Total DNA extraction. Total DNA was extracted from bacteria grown on Mueller-Hinton plates supplemented with 5% (bovine) blood. A tablet (Rosco Diagnostica, Taastrup, Denmark) containing 70 μg of vancomycin was placed on each plate. A 1-ml suspension of the resistant bacteria in phosphate-buffered saline was transferred to an Eppendorf tube, centrifuged at 20,000 rpm for 5 min, and then resuspended in 100 μl of Tris-EDTA buffer. Cells were lysed by boiling (for 10 min), and 2 μl was used for PCR amplification.

Pulsed-field gel electrophoresis (PFGE). For preparations of DNA embedded in agarose plugs, whole-cell DNA was prepared as previously described (28), with the following modifications: 3 ml of a solution containing 1 M NaCl, 10 mM Tris-HCl, 200 mM EDTA, 0.5% sodium lauryl sarcosine, 1.7 mg of lysozyme/ml, and 3.3 μg of Rnase A/ml was used as lysis buffer, and proteins were digested with 1 mg of proteinase K/ml and removed by a wash in 2 ml of Tris-EDTA buffer (10:1) containing 0.25 mg of phenylmethylsulfonyl fluoride/ml.

A small slice of the agarose plug was digested with 20 U of *Sma*I (Amersham Life Science) for a minimum of 4 h. Digested DNA was electrophoresed in a 1% agarose gel. Electrophoresis was performed in a Pharmacia LKB Gene Navigator unit by using the following settings: 2 s, 5 h, 5 s, 6 h, 9 s, 6 h, and 12 s, 5 h in 0.5× Tris-borate-EDTA buffer at 12°C at 180 V. DNA was transferred onto a nylon membrane (Hybond-N; Amersham International, Little Chalfont, Buckinghamshire,

TABLE 1. Vancomycin-resistant *E. faecium* isolates of animal and human origins

No.	Source	Country	Size of *vanA*-positive fragment[a] (kb)	Designation
1	Pony	England	<50	C15VF9
2	Duck	England	<50	C13VF7
3	Chicken	England	<50	C14VF8
4	Turkey	England	<50	C12VF4
5	Human	England	85	63910
6	Human	England	<48	67668
7	Human	England	100	GP3 61741
8	Pig	England	45	A10 Pig 2.19
9	Pig	England	160	A6 Pig22
10	Pig	England	160	A1 VF1
11	Human	England	60	60761(1)
12	Human	United States	Negative	EFM 10-601
13	Human	United States	55	EFM 11-803
14	Human	United States	200	EFM 12-901
15	Human	United States	38	EFM 15-1201
16	Human	United States	250	EFM 17-2302
17	Human	United States	200	EFM 19-2501
18	Human	United States	Negative	EFM 26-4507
19	Human	England	60	72801
20	Human	England	60	59479
21	Human	England	60	62899
22	Human	England	60	68521
23	Human	England	60	80103
24	Human	England	50	89407
25	Human	England	35	58538
26	Human	England	60	58155
27	Human	France	35	BM4147
28	Human	Denmark	180	H17575
29	Broiler	Denmark	50	73343-2-1
30	Broiler	Denmark	45	73281-7-2
31	Pig	Denmark	160	A17-SV1
32	Pig	Denmark	160	D1-SV1
33	Broiler	Denmark	45	73583-8-1
34	Broiler	Denmark	45	73449-1-2
35	Pig	Denmark	160	S3-SV1
36	Pig	Denmark	160	014-S2
37	Human	Denmark	29	5979
38	Broiler	Denmark	50	73452-4-2
39	Pig	Denmark	160	E8-SV3
40	Human	Denmark	145	H17243

[a] The collected strains were tested for the position of integration of Tn*1546*-like sequences by PFGE of total DNA digested with *Sma*I. This enzyme has no internal recognition site in the VanA gene cluster. Positive bands vary in size because, in addition to Tn*1546*, these fragments contain the flanking regions from the point of integration to the nearest *Sma*I sites. These areas vary as to the physical position of the target site on the chromosome.

United Kingdom) by capillary transfer and fixed by UV fixation. Hybridizations were performed by using a minihybridization oven (Hybaid Ltd., Teddington, Middlesex, England). A digoxigenin-labelled DNA *vanA* probe (535 bp) was used for hybridization.

PCR amplification of internal regions of Tn*1546*. DNA extractions and PCR amplification were performed according to the method of Aarestrup et al. (1). Primers were designed according to the published sequence of Tn*1546* (3, 6). All primers used are listed in Table 2. Both coding and noncoding areas were amplified. The positions and sizes of the PCR-amplified regions are indicated in Fig. 1. The melting temperatures for the individual primers were calculated by using the Tm DETERMINATION software (9) available on the Internet (http://alces.med.umn.edu/rawtm.html). All PCR amplifications were run with a $MgCl_2$ concentration of 1 mM.

Presence of IS sequences. The presence of published insertion element (IS) sequences in the VanA gene cluster was tested by designing one primer in the published sequence of Tn*1546* and one in the published IS sequence. All primers used are listed in Table 2. Only if the IS sequence is present at the published position in the VanA gene cluster can a positive PCR amplicon of the bridging area between the VanA gene cluster and the IS sequence be obtained. Table 3 lists positive and negative PCR amplification results as the numbers 1 and 0, respectively.

Sequencing. The nucleotide sequences of the amplification products were determined by cycle sequencing (30) using the Amplitaq FS dye terminator kit and a 373A automatic sequencer (Applied Biosystems, Perkin-Elmer, Foster City, Calif.). DNASIS software (Hitachi Software Engineering Co., Ltd.) was used for sequence analysis.

Hybridizations. Total DNA was digested with the restriction enzymes *Hin*dIII, *Bam*HI-*Bgl*II, *Bam*HI-*Eco*RV, and *Bam*HI-*Kpn*I. All enzymes were purchased from Amersham International. Fragments were separated in a 0.8% agarose gel. DNA was transferred to Hybond-N membranes (Amersham International).

Digoxigenin-labelled DNA probes for *vanR* (409 bp) and *vanX* (423 bp) were prepared by PCR amplification using the primers described in Table 2 and were subsequently labelled with the Boehringer Mannheim DNA labelling and detection kit. The obtained PCR products were purified by using Qiagen (Hilden, Germany) spin columns. The *vanR* probe was used for *Hin*dIII-digested total DNA, and the *vanX* probe was used for *Bam*HI-*Bgl*II-, *Bam*HI-*Eco*RV-, and *Bam*HI-*Kpn*I-digested total DNA.

RESULTS

All isolates were resistant to vancomycin (>64 μg/ml) and avoparcin (>16 μg/ml). Thirty-eight were resistant to teicoplanin (>16 μg/ml). Two isolates (no. 12 and 18) were sensitive to teicoplanin, indicating that these strains contain *vanB*. Of the

TABLE 2. Primers used to characterize Tn*1546* from vancomycin-resistant enterococci[a]

Primer	Sequence (5′→3′)	Position
VanA gene cluster		
Van A1	AAA TGT GCG AAA AAC CTT GC	7127–7146
Van A2	AAC AAC TAA CGC GGC ACT	7662–7645
VanS1	ATT GTT CAG CAT GGA GGG C	5696–5714
VanH2	GAG CAT GGA ATG CAT CTG CC	6081–6063
VanR1	AAA TAA GGG ACA AGC ACA CC	4125–4194
VanR2	CCC ATA TCT CAT GAA ATA GC	4534–4515
VanX1	ACT TGG GAT AAT TTC ACC GG	8082–8101
VanX2	TGC GAT TTT GCG CTT CAT TG	8505–8486
VanX3	CTC ATC ATG CGG CAA ATG G	8458–8476
VanY1	TGG GTA TTT TCA GAA GTC CC	9212–9193
VanY2-1	GTT TCC CGG ATC AAC ACA TAC TA	9927–9949
VanZ2-2	CCC AGT AGC AGT AAA TGG AGT CA	10262–10240
VanZ1	CTG GGA ATT TCA GAG AGA TG	10258–10277
VanZ2	AAT GGG TAC GGT AAA CGA GC	10581–10562
ORF12	CCA TTC CTC GTA TGT ATT CG	2318–2337
VanR2	CCC ATA TCT CAT GAA ATA GC	4534–4515
ORF14-2	GCC CTT AGG TTG GGA ACA TA	174–155
VanSma-2	ATA AAA TGA CTA ACG CCA CC	9446–9427
IS*1216V*–IS*3*-like		
IS1216V-1	AAA GCA ATT TCA GCA GGA TG	256–275
IS1216V-2	GTA CGA TGT TCT GTC CCT TG	711–692
IS3-like-1	ACT GGG TAT CGC CAA ATC CA	1251–1270
IS3-like-2	TTT GTC CCA TTG GTC AAC CG	1619–1600
IS3-like-2-1	CCA CAC TGA TTC ATA GCG AC	1748–1767
IS*1251*		
IS1251-3	GCA TCC ACT GTA AAC ACC AG	273–292
IS1251-4	CGC TGT GTT TGA CCA TCC AT	699–680
IS*1476*, IS1476-2-1	CTT TCG GGC ACG GAT CTA TT	1366–1385

[a] All primers were used for PCR amplification of internal areas of Tn*1546*. Positions refer to sequences published in GenBank; access numbers are M97297 for Tn*1546*, L40841 for IS*1216V*, L34675 for IS*1251*, and U63997 for IS*1476*. Correct positions of the IS sequences were determined by using one primer inside the IS sequence and one in the flanking region of Tn*1546*. For the position of IS*1251*, primers VanH2 and IS1251-4 were used; for linking of IS*1216V* to the IS*3*-like element, primers IS1216V-1 and IS3-like-2 were used; and IS3-like-2-1 and ORF14-2 were used to position this complex in the left end of Tn*1546*. Primers IS1476-2-1 and VanSma-2 were used to determine whether IS*1476* was present.

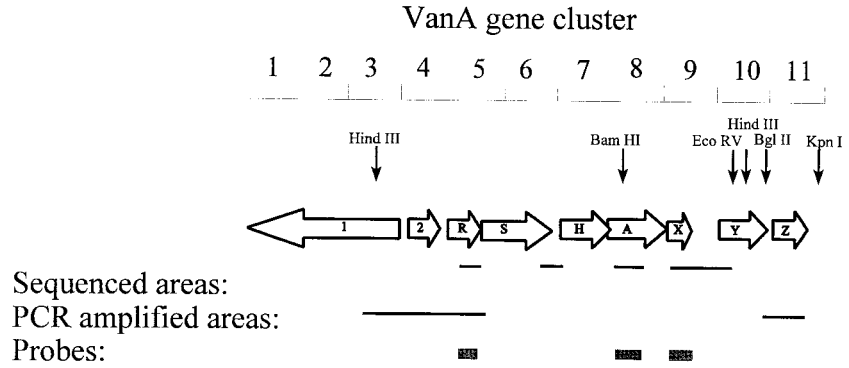

FIG. 1. Map of Tn*1546*. Numbers at the top represent kilobases. Open arrows represent coding sequences, and letters inside them stand for genes (e.g., R, *vanR*). Positions of recognition sites for selected restriction enzymes are marked by vertical arrows. Only essential sizes for the hybridizations are indicated.

40 isolates, 38 hybridized to the *vanA* probe and gave positive results in the PCR amplifications. The two negative isolates were identical to the teicoplanin-sensitive isolates (no. 12 and 18). Positive hybridization signals of different sizes were obtained (Table 1), indicating different positions of integration for Tn*1546* on the genome. This is an indication that the tested strains are not identical. The position of integration (target) could be identical for all strains, since several identical targets could exist at different locations on the chromosome. Isolates 12 and 18 gave no positive amplicons for *vanA* (Fig. 2A), *vanR*, or *vanX* (Fig. 2B). These isolates were later defined as *vanB* (28a).

Coding regions of Tn*1546*. PCR amplification products of *vanA* (457 bp) (Fig. 2A), *vanR* (303 bp), and *vanX* (354 bp) (Fig. 2B) from Tn*1546* were obtained for 38 isolates. All PCR products from the three replicons were of the expected sizes. These were sequenced, and only a single-nucleotide variation (T to G) was found in the *vanX* gene at position 8234. For *vanZ*, a PCR product of the expected size (323 bp) was obtained from all isolates. No further investigations of these PCR products were performed.

Intergenic area. PCR products of the expected size for the *vanXY* intergenic region (554 bp) were obtained from 38 isolates. No sequence or size variation was observed in the amplicons of the *vanXY* intergenic region (data not shown). PCR amplification of the *vanSH* intergenic region produced an amplicon of 318 bp for 35 isolates. For three isolates, an amplicon of approximately 1,900 bp was obtained. These three isolates (no. 15 through 17) (Fig. 2C) were all of human origin and all from the United States. For the region containing open reading frame 2 (ORF2), the ORF2-*vanR* intergenic region, and *vanR* (position 2318 to 4534), PCR products (2,216 bp) were obtained from 38 isolates. *Bgl*II digestion of the amplicons resulted in fragments of 1,085, 1,027, and 104 bp, respectively (data not shown). The *vanYZ* intergenic region was PCR amplified for 38 isolates, and no size variation in the PCR products was observed (data not shown).

IS sequences. The presence of the IS*1216V* (17) sequence was detected in all 40 isolates, but only in 16 isolates was it linked to the IS*3*-like sequence at the published position inside the VanA gene cluster (Table 3) (17). In the remaining 24 isolates, the IS*3*-like sequence was positioned outside Tn*1546*. IS*1251* (16) was detected in three human isolates from hospitals in the United States (no. 15 through 17) for which a larger DNA fragment was obtained from PCR amplification of the intergenic area (Fig. 2C), and its presence was confirmed by an amplicon positive for the presence of IS*1251* at this position (Table 3). IS*1476* (24) was not found in any of the isolates.

DNA hybridizations. A probe specific for the *vanR* gene hybridized to one band ranging from 4 to 11 kb of *Hin*dIII (positions 2455 and 9571)-digested total DNA (Table 3) in 35 isolates. In three of the isolates (no. 6, 15, and 24), two bands were detected (Table 3). A probe specific for the *vanX* gene hybridized to one band ranging from 3 to 7 kb of *Bam*HI (position 7295)-*Kpn*I (position 10849)-digested total DNA (Table 3) in 36 isolates. Isolates 6 and 24 each gave two bands. With the *vanX*-specific probe, bands ranging from 2.5 to 10.5 kb of *Bam*HI (position 7295)-*Bgl*II (position 9825)-digested total DNA were detected (Table 3); isolates 6 and 24 each gave two bands. *Bam*HI (position 7295)-*Eco*RV (position 9304)-digested total DNA of selected strains was hybridized with the *vanX*-specific probe. Positive bands ranging from 2.1 to 10.5 kb were detected (Table 3); isolates 6 and 24 each gave two bands.

DISCUSSION

Since the emergence of vancomycin-resistant *E. faecium*, several investigations have been performed to study the dissemination of glycopeptide in these strains. Analyses of bacteria isolated from hospitalized patients have indicated both clonal spread of vancomycin-resistant *E. faecium* and horizontal gene transfer of Tn*1546*. After the detection of vancomycin-resistant *E. faecium* in sewage plants, animals, healthy humans, and pet animals (12, 33), it was hypothesized that the hospital environment was creating vancomycin-resistant *E. faecium*, which subsequently spread to the environment. After the discovery that the glycopeptide avoparcin, commonly used as a growth promoter in agriculture, selected for vancomycin-resistant *E. faecium* in production animals (2, 22), the hypothesis that vancomycin-resistant *E. faecium* could spread from animals to humans via the food chain was raised (8). To test the transfer between animals and humans, several isolates of human and animal origins have been typed (8, 19, 20). These experiments indicated high diversity among the strains of vancomycin-resistant *E. faecium* defined by ribotypes, and the focus was turned to the dissemination of the genetic element for vancomycin resistance, Tn*1546*.

In the present study, the genetic elements for vancomycin resistance in 40 high-level vancomycin-resistant *E. faecium* isolates of animal and human origins from Europe and the United States were studied. For 38 isolates, an amplicon positive for *vanA* was obtained (Table 1). For two isolates (no. 12 and 18), a PCR amplicon positive for *vanB* was obtained (Table 1).

A *vanA* probe gave specific bands of different sizes when hybridized to DNA fragments from PFGE of *Sma*I-digested total DNA from 38 isolates. The variations in size of positive

TABLE 3. Genetic characterization of Tn*1546*-like elements from vancomycin-resistant enterococci by PCR and DNA hybridization

Isolate type[a]	Isolate no.	Source	PCR result			DNA hybridization signal[d] (kb)			
			M *vanX*[b]	IS*1216V*–IS*3*-like[c]	IS*1251*[c]	VanR (H3)	VanX		
							BHI-KI	BHI-BII	BHI-EV
1	1	Pony	G	0	0	7	3.5	2.5	
1	2	Duck	G	0	0	7	3.5	2.5	
1	3	Chicken	G	0	0	7	3.5	2.5	
1	4	Turkey	G	0	0	7	3.5	2.5	
1	13	Human	G	0	0	7	3.5	2.5	
1	27	Human	G	0	0	7	3.5	2.5	
1	30	Broiler	G	0	0	7	3.5	2.5	2.1
1	33	Broiler	G	0	0	7	3.5	2.5	
1	34	Broiler	G	0	0	7	3.5	2.5	
1	38	Broiler	G	0	0	7	3.5	2.5	
2	8	Pig	T	1	0	7	3.5	2.5	
2	9	Pig	T	1	0	7	3.5	2.5	
2	10	Pig	T	1	0	7	3.5	2.5	2.1
2	14	Human	T	1	0	7	3.5	2.5	
2	28	Human	T	1	0	7	3.5	2.5	2.1
2	31	Pig	T	1	0	7	3.5	2.5	
2	32	Pig	T	1	0	7	3.5	2.5	
2	35	Pig	T	1	0	7	3.5	2.5	
2	36	Pig	T	1	0	7	3.5	2.5	
2	39	Pig	T	1	0	7	3.5	2.5	
2	40	Human	T	1	0	7	3.5	2.5	
3	19	Human	G	0	0	8	3.0	10.5	10.5
3	20	Human	G	0	0	8	3.0	10.5	10.5
3	21	Human	G	0	0	8	3.0	10.5	10.5
3	22	Human	G	0	0	8	3.0	10.5	10.5
3	23	Human	G	0	0	8	3.0	10.5	10.5
3	26	Human	G	0	0	8	3.0	10.5	10.5
4	5	Human	G	1	0	7	3.5	2.5	
5	6	Human	G	1	0	7, 9	3.5, 4.4	2.5, 3.5	2.1, 2.9
6	7	Human	G	1	0	7	3.5	2.5	
7	11	Human	G	0	0	8	3.0	10.5	2.1
8	15	Human	T	0	1	4, 5	3.5	2.5	
9	16	Human	T	1	1	4	3.5	2.5	
9	17	Human	T	1	1	4	3.5	2.5	2.1
10	24	Human	G	0	0	8, 11	3.0, 7.0	5.5, 10.5	6.8, 10.5
11	25	Human	G	0	0	15	4.4	3.5	2.9
12	29	Broiler	G	0	0	8	4.4	3.5	2.9
13	37	Human	T	0	0	7	3.5	2.5	

[a] Isolates are divided into 13 types according to the results obtained.
[b] Sequence result of PCR-amplified region of the *vanX* gene. T, sequence homologous to published sequence of Tn*1546*; G, sequence with a single-nucleotide variation (T to G) at position 8234.
[c] Presence of the IS*1216V*–IS*3*-like element and of IS*1251* at the published positions in the left end of Tn*1546* and in the *vanSH* intergenic region, respectively. Symbols: 1, correct position; 0, absence of a positive PCR amplification.
[d] Where two values are given, two bands were detected. Abbreviations: H3, *Hin*dIII; BHI, *Bam*HI; KI, *Kpn*I; BII, *Bgl*II; EV, *Eco*RV.

bands (Table 1) indicated different positions of integration of Tn*1546*, suggesting that the isolates were nonidentical. For 7 of the 12 English human isolates and 7 of 8 porcine isolates, hybridization to fragments of similar sizes (Table 1) was detected.

PCR amplification and sequencing of internal areas of Tn*1546* in the 38 *vanA*-positive isolates confirmed high homology to the published sequence of Tn*1546* in coding regions. In total, 19% of Tn*1546* was sequenced, and only a single-base-pair difference was detected in the *vanX* gene (Table 3). The detected difference at position 8234 in the *vanX* gene changed the amino acid at this position from a lysine to an asparagine but apparently did not affect the phenotype. No sequence or size variation was seen in the *vanXY* intergenic region, contrary to published results (25). Since some of the isolates in the previous investigation (25) were not vancomycin-resistant *E. faecium*, and others originated from a hospital in England different from the ones from which isolates for this survey were taken, the result is not surprising. This just indicates that more isolates need to be tested in order to define all types.

Two previously published IS sequences were found to have integrated into Tn*1546*. IS*1216V* was present in all 40 strains, but only in 16 of the 38 *vanA*-positive isolates could the position of this IS sequence combined with the IS*3*-like element (17) be confirmed in the left end of Tn*1546* (Table 3). In the rest of the positive isolates, the IS*1216V*-like elements probably are positioned outside Tn*1546*. The presence of IS*1251* was detected in three human isolates from the United States (no. 15 through 17), positioned in the *vanSH* intergenic region (Table 3). Handwerger and Skoble (17) presumed that the presence of the IS*3*-like sequence positioned Tn*1546* on the chromosome. In the present work, no efforts were made to localize Tn*1546* on the chromosome or on a plasmid, but sizes of positive fragments from PFGE of *Sma*I-digested total DNA

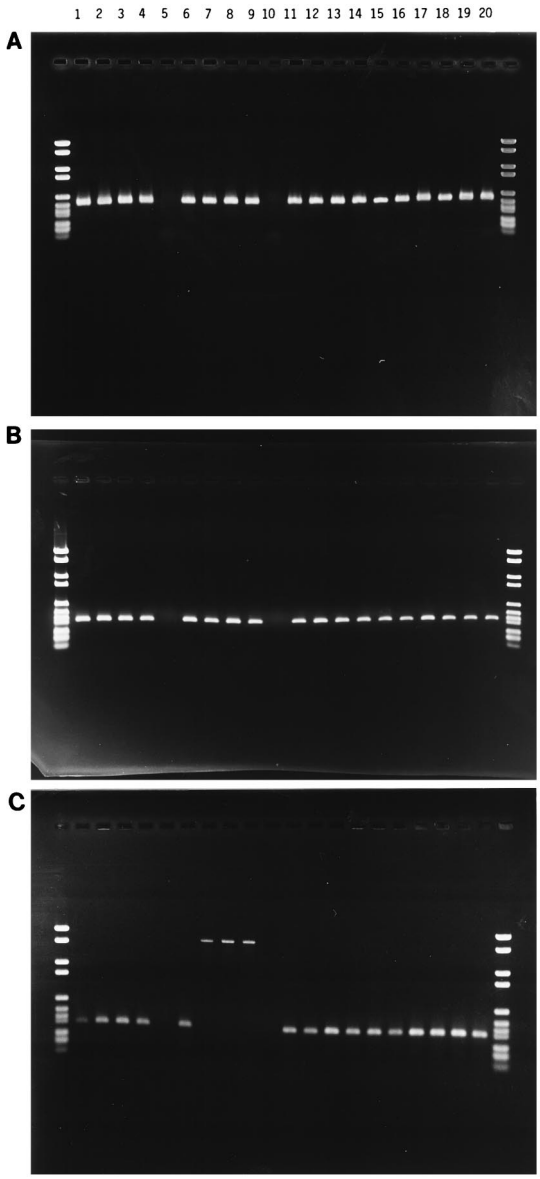

FIG. 2. PCR amplification of the internal region of the VanA gene cluster. (A) The *vanA* gene of selected isolates, from position 7266 to 7623. Isolates 12 and 18 (lanes 5 and 10) gave no positive product. (B) The *vanX* gene of selected isolates, from position 8129 to 8483. Isolates 12 and 18 (lanes 5 and 10) gave no positive product. (C) The *vanSH* intergenic region of selected isolates, from position 5715 to 6031. Isolates 12 and 18 (lanes 5 and 10) gave no positive product. Isolates 15 through 17 (lanes 7 through 9) gave a positive product of approximately 1,900 bp. The Boehringer IV (Boehringer Mannheim) was used for size determination. Sizes of markers are 2,176, 1,766, 1,230, 1,033, 653, 517, 453, 394, 298, 234, 220, and 154 bp. Results for isolates are shown as follows: lane 1, 5; lane 2, 6; lane 3, 7; lane 4, 11; lane 5, 12; lane 6, 14; lane 7, 15; lane 8, 16; lane 9, 17; lane 10, 18; lane 11, 22; lane 12, 24; lane 13, 25; lane 14, 27; lane 15, 28; lane 16, 29; lane 17, 30; lane 18, 34; lane 19, 37; lane 20, 40.

(Table 1) of IS*3*-like-positive clones were all large (>85 kb) except for isolate 6 (<48 kb) and isolate 8 (48 kb). This could indicate a chromosomal position.

Based solely on Southern blot analysis of total DNA digested with several restriction enzymes, creating fragments covering most of Tn*1546*, 38 isolates could be divided into nine unique types (Table 3). Type 1 consists of 24 isolates. These gave fragment sizes corresponding to Tn*1546* according to previous published sequences. Type 2 consists of two human clinical isolates from the United States (no. 16 and 17). These gave a smaller fragment. Type 3 consists of six human clinical isolates from England. These gave a larger fragment. These six isolates (no. 19 through 23 and no. 26) and also isolate 11 are believed to contain two uncharacterized size variations of approximately 8 kb. This is indicated by larger bands obtained from DNA hybridizations (Table 3). For isolate 11, a variation has been mapped between positions 9304 and 9825. For the rest, the variation has been mapped between positions 7295 and 9304 (Fig. 1 and Table 3). Since these areas contain the essential *vanA* and *vanX* genes, the *vanXY* intergenic area, and *vanY* (Fig. 1), and since no sequence variation was found in the *vanA* gene, the *vanR* gene, or the *vanXY* intergenic region, the insertions are believed to be in the *vanY* gene. Attempts to identify the insertions as the recently published IS*1476* (24) residing in the *vanY* gene (position 9333) by PCR amplification have failed. The *vanY* gene codes for a D,D-carboxypeptidase, which contributes to the hydrolysis of soluble peptidoglycan precursors, complementary to *vanX* dipeptidase hydrolysis, and is for that reason not essential for the phenotype of the VanA gene cluster (4, 5).

For three isolates (no. 15 through 17), the size of *Hin*dIII-digested total DNA obtained by using a *vanR*-specific probe can be explained by the presence of IS*1251*. This IS sequence contains an additional *Hin*dIII recognition site, thus creating a 4-kb band instead of the 7-kb band (Table 3). The additional 5-kb band obtained with isolate 15 cannot be explained. Isolates 6 and 24 each contain two nonidentical copies of Tn*1546*. This is, to our knowledge, the first time this has been described.

At least five additional size variations have been mapped in seven unique types to the area between positions 7295 and 9304. Most of this area has been sequenced (Fig. 1), and the variations detected by hybridizations could be located in *vanY* and might be explained by an IS sequence "hot spot" in this nonessential gene. This is at present being investigated.

Adding together the results obtained, no significant variations were found in the Tn*1546* elements. Only the presence or absence of IS sequences and a single difference in the *vanX* gene seem to differentiate the Tn*1546* elements observed in this study from the published sequence (3, 6). However, some variations were found; thus, a genetic characterization of Tn*1546* can provide information on dissemination of vancomycin resistance due to horizontal gene transfer.

In this study, the 38 *vanA*-positive isolates could be divided into 13 types based on hybridizations, obtained sequences, and the presence of IS sequences (Table 3). Types 1 and 2 consist of isolates of human and animal origins, suggesting that indistinguishable genetic elements for vancomycin resistance can be present in bacteria isolated from animals and humans. Type 1 includes the reference strain, BM4147 (isolate 27). Type 2 consists of isolates with the single-base-pair difference and the presence of the IS*1216V*–IS*3*-like sequence. Type 3 consists of clinical isolates of human origin from England and indicates an epidemiological relationship among the isolates. Type 9 consists of two human isolates from the United States. The last 10 unique types differ from the defined genetic subgroups by the presence of IS*1251* and detected size variations of unknown origin.

By hybridization with the *vanA*-specific probe to DNA fragments from PFGE of *Sma*I-digested total DNA, different integration positions of Tn*1546* were detected. This indicates that the strain does not originate from a clonal spread. Therefore, the existence of two genetic subgroups containing isolates of both human and animal origins indicates two possibilities. Either the isolates have obtained Tn*1546* from a common

reservoir or a horizontal gene transfer has occurred. These two possibilities cannot be distinguished, but both show that bacteria of human and animal origins can harbor similar resistance genes.

The work described in this article can be used to study the epidemiological spread of vancomycin-resistant *E. faecium* where horizontal transfer of Tn*1546* dominates the dissemination of vancomycin resistance. For this purpose it is essential to characterize all variations in Tn*1546* by investigation of a broad variety of strains. This work is being performed at present.

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