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Distribution of Insertion Sequences Associated with Tn*1546*-Like Elements among *Enterococcus faecium* Isolates from Patients in Korea

Ji Young Huh,[1] Wee Gyo Lee,[1]* Kyungwon Lee,[2] Wan Shik Shin,[3] and Jin Hong Yoo[3]

Department of Laboratory Medicine, Ajou University School of Medicine, Suwon,[1] and Department of Laboratory Medicine, Yonsei University School of Medicine,[2] and Department of Internal Medicine, Catholic University School of Medicine,[3] Seoul, Korea

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The *vanA* gene cluster is carried as a part of Tn*1546*-like elements. The genetic diversity in Tn*1546*-like elements has been documented previously. The differences described thus far have included the integration of insertion sequence (IS) elements IS*1216V*, IS*1251*, IS*1476*, and IS*1542*. Among these, IS*1216V* has been reported to be widespread in VanA enterococci of diverse geographic areas, whereas IS*1542* and IS*1476* have been reported only in the United Kingdom and Canada, respectively. We investigated the distribution of ISs among 20 *vanA*-containing *Enterococcus faecium* isolates from human patients in nine different university hospitals in Korea. Pulsed-field gel electrophoresis (PFGE) was performed to identify the clonality of the isolates. Moreover, PCR amplification of the internal regions of Tn*1546* was performed for structural analysis of the *van* gene, and both DNA strands of the PCR amplicons were directly sequenced by the dideoxy termination method. The PFGE patterns revealed a high degree of clonal diversity. Structural analyses of the *van* gene detected IS*1542* and IS*1216V* in the genomes of all 20 isolates, whereas it did not detect IS*1476* or IS*1251* in the genomes of any of the isolates. In addition, IS*19* was detected in the *vanS-vanH* intergenic region of one isolate. These data indicate that identification of the IS within a *vanA* gene cluster could be a useful tool in epidemiological investigations. In addition, the distribution of ISs associated with Tn*1546*-like elements among the Korean isolates is therefore similar to that among European vancomycin-resistant enterococci.

Recently, vancomycin-resistant enterococci (VRE) containing the *vanA* gene have been isolated from humans and animals worldwide (21). Epidemiologic studies of VRE indicate that there are geographic differences. In Europe, the evolution of VRE is related to the use of avoparcin, which has been used to promote animal growth, and this is consistent with the potential spread of VRE from animals to humans (16, 19). In contrast, in the United States, where avoparcin has not been approved for use, VRE have been isolated only from hospitalized patients and not from healthy individuals or animals.

In Korea, VRE were first detected in 1992 (10). Until 1997, the prevalence of VRE in Korea was low; however, it rapidly increased thereafter. Shin et al. (15) reported that only 8 VRE isolates among 5,275 enterococci were detected from 1995 to 1997. In contrast, from 1998 to 2000 the number of VRE significantly increased to 325 isolates among 5,705 enterococci. Moreover, until 1998 avoparcin had been used in Korea, as it had in Europe, to enhance animal growth. In this context, VRE in Korea have been isolated not only from hospitalized patients but also from animal feces and raw meat (23). Thus, it is important to understand the underlying molecular mechanisms for the 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. Recently, the horizontal transfer of a resistance gene cluster has also been regarded as an important mechanism in this. Pulsed-field gel electrophoresis (PFGE) has been widely carried out to obtain an understanding of the clonal dissemination of VRE, and recently, structural analyses of the *van* gene have been introduced to obtain an understanding of the horizontal transfer of the resistance gene cluster. It has been known that the *vanA* gene cluster is carried as a part of Tn*1546*-like elements, and this indicates that the horizontal transfer of Tn*1546*-like elements plays an important role in the spread of *vanA*-type VRE. Therefore, investigation of the genetic variations among Tn*1546*-like elements would be essential to providing an understanding of the mechanism of spread of VRE, particularly in the case of horizontal gene transfer. The majority of the variations comprise integration of insertion sequences (ISs) with or without a deletion at the insertion site, point mutations, and deletions (2). In this study, we therefore analyzed *vanA*-containing enterococci isolated from diverse geographic areas in Korea by PFGE and molecular analysis of the Tn*1546*-like elements to elucidate the mechanism of spread of VRE.

MATERIALS AND METHODS

Bacterial strains. From 2000 to 2002, 20 clinical isolates of *vanA*-containing *Enterococcus faecium* were collected from nine different university hospitals in diverse geographic areas in Korea. Previously characterized VRE strain *E. faecium* BM4147 served as a control (1). Organisms were identified by conventional biochemical reactions, with the Vitek identification system (bioMérieux, Hazelwood, Mo.), and with the API 20 Strep system (bioMérieux).

* Corresponding author. Mailing address: Department of Laboratory Medicine, Ajou University Hospital, San 5, Wonchun-Dong, Paldal-Gu, Suwon 442-749, South Korea. Phone: 82-31-219-5785. Fax: 82-31-219-5778. E-mail: weegyo@ajou.ac.kr.

TABLE 1. Nucleotide sequences of PCR primers used in this study

Gene specificity and primer	Sequences (5′→3′)	Location (positions)	Amplicon size (bp)	Reference or source
Tn*1546*-specific primers				
42F	ATT TTC CTG ACG AAT CCC TCG	42–62	308	14
349R	TCG GAA AAC AAG GTG AGC TTA GA	349–327		14
164F	AAC CTA AGG GCG ACA TAT GGT G	164–185	758	14
921R	AAA AGG AGC CAC CAT CTA CCG	921–901		14
170F	AGG GCG ACA TAT GGT GTA ACA	170–190	1,744	5
1913R	CGT CCT GCC GAC TAT GAT TAT TT	1913–1891		5
949F	GCA TGT AGT GAT GAA ACA CCT AGC TGC	949–975	2,028	14
2976R	TGA AGA TGA ATG GAT ACT GGG GAC C	2976–2952		14
1871F	ACC GTT TTT GCA GTA AGT CTA AAT	1871–1894	1,856	5
3726R	AGC CCT AGA TAC ATT AGT AAT T	3726–3705		5
3514F	ACT GTA ATG GCT GGT GTT AAC	3514–3534	465	15
3978R	CAT AGT TAT CAC CCC TTT CAC TAT	3978–3956		5
3907F	ATG CTT ATA AAT TCG GCC C	3907–3925	888	5
4794R	ATC CAA TCC CCA AGT TTC CC	4794–4775		5
3992F	TTA TTG TGG ATG ATG AAC ATG	3992–4012	520	15
4511R	TCG GAG CTA ACC ACA TTC	4511–4494		15
4676F	AAC GAC TAT TCC AAA CTA GAA C	4676–4697	1,094	5
5769R	GCT GGA AGC TCT ACC CTA AA	5769–5749		5
5235F	ATA TCA CGT TGG ACA AAG C	5235–5253	1,801	15
7035R	TTA CGT CAT GCT CCT CTG AG	7035–7017		15
8082F	ACT TGG GAT AAT TTC ACC GG	8082–8101	424	14
8505R	TGC GAT TTT GCG CTT CAT TG	8505–8486		14
8448F	GAT GAA CGC TCT CAT CAT GC	8448–8467	691	1
9138R	TTC CTG AGA AAA CAG TGC TTC A	9138–9117		1
8544F	GCA TAT AGC CTC GAA TGG	8544–8561	1,037	15
9580R	TCG TCA AGC TTG ATC CTA C	9580–9562		15
10446F	AAT ACT GTT GGA GGC TTT CTT GG	10446–10468	132	14
10577R	GGT ACG GTA AAC GAG CAA TAA TAC G	10577–10553		14
IS*1216V*-specific primers				
650F	ACC TTC ACG ATA GCT AAG GTT			This study
132R	AGG ATT ATA TAA GAA AAC CCG			This study

PFGE. PFGE was performed on a CHEF-DR III apparatus (Bio-Rad Laboratories, Richmond, Calif.), as described previously (11). After digestion with SmaI, genomic DNA was separated by electrophoresis, with ramped pulse times beginning with 5 s and ending with 30 s at 6 V/cm for 20 h. The banding patterns were interpreted by Dice analysis and analysis by the unweighted pair group method with arithmetic averages with Bio-Gene software (Vilber Lourmat Inc., Marne-la-Vallée, France).

DNA extraction and PCR. Extraction of bacterial DNA was performed with a Qiagen DNeasy kit (Qiagen GmbH, Hilden, Germany), according to the instructions of the manufacturer. The vancomycin resistance genotypes were determined by PCR with primers specific for the *vanA*, *vanB*, *vanC1*, and *vanC2-vanC3* gene sequences, as described previously (3, 6). For structural analysis of Tn*1546*-like elements, overlapping PCR amplification of internal regions of Tn*1546* was performed. The primer sequences and target locations for specific Tn*1546* regions are listed in Table 1. Primers ISV650F and ISV132R were designed according to the published sequence of IS*1216V* with the OLIGO program (version 6.0; National Biosciences Inc., Plymouth, Minn.). The melting temperatures of the individual primers were calculated by using the OLIGO program (National Biosciences Inc.). To determine the exact left end of the truncated Tn*1546*-like elements, genomic DNA from all isolates was amplified with a combination of Tn*1546*-derived primer 4511R and IS*1216V*-specific primers (primers ISV650F and ISV132R).

Sequence analysis. PCR amplicons larger than that of the prototype *vanA* gene cluster were purified with GeneClean kits (Qbiogene Inc., Carlsbad, Calif.). The purified PCR products were directly sequenced by using an ABI Prism 3100 DNA sequencer (Applied Biosystems, Foster City, Calif.). DNA fragments amplified with a combination of a Tn*1546*-specific primer and 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, Calif.) was used for sequence analysis.

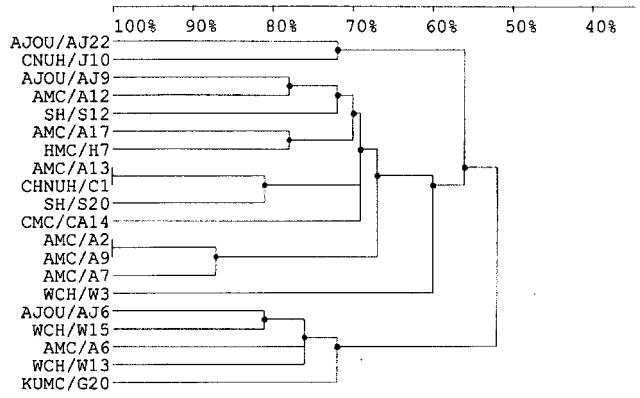

FIG. 1. Dendrogram produced following Dice analysis and analysis by the unweighted pair group method with arithmetic averages of the PFGE patterns of *E. faecium* isolates from hospitals.

RESULTS

PFGE. All isolates were largely heterogeneous in nature. The isolates formed only one cluster when a similarity cutoff of 85% was used (Fig. 1).

Structural analysis of Tn*1546*-like elements by PCR mapping and sequence analysis. All isolates were divided into three main types according to the distribution of ISs integrated into Tn*1546* elements (Table 2 and Fig. 2). 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 characterized by two copies of IS*1216V* at the left ends of Tn*1546*-like elements and in the *vanX-vanY* intergenic region as well as IS*1542* in the *orf2-vanR* intergenic region. Type III was characterized by the presence of IS*19* in the *vanS-vanH* intergenic region, in addition to IS*1542* in the *orf2-vanR* intergenic region and IS*1216V* in the *vanX-vanY* intergenic region. No isolates were identical to the prototype strain.

IS*1216V* was present in the *vanX-vanY* intergenic regions of the genomes of all 20 isolates, but at various points of integration. Among these 20 isolates, the insertions in 15 isolates were accompanied by small deletions adjacent to the insertion site (Fig. 2). Also, IS*1216V* was present at the left ends of Tn*1546*-like elements of 12 isolates (type II), with or without large deletions encompassing the *orf1* and/or *orf2* region. IS*1542* was detected in the *orf2-vanR* intergenic regions of all 20 isolates. The location of the IS*1542* insertion in this region was identical to that described previously (4, 14, 22), corresponding to nucleotide 3932 of Tn*1546* with an 8-bp duplication of the target sequence (CTATAATC). In type IIc isolates (Fig. 2), the 3′ end of IS*1542* was deleted at various points by an IS*1216V* insertion. IS*19* was detected in only one isolate and was integrated at nucleotide 5813 with an 8-bp duplication of the target sequence (GATGTATA).

DISCUSSION

After the first identification of VRE in Korea in 1992, several outbreaks of VRE associated with clonal spread were observed. Recently, however, the horizontal transfer of the resistance gene cluster has also been regarded as the main

TABLE 2. Structural analysis of Tn*1546*-like elements by overlapping PCR

Tn*1546*-like element type	No. of isolates	PCR product with the following primer pairs[a]:										
		42–349	164–921	170–1891	949–2976	1871–3726	3514–3978	3907–4794	3992–4511	4676–5769	5735–7035	8082–8505	8448–9138	8544–9580	10446–10577
BM4147	0	+	+	+	+	+	+	+	+	+	+	+	+	+	+
I	7	−	+	+	+	+	+	+	+	+	+	+	+	+	+
IIa	1	−	++	++	+	+	++	++	+	+	+	+	+	+	+
IIb	7	−	−	−	−	−	++	++	+	+	+	+	+	+	+
IIc	4	−	−	−	−	−	++	++	+	+	+	+	++	+	+
III	1	−	+	+	+	+	++	++	+	+	+	+	++	+	+

[a] + and ++, amplicons the same size as and larger than those obtained for *E. faecium* BM4147, respectively; −, absence of an amplicon with the particular pair of primers. The numbers in each primer pair correspond to the first numbers of the positions listed in Table 1.

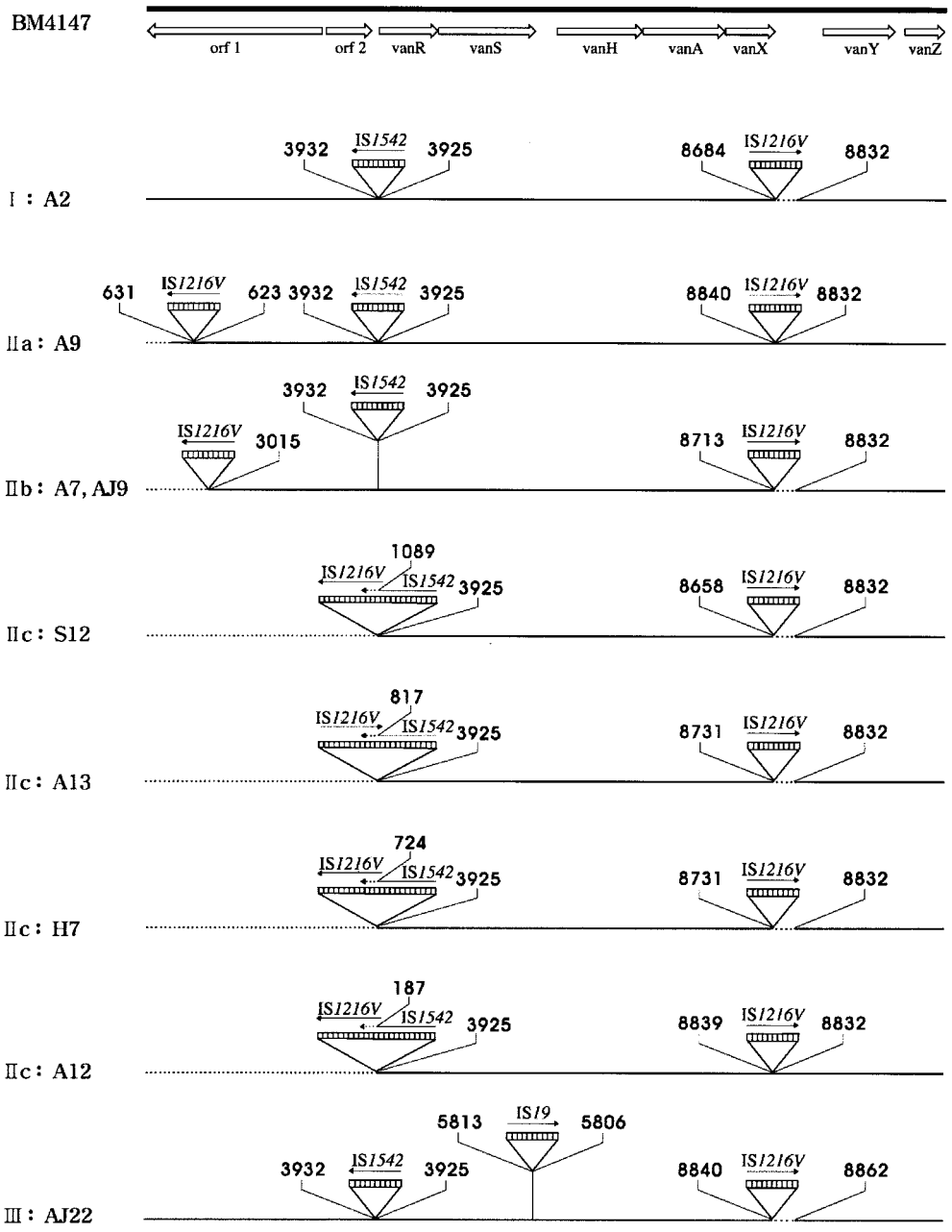

FIG. 2. Genetic maps of Tn*1546* types of *E. faecium* isolates from Korean hospitals. The positions of genes and open reading frames (*orf1* and *orf2*) and the direction of transcription are marked by open arrows at the top. Boxes with vertical lines represent IS elements. The positions of the first 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.

mechanism in the dissemination of the *van* gene (11, 15). VanB VRE were predominantly isolated in Korean hospitals in the initial years, whereas recent isolates (those recovered from 1998 to 2000) were mostly of the VanA type (11, 15). Therefore, structural analysis of the *vanA* gene cluster is critical to investigations of the epidemiology of *vanA*-containing enterococci from Korean hospitals.

The *vanA* gene cluster is carried as a part of Tn*1546*-like elements. The heterogeneity of Tn*1546* has previously been reported and comprises point mutations, deletions, and the integration of IS elements. Among these variations, the presence of IS elements accounts for much of the heterogeneity. Until recently, IS*1216V*, IS*1542*, IS*1251*, and IS*1476* were reported in VanA VRE. IS*1216V* is known to be ubiquitous in *vanA* elements (9, 20), whereas the other three IS elements appear to be geographically restricted. For example, IS*1542* is frequently found in clinical and poultry VRE isolates from the United Kingdom and Ireland (14, 22). IS*1251* and IS*1476* have been reported in the *vanA* elements of enterococci from the United States (5, 7) and Canada (12), respectively.

In this study we characterized the structures of the Tn*1546*-like elements among *E. faecium* isolates from patients admitted to hospitals in different parts of Korea and typed the strains by PFGE. By using a combination of overlapping PCR and sequencing analysis, three main types of *vanA* gene clusters were identified according to the distributions of ISs, i.e., IS*1216V*, IS*1542*, and IS*19*, in the Tn*1546*-like elements. In contrast, IS*1476* and IS*1251* were not detected in the Korean isolates. IS*1216V* and IS*1542* were identified in the genomes of all isolates from Korean hospitals. In type IIb and IIc isolates, IS*1216V* was inserted at the left ends of the *vanA* elements, with a deletion that included the *orf1* and/or *orf2* regions. IS*1542* was found in the Tn*1546*-like element at exactly the same position described previously (4, 14, 22). Interestingly, the 3′ end of IS*1542* belonging to type IIc isolates was deleted at various points by the IS*1216V* insertion. This finding suggests that IS*1216V* at the left end of Tn*1546* was acquired later than IS*1542*. Moreover, importantly, to our knowledge, our study is the first to demonstrate the presence of IS*19* in the *vanS-vanH* intergenic region of the *vanA* gene cluster. Perichon et al. (13) reported that IS*19* was inserted in the D-Ala–D-Ala ligase gene of VanD strain *E. faecium* BM4416, resulting in inactivation of the *ddl* ligase. However, IS*19* has never been documented in Tn*1546*-like elements.

The movement of ISs frequently causes structural alterations in Tn*1546*-like elements. Furthermore, several investigators have documented the functional changes associated with IS integration with or without the adjacent deletion, as in the loss of VanY activity by an IS*1476* insertion (12) and inactivation of the *ddl* ligase by IS*19* (13). In our study, it was unlikely that the integration of an IS would affect the function of the *vanA* gene cluster.

The genetic differences among Tn*1546*-like elements have been investigated in several previous studies (2, 8, 9, 17, 18, 20, 22). However, the Tn*1546* subtypes of the enterococci investigated were not comparable, since various molecular techniques were used. Before the use of overlapping PCR in order to analyze the structures of Tn*1546*-like elements, we performed long-PCR amplification with subsequent restriction fragment length polymorphism (RFLP) analysis, as described previously (17). Eleven of the 20 isolates failed to yield a product by long PCR, since they were types IIb and IIc by the overlapping PCR. Failure of long-PCR amplification was explained by deletions in the proximal *orf1* primer binding region of the *vanA* gene cluster. This result suggests that the long PCR with RFLP analysis is not suitable for typing of the VanA transposons of Korean isolates. By using overlapping PCR and sequencing analyses, the Tn*1546* subtypes could be compared with those described previously. To our knowledge, type I in this study likely corresponds to type B of the European isolates reported by Willems et al. (20), except for the IS*1542* insertion. Type II corresponds to type E. In particular, type IIc appears to be identical to subtype E13 (14, 20). All types of the Tn*1546*-like elements in this study harbored IS*1542*, which is restricted to Europe, mainly the United Kingdom. Thus, the distribution of ISs in Tn*1546*-like elements among Korean VRE isolates is similar to that among European VRE isolates rather than to that among American VRE isolates. The identification of ISs within the *vanA* gene cluster to analyze and compare the structures of Tn*1546*-like elements could be a useful tool in epidemiological studies.

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