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First isolation and characterization of vancomycin-resistant *Enterococcus faecium* harboring *vanD5* gene cluster recovered from a 79-year-old female inpatient in Japan

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ABSTRACT

This study reports the first isolation and characterization of a *vanD5* genotype vancomycin-resistant *Enterococcus faecium* strain (*E. faecium* IPHb306) recovered from a 79-year-old Japanese female inpatient. Species identification was determined by biochemical testing, matrix-assisted laser desorption ionization-time of flight mass spectrometry, and species-specific PCR. Susceptibility tests indicated that *E. faecium* IPHb306 was resistant to vancomycin but susceptible to teicoplanin. Southern hybridization analyses indicated that *E. faecium* IPHb306 harbored a *vanD5* gene cluster on chromosomal DNA. Growth curve analyses showed that a vancomycin resistance phenotype could be inducible. Sequencing analyses of the *vanD5* gene cluster and the $ddl_{E.\ faecium}$ gene demonstrated several point mutations were present. Because this strain belongs to ST203, a major hospital-adapted lineage, spread of the *vanD5* genotype *E. faecium* ST203 is considered a clinical threat in Japan.

1. Introduction

The normal intestinal flora of humans and animals contains enterococci, which are considered to have low pathogenic potential. However, the recent emergence of multi-antimicrobial resistant strains has led to serious clinical and nosocomial infections. Vancomycin, a glycopeptide antibiotic, is used to treat serious enterococcal infection; however, its therapeutic efficacy is limited against vancomycin-resistant enterococci (VRE). VanA, VanB, VanD, and VanM phenotype VRE produce peptidoglycan precursors ending in depsipeptide D-alanyl-D-lactate (D-Ala-D-Lac) mediated by the D-Ala-D-Lac ligases VanA, VanB, VanD, and VanM, respectively, which have a low affinity for glycopeptides (O'Driscoll and Crank, 2015). VanA and VanB are the most prevalent phenotypes and are primarily present in *Enterococcus faecium* and *E. faecalis*; however, VanD or VanM phenotype VRE are relatively rare (Fang, Hedin, Telander, Li, and Nord, 2007).

In Japan, *vanA*-positive *E. faecium* was first isolated in 1996, and the first VRE outbreak was caused by *vanB*-positive *E. faecalis* in 1999 (Fujita, Yoshimura, Komori, Tanimoto, and Ike, 1998; Zheng, Tomita, Inoue, and Ike, 2009). Although there are increasing numbers of reports on VRE, only 1 study has reported a nosocomial infection caused by VanD4-type VRE in *E. raffinosus* (Tanimoto et al., 2006). Here, we report the isolation and characterization of *E. faecium* harboring *vanD5*, which has been reported in only Canada and Sweden (Boyd et al., 2000; Fang, Hedin, Telander, Li, and Nord, 2007), recovered from a 79-year-old Japanese female inpatient. We also determined the structure of vancomycin resistance determinants and the genomic location of the vancomycin resistance gene in the isolate.

2. Material and methods

2.1. Patient

A Japanese female patient aged 79 years was hospitalized in the Nagoya Medical Center on December 4, 2017, because of reduced movement related to pain after a fall at home. Her vital signs were stable except for a body temperature of 37.0°C on admission. Laboratory tests showed a high white blood cell count of 26,800 cells per µL. Hepatic function disorder was determined by moderate increases in the levels of aspartate aminotransferase (AST, 157 IU/L), alanine aminotransferase (ALT, 89 IU/L), lactate dehydrogenase (LD, 413 IU/L), and alkaline phosphatase (ALP, 737 IU/L). In addition, a high C-reactive protein value (CRP, 26.0 mg/dL) was reported. Lumbar compression fracture was identified by computed tomography. Concurrently, a low-density area was observed in the posterior lobe of the liver (S6/7). She had a partial hepatectomy and choledochojejunostomy in 2015 and distal

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pancreatectomy and splenectomy in 2017 to treat the cholangiocarcinoma. A recurrence of the malignancy accompanied by a liver abscess was suspected. During the patient's clinical course, her blood cultures were examined by the BACTEC™ FX system using BACTEC™ Plus Aerobic/F and Anaerobic/F Media (Becton Dickinson, Tokyo, Japan). The characterization and potential drug resistance of bacterial species were examined by the Micro Scan WalkAway 96 SI/Plus System using Micro Scan Pos and Neg Combo Panels (Beckman Coulter Japan, Tokyo, Japan). Susceptibility of bacteria to antibiotics was determined according to the Clinical and Laboratory Standards Institute (CLSI) (2012) guidelines (M100-S22). Anaerobic bacteria were identified by VITEK2 using a VITEC2 ANC card (Sysmex, Kobe, Japan).

2.2. Reconfirmation and examination of vancomycin-resistant E. faecium

2.2.1. Reconfirmation of the bacterial species

To determine which enterococcal species were present in the isolate, Gram stain; acid formation from arabinose, xylose, and methyl-α-D-glucopyranoside; motility; NaCl tolerance; and esculin hydrolysis were investigated using standard conventional methods as previously described (Devriese, Pot, Kersters, Lauwers, and Haesebrouck, 1996; Facklam and Elliott, 1995). A BBL CRYSTAL RGP (Becton, Dickinson and Company, Sparks, MD, USA) was also used according to the manufacturer's protocol. In addition, matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) analyses were performed using a formic acid extraction method with pure cultures of the isolate as previously described (Alatoom, Cunningham, Ihde, Mandrekar, and Patel, 2011; Farfour et al., 2012). MALDI-TOF MS data analyses were performed using Biotyper 3.0 software (Bruker Daltonics, Bremen, Germany).

2.2.2. Species-specific PCR and multilocus sequence typing (MLST)

Species-specific PCR amplifications were performed using primers described previously (Depardieu, Perichon, and Courvalin, 2004). Multilocus sequence typing (MLST) was performed using a standard set of *E. faecium* MLST primers to amplify 7 housekeeping genes (*atpA*, *ddl*, *gdh*, *purK*, *gyd*, *pstS*, and *adk*), and the allele numbers and sequence types (STs) were determined using the *E. faecium* MLST database (http://efaecium.mlst.net/).

2.2.3. Susceptibility testing

Antimicrobial susceptibility was evaluated using the Kirby–Bauer disk diffusion method with commercial susceptibility disks (Becton, Dickinson and Company). The antibiotics used in this study were as follows: ampicillin (AMPC) (10 μg), tetracycline (TC) (30 μg), chloramphenicol (CP) (30 μg), ciprofloxacin (CPFX) (5 μg), penicillin (PCG) (10 U), erythromycin (EM) (15 μg), doxycycline (DOXY) (30 μg), minocycline (MINO) (30 μg), levofloxacin (LVFX) (5 μg), norfloxacin (NFLX) (10 μg), rifampin (RFP) (5 μg), vancomycin (5 μg), teicoplanin (TEIC) (30 μg), and linezolid (LZD) (30 μg). Minimum inhibitory concentrations (MICs) for vancomycin and TEIC were determined by the broth microdilution method using Dry Plate Eiken DP32 (Eiken Chemical Co., Ltd., Tokyo, Japan). Additionally, the E-test method (bioMérieux, Marcy l'Etoile, France) with Mueller–Hinton II agar (Becton, Dickinson and Company) was also performed to determine the vancomycin MIC. Susceptibility criteria established by the CLSI (2015) were used. *Staphylococcus aureus* ATCC 25923 and *E. faecalis* ATCC 29212 were used for quality control in the disk diffusion and in the broth microdilution analyses, respectively.

2.2.4. Detection of the vancomycin-resistance gene and $ddl_{E.\ faecium}$ gene

Vancomycin resistance genes were detected by multiplex PCR with a QIAGEN Multiplex PCR Plus Kit (Qiagen, Hilden, Germany) and primer pairs for *vanA*, *vanB*, *vanC1/C2*, *vanD*, *vanE*, and *vanG*, as described previously (Bell, Paton, and Turnidge, 1998; Depardieu, Perichon, and Courvalin, 2004). To prepare DNA templates, DNA was extracted from bacteria in pure cultures by alkaline lysis. Direct DNA sequencing of the amplified fragment was performed using the dideoxy chain termination method with an ABI PRISM 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Nucleotide searches of the obtained sequences were performed using the BLASTN program run on the National Centre for Biotechnology Information server (http://www.ncbi.nlm.nih.gov/BLAST/).

To detect the entire *vanD5* gene cluster and $ddl_{E.\ faecium}$ gene in the isolate, PCR and sequence analysis were conducted using the primers shown in Table 1. The primers used in this study were designed from reference sequences using Primer3Plus (Rozen and Skaletsky, 2000). Direct DNA sequencing of the amplified fragments was performed as

Table 1
Primers used to detect the *vanD5* gene cluster and $ddl_{E.\ faecium}$ of *E. faecium* IPHb306.

Primer	Sequence (5′ → 3′)	Position
vanD5		
AF130997-4F	AATCTGCATTGTTGTTTCATATCG	4–27[a]
AF130997-45F	AGACAGATTATCGCAATGTAAGGA	45–68[a]
vanD-36F	AGAATTGGCCGACTTAGTTGA	36–56[b]
vanD-517R	GCTCGCACAGATACCAAAGA	517–498[b]
vanD-519F	TCAGGGGACGGTTGTTTCTA	519–538[b]
vanD-936F	TGATAGCGGTTATGGGTGTG	936–955[b]
vanD-955R	CACACCCATAACCGCTATCA	955–936[b]
vanD-1199F	CCGCTTTCATCGGTTATAGG	1199–1218[b]
vanD-1319R	TCAGCTCTTCCAGACGTTCA	1319–1300[b]
vanD-1494F	ATGCCAACAAACTGCAGAGA	1494–1513[b]
vanD-1529F	CTGAGAAATGCCGTCAGCTA	1529–1548[b]
vanD-1668F	AGTTTTACCGCCTGGATGTG	1668–1687[b]
vanD-1748R	CCACAATCTCCCTTGCAATC	1748–1729[b]
vanD-1778F	AGCGAAAACGGTATCACCAG	1778–1797[b]
vanD-1814F	CCCACCGTAGGAAAATCGTA	1814–1833[b]
vanD-1819R	GGTGGGCAATGTAACCTCAA	1819–1800[b]
vanD-2004R	CTGCGGTTCCTTCCATACTG	2004–1985[b]
vanD-2053F	TGCTCGGTCTGCTCATAGTC	2053–2072[b]
vanD-2129R	TGGAAGAGCGGGATCTTTAC	2129–2110[b]
vanD-2178R	CGTCGGCCAGAAATGAATAG	2178–2159[b]
vanD-2205R	AAGCTGGAGACTCCTCCTCA	2205–2186[b]
vanD-2456F	GAAAGGCGAACCCTGACTG	2456–2475[b]
vanD-2573R	GCTATTATCACCCGCAATGG	2573–2554[b]
vanD-2920F	CAGCAGTGATTGACGGAGAA	2920–2939[b]
vanD-2939R	TTCTCCGTCAATCACTGCTG	2939–2920[b]
vanD-3211F	CATAAAGCGGAGCTGTCAGA	3211–3230[b]
vanD-3384R	GACGGTATAATCGGCCACAC	3384–3365[b]
vanD-3648F	GCATATTCCGTTGGCAGAAG	3648–3667[b]
vanD-3718R	CCTCCTGCTTCATCATTTCC	3718–3699[b]
vanD-4068F	CAGTGAAATCTGCGATGGAG	4068–4087[b]
vanD-4091R	CAACCTCCATCGCAGATTTC	4091–4072[b]
vanD-4410F	CGCTCGCTTATATGGTTGTG	4410–4429[b]
vanD-4485R	AGGCTGTCCCCCTTTTGTAG	4485–4466[b]
vanD-4802F	CCGGATGAGGTAAGAGAACG	4802–4821[b]
vanD-4949R	AAGTAAAACCCGGCATGGTA	4949–4930[b]
vanD-5236F	TTGCTCTTATGGGACGGCTA	5236–5255[b]
vanD-5303R	GGTTGGGAAGTCCAATTCAG	5303–5284[b]
vanD-5605R	AGACGTAATGCCACCATTCA	5605–5586[b]
AF175293-5781R	TTATGTTTCCACGCCAGATG	5781–5762[c]
AF175293-5887R	CATTCAAGGCACCTCCTCTG	5887–5868[c]
$ddl_{E.\ faecium}$		
ddl-30F	GTTATTCGTTTATTTTTGTTTTGTGC	30–55[d]
ddl-43F	TTTTGTTTTGTGCTAAAATATGAGA	43–67[d]
ddl-472R	GCCGACATAAGGCATATTCA	472–453[d]
ddl-496R	ACATGCACTGGTCAATACGC	496–477[d]
ddl-805F	AGACGTTCGGACGACTTTGC	805–824[d]
ddl-822F	TGCCTGGTGAAGTCGTAAAAG	822–842[d]
ddl-1237R	AGAATGAACGCGGTTACGC	1237–1219[d]
ddl-1240R	GCTAGAATGAACGCGGTTAC	1240–1221[d]

[a] The positions of the *vanD5* primers are based on the sequence of the *E. faecium* strain BM4339 *vanD* glycopeptide resistance gene cluster (GenBank accession no. AF130997).
[b] The positions of the *vanD5* primers are based on the sequence of the *E. faecium* strain N03-0072 *vanD* glycopeptide resistance gene cluster (GenBank accession no. AY489045).
[c] The positions of the *vanD5* primers are based on the sequence of the *E. faecium* strain N97-330 *vanD* glycopeptide resistance gene cluster (GenBank accession no. AF175293).
[d] The positions of the *ddlE. faecium* primers are based on the sequence of the *E. faecium* strain BM4339 truncated D-alanine:D-alanine ligase gene (GenBank accession no. AF130998).

described above. The obtained sequence data were compared with appropriate reference data using the Clustal W algorithm and GENETYX-win software (Version 11; GENETYX, Tokyo, Japan).

2.3. Expression analysis of vancomycin resistance

To determine whether vancomycin resistance of the isolate was inducible or constitutive, growth curves were calculated using the methods described by Ostrowsky et al. (1999) with slight modifications. Briefly, isolates were grown overnight in 40 mL brain heart infusion (BHI) broth with or without 16 μg/mL of vancomycin at 37 °C with shaking at 160 rpm using an incubator shaker (TAITEC BioShaker BR-300; TAITEC Co., Ltd., Saitama, Japan). Ten milliliters of the culture was centrifuged at 1740×*g* for 20 min, and the bacterial pellet was resuspended in 20 mL of fresh BHI broth. The suspension was diluted 20-fold with 38 mL of prewarmed BHI broth in a 200-mL flask and then grown at 37 °C with shaking at 160 rpm in an incubator shaker until the optical density at 600 nm reached 0.05–0.17. An appropriate flask culture was supplemented with 16 μg/mL of vancomycin, and the optical density was monitored at 20-min intervals with OD-MonitorA&S (TAITEC Co., Ltd.).

2.4. Genomic location of the vanD5 gene

To determine the genomic location of the *vanD5* gene cluster, we used the Southern hybridization methods described by Biavasco et al. (2007) with slight modifications described in Sections 2.4.1, 2.4.2, and 2.4.3.

2.4.1. Pulsed-field gel electrophoresis (PFGE) of S1 nuclease-digested total DNA

S1 nuclease digestion of total DNA was performed following the manufacturer's protocol (Takara). PFGE was performed on a 1.0% SeaKem Gold Agarose gel (Cambrex Bio Science, Rockland, ME, USA) and a CHEF-DRIII apparatus (Bio-Rad Laboratories, Hercules, CA, USA) with 0.5× Tris-borate-EDTA buffer at 14 °C and 6 V/cm. A linearly ramped switching time of 0.7 to 15 s was applied for 19 h. *Xba*I-digested DNA from the PFGE standard strain *Salmonella enterica* serovar Braenderup H9812 (from the PulseNet program, Enteric Diseases Laboratory Branch, Centers for Disease Control and Prevention (Atlanta, Georgia, USA), through the National Institute of Infectious Diseases (Tokyo, Japan)), was used as a molecular marker.

2.4.2. PFGE of I-CeuI digested total DNA

The I-*Ceu*I digestion of total DNA was performed as previously described (Biavasco et al., 2007). PFGE was performed as described above, and a linearly ramped switching time of 60 to 90 s was applied for 20 h.

2.4.3. Southern hybridization

Southern hybridization was performed as previously described (Hashimoto, Tanimoto, Ozawa, Murata, and Ike, 2000). Probes for *vanD5* and 16S rDNA were labeled using the PCR DIG Probe Synthesis Kit (Roche Products, Welwyn Garden City, UK) with the following primers: vanD-4410F and vanD-4949R for the *vanD5* gene (Table 1), and LPW 99 and LPW 273 for 16S rDNA (Woo, Tam, Lau, Fung, and Yuen, 2004).

3. Results

3.1. Microorganism analyses during the patient's clinical course

At the beginning of the patient's clinical course, conventional microorganism examination identified Gram-positive and -negative rods in the abscess smear. Her blood culture was positive for *Klebsiella pneumoniae*, *Aeromonas hydrophilia*, and *Clostridium perfringens*. Meropenem (MEPM), metronidazole, and sulbactam/ampicillin were administered when the patient presented with a fever and were discontinued after 1–3 weeks. On March 13, 2018, Gram-positive cocci and *Prevotella oris* were identified in her blood culture; *Enterobacter cloacae*, *E. raffinosus*, and *Candida albicans* were cultured from her bile; and *E. cloacae*, *E. raffinosus*, and *E. faecium* were isolated from an abscess culture. On this basis, vancomycin and MEPM were administered for 4 weeks, accordingly. In April 2018, *E. faecium*, *E. cloacae*, *K. aerogenes*, *C. tropicalis*, and *P. buccae* were cultured from drainage of her abscess; and from this, a vancomycin-resistant *E. faecium* strain was isolated. In addition, a stool culture was positive for *E. faecium* that demonstrated vancomycin resistance. Genetic analysis using the Gene Xpert system (Cepheid, Tokyo, Japan) revealed the VRE strain was negative for *vanA* and *vanB*. On May 8, 2018, *K. oxytoca*, *A. hydrophilia*, *E. faecalis*, *P. buccae*, and other Gram-negative rods were isolated from an abscess; of note, VRE was not isolated. The patient died of sepsis in May 10.

3.2. Enterococcal species determination and MLST analysis

The isolate was reconfirmed as enterococci by positive Gram staining, the absence of catalase activity, the ability to grow on bile esculin agar with esculin hydrolysis, or in BHI broth with 6.5% NaCl. Other biochemical tests indicated that the isolate formed acid from arabinose but not from xylose and methyl-α-D-glucopyranoside and was nonmotile. The BBL CRYSTAL RGP isolate profile was 2332777552, indicating it was *E. faecium*. MALDI-TOF MS analyses and species-specific PCR were also used to confirm the isolate was *E. faecium*. Based on these results, the strain was identified as *E. faecium* and designated IPHb306. In addition, MLST analysis revealed that IPHb306 belonged to Sequence Type (ST) 203.

3.3. Antimicrobial susceptibility tests

Antimicrobial disk susceptibility testing demonstrated that IPHb306 was only susceptible to CP, TEIC, and LZD. The Dry Plate Eiken DP32 showed that the isolate was resistant to vancomycin (MIC of >16 μg/mL) and susceptible to TEIC (MIC of 2 μg/mL). Additionally, a vancomycin MIC value of 128 μg/mL was obtained by E-test.

3.4. Detection and sequencing analysis of the vancomycin-resistance gene

Multiplex PCR was used to identify the vancomycin-resistance gene. A 500-bp amplicon was obtained from IPHb306, indicating the isolate harbored the *vanD* gene. Direct sequencing analysis of the 500-bp amplicon demonstrated that the amplicon sequences were identical to partial sequences of *vanD5* (GenBank accession no. AY489045). To determine whether IPHb306 harbored the *vanD5* gene cluster, a DNA template of the isolate was subjected to PCR using the *vanD5* gene cluster specific primer pairs listed in Table 1. PCR products of the expected molecular size (from 291 bp to 1636 bp) were successfully amplified using any combination of the forward and reverse primers from IPHb306, indicating the isolate contains the *vanD5* gene cluster. To determine the entire nucleotide sequence of the *vanD5* gene cluster of IPHb306, direct sequence analyses of the PCR products amplified using combinations of 24 *vanD5* gene cluster specific primers were performed. We found that IPHb306 harbored a *vanD5* cluster homologous to that of a reference strain, *E. faecium* N03-0072 (GenBank accession no. AY489045). In addition, the nucleotide sequences of $vanR_{D5}$, $vanH_{D5}$, and *vanD5* genes in IPHb306 were 100% identical to the reference gene sequences. However, the $vanS_{D5}$ sequence from IPHb306 differed from $vanS_{D5}$ of *E. faecium* N03-0072 by 2 substitutions at reference coordinate nucleotides 1677 (G → A) and 1692 (A → G). These substitutions caused 2 amino acid changes in $VanS_{D5}$ at positions 330 (Arg → His) and 335 (Gln → Arg). Similarly, the $vanY_{D5}$ sequence of IPHb306 differed from that of *E. faecium* N03-0072 by a 1-bp deletion at the reference coordinate nucleotide 2034 (Boyd, Kibsey, Roscoe, and Mulvey, 2004), leading to a frameshift in *vanY*$_{D5}$ of IPHb306. The IPHb306 *vanX*$_{D5}$ sequence differed from that of *E. faecium* N03-0072 by four bases at reference coordinate nucleotides 5646 (G → T), 5649 (G → C), 5654 (A → G), and 5655 (G → A). This resulted in 2 amino acid changes in VanX$_{D5}$ at positions 200 (Trp → Cys) and 201(Met→Ile).

3.5. Sequencing analysis of the ddl$_{E. faecium}$ gene

Compared with the *E. faecium* reference strain BM4524, a VanB strain with inducible resistance to vancomycin (GenBank accession no. AF550665) (Depardieu, Courvalin, and Msadek, 2003), IPHb306 had a single nucleotide change in the *ddl*$_{E. faecium}$ gene at position 322 (C → A), leading to an amino acid change in D-alanine:D-alanine (D-Ala-D-Ala) ligase at position 108 (Gln → Lys). Additionally, IPHb306 had a single nucleotide change at position 38 (G → A) compared with *ddl*$_{E. faecium}$ in the *E. faecium* strain A902 (GenBank accession no. AF215736), a VanD2 strain with inducible resistance to vancomycin. This led to a deduced amino acid change in D-Ala-D-Ala ligase at position 13 (Gly → Glu).

3.6. Nucleotide sequence accession numbers

The *ddl*$_{E. faecium}$ gene and *vanD5* gene cluster nucleotide sequence data of IPHb306 were deposited in the DDBJ nucleotide sequence database under accession numbers LC458861 and LC458862, respectively.

3.7. Growth curve analyses

The growth curves of IPHb306 in BHI broth with or without 16 μl/mL of vancomycin are shown in Fig. 1. After the isolate was preincubated in BHI broth without vancomycin overnight, the lag phase was at 15 to 17 h when cultured in BHI broth supplemented with vancomycin. In contrast, after overnight preincubation in BHI broth supplemented with vancomycin, no inhibition of growth was observed. These results suggest that vancomycin resistance of the isolate was inducible.

3.8. vanD5 gene hybridization analyses

To determine the genomic location of the *vanD5* gene in IPHb306, we performed hybridization analyses with S1 nuclease-digested and I-*Ceu*I-digested total DNA. Hybridization experiments with S1 nuclease showed no hybridization between the plasmid DNAs of IPHb306 and the *vanD5* gene probe (Fig. 2A, B, and C). However, with I-*Ceu*I digestion, the DNA of IPHb306 was hybridized with the 16S rRNA gene and *vanD5* probes (Fig. 2D, E, and F). Taken together, these data suggest the *vanD5* gene is located on chromosomal DNA in IPHb306.

4. Discussion

Five different *vanD* gene variants, *vanD1*, *vanD2*, *vanD3*, *vanD4*, and *vanD5*, were previously detected in enterococcal isolates (Boyd et al., 2000; Casadewall and Courvalin, 1999; Costa et al., 2000; Depardieu et al., 2009; Depardieu, Reynolds, and Courvalin, 2003; Tanimoto et al., 2006). In 2004, the VanD5 operon was first detected in *E. faecium* isolated from a female patient in Canada with a urinary tract infection complicated with renal stones. Since then, only 1 study has reported enterococcal isolates harboring the *vanD5* gene (Fang, Hedin, Telander, Li, and Nord, 2007). Although the VanD5 operon of IPHb306 has a structure characteristic of the first reported VanD5 operon (*vanRSYHDX*) in *E. faecium* N03-0072 (Boyd, Kibsey, Roscoe, and Mulvey, 2004), we observed 2 point mutations resulting in 2 amino acid changes in VanS$_{D5}$, a 1-bp deletion leading to a frameshift in VanY$_{D5}$, and 4 point mutations resulting in 2 amino acid changes in VanX$_{D5}$. A previous study reported a truncated form of the VanY$_{D5}$ protein in *E. faecium* N03-0072 harbored a 1-bp insertional mutation (Boyd, Kibsey, Roscoe, and Mulvey, 2004); however, this insertion was not detected in IPHb306, and VanY$_{D5}$ in this strain was the full-length nonmutated protein, which has 95.59% amino acid identity to VanY$_{D2}$ (GenBank accession no. ACM47285). Therefore, this protein may be characterized as the parent protein of VanY$_{D5}$, although further studies to determine the expression of this protein are required.

The VanD1, VanD3, and VanD4 operons are expressed constitutively because of mutations in their *vanS*$_D$ and *ddl* genes, which result in dysfunctional encoded proteins. Similarly, expression of the VanD5 operon first reported in *E. faecium* N03-0072 is constitutive because of the 1-bp frameshift at codon 322 in the *ddl* gene, which affects the function of D-Ala-D-Ala ligase, although no mutations were reported in the *vanS*$_{D5}$ or *vanR*$_{D5}$ genes (Boyd, Kibsey, Roscoe, and Mulvey, 2004). Of note, expression of the VanD2 operon in *E. faecium* A902 was reported to be inducible (Ostrowsky et al., 1999), in contrast to other VanD operons, such as VanD1, VanD3, VanD4, and VanD5. In the current study, growth curve analyses demonstrated the VanD5 operons of IPHb306 were expressed

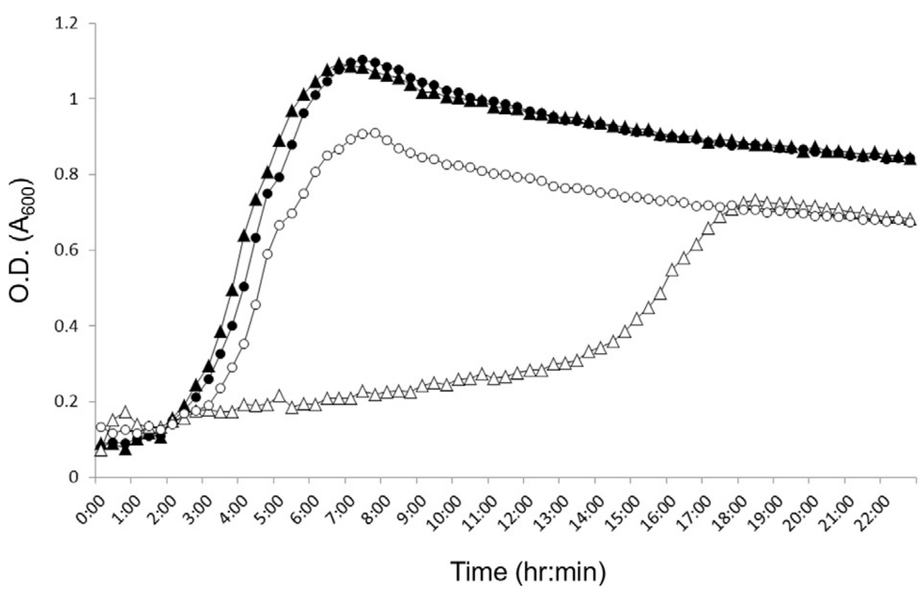

Fig. 1. Analysis of the growth characteristics of *Enterococcus faecium* IPHb306. Growth curves of *E. faecium* IPHb306 in the presence (△) or absence (○) of vancomycin after overnight preincubation in BHI broth. Growth curves of *E. faecium* IPHb306 in the presence (▲) or absence (●) of vancomycin after overnight preincubation in BHI broth containing vancomycin. The concentration of vancomycin was 16 μg/mL. Experiments were performed in triplicate, and the mean optical density is shown.

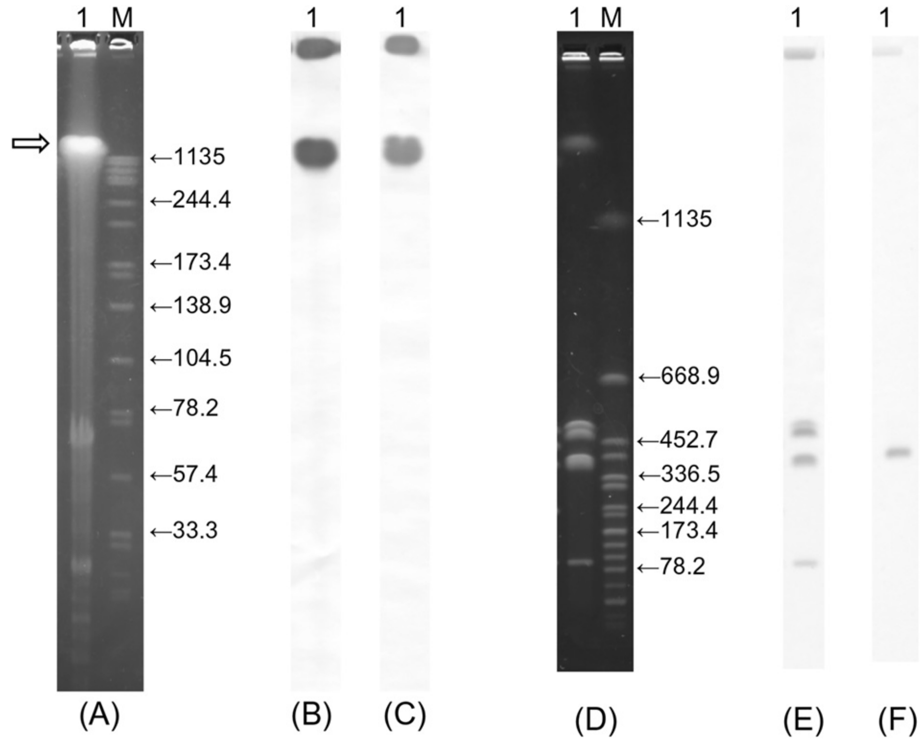

Fig. 2. Genetic analysis of the *vanD5* gene of *Enterococcus faecium* IPHb306. PFGE of S1 nuclease-digested total DNA (A) and corresponding Southern blot hybridizations using 16S rRNA (B) and *vanD5* gene probes (C). PFGE of I-*Ceu*I-digested DNA (D) and corresponding Southern blot hybridizations using 16S rRNA (E) and *vanD5* gene probes (F). Lane 1, *E. faecium* IPHb306; Lane M, *Xba*I-digested PFGE patterns of *S. enterica* serovar Braenderup H9812 DNA as a molecular size marker. Numbers on the right side of the gel images indicate the DNA molecular size in kilobase pairs. The white arrow indicates chromosomal DNA.

inducibly. Furthermore, sequence analysis demonstrated IPHb306 had no mutations in the $vanS_{D5}$ and $vanR_{D5}$ genes, and therefore, the function of the encoded proteins was unaffected, suggesting the 2-component regulatory system for vancomycin resistance may be functionally intact. The amino acid sequence of D-Ala-D-Ala ligase of IPHb306 is highly homologous to that of VanB *E. faecium* BM4525 and VanD2 *E. faecium* A902, which show inducible resistance to vancomycin. Taken together, these results suggest the D-Ala-D-Ala ligase of IPHb306 may remain functionally intact.

A general characteristic of *vanD* vancomycin-resistant enterococci is the chromosomal location of the *vanD* gene cluster, which is not transferable (Top et al., 2018). Southern hybridization analyses in this study revealed the *vanD5* gene cluster of IPHb 306 was located on chromosomal DNA, similar to many *vanD*-positive VRE. Recently, the whole-genome sequencing of *vanD*-positive *E. faecium* N15-508 isolated in Canada demonstrated the *vanD* gene cluster was located on a genomic island (IME*Efm15508*: integrative mobilizable element in *E. faecium* N15-508) of 66.7 kb, which might be acquired by horizontal transfer (Boyd, Lalancette, Lévesque, and Golding, 2016). Furthermore, a genomic comparison of 6 strains of Dutch *vanD*-positive *E. faecium* with 4 *vanD* gene clusters from other enterococcal species and anaerobic gut commensals demonstrated the *vanD* gene cluster was located on a genomic island of variable size, which was transferred horizontally between enterococci and anaerobic gut commensals (Top et al., 2018). To the best of our knowledge, this is the first report of the isolation of a *vanD5* genotype vancomycin-resistant *E. faecium* strain outside North America and the European Union. Therefore, the whole genome analysis of IPHb306 may help us understand the origin of the *vanD5* gene cluster in Japan.

MLST analysis of IPHb306 demonstrated this strain belongs to ST203, which is grouped into clonal complex 17 (CC17) associated with nosocomial *E. faecium* infections worldwide (Top, Willems, and Bonten, 2008). Recent epidemiological studies of VRE reported that vancomycin-resistant *E. faecium* ST203 harboring a *vanA* or *vanB* gene have become more predominant in Australia, Brazil, and Denmark (Hammerum et al., 2017; Lam et al., 2013; Sacramento et al., 2017). Notably, in Australia, *vanB* genotype *E. faecium* ST203 has caused a sustained increase in bloodstream infections (Lam et al., 2013). The first nosocomial outbreak of *vanD* genotype *E. faecium* ST117, which belongs to CC17, was reported in Canada in 2018 (Lam et al., 2018). *E. faecium* belonging to CC17, including ST203, which are resistant to aminoglycoside or macrolide (Isogai et al., 2013; Watanabe et al., 2009), have been detected in Japanese hospitals. Although the prevalence of VRE was presumed to be low in Japan according to the Japan Nosocomial Infections Surveillance (2016) when compared with other countries (Kudo, Nomura, Yomoda, Tanimoto, and Tomita, 2014; Suzuki et al., 2014), the *vanD5* genotype *E. faecium* ST203 might be an immediate threat as a causative agent of healthcare-associated infections in Japan.

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