Heteroresistance to Teicoplanin in *Enterococcus faecium* Harboring the *vanA* Gene[▽]

Hospital-acquired infections caused by vancomycin-resistant enterococci (VRE) are increasing. The resistance of VRE to multiple antibiotics makes them clinically challenging. There are at least six phenotypes of VRE, including VanA, VanB, VanC, VanD, VanE, and VanG (2, 8). These phenotypes correspond with the genotypes *vanA*, *vanB*, *vanC*, *vanD*, *vanE*, and *vanG*. VanA and VanB phenotypes are common among VRE isolates. In general, *vanA* genotype VRE shows VanA phenotype with high-level resistance to both vancomycin and teicoplanin, whereas *vanB* genotype VRE shows VanB phenotype characterized by various levels of resistance to vancomycin but susceptibility to teicoplanin (2). Interestingly, *vanA* genotype VRE strains were recently found to be susceptible to teicoplanin in East Asia (Japan, South Korea, Taiwan, and even mainland China) and named VanB phenotype-*vanA* genotype VRE (3, 4, 6, 7, 11). Until now, the mechanism for this phenomenon has not been clear. Among these strains, point mutations in the key region of the *vanS* gene or impairment of *vanY* or *vanZ* in the Tn*1546*-like element were discovered (3, 4, 7, 11). Also, several vancomycin-heteroresistant *Enterococcus faecium* isolates with the *vanA* genotype have been reported since 2001 (1, 5). Several novel point mutations in the *vanR*, *vanS*, *vanH*, *vanA*, *vanX*, and *vanY* genes of Tn*1546* were also discovered, resulting in amino acid replacements of VanR, VanS, and VanH. It was concluded that independent novel mutations can give rise to polymorphism, heteroresistance, and clonal diversity among VRE strains.

In the present study, we isolated two clinical VRE strains of *E. faecium*, ZP2298 and ZP2171, with a high level of resistance to vancomycin (MICs > 256 μg/ml) and teicoplanin (MICs of 96 and >256 μg/ml, respectively) from sputum samples from patients in a teaching hospital in mainland China. No prior exposure to glycopeptides was found in the isolates. Filter mating was performed using the two clinical VRE strains of *E. faecium* ZP2298 and ZP2171 as donors and *E. faecium* BM4105RF (Fus^r Rif^r) as the recipient as previously described (9). Transconjugants were selected on brain heart infusion (BHI) agar plates containing vancomycin (32 μg/ml) and rifampin (rifampicin) (256 μg/ml). After conjugation, the transconjugants of *E. faecium* ZP2298 and ZP2171, named *E. faecium* FB2298 and FB2171, respectively, exhibit high-level resistance to vancomycin (MICs > 256 μg/ml), but subcolonies were within the zone of inhibition around the teicoplanin Etest strip described for teicoplanin-heteroresistant isolates with teicoplanin MICs of 96 and >256 μg/ml, respectively (Fig. 1). The subcolonies, named *E. faecium* FBV2298 and FBV2171, were selected and showed high-level resistance to both vancomycin and teicoplanin. *E. faecium* FBV2171 maintained high level of resistance to vancomycin (MIC > 256 μg/ml) and was heteroresistant to teicoplanin after three serial passages on the BHI agar without vancomycin (named FBG2171). However, the resistance profile of *E. faecium* FBV2298 did not change after three serial passages on the BHI agar without vancomycin (named FBG2298). This is the first report of a teicoplanin-heteroresistant *E. faecium* isolate.

Pulsed-field gel electrophoresis (PFGE) of SmaI-digested genomic material from the recipient strain (*E. faecium* BM4145),
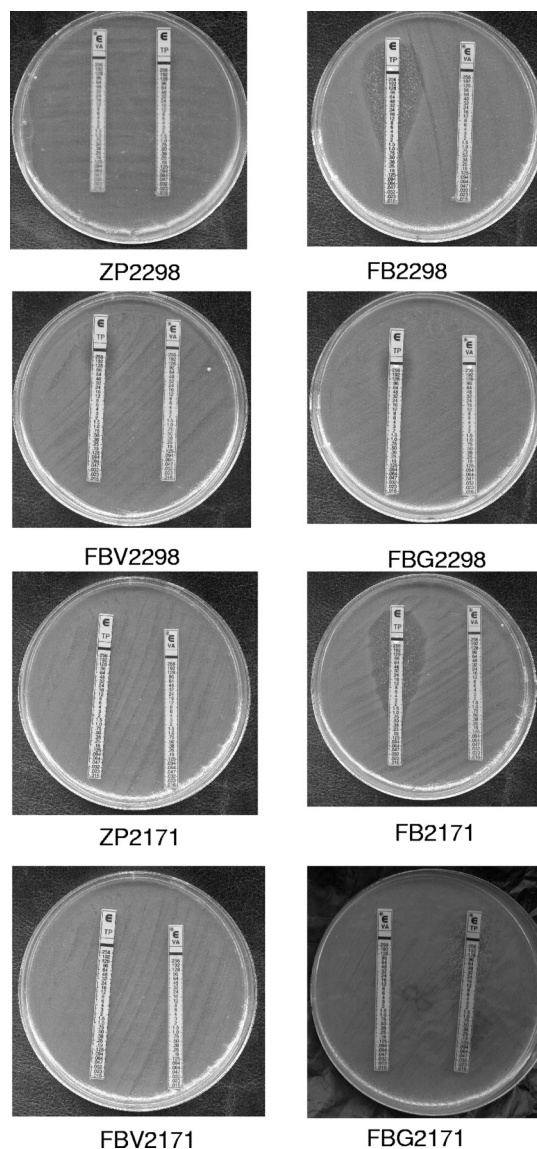

FIG. 1. Vancomycin and teicoplanin Etest results for VREF isolates. The two clinical VREF strains of *E. faecium*, ZP2298 and ZP2171, had a high level of resistance to vancomycin and teicoplanin (MICs > 256 μg/ml). After conjugation, the transconjugants of these two strains of *E. faecium*, FB2298 and FB2171, exhibit high-level resistance to vancomycin (MICs > 256 μg/ml), with resistant subcolonies present in the clear zone of inhibition around the teicoplanin Etest strip, described for teicoplanin-heteroresistant isolates. The resistant subcolonies, *E. faecium* FBV2298 and FBV2171, had teicoplanin MICs of 96 and >256 μg/ml, respectively. *E. faecium* FBG2298 and FBG2171 strains were generated after three serial passages of *E. faecium* FBV2298 and FBV2171, respectively.
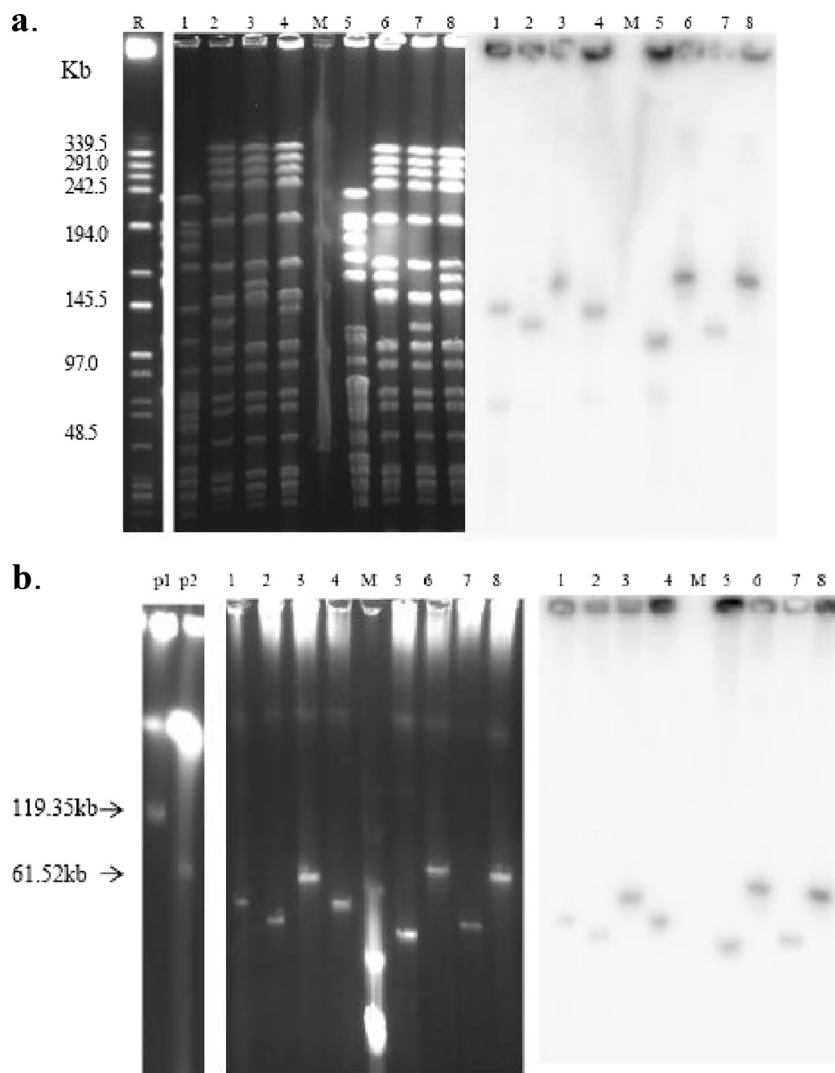
FIG. 2. (a) PFGE of genomic DNA and hybridization profiles of the *vanA* gene. (b) Undigested PFGE to identify plasmids and hybridization profiles of the *vanA* gene. Lanes: R, the recipient strain, *E. faecium* BM4105RF (Fus^r Rif^r); 1, *E. faecium* ZP2298 (clinical VRE isolate); 2, *E. faecium* FBV2298, which showed teicoplanin-resistant subcolonies present in the clear zone of inhibition; 3, transconjugant *E. faecium* FB2298 (teicoplanin heteroresistant); 4, FBG2298, strain after three serial passages on the BHI agar without vancomycin from FBV2298; M, lambda ladder; 5, *E. faecium* ZP2171 (clinical VRE isolate); 6, *E. faecium* FBV2171, which showed teicoplanin-resistant subcolonies present in the clear zone of inhibition; 7, transconjugant *E. faecium* FB2171 (teicoplanin heteroresistant); 8, FBG2171, strain after three serial passages on the BHI agar without vancomycin from FBV2171; p1, plasmid R448 (119.35 kb); p2, plasmid R626 (61.52 kb). Preparations were run in 1% agarose under 6.0 V/cm for 22 h with a pulsing time linearly ramped from 3 to 20 s.

transconjugant colonies, and variants (*E. faecium* FB2298, FB2171, FBV2298, FBV2171, FBG2298, and FBG2171) produced patterns with significant similarity, suggesting a genetic relationship (Fig. 2a). Minor differences (only one or two bands) were observed (Fig. 2a). It eliminated the possibility that mixed cultures were responsible for the findings observed. These patterns were distinct from those for the two clinical vancomycin-resistant *E. faecium* (VREF) strains (*E. faecium* ZP2298 and ZP2171) identified so far (Fig. 2a).

Undigested PFGE plugs were used to identify low- and high-molecular-weight plasmids. The *vanA* gene was the glycopeptide resistance determinant found in all of the VRE isolates by PCR. The *vanA* hybridization results of SmaI-digested PFGE absolutely corresponded with the plasmids identified by PFGE analysis of undigested DNA (Fig. 2a and b).

Interestingly, the *vanA*-containing plasmids with different sizes were identified by undigested PFGE among these VREF strains, including *E. faecium* ZP2298, ZP2171, FB2298, FB2171, FBV2298, FBV2171, FBG2298, and FBG2171 (Fig. 2b). No plasmids were identified in *E. faecium* BM4145. However, it is difficult to find the different sizes of plasmids extracted by Qiagen kit by conventional electrophoresis (data not shown). It is suggested that the differences in the PFGE and undigested PFGE profiles were due to the change in size of plasmids containing the *vanA* gene.

A more thorough investigation of the Tn*1546*-like elements harboring *vanA* in these isolates was performed by using PCR mapping and DNA sequencing (10). Sequence analysis of the *van* operon revealed a 1.5-kb IS*1485* insertion element between the *vanX* and *vanY* genes in all the original and
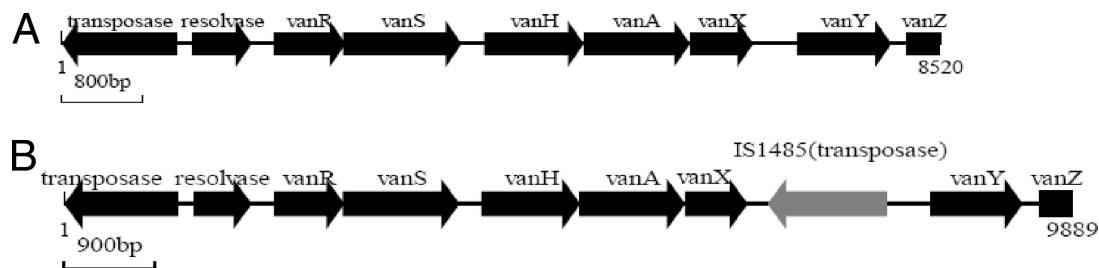
FIG. 3. Genetic maps of Tn*1546* types of *E. faecium* isolates in this study. (A) Typical Tn*1546*. (B) Tn*1546*-like element with IS*1485* inserted between *vanXY*. VRE isolates in this study included *E. faecium* ZP2298 and ZP2171, FB2298, FB2171, FBV2298, FBV2171, FBG2298, and FBG2171. The open reading frames are shown by boxes with arrows, with the arrow indicating the direction of transcription. The arrow on the gray box indicates the transcriptional orientation of the inserted IS*1485* element.

transconjugant VRE strains (Fig. 3). The locations of the IS*1485* insertion in this region were identical, corresponding to bp 8698 of Tn*1546* (GenBank accession no. M97297) with a 3-bp duplication of the target sequence (CTT). This is the first time the presence of IS*1485* in the *vanX-vanY* intergenic region of the *vanA* gene cluster has been demonstrated. No point mutations in the *vanR*, *vanS*, *vanH*, *vanA*, *vanX*, and *vanY* genes were discovered. The nucleotide sequences of the Tn*1546*-like element were submitted to GenBank (GenBank accession no. EU599211). The *vanA*-containing transposons in the teicoplanin-heteroresistant isolates had no detectable changes compared to the teicoplanin-resistant isolates. However, it was difficult to amplify the *vanZ* genes from the teicoplanin-heteroresistant transconjugant VREF strains by PCR. Also, it was shown that the sizes of the plasmids containing the *vanA* gene changed after conjugation and serial passages. It is presumed that the resistance phenotypic changes may be due to the size changes of plasmids containing the *van* operon. However, we could not determine what change happened among these plasmids, which needs to be studied further. No independent novel mutations in the *van* operon previously reported in the polymorphism, heteroresistance, and clonal diversity of VRE strains were found (3, 4, 7, 11). These teicoplanin resistance phenotypic changes were observed during the conjugation experiment and serial passages. It is concluded that the teicoplanin resistance phenotype of VRE may change during natural conjugation and serial passages. The emergence of VanB phenotype-*vanA* genotype VRE isolates may be related to conjugation and serial passages. However, we yet do not know the mechanism for the emergence of teicoplanin-heteroresistant *vanA* genotype VREF isolates. Further investigation to elucidate other molecular mechanisms of these strains is under way.

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