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Molecular Characterization of Vancomycin-Resistant *Enterococcus faecium* Isolates from Mainland China▽

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Little is known about vancomycin-resistant enterococci in China. Thirteen pulsed-field gel electrophoresis-confirmed heterogeneous VanA-type vancomycin-resistant *Enterococcus faecium* (VRE) isolates were obtained from five Chinese hospitals from 2001 to 2005. The isolates were typed by multilocus sequence typing into nine different sequence types (STs), including five new STs (ST18, ST25, ST78, ST203, ST320, ST321, ST322, ST323, and ST335). Vancomycin resistance in each isolate was encoded on conjugative plasmids; two of the plasmids, pZB18 (67 kbp) and pZB22 (200 kbp), were highly conjugative and were able to transfer at high frequencies of around 10^{-4} and 10^{-7} per donor cell in broth mating, respectively. None of the plasmids identified in these isolates carried *traA*, which is usually conserved in the pMG1-like highly conjugative plasmid for *E. faecium*, implying that pZB18 and pZB22 were novel types of a highly conjugative plasmid in enterococci. Thirteen Tn*1546*-like elements encoding VanA-type VRE on the conjugative plasmids were classified into six types (types I to VI), and most of them contained both IS*1216V* and IS*1542* insertions. The isolates carrying the type II element were predominant. The six type elements were different from that of a VanA-type *Enterococcus faecalis* strain isolated from Chinese chicken meat. The results suggested that the disseminations of VRE in these areas were by Tn*1546*-like elements being acquired by the conjugative plasmids and transferred among *E. faecium* strains.

The isolation of vancomycin-resistant enterococci (VRE) was first reported in 1988 in the United Kingdom and France (17, 29) and then in hospitals in the United States (20). VRE are now encountered in many countries, especially in Europe and the United States (2). There are several reports describing the isolation of VRE in East Asian regions and countries, including Japan, Korea, and Taiwan, and isolation frequencies of VRE from patients and food animals have been increasing both in Korea and in Taiwan (15, 34). Since the first Japanese report of a VanA-type VRE (*Enterococcus faecium*) clinical isolate in 1996, the frequencies of VRE isolation from patients have also increased (7, 18). However, little information is available on the prevalence of VRE and their molecular makeup from mainland China, although glycopeptide antimicrobials have been used there for decades.

VanA-type resistance, characterized by high-level inducible vancomycin resistance (MICs of 64 to >1,024 μg/ml) and teicoplanin resistance (MICs of 16 to >512 μg/ml), is most frequently encountered (19). The genes encoding VanA-type vancomycin resistance are located on mobile Tn*1546*-like elements; therefore, the horizontal transfer of resistance genes among enterococci has a more significant impact on the dissemination of VRE than does the clonal spread of resistant enterococci (14). Epidemiological studies of VanA-type enterococci indicate that there are geographic differences (22). Considerable diversity has been identified in the Tn*1546*-related elements. This variation, in the form of point mutations, insertion sequence (IS) elements, and deletions, has been exploited in several epidemiological studies (33, 31).

Vancomycin has been used in patient care in mainland China for 40 years, and its usage is increasing. Our group has previously described vancomycin-dependent VanA-type VRE strains isolated in Japan from retail chicken meat imported from China (25). Clinical isolates of VanA-type VRE (*E. faecium*) are rarely obtainable from China. Over the past five years, we have obtained a total of 13 clinical strains of VanA-type VRE (*E. faecium*) from China. The current report is the first to describe molecular characterization of VanA-type VRE from mainland China.

MATERIALS AND METHODS

Bacterial strains and culture media. Thirteen clinical isolates of vancomycin-resistant *E. faecium* recovered from blood cultures and urine and sputum samples from patients in China were used in this study (Table.1). VanA-type vancomycin-resistant *Enterococcus faecalis* strain KC122.1, isolated in Japan from chicken meat imported from China in 2001, was used for the comparative analysis of Tn*1546*-like elements (25). *E. faecium* strains BM4105RF and BM4105SS were used as recipient strains for transfer experiments (13). Enterococci were grown in Todd-Hewitt broth (THB).

Antimicrobial susceptibility testing. Glycopeptide resistance levels were determined by the agar dilution method. An overnight pure culture of each strain grown in Mueller-Hinton broth (Nissui, Tokyo, Japan) was diluted 100-fold with fresh broth. An inoculum of approximately 5×10^5 cells was plated on a series of Mueller-Hinton agar plates (Eiken, Tokyo, Japan) containing a range of concentrations of the test drug. The plates were incubated at 37°C, and the susceptibility results were finalized at 24 h of incubation. Susceptibility testing and interpretation of results were in compliance with standards recommended by

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TABLE 1. Chinese VanA-type vancomycin-resistant *E. faecium* clinical isolates[a]

Strain	Hospital (city)	Date of isolation (yr/mo/day)	Source	Diagnosis/underlying disease[b]	Antibiotic(s) used	MLST result[c]							
						Sequence type (nearest)	Clonal complex	Allelic profile					
								atpA	*ddl*	*gdh*	*purK*	*gyd*	*pstS*	*adkC*
I125	E (Dalian)	2001/12/9	Sputum	Cachexia/gastric cancer	CEPs, CLI	ST78	CC78	15	1	1	1	1	1	1
C264	A (Beijing)	2003/8/21	Bile	Cholangitis/hepatocirrhosis	VAN, CEPs	ST320* (ST80)	CC117	9	1	1	1	12	29	1
ZB11	A (Beijing)	2004/1/15	Urine	Pyelitis/gastric cancer	VAN	ST335* (ST172)	CCS	45*	13	34*	15	19	29	18*
ZB14	B (Beijing)	2004/2/13	Urine	Pyelitis/leukemia	CEPs	ST321* (ST31)	CC280	7	3	1	1	1	1	3
ZB15	B (Beijing)	2004/2/27	Urine	Pyelitis/kidney dysfunction	TEC	ST78	CC78	15	1	1	1	1	1	1
ZB16	B (Beijing)	2004/4/4	Urine	Pyelitis/hepatocirrhosis	CEPs	ST18	CC18	7	1	1	1	5	1	1
ZB18	B (Beijing)	2004/4/9	Blood	Sepsis/leukemia	VAN, CEPs	ST25	CC25	9	3	1	6	1	1	1
ZB19	C (Beijing)	2005/10/18	Sputum	Pneumonia/pulmonary dysfunction	VAN, CEPs	ST322* (ST262)	CC18	7	1	6	1	5	7	1
ZB20	C (Beijing)	2005/10/20	Urine	Pyelitis/rectal cancer	VAN, CEPs	ST203	CC78	15	1	1	1	1	20	1
ZB21	D (Tianjin)	2005/10/12	Blood	Sepsis/hepatocellular cancer	VAN, MEM	ST78	CC78	15	1	1	1	1	1	1
ZB22	B (Beijing)	2005/10/28	Sputum	Pneumonia/COPD	VAN, MEM	ST323* (ST17)	CC17	5	1	1	1	1	1	1
ZB23	C (Beijing)	2005/11/7	Sputum	Pneumonia/bronchiectasis	CEPs	ST203	CC78	15	1	1	1	1	20	1
ZB24	C (Beijing)	2005/11/8	Urine	Pyelitis/COPD	CEPs	ST203	CC78	15	1	1	1	1	20	1

[a] Drug abbreviations: CEPs, broad-spectrum cephalosporins; CLI, clindamycin; VAN, vancomycin; TEC, teicoplanin; MEM, meropenem; AMP, ampicillin; GEN, gentamicin; STR, streptomycin; TET, tetracycline; ERY, erythromycin; CHL, chloramphenicol; RIF, rifampin; LVX, levofloxacin; LZD, linezolid.
[b] COPD, chronic obstructive pulmonary disease.
[c] *, new ST/allele in this study.
[d] The *esp* gene was detected by PCR amplification, as described in the text. P, positive; N, negative.
[e] Tn*1546*-like elements were typed by DNA sequence structure, as shown in Fig. 4.
[f] The wild-type strain and *E. faecium* BM4105RF were used as donor and recipient, respectively (13).
[g] Plasmids were identified in our previous report (35).

Clinical Laboratory Standards Institute (formerly NCCLS). *E. faecium* ATCC 9790 was used as a control strain.

Plasmid DNA methods. Recombinant DNA methodology, analyses of plasmid DNA with restriction enzymes, and agarose gel electrophoresis were carried out by standard methods (21). PCR was performed with a Perkin-Elmer Cetus apparatus. Specific primers for DNA sequencing of the Tn*1546*-like element and insertion sequences were designed as previously reported and purchased from Invitrogen (11, 31, 22, 34). Sequence analysis was performed with a dye terminator cycle sequencing kit (Applied Biosystems) and a model 310 gene analyzer (ABI PRISM).

Conjugation experiments. Broth matings were performed as previously described with a donor/recipient ratio of 1:10 (5, 12). Overnight cultures of 0.05 ml of the donor and 0.45 ml of the recipient were added to 4.5 ml of fresh THB, and the mixtures were incubated at 37°C with gentle agitation for the appropriate times and then vortexed. Portions of the mixed cultures were then plated on solid media with appropriate selective antibiotics. Colonies were counted after 48 h of incubation at 37°C. Filter matings were performed as previously described with a donor/recipient ratio of 1:10 (6). Overnight cultures were prepared, 0.05 ml of the donor and 0.5 ml of the recipient were added to 4.5 ml of fresh THB, and the cells were then trapped on a membrane filter (Millipore, Bedford, MA). The cells on the filters were incubated at 37°C overnight and were then suspended in 1 ml of THB. Appropriate dilutions of the mixture were transferred to plates of solid medium containing selective antibiotics. Throughout the mating experiments, the antibiotic concentration used for the selection of vancomycin-resistant transconjugants was 6 μg/ml. The antibiotic concentrations used for the selection of rifampin- and fusidic acid-resistant recipient strains or streptomycin- and spectinomycin-resistant recipient strains were 25 and 25 μg/ml or 250 and 250 μg/ml, respectively.

PFGE. Pulsed-field gel electrophoresis (PFGE) was then carried out in a 1% agarose gel with 0.5% Tris-borate-EDTA buffer, and the following settings were applied: 1 to 23 s, 6 V/cm, and 22 h (with the CHEF Mapper system [Bio-Rad]) (18).

DNA-DNA hybridization. Southern hybridization was performed with the digoxigenin-based nonradioisotope system of Boehringer GmbH (Mannheim, Germany), and all procedures were based on the manufacturer's manual (21).

MLST analysis. Multilocus sequence typing (MLST) analysis of *E. faecium* isolates was performed as previously reported (10, 32). The alleles and sequence types (STs) were analyzed and determined through the MLST database (http://efaecium.mlst.net/). The new alleles and new STs identified in this study have been deposited in the database.

Detection of the *esp* gene. To detect the *esp* gene of the *E. faecium* isolates, PCR amplification was performed as previously described with specific primers (16).

Detection of the *traA* gene of a pMG1-like plasmid. To detect the *traA* gene in the conjugative plasmids, PCR amplification was performed with specific primers (traA-F, TGAGAAAGAAATCGCTGATG; traA-R, TGAAGGCGTTCTTCT TTCAG), as previously described (24, 28).

RESULTS AND DISCUSSION

Isolation and characterization of VanA-type vancomycin-resistant *E. faecium*. The characteristics of the 13 isolates of vancomycin-resistant *E. faecium* are listed in Table 1. Each VRE strain was isolated from an individual patient in the hospital. In all cases, glycopeptide antibiotics and/or broad-spectrum cephalosporins were administrated to the patient before the isolation of VRE. All isolates were multidrug resistant, with MICs of 256 to 512 μg/ml and 16 to 512 μg/ml for vancomycin and teicoplanin, respectively. All 13 *E. faecium* isolates were resistant to erythromycin, and 11 isolates (85%) also showed high-level resistance to ampicillin and gentamicin. All isolates were sensitive to linezolid.

The PFGE profiles of SmaI-digested chromosomal DNA demonstrated that strain ZB23 was closely related to strain ZB24, differing by only one band; the other 11 isolates were largely heterogeneous in nature (Fig. 1), indicating that these isolates were unrelated and suggesting that vancomycin resistance was able to emerge in different *E. faecium* strains.

MLST analysis. All isolates were analyzed by the MLST scheme for *E. faecium* described previously (10, 32; http://www.mlst.net). Allelic profiles of these *E. faecium* isolates were obtained by sequencing of internal fragments of seven housekeeping genes—*atpA*, *ddl*, *gdh*, *purK*, *gyd*, *pstS*, and *adkC*—and STs were determined (Table 1). In strain ZB18, three of seven alleles, *atpA*, *adh*, and *pdkC*, belonged to the new alleles, and the closest homologue alleles found were between *atpA* and *atpA15* (81% identity), *adh* and *gdh19* (99% identity), and *pdkC* and *adkC8* (81% identity). Based on the allelic profiles of the 13 isolates, three belonged to ST78 and three to ST203,

TABLE 1—*Continued*

esp status[d]	Tn*1546*-like element		Transfer frequency of vancomycin resistance[f] (per donor cell)		MIC (μg/ml)										
	Type[e]	Location	Broth mating (4 h)	Filter mating (16 h)	VAN	TEC	AMP	GEN	STR	TET	ERY	CHL	RIF	LVX	LZD
N	VI	Plasmid (pI125V[g])	$<10^{-7}$	1.6×10^{-4}	512	16	128	>1,024	2048	0.5	512	8	4	16	2
N	V	Plasmid (pC264V[g])	$<10^{-7}$	2.3×10^{-7}	256	32	128	>1,024	64	0.5	512	32	4	32	2
N	II	Plasmid	$<10^{-7}$	3.0×10^{-5}	512	256	0.25	>1,024	32	0.5	512	16	512	1	2
N	II	Plasmid	$<10^{-7}$	3.7×10^{-6}	512	256	128	>1,024	>4,096	0.25	512	8	4	4	2
N	II	Plasmid	$<10^{-7}$	1.4×10^{-3}	512	256	128	>1,024	32	0.25	512	16	4	64	2
N	II	Plasmid	$<10^{-7}$	5.7×10^{-6}	512	256	128	>1,024	16	8	512	8	4	64	2
N	I	Plasmid (pZB18)	3.1×10^{-4}	7.0×10^{-1}	512	256	2	16	2,048	256	256	64	<0.1	2	2
P	II	Plasmid	$<10^{-7}$	1.7×10^{-5}	512	512	128	>1,024	32	0.25	512	16	8	128	2
P	III	Plasmid	$<10^{-7}$	1.8×10^{-5}	256	64	128	>1,024	64	64	512	16	8	32	2
P	IV	Plasmid	$<10^{-7}$	2.8×10^{-6}	512	16	128	128	32	0.25	512	16	4	64	2
P	II	Plasmid (pZB22)	4.2×10^{-7}	8.5×10^{-3}	512	512	128	>1,024	32	64	512	4	4	64	2
P	II	Plasmid	$<10^{-7}$	2.7×10^{-6}	512	256	128	>1,024	32	0.5	512	16	<0.1	64	2
P	II	Plasmid	$<10^{-7}$	3.1×10^{-6}	512	256	128	>1,024	32	0.25	512	16	<0.1	64	2

and one isolate belonged to ST18 and another to ST25. The remaining five isolates were new STs, designated as ST320, ST335, ST321, ST322, and ST323 (Table 1; http://efaecium.mlst.net/ [accessed 10 February 2007]).

The nearest relation to each of the five new STs was ST80, ST172, ST31, ST262, and ST17, respectively. In total, nine STs (ST323, ST18, ST322, ST25, ST78, ST203, ST320, ST321, and ST335) of the Chinese isolates were categorized into seven clonal complexes (CC), as follows; CC17 (ST323), CC18 (ST18 and ST322), CC25 (ST25), CC78 (ST78 and ST203), CC117 (ST320), CC280 (ST321), and CCS (ST335). Previous reports showed that CC17 strains, which are frequently isolated as hospital outbreak strains, have a genetic lineage to ampicillin resistance and pathogenicity islands containing the *esp* gene (16, 30, 32). The clonal complexes of the Chinese isolates were genetically linked to each other and were close to CC17, except for CCS. All isolates were ampicillin resistant, except for two isolates belonging to CCS and CC25, which were relatively far from the other CCs. Most previously reported hospital outbreak isolates are ampicillin resistant and positive for the *esp* gene (16). However, only 6 of the 11 ampicillin-resistant isolates in this study were found to carry the *esp* gene.

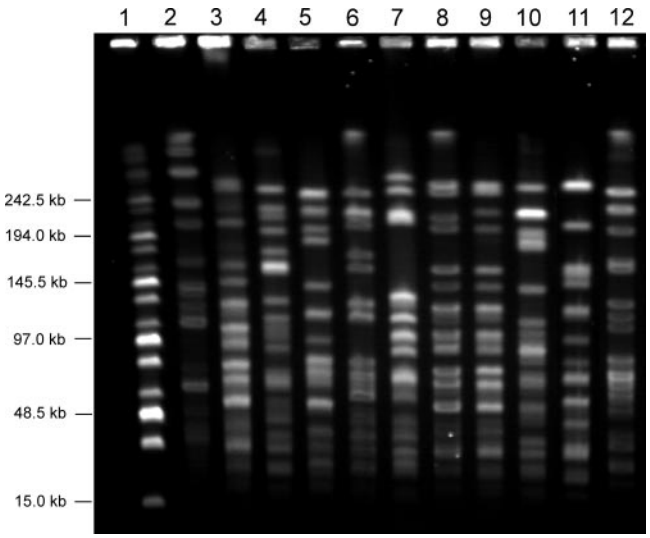

FIG. 1. PFGE of SmaI-digested chromosomal DNAs. Lane 1, molecular mass marker (Midrange Molecular Marker; New England Biolabs); lanes 2 to 12, plasmid DNAs from strains ZB11, ZB14, ZB15, ZB16, ZB22, ZB19, ZB23, ZB24, ZB21, ZB18, and ZB20, respectively.

Analysis of VanA-type vancomycin resistance genes encoded on Tn*1546*-like elements. Since, most Tn*1546*-like elements encoding VanA-type vancomycin resistance are plasmid borne (4), all of the isolates in this study were examined for plasmid content and location of the *vanA* gene by Southern hybridization. The EcoRI restriction profiles of total plasmid DNAs isolated from the VRE strains showed that the plasmids of strain ZB23 were identical to those of strain ZB24 and that the plasmids of strain ZB14 were closely related to those of strain ZB15 (Fig. 2A). Other strains showed heterogeneous plasmid patterns. The *vanA* probe hybridized to an EcoRI fragment in plasmid DNA from each of the strains (Fig. 2B). These results indicated that all of the VanA determinants (Tn*1546*-like elements) were carried on plasmids in these VRE strains. The transferabilities of the vancomycin-resistant traits of the strains were examined by broth mating and filter mating with *E. faecium* BM4105RF used as the recipient strain (Table 1). Vancomycin resistance was transferred at frequencies from 10^{-1} to 10^{-7} per donor cell by filter mating among the strains studied. The vancomycin resistance of ZB18 and ZB22 was transferred at frequencies of 10^{-4} and 10^{-7} per donor cell by broth mating, respectively. All of the vancomycin resistance plasmids were self-transferable or mobile. Two highly conjugative vancomycin resistance plasmids, pZB18 (67 kbp) and pZB22 (200 kbp), were isolated from strains ZB18 and ZB22, respectively (Fig. 3). There are two kinds of highly conjugative plasmids found in enterococci, including pheromone-responsive plasmids of *E. faecalis* and pMG1-like plasmids of *E. faecium* (3, 13). We previously discovered the highly conjugative gentamicin resistance plasmid pMG1 (65 kbp) from an *E. faecium* clinical isolate in Japan (13). pMG1-like plasmids were widely disseminated in vancomycin-resistant *E. faecium* clinical isolates obtained from a hospital in the United States (27). Recently, we also isolated pMG1-like plasmids carrying Tn*1546*-like transposons that encode vancomycin resistance in *E. faecium* clinical isolates in Japan (26, 28). All of the pMG1-like plasmids carry a conserved *traA* gene which is involved in the *tra* gene

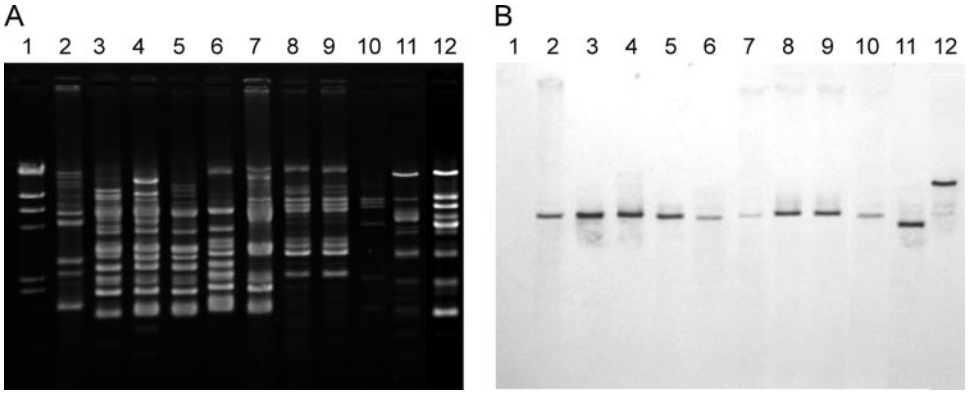

FIG. 2. Agarose gel electrophoresis of EcoRI-digested plasmid DNAs from *E. faecium* isolates (A) and Southern hybridization analysis with the *vanA* probe (B). Lanes 1, HindIII-digested lambda DNA; lanes 2 to 12, plasmid DNAs from strains ZB11, ZB14, ZB15, ZB16, ZB22, ZB19, ZB23, ZB24, ZB21, ZB18, and ZB20, respectively.

system for conjugation and is pMG1 specific (24). pZB18 and pZB22 were examined by PCR amplification to determine whether *traA* was conserved in each of these plasmids. Neither of plasmids carried the *traA* gene. The result implied that both pZB18 and pZB22 were different from the pMG1-like plasmids and could be a new type of highly conjugative *E. faecium* plasmid, as previously reported (23).

The DNA sequences of the Tn*1546*-like elements encoding the *vanA* operon for vancomycin resistance on the plasmids were determined (Fig. 4) (1). Specific primers for the insertion sequences IS*1216V* (809bp) and IS*1542* (1,324bp), which are often found in Tn*1546*-like elements, were used in the sequence analysis (8, 31, 33).

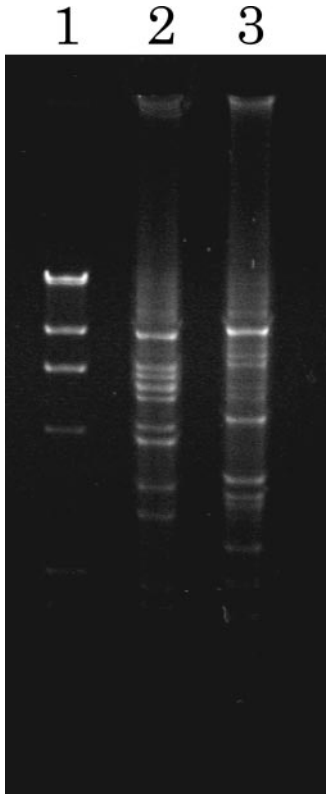

FIG. 3. Agarose gel electrophoresis of the EcoRI-digested highly conjugative plasmid DNA of pZB18 and pZB22. Lane 1, HindIII-digested lambda DNA; lane 2, pZB18; lane 3, pZB22.

A summary of the sequence analysis of the plasmid Tn*1546*-like elements and their comparison to the prototype element (designated type I in this study) are shown in Fig. 4. The *vanS* genes of all of the Chinese isolates were identical to that of the BM4147 strain and had no substitutions. Three specific substitutions within VanS result in low-level teicoplanin resistance, which is frequently found in East Asian VRE isolates (9, 15, 18, 34). The Tn*1546*-like elements of the 13 isolates were classified into six types based on sequence analysis and were designated type I to type VI (Fig. 4). We have reported two VanA-type VRE (*E. faecium*) clinical isolates, C264 and I125, which were originally isolated from patients in China (35). The Tn*1546*-like elements of both strains contained the insertion sequences IS*1216V* and IS*1542* and are classified as type V and type VI, respectively (Fig. 4).

Our group reported the first case of VanA-type *Enterococcus faecalis*: strain KC122.1, isolated in Japan from chicken meat imported from mainland China in 2001 (25). The Tn*1546*-like element was encoded on a conjugative plasmid and had three specific substitutions of VanS, resulting in low-level teicoplanin resistance, as mentioned above. The Tn*1546*-like element of KC122.1 was also examined in this study (Fig. 4). The element was identical to the prototype (type I) except for five point mutations, including the three substitutions within VanS, and had no insertions, suggesting that the origins of the elements of VRE clinical isolates were different from that of the VRE isolate from food animals.

Analytical data for the Tn*1546*-like elements of Chinese VRE isolates can be summarized as follows: (i) all of the elements are plasmid borne; (ii) 12 of the 13 isolates had multiple insertions of IS*1216V* and IS*1542* in the Tn*1546*-like elements; (iii) IS*1542* was inserted into the 8-bp target sequence CTATAATC, from bp 3817 to 3924 of Tn*1546*; (iv) the origins of the two IS*1216V* elements differed from each other, and the IS*1216V* in the *vanXY* region had one base pair substitution (T662C); (v) the distributions and insertions of IS*1216V* and IS*1542* associated with the Tn*1546*-like elements of the Chinese isolates were similar to those previously re-

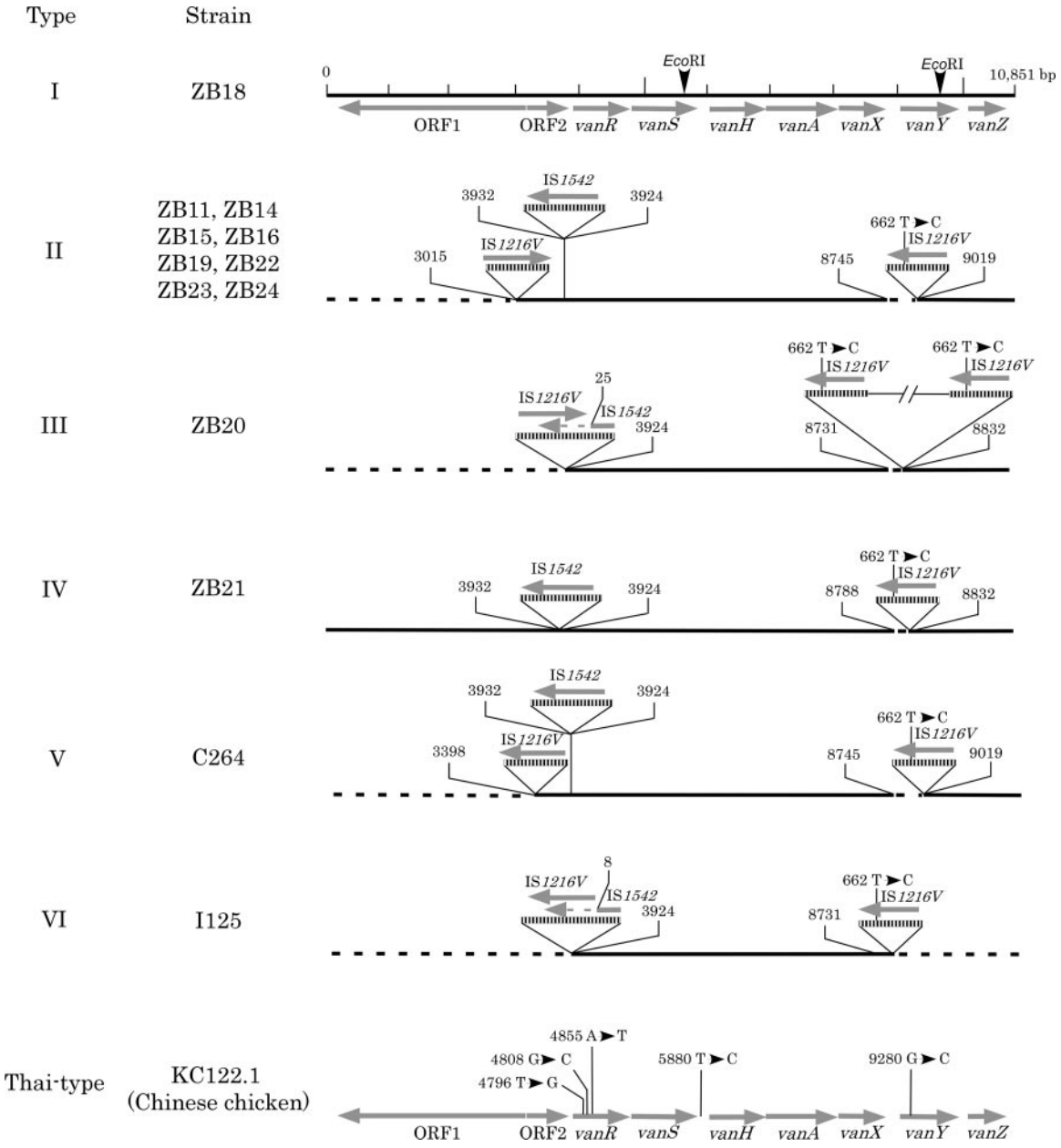

FIG. 4. Genetic organization and typing of Tn*1546*-like elements found in Chinese clinical isolates. The upper horizontal arrows show the genes and open reading frames (ORFs) encoded on the prototype Tn*1546* element of plasmid pIP816 (1). Boxes with vertical lines represent IS elements. The numbers at the IS insertions show the positions of the first nucleotides upstream and downstream of the inserts. The horizontal arrows on the IS elements indicate the transcriptional orientation of the transposase encoded on the ISs. The dotted horizontal lines indicate the deleted region. KC122.1 is a VanA-type *E. faecalis* strain isolated from chicken meat imported from mainland China (25). The Tn*1546*-like element of KC122.1 was temporarily classified as Thai-type, which is often found in VRE isolates from chicken meats imported from Thailand (9, 18).

ported for European and Korean VanA-type VRE isolates (11, 22, 31, 34); (vi) Tn*1546*-like elements were classified into six types, based on DNA sequencing (type I to VI), and type II elements were predominantly isolated from hospitals in Beijing and could be disseminated among different *E. faecium* strains; and (vii) there was no linkage between VRE isolates from humans (patients) and the VRE isolate from a food animal (chicken meat).

This study is the first to provide detailed molecular analyses of VRE clinical isolates from mainland China. To further elucidate the characteristics of Chinese VRE strains, a nationwide surveillance of VRE and systemic analyses of other types of VRE strains are necessary. In the meantime, the current recommended hospital infection control measures for developed countries may be readily implemented to prevent further spread of VRE in mainland China.

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