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Genetic Rearrangements of Tn*1546*-Like Elements in Vancomycin-Resistant *Enterococcus faecium* Isolates Collected from Hospitalized Patients over a Seven-Year Period[▽]

Il Joong Park, Wee Gyo Lee,* Young Ae Lim, and Sung Ran Cho

Department of Laboratory Medicine, Ajou University School of Medicine, Suwon, Korea

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The heterogeneity of Tn*1546* results from point mutations, deletions, and the integration of insertion sequence (IS) elements. Among these variations, the presence of IS elements accounts for much of the heterogeneity. Such a rearrangement could play a key role in the evolution of the *vanA* gene cluster, and hence, it may modify its transferability. In this study, we characterized the consequence of Tn*1546* in *vanA*-containing *Enterococcus faecium* isolates collected from patients over time. From 1998 to 2004, 57 *vanA*-containing *E. faecium* isolates were collected from hospitalized patients at Ajou University Hospital in Korea. PCR amplification of internal regions of Tn*1546* was performed, and both DNA strands were directly sequenced by the dideoxy termination method. All isolates were divided into three main types, including the prototype, according to the distribution of IS elements integrated into Tn*1546* elements. Type I was characterized by an IS*1542* insertion in the *orf2-vanR* intergenic region and an IS*1216V* insertion in the *vanX-vanY* intergenic region. Type II was represented by the presence of two copies of IS*1216V* at the 3′ end of IS*1542* and in the *vanX-vanY* intergenic region, as well as IS*1542* in the *orf2-vanR* intergenic region. Seventeen strains isolated from 1998 to 2000 represented type I, and 38 strains isolated from 2000 to 2004 represented type II. The remaining two isolates were the prototype. The tendency for the rearrangement of Tn*1546* was that the sequences were shortened as time passed, especially at the left or the right end, and hence, this could gradually modulate their transferability.

In Korea, vancomycin-resistant enterococci (VRE) were first detected in 1992 (9). The prevalence of VRE in Korea had been low until 1997; however, since then it has increased dramatically. Shin et al. reported only 8 VRE isolates among 5,275 enterococci collected during 1995 and 1997 (13). In contrast, from 1998 to 2000 the number of VRE significantly increased to 325 isolates among 5,705 enterococci. Thus, it is very important to obtain an understanding of the molecular mechanisms underlying the rapid dissemination of VRE in Korea.

Generally, the main mechanism for the dissemination of the *van* gene in enterococci could be the clonal dissemination of VRE, but the horizontal transfer of a resistance gene cluster has also been suggested to be a significant mechanism (6, 10, 11). Pulsed-field gel electrophoresis (PFGE) has been widely employed to obtain an understanding of the clonal dissemination of VRE, and structural analyses of the Tn*1546*-like element have recently been introduced to gain an understanding of the horizontal transfer of the resistance gene cluster. The heterogeneity of the Tn*1546*-like element results from point mutations, deletions, and the integration of insertion sequence (IS) elements (16, 17); and among these variations, the presence of IS elements accounts for much of the heterogeneity (3). Such a rearrangement has been shown to play a key role in the evolution of the *vanA* gene cluster and may modify its transferability (7, 8, 11). In the present study, we characterized the consequence of Tn*1546* in *vanA*-containing *Enterococcus faecium* isolates collected from patients over time passed to obtain an understanding of the hospital dissemination of VRE over time.

MATERIALS AND METHODS

Patient population and bacterial strains. The study was carried out at Ajou University Hospital (Suwon, Korea), which is a 1,000-bed university hospital with an average of 330,000 patient discharges per year. From January 1998 to December 2004, we collected 57 isolates of *vanA*-containing *E. faecium* from 48 hospitalized patients at Ajou University Hospital (Table 1). In order to focus on strain-independent variability, PFGE was used to exclude duplicate isolates. All 57 isolates of *vanA*-containing *E. faecium* had seven or more band differences, since we regarded the strains of VRE as "genetically unrelated" if they revealed seven or more band differences by PFGE, according to Tenover et al. (14). The 57 isolates genetically unrelated according to their PFGE patterns were included in our study. PFGE was performed on a CHEF-DR III apparatus (Bio-Rad Laboratories, Richmond, CA), as described previously (10). A previously characterized VRE strain, *E. faecium* BM4147, served as the control (2). The organisms were identified by using conventional biochemical reactions and the Vitek identification system (bioMérieux, Hazelwood, MO). The medical records of all 48 patients were reviewed as part of an epidemiological investigation. Data regarding age, gender, service, the ward location at the time of the first culture positive for VRE, and all previous admissions were collected (Table 1).

DNA extraction and PCR. Bacterial DNA was extracted by use of a DNeasy kit (Qiagen GmbH, Hilden, Germany), according to the manufacturer's instructions. For structural analysis of Tn*1546*-like elements, PCR amplification of overlapping internal regions of Tn*1546* was performed as described previously (8).

Sequence analysis and DNA hybridization. PCR amplicons larger than that of the prototype *vanA* gene cluster were purified with GeneClean kits (Qbiogene Inc., Carlsbad, CA) for examination of the types of ISs inserted and the insertion sites of the ISs. The purified PCR products were directly sequenced by using an ABI Prism 3100 DNA sequencer (Applied Biosystems, Foster City, CA). DNA

* Corresponding author. Mailing address: Department of Laboratory Medicine, Ajou University Hospital, San 5, Wonchun-Dong, Yeongtong-Gu, Suwon 443-721, South Korea. Phone: 82-31-219-5785. Fax: 82-31-219-5778. E-mail: weegyo@ajou.ac.kr.
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TABLE 1. Characteristics of 57 VRE isolates from 48 patients[e]

Strain identifier	Age (yr)/sex[a]	Service/floor[b]	Specimen type[c]	Date of isolation (mo/day/yr)	No. of days to isolation	No. of previous admissions	Mating frequency (no. of transconjugant/donor)	Tn*1546* type[d]
D43	1/F	ped/nicu	U	04/27/98	33	0	1.29×10^{-6}	Ia
D45	2/M	gs/gsw	W	05/03/98	7	0	3.50×10^{-7}	Ib
D3	64/M	ca/micu	C	06/15/98	40	2	2.00×10^{-5}	Ic
D49	67/M	ns/ncu	W	08/28/98	60	3	1.11×10^{-8}	Id
D6	75/F	ns/nsw	U	01/07/99	387	0	1.67×10^{-7}	Ic
D7	67/F	ns/ncu	C	01/17/99	167	0	3.00×10^{-7}	Ic
D52	22/F	ns/nsw	W	04/02/99	22	0	7.14×10^{-8}	Ic
D8	60/F	pul/mw	U	07/01/99	70	0	1.50×10^{-6}	Ib
D9	62/F	hema/mw	R	08/17/99	36	0	1.13×10^{-6}	Ic
D53	54/M	reh/nw	U	08/26/99	53	0	3.00×10^{-8}	Ib
D10	31/F	hema/mw	U	11/15/99	190	1	1.48×10^{-5}	Ib
D55	71/F	pul/micu	B	12/02/99	334	3	8.75×10^{-8}	Ic
D11	10/M	ped/sicu	P	02/08/00	10	0	2.67×10^{-7}	Ic
D57	21/M	pul/nsw	B	03/04/00	26	1	8.33×10^{-8}	Ie
D58	34/F	gs/mw	R	04/03/00	45	0	2.00×10^{-7}	Ic
D13	54/M	hema/gsw	P	05/13/00	36	2	2.44×10^{-5}	Ic
D14	75/M	cs/csw	B	06/19/00	21	0	3.75×10^{-5}	IIa
D59	34/M	ns/nsw	U	08/03/00	6	0	2.86×10^{-5}	Id
D15	73/F	pul/mw	B	08/19/00	66	0	2.50×10^{-7}	IIa
D60	69/F	pul/micu	B	11/06/00	181	0	1.00×10^{-5}	IIa
D16	65/F	pul/micu	R	01/02/01	76	1	4.62×10^{-5}	IIa
D61	87/F	ca/ccu	U	01/30/01	393	3	1.27×10^{-7}	IIb
D17	55/F	hema/micu	B	02/07/01	22	0	9.00×10^{-4}	IIa
D62	49/F	hema/mw	W	02/09/01	46	0	5.56×10^{-6}	IIa
D63	61/F	ge/mw	U	04/26/01	90	0	6.25×10^{-6}	IIa
D65	76/M	neph/micu	W	06/14/01	10	0	0	P
D19	76/M	neph/micu	R	07/09/01	35	0	8.89×10^{-5}	IIb
D20	76/M	neph/micu	U	07/18/01	44	0	4.29×10^{-7}	IIb
D21	76/M	neph/micu	R	08/13/01	70	0	3.00×10^{-8}	IIb
D22	76/M	neph/micu	U	08/17/01	74	0	2.00×10^{-7}	IIb
D23	76/M	neph/micu	R	08/20/01	77	0	7.14×10^{-7}	IIb
D24	57/M	pul/micu	Bo	09/07/01	43	7	2.14×10^{-8}	IIb
D66	72/M	ge/er	W	09/24/01	29	0	1.00×10^{-4}	IIb
D25	76/M	neur/nw	S	09/25/01	24	0	3.57×10^{-8}	IIb
D67	59/M	hema/micu	S	10/20/01	13	2	2.00×10^{-6}	IIb
D68	29/M	os/osw	R	11/05/01	127	0	1.54×10^{-7}	IIb
D70	48/M	ns/ncu	W	12/17/01	70	0	7.69×10^{-8}	IIb
D69	38/M	ca/mw	C	12/19/01	13	0	6.36×10^{-5}	IIa
D26	32/M	gs/sicu	Pe	12/20/01	41	0	1.33×10^{-8}	IIb
D27	32/M	gs/sicu	U	12/27/01	48	0	2.50×10^{-8}	IIb
D71	71/F	neph/mw	R	03/02/02	120	1	1.75×10^{-7}	IIb
D28	48/M	ns/ncu	R	03/02/02	145	0	4.00×10^{-5}	IIb
D29	48/M	ns/ncu	R	03/11/02	154	0	3.80×10^{-5}	IIb
D72	70/M	pul/er	C	05/16/02	48	0	1.38×10^{-6}	IIb
D30	18/M	pul/micu	R	06/05/02	278	0	4.71×10^{-7}	IIb
D73	41/M	hema/mw	U	08/18/02	27	5	5.00×10^{-7}	IIb
D31	69/F	pul/er	U	10/11/02	112	3	8.18×10^{-6}	IIc
D32	58/M	pul/mw	W	10/17/02	85	1	2.00×10^{-7}	IIb
D33	58/M	pul/mw	R	10/23/02	91	1	5.40×10^{-6}	IIb
D74	47/M	cs/sicu	W	11/16/02	24	0	0	P
D34	73/F	neur/er	U	01/31/03	44	1	1.64×10^{-6}	IIb
D37	60/M	pul/mw	U	10/23/03	71	0	1.54×10^{-7}	IId
D35	41/M	ca/ccu	C	10/26/03	43	0	2.22×10^{-5}	IIe
D36	62/F	ps/psw	C	10/31/03	41	0	1.30×10^{-7}	IIf
D38	1/M	ped/pedsw	R	01/11/04	81	0	7.27×10^{-5}	IIg
D39	31/M	reh/nw	W	02/09/04	28	0	1.43×10^{-8}	IIe
D40	55/M	gs/sicu	Pe	02/15/04	5	3	2.50×10^{-7}	IIh

[a] F, female; M, male.

[b] ped, pediatrics; gs, general surgery; ca, cardiology; ns, neurosurgery; pul, pulmonology; hema, hematology; reh, rehabilitation; cs, chest surgery; ge, gastroenterology; neph, nephrology; neur, neurology; os, orthopedic surgery; ps, plastic surgery; nicu, neonatal intensive care unit; gsw, general surgery ward; micu, medical intensive care unit; ncu, neurosurgical intensive care unit; nsw, neurosurgery ward; mw, medical ward; nw, neurology ward; sicu, surgical intensive care unit; csw, chest surgery ward; ccu, coronary care unit; er, emergency room; osw, orthopedic surgery ward; psw, plastic surgery ward; pedsw, pediatric surgery ward.

[c] U, urine; W, wound; C, catheter; R, rectal swap; B, blood; P, pleural fluid; BO, bone; S, sputum; Pe, peritoneal fluid.

[d] The 57 isolates were typed according to the distributions and positions of the IS elements and were named by their order of occurrence.

[e] Among 48 patients, two genetically unrelated VRE isolates were obtained from the same individual (a 32-year-old man on the general surgery service of the surgical intensive care unit and a 58-year-old man on the pulmonary service of the medical ward), three genetically unrelated VRE isolates were obtained from same individual (a 48-year-old man on the neurosurgery service of the neurosurgical intensive care unit), and six genetically unrelated VRE isolates were obtained from the same individual (a 76-year-old man under the care of the nephrology service section of the medical intensive care unit).

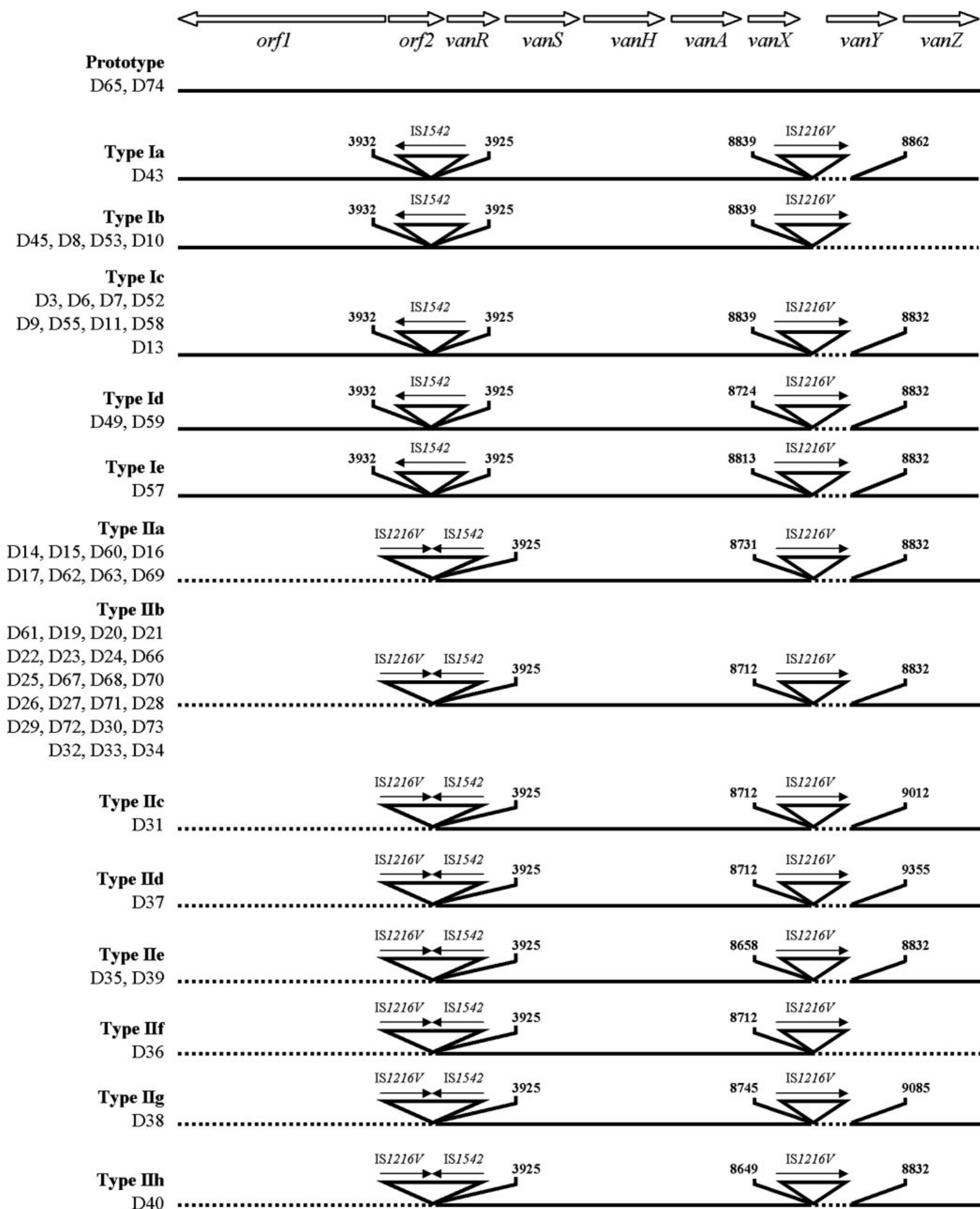

FIG. 1. Genetic maps of Tn*1546* types of *Enterococcus faecium* isolated at Ajou University Hospital. The positions of the genes and open reading frames (*orf1* and *orf2*) and the direction of transcription are marked by open arrows at the top. Inverted triangles represent IS elements. The positions of the first nucleotide upstream and the first nucleotide downstream from the IS insertion sites are depicted. Solid arrows indicate the transcriptional orientation of the inserted IS elements. Deletions are indicated by dotted lines.

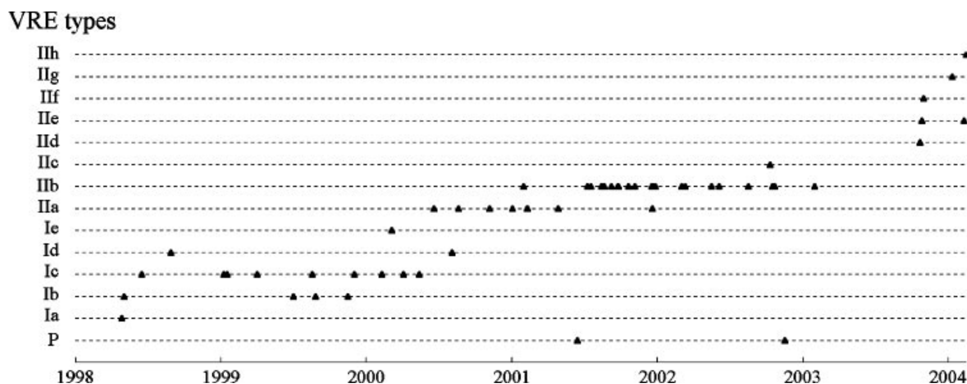

FIG. 2. Development of VRE isolates by year of this study (from 1998 to 2004). Each VRE isolation is represented by triangle.

fragments amplified with a combination of a Tn*1546*-specific primer and an IS*1216V*-specific primer were also purified and subsequently sequenced to determine the exact integration site and orientation of the IS*1216V* insertion. The DNASIS program for Windows (version 2.6; Hitachi Software Engineering, South San Francisco, CA) was used for sequence analysis. Hybridization was performed as described previously (10, 18) with probes specific for *vanY* and *vanZ*.

Transfer of resistance genes. Filter matings were performed by using *Enterococcus faecalis* JH2-2 as the recipient and all isolates as the donors, as described previously (15). Transconjugants were selected on brain heart infusion agar plates containing 50 μg/ml of rifampin, 20 μg/ml of fusidic acid, and 10 μg/ml of vancomycin. The transconjugants were examined for structural analysis of Tn*1546* by PCR of overlapping internal regions.

Annual vancomycin usage and VRE prevalence. We reviewed the total number of inpatients, the annual vancomycin usage, and annual rate of VRE from 1998 to 2004 using a laboratory information system.

RESULTS

PFGE. The total number of VRE isolates collected in Ajou University Hospital between 1998 and 2004 was 1,750. Among them, we selected 57 genetically unrelated VRE strains, as determined by PFGE typing (Table 1). According to Tenover et al. (14), we regarded the strains of VRE as "genetically unrelated" if they revealed seven or more band differences by PFGE.

Patient population and bacterial strains. The mean age of the patients was 52.8 ± 21.7 years, with a ratio of males to females of 6.5:3.5. A total of 57 VRE were recovered from cultures of urine, rectal swab, wound, blood, catheter tip, pleural fluid, peritoneal fluid, sputum, and bone from 48 different patients over a 7-year period (Table 1).

Structural analysis of Tn*1546*-like elements by PCR mapping and sequence analysis. Fifty-seven isolates were divided into three main types, including the prototype, according to the distribution of IS elements integrated into Tn*1546* elements. Each type was further divided according to the position of the insertion site of the IS elements. The subtypes were named by their order of occurrence (Fig. 1).

Seventeen strains isolated from 1998 to 2000 belonged to type I, which was characterized by an IS*1542* insertion in the *orf2-vanR* intergenic region and an IS*1216V* insertion in the *vanX-vanY* intergenic region. Type Ia had one copy of IS*1216V* at position 8839 and lost nucleotides 8840 to 8861. Type Ib had one copy of IS*1216V* at position 8839, with a total deletion adjacent to the insertion site of IS*1216V* at the right end of Tn*1546*. Type Ic had one copy of IS*1216V* at position 8839 and an 8-bp duplication of the target sequence. Type Id had one copy of IS*1216V* at position 8724 and lost nucleotides 8725 to 8831. Type Ie had one copy of IS*1216V* at position 8813 and lost nucleotides 8814 to 8831 (Fig. 1 and 2). Type Ic was the most prevalent among the isolates.

Thirty-eight strains isolated from 2000 to 2004 belonged to type II, which had two copies of IS*1216V* at the 3′ end of IS*1542* and in the *vanX-vanY* intergenic region, as well as IS*1542* in the *orf2-vanR* intergenic region. Eight of 38 isolates were type IIa, which had one copy of IS*1216V* at position 8731 and which lost nucleotides 8732 to 8831. Twenty-three of 38 isolates were type IIb, which had one copy of IS*1216V* at position 8712 and which lost nucleotides 8713 to 8831. The remaining isolates were types IIc, IId, IIe, IIf, IIg, and IIh, according to the position of the insertion site of IS*1216V* (Fig. 1 and 2). Type IIb was the most prevalent among the isolates.

The sequences of the remaining two strains, isolated in 2001 and 2002, respectively, were consistent with the Tn*1546* sequence of *E. faecium* BM4147 (Fig. 1 and 2).

Transfer of resistance genes. All 17 of the type I donors transferred vancomycin resistance at a mean frequency of 5.48×10^{-6} transconjugants per donor, and all 38 of the type II donors transferred vancomycin resistance at a mean frequency of 3.82×10^{-5} transconjugants per donor (the difference was not statistically significant). The structure of Tn*1546* of the transconjugants was indistinguishable from that of the donors (data not shown). No transconjugants were obtained by using the remaining two isolates of the prototype as donors (Table 1).

Annual usage of vancomycin and VRE prevalence. The annual usage of vancomycin had abruptly increased 10 times (i.e., from 27.4 mg per inpatient in 1998 to 275.2 mg per inpatient in 2000) (Fig. 3A). From 1998 to 2000, the prevalence of VRE also increased from 6% to 13.9% (Fig. 3B). It is remarkable that type II has been the main type that has been isolated since 2000 (Fig. 2).

DISCUSSION

The *vanA* gene cluster is carried as part of Tn*1546*-like elements (2). The genetic diversity in Tn*1546*-like elements, including the integration of IS elements IS*1216V*, IS*1251*, IS*1476*, and IS*1542*, has been documented previously (16, 17). Analysis of the IS insertion within a *vanA* gene cluster is a

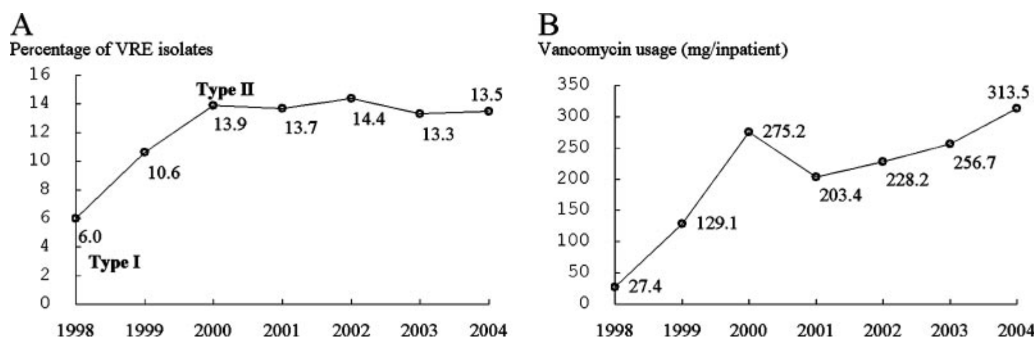

FIG. 3. (A) Percentage of VRE isolates from 1998 to 2004. Type I was the major type isolated from 1998 to 2000, and type II was the main type isolated since 2000. (B) Annual vancomycin usage from 1998 to 2004.

useful tool in epidemiological investigations (8). Tn*1546*-like elements may move more into previously susceptible enterococcal strains via plasmids or transposons (1, 4), particularly when VRE become endemic in a hospital (5, 11). The reason for the rapid spread of VRE can possibly be explained by such a mobility of Tn*1546*-like elements that have adapted to adverse environmental conditions (6).

In this study, the rearrangement of Tn*1546* appeared to have a tendency to reduce the lengths of their sequences by IS insertion as time passed. Type I, which had been detected from 1998 to 2000, was characterized by IS*1542* and IS*1216V* without a total deletion adjacent to the insertion site, except in type Ib. From 2000 to 2004, most VRE were found to be type II. Type II probably evolved from type I through a rearrangement of Tn*1546*. Type II strains have another insertion of IS*1216V* at the 3′ end of the IS*1542* of type I strains and a total deletion adjacent to the insertion site of IS*1216V*. With sequence shortening, the mean frequency of transfer in type II VRE was about sevenfold higher than that in type I. It is possible that such characteristics of type II VRE have played a role in the rapid dissemination of VRE at Ajou University Hospital since 2000.

Geographically, a difference in the genetic rearrangement of Tn*1546* by IS insertion is well known worldwide, including in Korea (8, 12, 17), and hence, the difference could be a useful molecular marker for local epidemiological studies. Furthermore, we discriminated the horizontal dissemination of VRE in more detail according to the position of the insertion site of IS*1216V*. At Ajou University Hospital, two subtypes, type Ib and type Ic, appeared to have played major roles in the hospital dissemination of VRE from 1998 to 2000, and type Ic was more frequent than type Ib. After June 2000, type IIa became the main type, followed by type IIb, with a period of overlap of the two subtypes of about 1 year. Interestingly, during the transition period in 2000, two strains revealed that types Id and Ie were isolated from patients hospitalized in the neurosurgery unit, which was on the floor where VRE were endemic. This was most likely due to a genetic transition which resulted from adaptation to the massive use of vancomycin in 2000. Likewise, six strains revealed that types IId through IIh were isolated from patients hospitalized on the coronary care unit, plastic surgery unit, and pediatric unit, which were not on the floor where VRE were endemic in 2004. No clear epidemiological links among the patients could be established, however, suggesting that no genetic rearrangement but, rather, that sporadic events had occurred. Further genetic analysis of VRE isolates collected after 2004 is needed.

Two isolates, isolates D65 and D74, represented the prototype. Strain D65 was isolated from a wound from a 76-year-old patient on the nephrology unit who did not receive vancomycin. Strain D74 was isolated from a wound from a 47-year-old patient on the chest surgery unit who received vancomycin for 10 days. However, there was no epidemiological link between the two patients, indicating that these two events could not be due to intrahospital transmission but, rather, were due to transmission from other sources, e.g., community acquisition or acquisition in another hospital.

In summary, the dominant type of the Tn*1546*-like element among strains of VRE disseminated in Ajou University Hospital was found to have changed from type Ic to type IIb, with a transient period of overlap. The rearrangement of Tn*1546* tended to reduce the lengths of their sequences as time passed, especially at the left or the right end, and may modulate their transferability.

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