Characteristics of vancomycin-resistant enterococci (VRE) isolated from medical fields in Japan

日本国内の医療現場より分離された

バンコマイシン耐性腸球菌に関する解析

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resistant *Enterococcus faecium* clinical isolates from a fecal sample from a same patient after vancomycin therapy.

Characteristics of vancomycin-resistant enterococci (VRE) isolated from medical fields in Japan

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Introduction

Enterococci are Gram-positive bacteria and usually colonize gastrointestinal tracts in human. They are typical opportunistic pathogens and sometimes cause bloodstream infections, urinary tract infections and cholangitis in compromised hosts.

Vancomycin (VAN) is a glycopeptide antibiotic commonly used to treat severe Gram-positive bacterial infections. Since vancomycin resistant enterococcus (VRE) was first reported in 1998, it arose as a global problem in medical fields. Due to the limited options for treatment, Center for Disease Control and Prevention (CDC) regarded VRE as a serious hazard-level threat.

Under the Infectious Disease Control Law in Japan (Kansenshō-Hō, Act on Prevention of Infectious Disease and Medical Care for Patients Suffering Infectious Disease of Japan), notifiable VRE is defined as enterococcal clinical isolates causing infectious disease and showing VAN MIC≥16mg/L. Since only approximately 100 cases are reported every year, the prevalence of VRE is presumed to be low in comparison with other countries.

Here, we described VanB-type and VanD-type enterococci isolated in medical fields in Japan.

These VanB-type *Enterococcus faecium* were obtained from different patients. These isolates showed too low-level vancomycin resistance (VAN MIC 3mg/L) and genetically high relatedness. In addition, these low-level VAN resistant *vanB* gene clusters had the capability to convert to a higher-level VAN resistant phenotype.

VanD-type vancomycin resistance is a relatively rare type among nine types of vancomycin-resistance. Until now, only dozens of VanD-type isolates have been reported in the world. We described three VanD-type vancomycin resistance *E. faecium* isolates which were isolated from a same patient who had received a long-term vancomycin (VAN) treatment. The nucleotide sequence analysis of *vanD* gene clusters showed high identities with *Ruminococcus* spp. which might be a reservoir of *vanD*

gene cluster in human fecal flora, suggesting a possibility of horizontal transfer of *vanD* gene cluster.

In this thesis, we pose potential threats of VRE in clinical settings in Japan.

Dissemination and genetic analysis of the stealthy *vanB* gene clusters of *Enterococcus faecium* clinical isolates in Japan

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Abstract

Background

VanB-type vancomycin (VAN) resistance gene clusters confer VAN resistance on *Enterococcus* spp. over a wide range of MIC levels (MIC=4–1000 mg/L). However, the epidemiology and the molecular characteristics of the low-level VAN resistant VanB-type *Enterococcus* still remains unclear.

Results

We characterized 19 isolates of VanB-type *Enterococcus faecium* that were not resistant to VAN (MIC=3 mg/L) obtained from two hospitals in Japan between 2009 and 2010. These isolates had the same nucleotide sequences for the *vanB* gene cluster and showed identical multilocus sequence typing (MLST) (ST78) with the highly related profiles in pulsed-field gel electrophoresis (PFGE). The *vanB* gene cluster was located on a plasmid, and was transferable to *E. faecium* and *E. faecalis*. Notably, these low-level VAN resistant *vanB* gene clusters had the capability to convert to a higher-level VAN resistant phenotype (MIC≥16 mg/L) with a frequency of 10^{-6}–10^{-7}/parent cell *in vitro*. Most of these revertants acquired genetic mutations in the $vanS_B$ gene, while the remainder of the revertants probably had other mutations outside of the *vanB* gene cluster. All of the revertants we tested showed increases in the VAN-dependent expression of the

vanB gene cluster, suggesting that the genetic mutations affected the transcriptional activity and reversed the VAN resistance. Targeted mutagenesis revealed that three unique nucleotide substitutions in the *vanB* gene cluster of these strains attenuated vancomycin resistance.

Conclusions

In summary, this study indicated that stealthy VanB-type *E. faecium* strains that have the potential ability to become resistance to VAN could exist in clinical settings.

Keywords

VRE, antibiotic resistance, outbreak, conjugation, reversion

Background

Enterococcus spp. is a typical opportunistic pathogen causing urinary tract infections, bloodstream infections and surgical site infections in compromised hosts. Vancomycin (VAN) is a glycopeptide antibiotic that inhibits peptidoglycan synthesis and is used to treat severe Gram-positive bacterial infections [1]. VAN resistant clusters were distributed among several enterococcal species [2-4]. Notably, VAN resistant enterococci (VRE) almost consist of *Enterococcus faecali*s and *Enterococcus faecium*. Recent reports have documented that VRE was first reported in England and France in 1986 [5, 6] and is now one of the major nosocomial pathogens in the world [2]. Due to the limited options for treatment, invasive infections by this pathogen are important causes of morbidity and mortality. For that reason, active surveillance is being carried out to prevent the spread of VRE [7]. The VAN resistance in VRE are classified into eight acquired gene clusters. These are *vanA, vanB, vanD, vanE, vanG, vanL, vanM* and *vanN* [3]. VanA- and VanB-type VRE are the major and most important genotypes in clinical settings. VanA-type VRE shows high resistant levels to both VAN (MIC=64–100 mg/L) and teicoplanin (MIC=16–512 mg/L), whereas VanB-type VRE shows susceptibility to teicoplanin (MIC=0.5–1 mg/L) and various levels of resistance to VAN (MIC=4–1000 mg/L) [7]. In recent years, outbreak of VanB-type VRE occurred in Europe, USA and worldwide [2, 4,

8]. The *vanB* gene cluster consists of a two-component regulatory system (*vanR*$_B$, *vanS*$_B$) and five resistance genes (*vanY*$_B$, *vanW*, *vanH*$_B$, *vanB*, *vanX*$_B$) (Fig. 1) [9]. Contrary to the highly conserved resistance genes, the amino acid sequences of $VanS_B$ and $VanR_B$ show less similarity to those of VanA-type VanS and VanR with 34% and 23% identities, respectively [10]. These differences are suspected as being responsible for the glycopeptide-resistant characteristics of VanB-type VRE [10]. The wide range in the level of VAN resistance of VanB (MIC=4–1000 mg/L) increases the difficulties in detecting it in clinical settings. A previous study reported that 55% of outbreak VanB-type isolates in single neonatal ICU of a hospital in Germany showed MICs of VAN that were less than 4 mg/L [8]. Meanwhile, there is concern that such low-level VRE may cause treatment failure due to its conversion to a high level of resistance [11]. Indeed, in VanA-type *E. faecium*, it has been reported that low-level VAN resistant *E. faecium* converted to the VAN resistant phenotype during antibiotic therapy and this was named vancomycin-variable *E. faecium* (VVE) [12-14]. In Japan, the first isolation of VRE was VanA-type *E. faecium* in 1996 [15] and the first outbreak of VRE was caused by VanB-type *E. faecium* [16]. Although the prevalence of VRE is presumed to be low in Japan in comparison with other countries, little is known about the prevalence of low-level VanB-type VRE. In this study, we characterized *E. faecium* isolates that harbor the *vanB* gene

cluster but were not resistant to VAN, and performed genetic analysis to assess the responsible determinants for the too low-level VAN resistant phenotype of their *vanB* gene cluster.

Materials and Methods

Bacterial strains, plasmids, growth condition, oligonucleotides, media, and antimicrobial reagents

The bacterial strains and plasmids used in this study are shown in Table 1. *E. faecium* M1–10 strains and Y7–12 strains were provided by MIROKU Medical Laboratory Co. (Nagano, Japan) and Yamaguchi Prefectural Institute of Public Health and Environment, respectively. pMG2200 is the pheromone-responsive plasmid isolated from *E. faecalis* clinical isolates harboring the *vanB* gene cluster. pMG2200 conferred VAN resistance on the host strain, showing an MIC for VAN of more than 64 mg/L [16]. The oligonucleotides used in this study are shown in Additional file 8: Table. S3. Enterococcal strains were routinely grown in Todd-Hewitt broth (THB; Difco, Detroit, MI) at 37°C. *Escherichia coli* strains were grown in Luria-Bertani (LB; Difco) at 37°C. All antibiotics were obtained from Sigma Co. (St. Louis, MO).

Antibiotic susceptibility test

MICs were determined by the agar dilution method according to Clinical and Laboratory Standards Institutes (CLSI) guidelines (http://clsi.org/). After each strain was grown overnight in Mueller-Hinton broth (MHB; Nissui, Tokyo, Japan), the cultures were diluted 100-fold with fresh MHB. An inoculum of approximately $5x10^5$ cells (5µl) was

spotted onto a series of Mueller-Hinton agar (Eiken, Tokyo, Japan) plates containing a range of concentrations of the test drug. After incubation at 37°C for 24 hours, the susceptibility was determined. The interpretation of the results was in compliance with standards recommended by CLSI. The breakpoints of MICs for resistance to antibiotics were defined as follows (mg/L); vancomycin (VAN), ≥16; teicoplanin (TEC), ≥16; ampicillin (AMP), ≥12.5; gentamicin (GEN), >500; kanamycin (KAN), ≥1024; streptomycin (STR), >1000; chloramphenicol (CHL), ≥32; tetracycline (TET), ≥16; erythromycin (ERY), ≥8; ciprofloxacin (CIP), ≥4 (Table 1). *E. faecalis* V583 and ATCC29212 were used as controls.

Pulsed-field gel electrophoresis (PFGE) analysis and dendrogram

PFGE analysis was performed as previously described [40]. Briefly, enterococci DNA embedded in an agarose plug was digested overnight at 37°C using *Sma*I (Roche, Basel, Switzerland), and then subjected to PFGE using a CHEF-MAPPER (Bio-Rad, CA) according to the manufacture's protocol. The guidelines proposed by Tenover et al. were used for the interpretation of PFGE results [41]. The genetic relatedness was analyzed using the Dice coefficient and the dendrogram and was calculated with the clustering algorithm of Unweighted Pair-Group Method with an Arithmetic Mean (UPGMA) using FP Quest Software (Bio-Rad) [42, 43]. A lambda PFG Ladder (New England

BioLabs, MA) was used as the Molecular Marker (MM).

Southern transfer and hybridization analysis

PFGE analyses with S1 nuclease or I-*Ceu*I were performed as described above. Briefly, enterococci DNA embedded in agarose plug was digested for 20 minutes at 37°C with S1 nuclease (Promega, WI) or overnight at 37°C using I-*Ceu*I (New England BioLabs, MA), the DNAs were then subjected to PFGE using a CHEF-MAPPER (Bio-Rad, CA) according to the manufacture's protocol. Southern hybridization was performed with the digoxigenin-based non-radioisotope system of Boehringer GmbH (Mannheim, Germany), and Southern transfer and the hybridization procedure were carried out according to the manufactures`s manual and standard protocol [44]. Specific probes for *vanB* gene and the 23S rRNA gene of *E. faecium* were used [45].

Multilocus sequence typing (MLST) analysis

MLST was performed as previously described [27]. The house keeping genes *atpA*, *ddl*, *gdh*, *purK*, *gyd*, *pstS* and *adk* were sequenced and STs were determined according to MLST.net (http://efaecium.mlst.net/).

Conjugation experiment

Filter mating was performed as described previously [46]. Briefly, an overnight culture of the bacteria diluted 50-fold in 5 ml of fresh THB broth and pre-cultured until

the end of the exponential phase. 100 µl of the donor culture and 100 µl of the recipient culture were mixed in 5 ml of THB broth. FA2-2 or BM4105RF and wild M1 or Y7 were used as the recipients and donors, respectively. The donor and recipient cell mixture was allowed to mate at 37°C for 5 hours on 0.45 µm nitrocellulose membrane (Merck Millipore, Darmstadt, Germany). The mating mixture was plated on selective THB agar containing the appropriate antibiotics. After incubation at 37°C for 48 hours, the colonies that grew were isolated and purified at least twice. The frequency of conjugation was calculated as the number of transconjugants per donor cell.

Isolation of the VAN-resistant revertants

Cultures of each strain grown in THB broth at 37°C for 24 hours were plated onto a THB agar plate with or without VAN at concentration of 16 mg/L. Colonies grown after 24 hour of incubation at 37°C were counted and the frequency of the rate of reversion was estimated from the colony-forming unit (CFU) ratio of resistant strains to total strains. The values were the average of three independent experiments with standard error. Colonies grown on THB agar plates with VAN (16 mg/L) were isolated on new THB agar plates containing VAN (16 mg/L). Single colony isolations were performed at least twice for each strain. The MICs of the resulting revertants were determined by the agar dilution method as described above. The nucleotide sequences of the *vanB* gene cluster was

determined as described above.

qRT-PCR analysis

An overnight bacterial culture was diluted 100-fold in brain heart infusion (BHI) medium with or without VAN (1 mg/L) and incubated at 37°C until exponential phase. After collection of the bacterial cells by centrifugation for 5min at 12,000 rpm, total RNA was prepared using a Fast RNA Pro Red Kit (Q-Biogene, Inc) and Fast Prep disintegrator (40 sec, speed: 6.0). The resulting RNA was further extracted with chloroform, precipitated with ethanol and resuspended in 0.05ml diethylpyrocarbonate (DPEC)-treated water. Total RNA (30 μg) was incubated with recombinant DNase1 (RNase-free) and RNase inhibitor (Takara Bio Inc, Shiga, Japan) at 37°C for 30 min. After extraction with phenol-chloroform-isoamyl alcohol (25:24:1) and chloroform-isoamyl alcohol (24:1), the samples were precipitated with ethanol and resuspended with 20 μl of DPEC-treated water. The concentration of the RNA solution was determined by a fluorescence-based assay with Qubit 3.0 (Thermo Fisher Scientific Inc, MA). Reverse transcription was carried out with the PrimeScript RT Master Mix (Takara Bio Inc). Real-time PCR was carried out with the Thunderbird SYBR qPCR Mix (Toyobo Co., Tokyo, Japan) using an ABI 7500 Fast RT-PCR instrument (ABI, CA). Real-time-PCR cycled at 1min at 95°C, followed by 40 cycles of 15 sec at 95 °C, 15 sec at 55 °C, 1 min at 72 °C. The real-time

PCR primers were designed by Primer3Plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) (Additional file 8: Table S3).

Kinetics of cell growth.

Overnight bacterial cultures were diluted 100-fold in fresh THB broth with or without VAN at concentration of 1 mg/L. The culture was incubated at 37°C and the turbidity was measured at an optical density of 620 nm at each time point using a Multiskan FC Microplate Photometer (Thermo Fisher Scientific Inc).

Plasmid construction and targeted mutagenesis of the *vanB* gene cluster

Targeted mutagenesis of the $vanS_B$, *vanB*, *vanW* genes in the *vanB* gene cluster was performed as previously described [47]. Briefly, the DNA fragments to be inserted were constructed by PCR using the corresponding primers, as indicated in Additional file 8: Table S3, and inserted into the pCJK47 vector using the restriction enzyme *Eco*RI (Roche) and a DNA Ligation Kit (Takara Bio Inc.), as described previously. After transformation into *E. coli* EC1000 as previously described elsewhere, the recombinant-expressing *E. coli* strains were incubated in 5ml of LB containing 300 mg/L erythromycin at 37°C with shaking. Recombinant plasmid DNA was extracted using the QIAprep Spin Miniprep Kit (QIAGEN, Hilden, Germany). The constructed plasmids were sequenced to confirm that the desired sequence had been inserted. Electrotransformation into

CK111/pCF10-101 was performed as previously described. Overnight cultures of the donor strain (CK111/pCF10-101, pCJK47-derivatives) and recipient strain (M1TC; *E. faecalis* FA2-2 transconjugants of M1 strain) were diluted 100-fold into fresh THB broth and incubated separately at 37°C for 1 hour. 100µl aliquots of the donor and recipient cultures were mixed with 800µl of fresh THB broth and incubated at 37°C with shaking at 150rpm for 12 hours, and were then spread on a BHI plate (rifampicin 25mg/L, fusidic acid 25mg/L, erythromycin 10mg/L and X-gal 100mg/L). After incubation at 37°C for 32-48 hour, colonies that were blue in color were isolated and purified as these were expected to be integrant clones where the pCJK47-derivative plasmid had integrated into the chromosomal target locus of the recipient strain. These integrant clones were inoculated in THB and incubated at 37°C for 12 hour. After a 100-fold dilution, 100µl of the culture broth was plated on an MM9YEG plate supplemented with 10mM 4-Chloro-DL-phenylalanine (SIGMA-ALDRICH Co., MO) and 40mg/L X-gal, followed by incubation at 37°C for 12 hours. White colonies were then isolated and purified as potential candidates carrying mutations within the target genes. The nucleotide sequences were checked to determine whether mutagenesis had occurred in the target sequences.

Results

Isolation of too low-level VAN resistant *E. faecium* harboring the *vanB* gene cluster

In 2009 and 2010, a total of nineteen VAN susceptible *E. faecium* strains that harbored the *vanB* gene cluster were isolated from the feces of individual in-patients during a VRE screening test at two hospitals located in adjacent prefectures in Japan. We designated these isolates M1–10 (Hospital A) and Y7–12 (Hospital B), respectively (Table 1). All the patients did not show any significant symptoms of bacterial infectious diseases and were carriers. The VanB-type VAN resistance genes were detected by PCR. All of the 19 isolates were susceptible to VAN (MIC=3 mg/L) and teicoplanin (MIC=1 mg/L), and resistant to ampicillin (MIC≥128 mg/L), tetracycline (MIC=64 mg/L), erythromycin (MIC>256 mg/L) and ciprofloxacin (MIC=64 mg/L) (Table 1). To determine the correlation between their genetic backgrounds, we performed PFGE and MLST analysis (Fig. 1, Table 1). Based on the results from PFGE analysis, using a 85% similarity value as cutoff point, these 19 isolates were classified into three main clusters (I, II, and III). Clusters I and II were further divided into two sub-clusters (I-A/I-B and II-A/II-B), respectively, based on 90% of similarity value. Y7–15 strains obtained from hospital B were all categorized into cluster II, showing that they were highly related PFGE pattern. For M1–10 strains obtained from hospital A, although cluster I-A and I-B strains showed

more than a 85 % similarity value respectively, there was a genetic variation compared with the Y-series strains. MLST showed that these isolates belonged to a ST78 lineage (allelic profile, 15-1-1-1-1-1-1), indicating that all these too low-level VAN resistant *E. faecium* strains with the *vanB* gene cluster had a similar genetic background, but there is a slight genetic variation.

Single nucleotide variations (SNVs) in *vanB* gene cluster of too low-level VAN resistant isolates

To identify the determinants for too low-level VAN resistance in the M1–10 and Y7–12 strains, we analyzed the nucleotide sequences of the *vanB* gene clusters in M1 and Y7, which were the first isolates to be detected in the respective hospitals (Fig. 2, Table 2). The nucleotide sequences of the *vanB* gene clusters of these strains were completely identical. Then we compared the nucleotide sequence of the *vanB* gene cluster of M1 and Y7 with that of VanB-type *E. faecium* BM4661, which shows a high-level VAN resistance (MIC=256 mg/L) [17]. Compared to the *vanB* gene cluster of BM4661, M1 and Y7 had the same single nucleotide variations leading to six amino acid substitutions in the *vanB* gene cluster (Fig. 2, Table 2). Similarly, we compared the sequences of these isolates with those of other VanB-type VRE in the NCBI database displaying MIC-values of VAN greater than 16 mg/L [16-21]. Despite various levels of resistance to VAN, those strains

showed high sequence identities in the *vanB* gene cluster (Additional file 1: Table S1). However, a total of three unique substitutions in $VanS_B$ (Ala124Glu), VanW (Thr92Pro) and VanB (Ala128Val) were detected in M1 and Y7 (Fig. 2, Table 2, Additional file 2-4: Fig. S1–3).

The *vanB* gene cluster of M1 or Y7 is mobilized to *E. faecalis and E. faecium*

The *vanB* gene cluster is reported to be encoded on a mobile genetic element such as the Tn*1549*, Tn*1547*, Tn*5387* conjugative transposon [22-24]. To test the effect of the host genetic background on the attenuated VAN-resistance, conjugative transfer experiments of the *vanB* gene cluster were carried out. The *vanB* gene cluster of M1 or Y7 was not transferred by broth mating (data not shown), but was successfully transferred by filter mating to the recipient strain *E. faecalis* FA2-2 together with gentamicin, kanamycin and tetracycline resistances, and to *E. faecium* BM4105RF together with gentamicin and tetracycline resistances (Table 1). The resistance gene against kanamycin was thought to be co-transferred to BM4105RF. However, we could not detect this because this strain showed native resistance to kanamycin. The *vanB* gene cluster of M1 and Y7 transferred at frequencies of around 10^{-6} to 10^{-7} per donor cell between FA2-2 or around 10^{-7} to 10^{-8} per donor cell between BM4105RF during filter mating. We identified the nucleotide sequence of the *vanB* gene cluster of the representative transconjugants

(recipient; FA2-2), designated as M1TC (donor; M1) or Y7TC (donor; Y7). It was confirmed that the sequence of the *vanB* gene clusters in M1TC or Y7TC and the respective donor strain coincided. The transconjugants showed low MIC values for VAN and teicoplanin as well as the donor strain (Table 1), indicating that neither the *vanB* gene cluster from M1 nor Y7 confers the glycopeptide resistance to the FA2-2 and the BM4105RF strains or to the original *E. faecium* host background.

The *vanB* gene cluster of M1 or Y7 is located on a plasmid and transferred to a chromosome of the recipient

To determine the localization of the *vanB* gene cluster in these strains, we performed Southern transfer and a hybridization analysis. Southern hybridization of the *vanB* gene probe to the ca. 330 kb and ca. 100 kb S1 nuclease-treated DNAs of M1 and Y7 indicated a plasmid-localization for the *vanB* gene cluster (Additional file 5: Fig. S4). However, co-hybridization with the *vanB* gene probe and 23S rRNA gene probe to I-*Ceu*I-digested DNAs were identified in M1TC and Y7TC (Additional file 6: Fig. S5). Furthermore, we could not identify the transfer of the original plasmid to M1TC or Y7TC (Additional file 5: Fig. S4). These results suggested that the mobile elements including the *vanB* gene cluster were inserted on the chromosomes of the transconjugants.

Reversion of the too low-level VAN resistance of *vanB* gene cluster to the higher-level

VAN resistance

To test whether the *vanB* gene cluster in M1 and Y7 was able to revert its too low-level VAN resistance to the higher-level VAN resistance, we performed reversion experiments. Each 24 hour culture of M1, Y7, M1TC and Y7TC strains without drug was inoculated onto a THB agar plate containing VAN at concentration of 16 mg/L, following incubation at 37 °C. Spontaneous VAN-resistant derivative mutants (revertants) were obtained with a frequency of 10^{-6}–10^{-7} /cell (Table 3). The frequencies of reversion in the transconjugants M1TC and Y7TC were almost as same as those of M1 and Y7. To examine the genetic changes in the *vanB* gene cluster, we first checked the nucleotide sequence of the *vanB* gene cluster of the revertants derived from M1TC or Y7TC (identical FA2-2 genetic host background). The mutations found in the *vanB* gene cluster in these revertants, designated as M1TCR1–10 and Y7TCR1–4, were concentrated in the intracytoplasmic domain of $VanS_B$ (Fig. 3, Table 4). Four out of ten of the M1TCR-strains (40%) and three out of four Y7TCR-strains (75%) had the *vanS*$_B$ gene mutation. All of the revertants showed the higher-level VAN resistance (MIC>16 mg/L). Among them, M1TCR6 was the only strain showing both the VAN and teicoplanin resistant phenotype. This revertant had an insertion of 21bp (7 a. a.) in the intracytoplasmic domain of $VanS_B$ (Fig. 3, Table 4). Furthermore, two revertants with the *vanB* gene mutation were identified

in M1TCR2 and Y7TCR4. We also examined the sequence of the intracytoplasmic domain of the *vanS*$_B$ gene in the M1-derived revertants (M1R1─17) and Y7-derived revertants (Y7R1─7). Of these revertants, 17 revertants (71%) had mutations in the intracytoplasmic domain of VanS$_B$ (Fig. 3, Additional file 7: Table S2). We could not obtain revertants with any mutations in the *vanR*$_B$, *vanW*$_B$, *vanH*$_B$ or the *vanX*$_B$ genes. The *vanS*$_B$ gene encodes part of the two-component regulatory system and regulates transcription level of the *vanB* gene clusters. This result suggested that the too low-level VAN resistant phenotype was caused by a change in the transcription level of the *vanB* gene cluster.

Increased expression level of the *vanB* gene cluster in the revertants

The transcription of the *vanB* gene cluster is strictly suppressed in the absence of VAN, and induction by VAN via the VanS$_B$/R$_B$ pathway is essential for mediating VAN resistance [9]. To test whether the reversion to increased-level VAN resistance was due to the change in the transcriptional level of the *vanB* gene cluster, we examined the expression level of the *vanX*$_B$ gene as a representative of the *vanB* gene cluster of these strains by real-time PCR analysis (Table 4). Gene expression of *vanX*$_B$ was not detected in the absence of VAN, except for M1TCR6. In the presence of VAN, there was an approximately 4-fold decrease of *vanX*$_B$ gene expression in M1TC compared to the VAN-

resistant prototype strain FA2-2/pMG2200 (data not shown). It was previously reported that pMG2200 has the Tn*1549*-like transposon encoding *vanB* gene cluster and that pMG2200 conferred high-level VAN resistance (MIC≥64 mg/L) [16]. In contrast, there was ca. 6-fold increase in M1TCR3 and ca. 40-fold increase in M1TCR6 compared with M1TC. Additionally, the constitutive expression of $vanX_B$ gene in M1TCR6 was detected even in the absence of VAN (Table 4). These data suggested that the increased expression levels of the *vanB* gene cluster were associated with the higher-level VAN resistance phenotypes of the revertants even in M1TCR1 and M1TCR5 which had no mutations in *vanB* gene cluster.

No burden on bacterial growth by carriage of the *vanB* gene cluster in the transconjugants and its inducible revertants

The drug resistant determinants impose a burden on cell growth, and the biological cost for expressing the drug resistance is an important factor for their prevalence in host human tissue or any clinical setting [25, 26]. To examine the effect of the *vanB* gene clusters with various level of VAN resistances on bacterial growth, FA2-2, FA2-2/pMG2200, M1TC, M1TCR3 or M1TCR6 was individually incubated at 37°C in THB and their growth curves were analyzed (Fig. 4). Individual growth curves were similar to each other, except for M1TCR6 in the absence of VAN. These results indicated that the

mobile element including the *vanB* gene cluster, if the genes were inducible and not constitutively expressed, did not reduce bacterial growth *in vitro*.

Targeted mutagensis of *vanB* gene cluster restored the resistance to VAN

To examine whether single nucleotide variations (Fig. 2, Table 2) in the M1/Y7-derived *vanB* gene cluster are responsible for the attenuated resistance to VAN, we carried out targeted mutagenesis by homologous recombination with pCJK47.

A total of three unique substitutions, in $VanS_B$ (Ala124Glu), VanW (Thr92Pro) and VanB (Ala128Val), were detected in M1 and Y7 (Table 2). We exchanged these three unique nucleotide variations to those of VanB-type VRE displaying MIC-values of VAN greater than 16 mg/L (Table 2, Additional file 2-4: Fig. S1-3). The mutant strain with VanW (274C>A; Pro92Thr) and/or VanB (383T>C; Val128Ala) did not show any increased-level of VAN resistance (MIC=3mg/L). However, the MIC values of VAN in the mutants with $VanS_B$ (371A>C; Glu124Ala) and VanW (274C>A; Pro92Thr) or VanB (383T>C; Val128Ala) were increased (MIC=8mg/L). Exchanges of all these three single nucleotide variations in M1TC restored VAN resistance (MIC=16mg/L) (Table 5). In particular, $VanS_B$ (371A>C; Glu124Ala) appeared to be the key substitution required for vancomycin resistance.

Discussion

Epidemiology of M1–10 and Y7–12 *E. faecium* strains

In this study, we genetically characterized the *E. faecium* isolates M1–10 and Y7–15 strains, which were susceptible to VAN despite possessing the *vanB* gene cluster (Table 1). These strains were derived from two unrelated hospitals, but showed similar genetic backgrounds belonging to ST78 (Fig. 1, Table 1). Recently, it was reported that clonal lineage was disseminated as a consequence of antibiotic pressure in clinical settings [27]. The ST78 in *E. faecium* is one of the important group which has spread hospitalized patients and belongs to Bayesian analysis of population structure (BAPS) 2-1. The BAPS 2-1 is a hospital-adapted lineage and associated with farm animals [28]. The PFGE patterns of the Y7–15 strains showed highly related PFGE types, suggesting the clonal dissemination of these isolates in the hospital. Meanwhile, M1–10 strains showed a relatively small genetic variation in their PFGE pattern, and the PFGE profiles of M5 and M9 showed 70% similarities with those of the other M-series strains. The recombination occurs more frequently and has an important role in a genetic diversity in *E. faecium* [28, 29]. During a nosocomial outbreak, de novo generation of vancomycin-resistant *E. faecium* by mobile element including *vanB* gene cluster was more frequently than cross-transmission [30]. Taken together with the fact that M1–10 and Y7–12 strains shared an

completely identical *vanB* gene cluster and similar antimicrobial susceptibility profile, there seemed to be a possibility of a horizontal gene transfer and/or a long-term colonization sufficient for genetic rearrangement in Hospital A and a transmission between patients in Hospital B.

Stealth behavior of potential VAN-resistance enterococci

About a hundred cases of VRE infection are reported in Japan annually by National Institute of Infectious Disease (NIID) surveillance but there are few investigations of the prevalence of VRE (http://www.nih.go.jp/niid/ja/). Matsumoto *et al.* reported that among 24,297 clinical samples that included feces, urine, blood, sputum, decubitus and pus, neither VanA- nor VanB-type VRE was detected in Kitakyusyu city in 2002 [31]. Matsushima *et al.* reported the regional spread of VRE in Kyoto prefecture [32]. However, because of the VAN-concentrations used for the VRE screening (6 mg/L and 16 mg/L, respectively), there is the possibility of missing low-level VAN resistant isolates [32]. Under the Infectious Disease Control Law in Japan (Kansenshō-Hō, Act on Prevention of Infectious Disease and Medical Care for Patients Suffering Infectious Disease of Japan), notifiable VRE is defined as enterococcal clinical isolates causing infectious disease and showing VAN MIC≥16mg/L. The *E. faecium* isolates we reported here showed too low-level VAN resistance (MIC=3mg/L) that is outside the scope of this

definition for notifiable VRE in Japan (Table 1). Notably, we also demonstrated that spontaneous reversion to the higher-level VAN resistance of the too low-level resistant *vanB*-carrying *E. faecium* could occur with a frequency of 10^{-6}–10^{-7}/parent cell (Table 3). This frequency seems to be relatively high in comparison with the usual frequency of reversion to antibiotics in other bacteria [33]. These barely detectable too low-level resistant strains have the capacity for long-term colonization in the human gut without producing any symptoms, because the too low-level resistance to VAN does not affect bacterial fitness even in the absence of VAN (Fig. 4). Consistent with our results, Marie-Laure Foucault *et al.* has reported that inactivated or inducible Tn*1549* carrying the *vanB* gene cluster produces no additional fitness cost [26]. Furthermore, the transfer of this *vanB* gene cluster on the plasmid of M1 or Y7 to the chromosome of other enterococci indicated the risk of efficient dissemination of a resistance gene. Therefore, it should be considered that this “stealth behavior” of too low-level resistance *vanB* gene clusters is subjected to the active surveillance by the standard VAN screening method.

Genetic mutations leading to the reversion phenotype

It has been reported elsewhere that enterococcus strains harboring the VanB-type VAN resistance gene cluster showed various MIC level for VAN or TEC [7, 9]. However, the determinants involved in this variation of MIC is not understood [8]. We detected the

experimental reversion to the higher VAN resistance phenotype in M1 and Y7 strains, or in their corresponding transconjugants M1TC and Y7TC (Table 1, 3). For the genetic regulation of the *vanB*-mediated VAN resistance, the $VanS_B$ sensor protein has a pivotal role of both kinase and dephosphatase activity that modulates the phosphorylation level of $VanR_B$. When $VanS_B$ senses VAN, the $VanS_B/R_B$ system activates transcription of the *vanB* gene cluster and then mediates the VAN resistance [9]. Actually, the VAN-induced expression levels of *vanB* gene cluster were increased (by 3.6 to 39.5 fold) in the revertants compared with each parent strain in our investigations (Table 4). Therefore, the reversion to the higher-level VAN resistance appeared to be mediated by the increased expression level of the *vanB* gene cluster. It was previously reported that various mutations in *the* $vanS_B$ gene changed the glycopeptide resistance phenotype [6, 9, 34]. Consistent with this, 24 strains of the 38 revertants (63%) that were tested carried the novel mutations in intracytoplasmic domain of $vanS_B$ gene (Fig. 3, Table 4 and Additional file 7: Table S2). In general, the high frequency of reverting to antibiotics resistance and the concentration of mutations in “one gene” indicated that the mechanism of reversion was loss of function of the gene rather than gain of function [33]. These mutations appeared to lead to the attenuated dephosphatase function of $VanS_B$. Meanwhile, the rest of the revertants derived from M1TC or Y7TC did not harbor any genetic mutation in the

vanB gene cluster containing $vanR_B$, $vanY_B$, *vanW*, $vanH_B$, *vanB*, $vanX_B$ and or the sensor domain of $vanS_B$, except for M1TCR2 and Y7TCR4 carrying the mutations in the *vanB* gene (Table 4). However, these revertants with no mutation, such as M1TCR1 or M1TCR5 still showed increased expression of the *vanB* gene cluster in the presence of VAN (Table 4), indicating that genetic mutations outside of the *vanB* gene cluster must affect the transcriptional activity of *vanB* genes. Additionally, M1TCR2 had an amino acid substitution in the *vanB* gene (Table 4). According to previous reports, this amino acid substitution of VanB did not seem to be important for ligase function [35-37]. Therefore, the mechanism of reversion of M1TCR2 was presumed to be the same as that of M1TCR1 or M1TCR5. The possible cross talks between $VanR_B$ and cognate enterococcal kinase were presumed to be responsible in the previous study [38]. This report may provide clues about the mechanism of reversion without a mutation in the *vanB* gene cluster including the P_{RB}/P_{YB} promotor region. A further study is underway to clarify this point. Collectively, reversion from too low-level VAN resistance mediated by the *vanB* gene cluster to a higher level of resistance appears to be associated with the increased transcriptional expression of the *vanB* gene cluster, but the genetic mutation or the mechanism responsible for the reversion appears to be varied and not limited in the *vanB*-related genes.

Single nucleotide variations in the *vanB* gene cluster attenuated resistance to VAN

In addition to this revertant analysis, we carried out targeted mutagenesis to examine whether the single nucleotide variations (Table 2) in the M1/Y7-derived *vanB* gene cluster are responsible for the attenuated resistance to VAN. There were three unique amino acid substitutions (Ala124Glu in $VanS_B$, Thr92Pro in VanW and Ala128Val in VanB) in the M1/Y7 strain (Table 2). It was reported that the essential genes of VanB-type VAN resistance were *vanH*$_B$, *vanB* and *vanX*$_B$ [1] and deletion of *vanW*$_B$ in V583 did not affect the VAN resistance [39]. In addition, it has been reported previously that the amino acid substitution in VanB (Ala128Val) did not have an impact on ligase function [35-37]. However, our experiment suggested that three single nucleotide variations resulting in an amino acid change in the *vanB* gene cluster appeared to have a combined impact on vancomycin resistance (Table 5). The MIC-values of VAN in the mutant strains without $VanS_B$ (371A>C; Glu124Ala) were equal to that in the parent strain (MIC=3mg/L). Therefore, the nucleotide sequence variation in *vanS*$_B$ gene is assumed to be the main factor, but all of the three variations lead to too low-level VAN resistance.

Conclusions

It has been thought that VVE means only VanA-type VAN susceptible enterococci that can revert to the resistant phenotype [14]. As shown in this study, these too low-level VAN resistant VanB-type isolates also have the ability to revert to the higher-level VAN resistant phenotype. Due to the potential threat in clinical settings and the risk of treatment failure, these strains should be included in VVE. Further study is required to understand by what molecular mechanism(s) the stealthy *vanB* gene cluster converts to the higher-level resistance and the responsible determinant(s) for the varied range of glycopeptide MIC in the VanB-type resistance enterococci.

Ethics approval and consent to participate

Not applicable.

Availability of data and material

All data and materials are provided in the article (and its supplementary information).

Competing interests

The authors declare that they have no competing interests.

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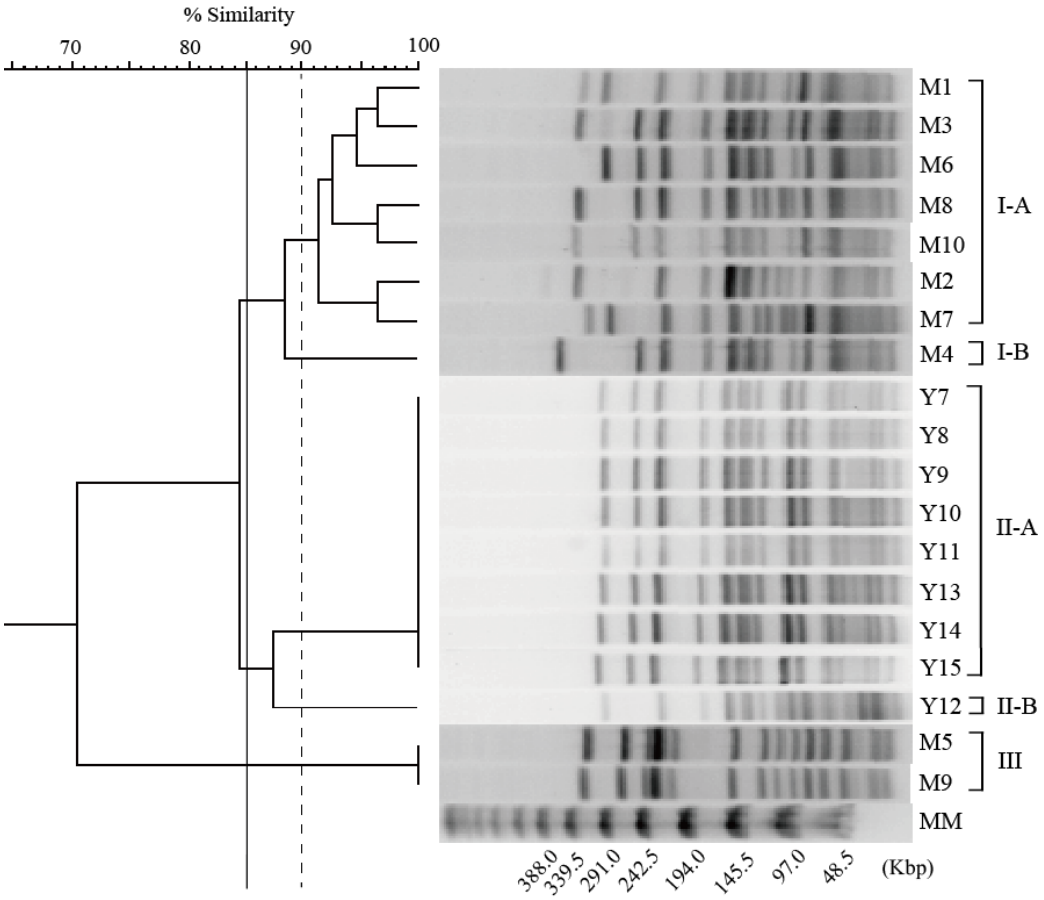

Figure 1. PFGE profiles and dendrogram of the low-level VAN resistant *E. faecium* isolates. Bacterial DNAs were digested with *Sma*I and separated by pulsed-field gel electrophoresis (PFGE). The genetic relatedness was analyzed using the Dice coefficient and the dendrogram was constructed with the clustering algorithm of Unweighted Pair-Group Method with an Arithmetic Mean (UPGMA) using FP Quest Software (Bio-Rad). Major clusters and subclusters of the isolates were delineated with 85% and 90% similarity cutoff values for PFGE as indicated by the vertical solid line and dotted line, respectively. A lambda PFG Ladder (New England BioLabs, MA) was used as the Molecular Marker (MM).

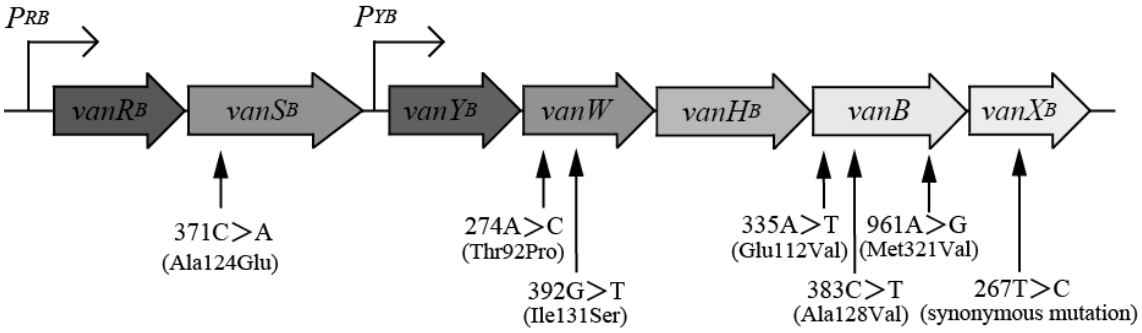

Figure 2. Schematic representation of the *vanB* gene cluster and single nucleotide variations in M1 and Y7 compared with the *vanB* gene cluster in BM4661.

Expression of the *vanB* cluster genes is regulated by the two-component regulatory system of VanS$_B$/VanR$_B$. In the presence of VAN, the histidine kinase VanS$_B$ senses VAN and activates the transcriptional activator VanR$_B$. Consequently, the two- component regulatory genes (*vanR$_B$* and *vanS$_B$*) and the resistance genes (*vanY$_B$*, *vanW*, *vanH$_B$*, *vanB* and *vanX$_B$*) are transcribed from the VanR$_B$-driven promoters *P$_{RB}$*/*P$_{YB}$* to mediate the VAN resistance [10]. The arrows indicate the location of nucleotide variations (and resulting amino acid substitutions) identified in the *vanB* gene cluster of M1/Y7 compared with that of BM4661.

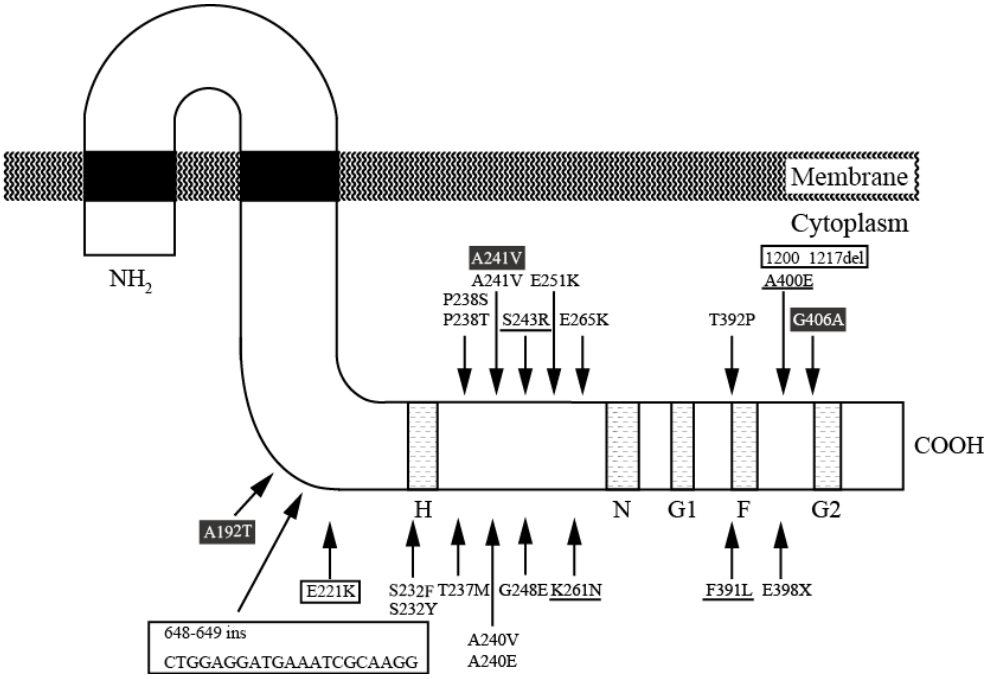

Figure 3. Schematic representation of novel mutations in the *vanS$_B$* gene of the revertants. VanS$_B$ contains the motifs designated as H, N, G1, F, G2 (lightly shaded boxes) that are the conserved motifs of histidine protein kinases [48]. The His residue (233) in the H box is the primary autophosphorylation site [34].The arrows and characters in black boxes represent mutations found in M1R, the underlined characters represent those in Y7R, those in boxes represent mutations in M1TCR and characters by themselves represent mutations in Y7TCR, respectively.

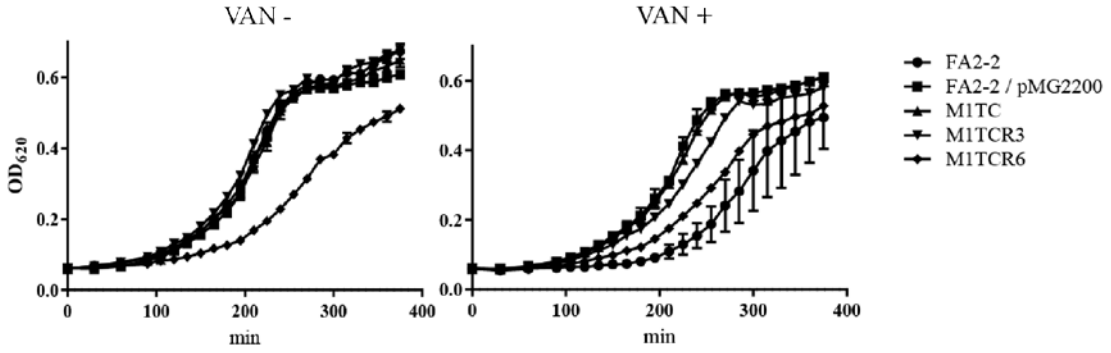

Figure 4. Growth curves of the recipient, transconjugant and revertants in the presence or absence of VAN. Overnight bacterial cultures were diluted 100-fold in fresh THB broth with (Right) or without (Left) 1 mg/L VAN. The culture was incubated at 37 °C and the turbidity was measured at an optical density of 620 nm (OD_{620}) at each time point. The values were the mean of three independent experiments with standard error, each experiment was performed in triplicate.

Table 1. Bacterial strains used in this study.

Strain/plasmid	Species	Source	Date of Isolation (mo/yr)	Hospital	VRE genotype	Multilocus sequence typing	PFGE types	MIC (mg/L)[a]									
								VAN	TEC	AMP	GEN	KAN	STR	CHL	TET	ERY	CIP
M1	*E. faecium*	feces	09/09	A	VanB	ST78	I-A	3	1	256	>1024	>1024	32	8	64	＞256	64
M2	*E. faecium*	feces	10/09	A	VanB	ST78	I-A	3	1	256	>1024	>1024	32	8	64	＞256	64
M3	*E. faecium*	feces	10/09	A	VanB	ST78	I-A	3	1	256	>1024	>1024	32	8	64	＞256	32
M4	*E. faecium*	feces	10/09	A	VanB	ST78	I-B	3	1	256	8	>1024	32	8	64	＞256	64
M5	*E. faecium*	feces	10/09	A	VanB	ST78	III	3	1	128	8	128	256	8	64	＞256	64
M6	*E. faecium*	feces	10/09	A	VanB	ST78	I-A	3	1	256	8	>1024	32	8	64	＞256	64
M7	*E. faecium*	feces	10/09	A	VanB	ST78	I-A	3	1	256	>1024	>1024	32	8	64	＞256	64
M8	*E. faecium*	feces	10/09	A	VanB	ST78	I-A	3	1	256	>1024	>1024	32	8	64	＞256	64
M9	*E. faecium*	feces	10/09	A	VanB	ST78	III	3	1	128	8	128	256	8	64	＞256	64
M10	*E. faecium*	feces	10/09	A	VanB	ST78	I-A	3	1	256	>1024	>1024	32	8	64	＞256	64
Y7	*E. faecium*	feces	02/10	B	VanB	ST78	II-A	3	1	256	>1024	>1024	32	8	64	＞256	64
Y8	*E. faecium*	feces	02/10	B	VanB	ST78	II-A	3	1	256	>1024	>1024	32	8	64	＞256	64
Y9	*E. faecium*	feces	02/10	B	VanB	ST78	II-A	3	1	256	>1024	>1024	32	8	64	＞256	64
Y10	*E. faecium*	feces	02/10	B	VanB	ST78	II-A	3	1	256	>1024	>1024	32	8	64	＞256	64
Y11	*E. faecium*	feces	02/10	B	VanB	ST78	II-A	3	1	256	>1024	>1024	32	8	64	＞256	64
Y12	*E. faecium*	feces	02/10	B	VanB	ST78	II-B	3	1	128	>1024	>1024	32	8	64	＞256	64
Y13	*E. faecium*	feces	03/10	B	VanB	ST78	II-A	3	1	256	>1024	>1024	32	8	64	＞256	64
Y14	*E. faecium*	feces	03/10	B	VanB	ST78	II-A	3	1	256	>1024	>1024	32	8	64	＞256	64
Y15	*E. faecium*	feces	03/10	B	VanB	ST78	II-A	3	1	256	>1024	>1024	32	8	64	＞256	64
FA2-2	*E. faecalis*	-	-	-	-	-	-	1	1	2	32	128	64	8	2	0.5	1
FA2-2/pMG2200	*E. faecalis*	-	-	-	VanB	-	-	64	1	2	32	128	64	8	2	0.5	1
M1TC[b]	*E. faecalis*	-	-	-	VanB	-	-	3	1	2	>1024	>1024	64	8	64	0.5	1
Y1TC[b]	*E. faecalis*	-	-	-	VanB	-	-	3	1	2	>1024	>1024	64	8	64	0.5	1
BM4105RF	*E. faecium*	-	-	-	-	-	-	1	1	3	8	>1024	32	2	1	0.5	2
M1TC2[c]	*E. faecium*	-	-	-	VanB	-	-	3	1	3	>1024	>1024	32	2	128	0.5	2
Y7TC2[c]	*E. faecium*	-	-	-	VanB	-	-	3	1	3	>1024	>1024	32	2	128	0.5	2

[a]VAN, vancomycin; TEC, teicoplanin; AMP, ampicillin; GEN, gentamicin; KAN, kanamycin; STR, streptomycin; CHL, chrolamphenicol; TET, tetracycline; ERY, erythromycin; CIP, ciprofloxacin. [b]Corresponding transconjugants of FA2-2 obtained by filter mating with the donor M1 or Y7 strain, respectively. [c]Corresponding transconjugants of BM4105RF obtained by filter mating with the donor M1 or Y7 strain, respectively.

Table 2. Single nucleotide variations found in the vanB gene cluster of *E. faecium* M1 and Y7 compared to that of BM4661a.

Gene	Sunstitutions[b]	
	BM4661>M1/Y7	
vanS$_B$	371C>A	(Ala124Glu)[d]
vanW	274A>C	(Thr92Pro)[d]
vanW	392G>T	(Ile131Ser)
vanB	335A>T	(Glu112Val)
vanB	383C>T	(Ala128Val)[d]
vanB	961A>G	(Met321Val)
vanX$_B$	267T>C	SM[c]

[a]The genetic information for BM4661 (accession no.; FJ767776.1) was obtained from the genome database in NCBI (http://www.ncbi.nlm.nih.gov/).

[b]The nucleotide sequences of vanB gene clusters of M1 and Y7 strains were identical. The brackets indicate amino acid substitutions in vanB gene clusters. The numbers of substitusions represent the location of each gene and protein.

[c]SM, synonymous mutation.

[d]Unique substitusions for M1 and Y7 strains (supplementary data Figure S1, S2, S3).

Table 3. Frequency of reversion to VAN-resistant phenotype.

Parent strain	The frequency of reversion[a]
M1	$(4.2 \pm 2.0) \times 10^{-7}$
Y7	$(1.7 \pm 1.2) \times 10^{-6}$
M1TC[b]	$(2.4 \pm 0.5) \times 10^{-7}$
Y7TC[b]	$(3.5 \pm 1.0) \times 10^{-7}$

[a]Frequency was estimated from the colony-forming unit (CFU) ratio of resistant strains to total strains.

[b]Corresponding transconjugants of M1 or Y7 strain.

Table 4. Glycopeptide MICs and detected mutations in vanB gene cluster of the VAN-resistant revertants obtained from M1TC and Y7TC.

Strain	Mutation[a]		MIC (mg/L)[b]		RT-PCR (Fold change (±SE)[c]	
	vanS$_B$ gene	*vanB* gene	VAN	TEC	VAN (-)	VAN (+)
M1TC	-	-	3	1	N. D.	1
M1TCR1	N. D.	N. D.	16	1	N. D.	4.4 (±1.0)
M1TCR2	N. D.	574G>A(Ala192Thr)	16	1	N. D.	3.6 (±0.5)
M1TCR3	661G>A(Glu221Lys)	N. D.	16	1	N. D.	5.8 (±1.2)
M1TCR4	N. D.	N. D.	16	1	-	-
M1TCR5	N. D.	N. D.	16	1	N. D.	4.9 (±1.4)
M1TCR6	648-649 ins CTGGAGGATGAAA	N. D.	128	128	36.1 (±8.0)	39.5 (±13.9)
M1TCR7	1200-1217 del	N. D.	16	1	-	-
M1TCR8	N. D.	N. D.	16	1	-	-
M1TCR9	N. D.	N. D.	16	1	-	-
M1TCR10	661G>A(Glu221Lys)	N. D.	32	1	-	-
Y7TC	-	-	3	1		
Y7TCR1	722C>T(Ala241Val)	N. D.	16	1	-	-
Y7TCR2	N. D.	N. D.	16	1	-	-
Y7TCR3	574G>A(Ala192Thr)	N. D.	64	1	-	-
Y7TCR4	1218G>C(Gly406Ala)	317C>A(Ala106Glu)	16	1	-	-

[a]The sequence was compared with the vanB gene cluster of the cognate parent strain, M1TC or Y7TC.

[b]VAN: vancomycin, TEC: teicoplanin.

[c]Real-time PCR data represent the fold changes in vanXB transcriptional level relative to that of M1TC in the presence of vancomycin (1 mg/L; VAN (+)). The values were the means of three independent experiments with standard error, each experiment performed in duplicate. N. D.; Not detected.

Table 5. Glycopeptide MICs of the MITC derivatives that had been introduced mutations in vanB gene cluster.

Strain	MIC (mg/L)[a]	
	VAN	TEC
M1TC	3	1
M1TC *vanS*$_B$_E124A[b]	4	1
M1TC *vanB* V128A[b]	3	1
M1TC *vanW* P92T[b]	3	1
M1TC *vanS*$_B$ E124A/*vanB*_ V128A[b]	8	1
M1TC *vanS*$_B$ E124A/*vanW*_ P92T[b]	8	1
M1TC *vanB* V128A/*vanW* P92T[b]	3	1
M1TC *vanS*$_B$ E124A/*vanB*_ V128A/*vanW*_ P92T[b]	16	1

[a]VAN, vancomycin; TEC, teicoplanin

[b]Three unique substitusions for M1 and Y7 strains were exchanged to that of the reference gene of pMG2200 by pCJK47-used homologous recombination.

Additional materials

Figure S1. Amino acid sequence alignment of $VanS_B$ encoded by M1/Y7 and the other VanB-type VRE

The genetic information for VanB-type vancomycin resistant enterococci was obtained from the genome database in NCBI (http://www.ncbi.nlm.nih.gov/). Alignments of $VanS_B$ amino acid sequence of wild M1/Y7 with respect to typical VanB-type vancomycin resistant enterococci such as MLG229 (accession no.; AY655721.2), UW7606x64/3 TC1 (accession no.; CP013009.1), V583 (accession no.; NC_004668), Aus0085 (accession no.;NC_021994.1) and BM4661 (accession no.; FJ767776.1) were carried out using ClustalW. A box indicated the unique substitution to M1 /Y7 strains.

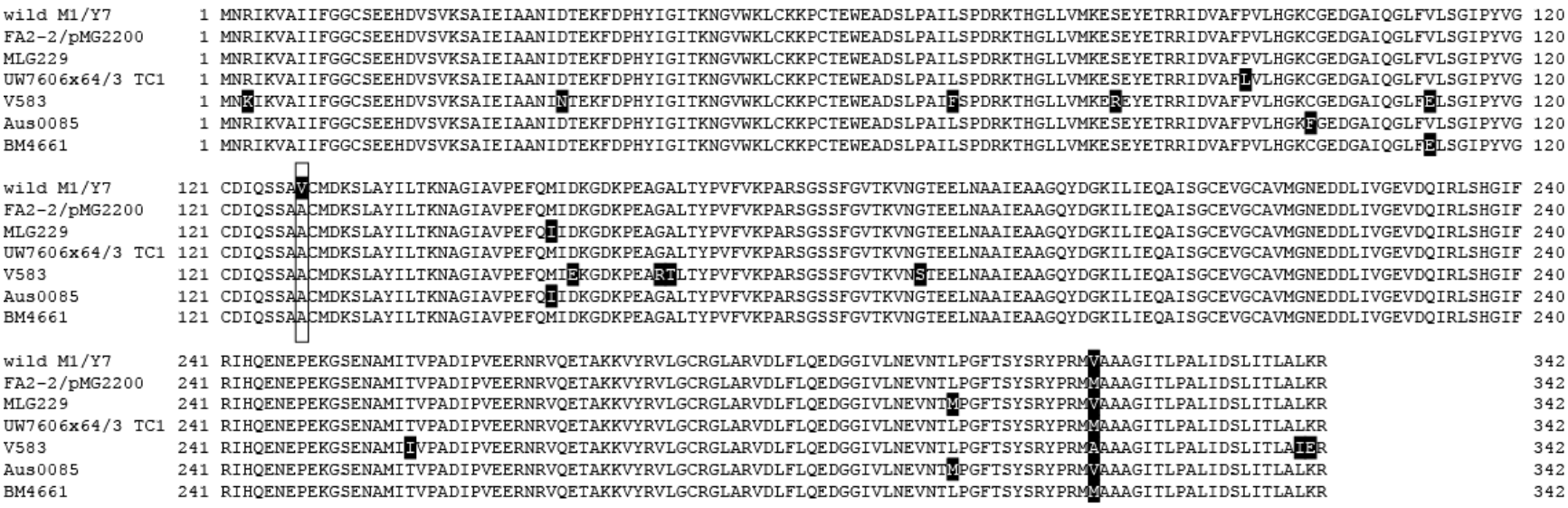

Figure S2. Amino acid sequence alignment of VanB encoded by M1/Y7 and the other VanB-type VRE

The genetic information for VanB-type vancomycin resistant enterococci was obtained from the genome database in NCBI (http://www.ncbi.nlm.nih.gov/). Alignments of VanB amino acid sequence of wild M1/Y7 with respect to typical VanB-type vancomycin resistant enterococci such as MLG229 (accession no.; AY655721.2), UW7606x64/3 TC1 (accession no.; CP013009.1), V583 (accession no.; NC_004668), Aus0085 (accession no.;NC_021994.1) and BM4661 (accession no.; FJ767776.1) were carried out using ClustalW. A box indicated the unique substitution to M1 /Y7 strains.

Figure S3. Amino acid sequence alignment of VanW encoded by M1/Y7 and the other VanB-type VRE

The genetic information for VanB-type vancomycin resistant enterococci was obtained from the genome database in NCBI (http://www.ncbi.nlm.nih.gov/). Alignments of VanW amino acid sequence of wild M1/Y7 with respect to typical VanB-type vancomycin resistant enterococci such as MLG229 (accession no.; AY655721.2), UW7606x64/3 TC1 (accession no.; CP013009.1), V583 (accession no.; NC_004668), Aus0085 (accession no.;NC_021994.1) and BM4661 (accession no.; FJ767776.1) were carried out using ClustalW. A box indicated the unique substitution to M1 /Y7 strains.

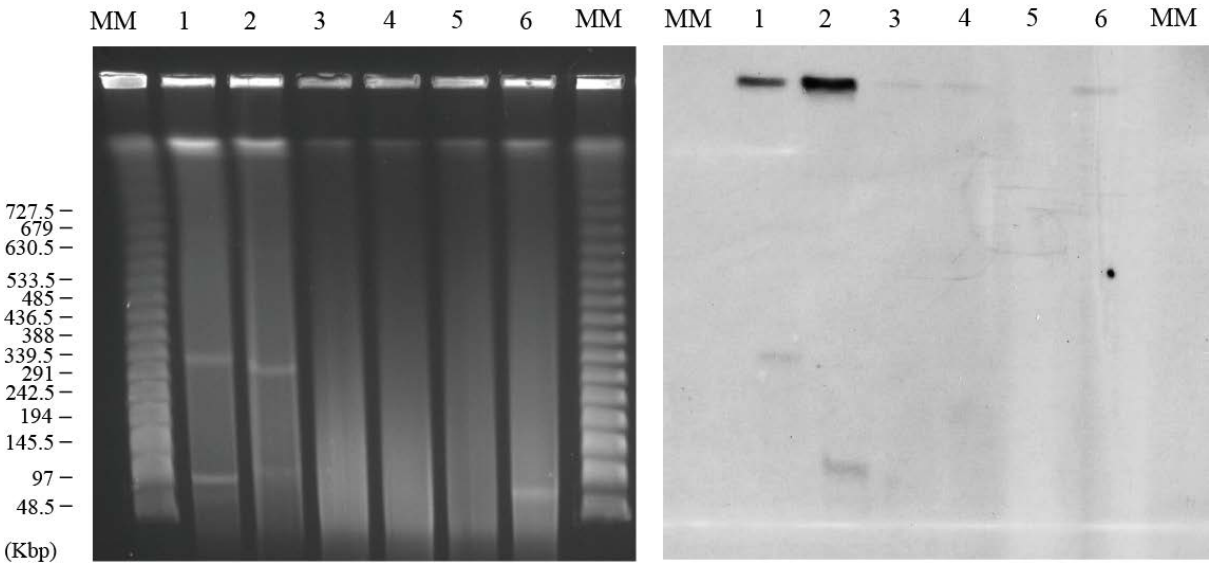

Figure S4. PFGE of S1 nuclease-treated DNA and hybridization with *vanB* gene probes

PFGE of S1 nuclease-treated DNAs isolated from M1, Y7, M1TC, Y7TC, FA2-2 and V583 was performed (Left) and gel was Southern blotted and hybridized to *vanB* gene (Right). Lanes: MM, Lambda Ladder PFG Marker (New England BioLabs, MA); 1, M1; 2, Y7; 3, M1TC; 4, Y7TC; 5, FA2-2; 6, V583.

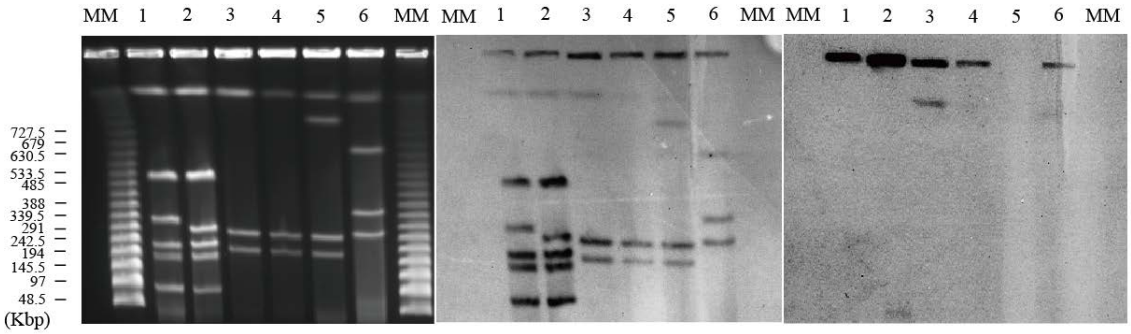

Figure S5. PFGE of I-*Ceu*I-digested DNA and hybridization with 23S rRNA gene and *vanB* gene probes

PFGE of I-*Ceu*I-digested DNAs isolated from M1, Y7, M1TC, Y7TC, FA2-2 and V583 was performed (Left) and gel was Southern blotted and hybridized to 23S rRNA gene (Middle) and *vanB* gene (Right). Lanes: MM, Lambda Ladder PFG Marker (New England BioLabs, MA); 1, M1; 2, Y7; 3, M1TC; 4, Y7TC; 5, FA2-2; 6, V583.

Table S1. Oligonucleotides used in this study.

Names	Sequence (5'-3')[a]	Description	Reference
*E.faecalis*_ DDL_ E1	ATCAAGTACAGTTAGTCT	species specific *ddl* of *E. faecalis*	(46)
*E.faecalis*_ DDL_ E2	ACGATTCAAAGCTAACTG	species specific *ddl* of *E. faecalis*	(46)
*E.faecium*_ DDL_ F1	TAGAGACATTGAATATGCC	species specific *ddl* of *E. faecium*	(46)
*E.faecium*_ DDL_ F2	TCGAATGTGCTACAATC	species specific *ddl* of *E. faecium*	(46)
VanB_B1	ATGGGAAGCCGATAGTC	*vanB* gene of *vanB* gene cluster	(46)
VanB_B2	GATTTCGTTCCTCGACC	*vanB* gene of *vanB* gene cluster	(46)
VanS_E124A_*EcoR*I_F3	tagataGAATTCCAAGGCAGGTGTGGGATTG	construction of pCJK47_*vanS*$_B$_Glu124Ala	This study
VanS_E124A_*EcoR*I_R3	atagatGAATTCATCTCTGTGGCAGCTTCGATG	construction of pCJK47_*vanS*$_B$_Glu124Ala	This study
VanB_V128A_*EcoR*I_F3	atctatGAATTCTTGGAAAGCGGAAAGCTGG	construction of pCJK47_*vanB*_Val128Ala	This study
VanB_V128A_*EcoR*I_R3	tgagatGAATTCTGTACGATGTAAAACCGGGC	construction of pCJK47_*vanB*_Val128Ala	This study
VanW_A92T_*EcoR*I_F3	tcacgtGAATTCGAGACTGGCTTCCGTATTGG	construction of pCJK47_*vanW*_Pro92Thr	This study
VanW_A92T_*EcoR*I_R3	tccataGAATTCTAACGCATTGATTGCCAGC	construction of pCJK47_*vanW*_Pro92Thr	This study
VanXB_38476F_RT-PCR	GAAAT GTTGCATGGC GTCCG	real time PCR for *vanX*$_B$	This study
VanXB_38611R_RT-PCR	TTTGTGCCTCGCGCAGAG	real time PCR for *vanX*$_B$	This study
GyrB_*E. faecalis*_1F	GGCTTTCTTCACGAGCTTTG	real time PCR for *gyrB*	This study
GyrB_*E. faecalis*_1R	TTGTCGATAACGTCGTAGGC	real time PCR for *gyrB*	This study

[a]Small letters indicate additional tag-nucleotides for plamid construction to be digested by restriction.

Table S2. Mutations in intracytoplasmic domain of VanSB and glycopeptides MICs of the VAN-resistant revertants.

Strain[a]	Mutations in intracytoplasmic domain of VanSB[b]	MIC (mg/L)[c]	
		VAN	TEC
M1R1	695C>T(Ser232Phe)	32	1
M1R2	N. D.	32	1
M1R3	710C>T(Thr237Met)	32	1
M1R4	1192C>T(Glu398X)	32	1
M1R5	793G>A(Glu265Lys)	32	1
M1R6	695C>A(Ser232Tyr)	32	1
M1R7	719C>T(Ala240Val)	128	1
M1R8	751G>A(Glu251Lys)	32	1
M1R9	N. D.	128	1
M1R10	1174A>C(Thr392Pro)	32	1
M1R11	712C>A(Pro238Thr)	32	2
M1R12	N. D.	32	1
M1R13	712C＞T Pro238Ser	32	2
M1R14	N. D.	32	1
M1R15	722C＞T(Ala241Val)	32	1
M1R16	743G>A(Gly248Glu)	32	1
M1R17	719C>A(Ala240Glu)	32	1
Y7R1	N. D.	32	1
Y7R2	1199C>A(Ala400Glu)	16	1
Y7R3	1173C>A(Phe391Leu)	32	1
Y7R4	729C>A(Ser243Arg)	32	1
Y7R5	783G>T(Lys261Asn)	32	1
Y7R6	N. D.	16	1
Y7R7	N. D.	32	1

[a]M1R1—M1R12; revertants from M1, Y7R1—7; revertants from Y7.

[b]The sequence was compared with the vanB gene cluster of the cognate parent strain, M1 or Y7.

[c]VAN: vancomycin, TEC: teicoplanin.

N. D.; Not detected.

Table S3. Oligonucleotides used in this study.

Names	Sequence (5'-3')[a]	Description	Reference or source
*E.faecalis*_DDL_E1	ATCAAGTACAGTTAGTCT	species specific *ddl* of *E. faecalis*	(46)
*E.faecalis*_DDL_E2	ACGATTCAAAGCTAACTG	species specific *ddl* of *E. faecalis*	(46)
*E.faecium*_DDL_F1	TAGAGACATTGAATATGCC	species specific *ddl* of *E. faecium*	(46)
*E.faecium*_DDL_F2	TCGAATGTGCTACAATC	species specific *ddl* of *E. faecium*	(46)
VanB_B1	ATGGGAAGCCGATAGTC	*vanB* gene of *vanB* gene cluster	(46)
VanB_B2	GATTTCGTTCCTCGACC	*vanB* gene of *vanB* gene cluster	(46)
VanS_E124A_*EcoR*I_F3	tagataGAATTCCAAGGCAGGTGTGGGATTG	construction of pCJK47_$vanS_B$_Glu124Ala	This study
VanS_E124A_*EcoR*I_R3	atagatGAATTCATCTCTGTGGCAGCTTCGATG	construction of pCJK47_$vanS_B$_Glu124Ala	This study
VanB_V128A_*EcoR*I_F3	atctatGAATTCTTGGAAAGCGGAAAGCTGG	construction of pCJK47_*vanB*_Val128Ala	This study
VanB_V128A_*EcoR*I_R3	tgagatGAATTCTGTACGATGTAAAACCGGGC	construction of pCJK47_*vanB*_Val128Ala	This study
VanW_A92T_*EcoR*I_F3	tcacgtGAATTCGAGACTGGCTTCCGTATTGG	construction of pCJK47_*vanW*_Pro92Thr	This study
VanW_A92T_*EcoR*I_R3	tccataGAATTCTAACGCATTGATTGCCAGC	construction of pCJK47_*vanW*_Pro92Thr	This study
VanXB_38476F_RT-PCR	GAAAT GTTGCATGGC GTCCG	real time PCR for $vanX_B$	This study
VanXB_38611R_RT-PCR	TTTGTGCCTCGCGCAGAG	real time PCR for $vanX_B$	This study
GyrB_*E. faecalis*_1F	GGCTTTCTTCACGAGCTTTG	real time PCR for *gyrB*	This study
GyrB_*E. faecalis*_1R	TTGTCGATAACGTCGTAGGC	real time PCR for *gyrB*	This study

[a]Small letters indicate additional tag-nucleotides for plamid construction to be digested by restriction.

Molecular characterization of the VanD-type vancomycin-resistant *Enterococcus faecium* clinical isolates from a fecal sample from a same patient after vancomycin therapy.

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Abstract

VanD type resistance was a relatively rare type among nine types of vancomycin-resistance. Until now, several reports have described VanD-type Enterococci. In general, it is characterized by constitutive expression of the resistance genes due to mutations of *vanS*$_D$ or *vanR*$_D$ gene and host *ddl* mutations.

In this report, we described three VanD-type vancomycin resistance *E. faecium* isolates that were isolated from a same patient who had received a long-term vancomycin (VAN) treatment. Multilocus sequence typing (MLST) revealed that these isolates belonged to a same lineage. Pulsed-field gel electrophoresis (PFGE) of these isolates showed the same profiles, suggesting that these were clonal isolates. In addition, a phylogenetic analysis based on the nucleotide sequences of *vanD* gene cluster showed relative relationship between these isolates and *Ruminococcus* spp..

Of three, we identified a 12-bp deletion close to a important site of *ddl* gene of AA620. In contrast, AA622 and AA624 had the intact gene. In addition, 1bp deletion resulted in a frameshift in VanS$_D$ sensor protein of AA620. These results indicated that the majority of peptidoglycan precursors in AA620 was $_D$-Ala-$_D$-Lac, and this led to the higher-level resistant phenotype than in AA622/624. According to the analysis of the molecular epidemiology, these findings indicated that AA620 and AA622/AA624 strains were

originated separately from a same parent under a pressure of VAN. Further, there might be a horizontal transfer of *vanD* gene cluster between *E. faecium* and anaerobic bacteria.

Background

Enterococci is an opportunistic pathogen that may cause urinary tract infections and endocarditis in compromised host. Vancomycin resistance enterococci (VRE) is now one of the hospital-acquired pathogen in the world (1). Although there are nine types of vancomycin resistance, predominant types in clinical setting are VanA and VanB.

VanD type *E. faecium* was relatively rare, and first isolated in 1991 (2). Until now, there are several reports with VanD-type enterococci. VanD-type resistance had been reported in *Enterococcus faecium, E. faecalis, E. gallinarum, E. raffinosus* and *E. avium*. (3-7). VanD-type resistance genes produced the precursor ending in D-Alanine-D-Lactate and conferred VAN resistance on *Enterococcus* spp (8). In general, it is characterized by constitutive expression of the resistance genes due to mutations of $vanS_D$ or $vanR_D$ gene that were composed of two component regulatory systems (5, 6), and host *ddl* mutations except some isolates (4, 9). In this report, we described three VanD-type vancomycin resistance *E. faecium* isolates which were isolated from a same patient who had received a long-term vancomycin (VAN) treatment, but showed different MICs of VAN and TEC.

Materials and methods

Bacterial strains, growth condition, media, and antimicrobial reagents

The bacterial strains used in this study are shown in Table 1. Enterococcal strains were routinely grown in Todd-Hewitt broth (THB; Difco, Detroit, MI) at 37°C.

Antibiotic susceptibility test

MICs were determined by the agar dilution method according to Clinical and Laboratory Standards Institutes (CLSI) guidelines (http://clsi.org/). After each strain was grown overnight in Mueller-Hinton broth (MHB; Nissui, Tokyo, Japan), the cultures were diluted 100-fold with fresh MHB. An inoculum of approximately $5x10^5$ cells (5µl) was spotted onto a series of Mueller-Hinton agar (Eiken, Tokyo, Japan) plates containing a range of concentrations of the test drug. After incubation at 37°C for 24 hours, the susceptibility was determined. The interpretation of the results was in compliance with standards recommended by CLSI. The breakpoints of MICs for resistance to antibiotics were defined as follows (mg/L); vancomycin (VAN), ≥16; teicoplanin (TEC).

Pulsed-field gel electrophoresis (PFGE) analysis and dendrogram

PFGE analysis was performed as previously described (10). Briefly, enterococci DNA embedded in an agarose plug was digested overnight at 37°C using *Sma*I (Roche, Basel, Switzerland), and then subjected to PFGE using a CHEF-MAPPER (Bio-Rad, CA)

according to the manufacture's protocol. The guidelines proposed by Tenover et al. were used for the interpretation of PFGE results (11). A lambda PFG Ladder (New England BioLabs, MA) was used as the Molecular Marker (MM).

Southern transfer and hybridization analysis

PFGE analyses with S1 nuclease or I-*Ceu*I were performed as described above. Briefly, enterococci DNA embedded in agarose plug was digested for 20 minutes at 37°C with S1 nuclease (Promega, WI) or overnight at 37°C using I-*Ceu*I (New England BioLabs, MA), the DNAs were then subjected to PFGE using a CHEF-MAPPER (Bio-Rad, CA) according to the manufacture's protocol. Southern hybridization was performed with the digoxigenin-based non-radioisotope system of Boehringer GmbH (Mannheim, Germany), and Southern transfer and the hybridization procedure were carried out according to the manufactures`s manual and standard protocol (12). Specific probes for *vanB* gene and the 23S rRNA gene of *E. faecium* were used (13).

Multilocus sequence typing (MLST) analysis

MLST was performed as previously described (14). The house keeping genes *atpA, ddl, gdh, purK, gyd, pstS* and *adk* were sequenced and STs were determined according to MLST.net (http://efaecium.mlst.net/).

Results and Discussion

We obtained three VanD-type *E. faecium* strains from urine and stool specimens in a same patient. She was a 82 year-old surgical patient with abdominal aortic aneurysm. Perioperative antibiotic (cefmetazole, CMZ) was administered during the blood vessel prosthesis implantation. After the operation, she developed a sepsis and an acute respiratory distress syndrome (ARDS). The intravenous antibiotic treatment, Tazobactam/Piperacillin (TAZ/PIPC) was started to control the infection. Because of inefficiency of the treatment, the intravenous antibiotic therapy was changed to Doripenem (DRPM), followed with VAN and levofloxacin (LVFX).

The VanD-type strain AA620 was isolated from an urine specimen 3 weeks after discontinuation of the administration. AA622 and AA624 were also recovered from the stool specimen of the same patient several weeks after the isolation of AA620. The VanD-type resistance genes were detected by PCR in three isolates. However, these isolates showed different MICs of VAN and TEC. AA620 was moderate resistant to VAN (MIC=64 mg/L) and susceptible to teicoplanin (MIC=2 mg/L) (TEC). Compared with AA620, both AA622 and AA624 were relatively lower resistant to VAN (MIC=16 mg/L) and susceptible to TEC (MIC=1 mg/L) (Table 1).

We performed MLST and PFGE analysis. MLST showed these isolates belonged to a

ST17 or a ST78 (allelic profile, 1 or 15-1-1-1-1-1-1) (Table2). Nucleotide sequence spikes in *atpA* gene of these strains overlaps, causing an allelic profile of *atpA* belonged to 1 or 15. PFGE types generated using *Sma*I showed the same pattern (Fig. 1). According to these results, these isolates recovered from a same patients within 6-month period were interpreted as indistinguishable isolates (15).

To detect the location of *vanD* gene cluster, we used the Southern blotting Co-hybridization of both *vanD* gene probe and 23S rRNA gene probe to I-*ceu*I-digested DNAs was identified (Fig. 2). These results suggested that *vanD* gene cluster was located on the chromosome. In addition, we performed PFGE of S1 nuclease-treated DNAs of these isolates. The plasmid profile were different between AA620 and AA622/624 (Fig. 3). However we performed the conjugative experiment, we didn't obtain the conjugant strain.

In general, VanD-type resistance is characterized by constitutive expression of the resistance genes and most of isolates that harbor *vanD* gene cluster have genetic mutations for *ddl* gene (4). We analyzed the nucleotide sequence of the *vanD* gene clusters and *ddl* genes in AA620, AA622 and AA624. We constructed a phylogenetic tree and calculated identities for the nucleotide sequences of *vanD* gene clusters from some bacteria that had been reported harboring *vanD* gene cluster in NCBI database (Fig. 4, Table 3). These

results showed relative relationship between the nucleotide sequences of these strains and those of the *vanD1* operon from BM4339 and CCRI-16110, compared with other strains (Fig. 4, Table 3). It has already reported that anaerobic bacteria were reservoirs for glycopeptide resistance genes such as *vanD* gene cluster (16). Of them, *Ruminococcus* species (strain CCRI-16110) harbored a *vanD* gene cluster and integrase-like protein gene (*intD*) which were similar to that of BM4339 (17). In AA620, AA622 and AA624, the gene order of the *vanD* gene cluster and *intD* was in accord with CCRI-16110 (Fig. 5) and the nucleotide sequences of *vanD* gene cluster were similar each other (Table 3) (17).

We identified a 12-bp deletion in *ddl* gene of AA620 (Fig. 6). This deletion was located close to an important site for enzymatic activity (18). It suggested that this deletion led to reduce or impaired Ddl ligase activity. In Contrast to AA620, the nucleotide sequence of *ddl* gene in AA622 and AA624 coincided with that of *E. faecium* BM4524 that had been shown Ddl ligase activity (19). Moreover, 1bp deletion resulted in a frameshift in $VanS_D$ sensor protein of AA620 (Fig. 7). This frameshift led to a truncated protein that didin't have four conserved domains for kinase/phosphatase activities. The similar mutation had been already reported (4), and this might cause constitutive expression of resistance genes. Contrary to AA620, 3bp insertions in $vanS_D$ gene had been founded in AA622/624 (Fig. 7). These findings indicated that the majority

of peptidoglycan precursors in AA620 was $_{D}$-Ala-$_{D}$-Lac, and this led to the higher-level resistant phenotype than in AA622/624.

Here, we described three VanD-type vancomycin resistance *E. faecium* isolates that were isolated from a same patient who had received a long-term vancomycin (VAN) treatment. According to the analysis of the molecular epidemiology, these findings indicated that AA620 and AA622/AA624 strains were originated separately from a same parent under a pressure of VAN. The nucleotide sequence analysis of *vanD* gene cluster and *intD* in these strain showed high nucleotide sequence identities with that of CCRI-16110. This strain was *Ruminococcus* spp. and might be a reservoir of *vanD* gene cluster (17). We couldn't obtain the other bacteria like *Ruminococcus* spp. that harbored *vanD* gene cluster from this patient, but this doesn't rule out the possibility of the transfer of vanD gene cluster in this patient. Further

Conclusion

Because of the high prevalence of *vanD* gene cluster in human fecal flora (16), we think a surveillance for enterococci harboring *vanD* gene cluster is required in a medical field.

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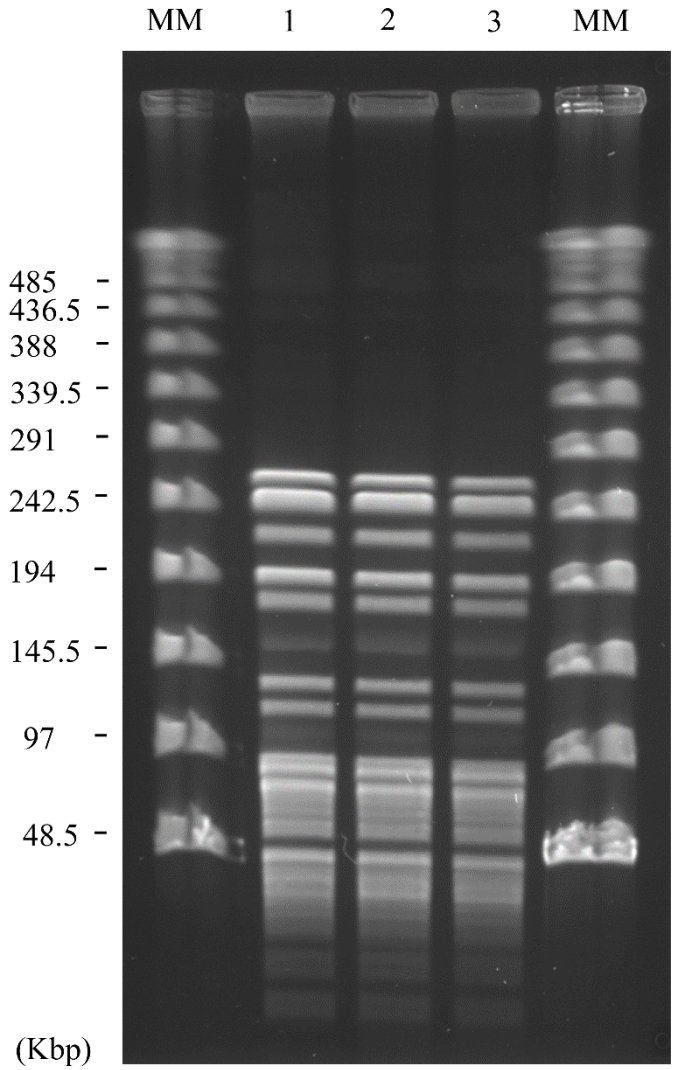

Figure 1. PFGE of *sma*I-digested DNA.

PFGE of *sma*I-digested DNAs isolated from AA620, AA622 and AA624 was performed. MM, Lambda Ladder PFG Marker (New England BioLabs, MA); 1, AA620; 2, AA622; 3, AA624.

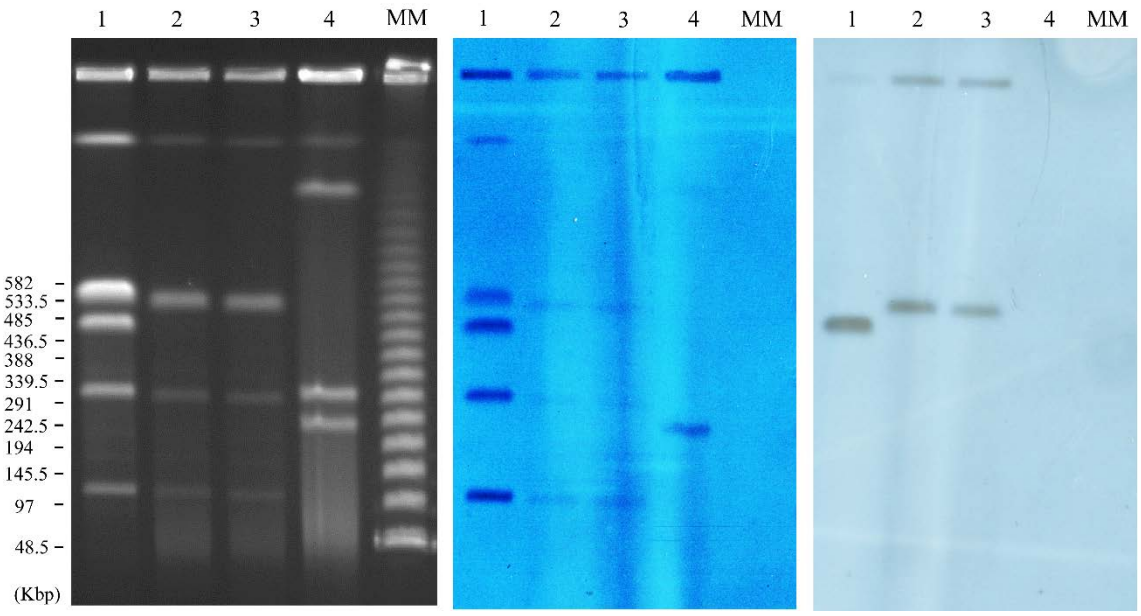

Figure 2. PFGE of I-*Ceu*I-digested DNA and hybridization with 23S rRNA gene and *vanD* gene probes.

PFGE of I-*Ceu*I-digested DNAs isolated from AA620, AA622, AA624 and FA2-2 was performed (Left) and gel was Southern blotted and hybridized to 23S rRNA gene (Middle) and *vanD* gene (Right). Lanes: MM, Lambda Ladder PFG Marker (New England BioLabs, MA); 1, AA620; 2, AA622; 3, AA624; 4, FA2-2.

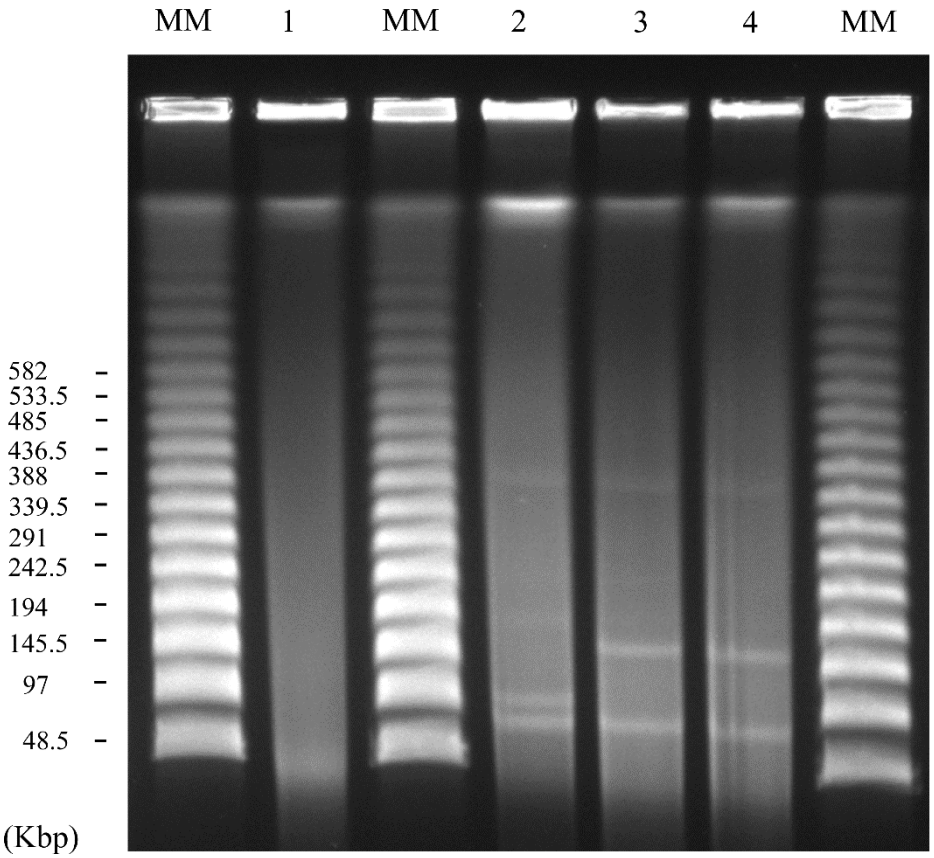

Figure 3. PFGE of S1 nuclease-treated DNA

PFGE of S1 nuclease-treated DNAs isolated from AA620, AA622, AA624 and FA2-2 was performed. Lanes: MM, Lambda Ladder PFG Marker (New England BioLabs, MA); 1, FA2-2; 2, AA620; 3, AA622; 4, AA624.

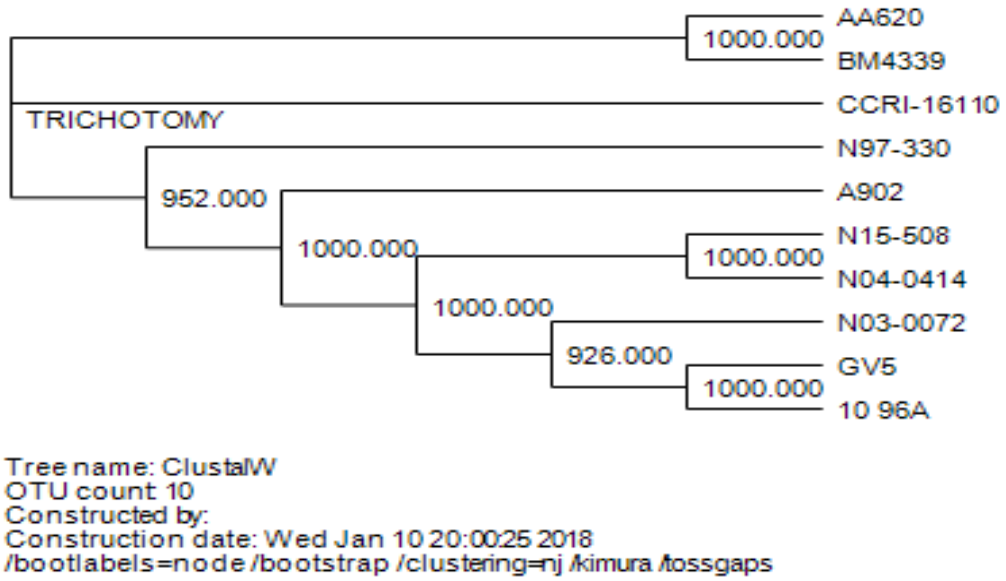

Figure 4. Phylogenetic tree based on the sequences of *vanD* gene clusters.

The genetic information for VanD-type vancomycin resistant enterococci was obtained from the genome database in NCBI (http://www.ncbi.nlm.nih.gov/). Phylogenetic tree based on *vanD* gene cluster sequences of AA620 with respect to typical strains harboring *vanD* gene cluster such as BM4339 (accession no.;AF130997.1), CCRI-16110 (accession no.;EF508033.1), N97-330 (accession no.;AF175293.1), A902 (accession no.;EU999036.1), N03-0072 (accession no.;AY489045.1), GV5 (accession no.;AB242319.1), N15-508 (accession no.; KT825491.1), N04-0414 (accession no.; DQ172830.1) and 10/96A (accession no.; AY082011.1) were carried out using Neighbor-joining method.

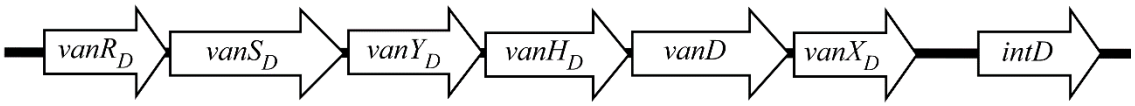

Figure 5. Schematic representation of the *vanD* gene cluster of AA620, AA622 and AA624.

The *vanD* cluster harbored the two-component regulatory system genes ($vanR_D$ and $vanS_D$) and the resistance genes ($vanY_D$, $vanH_D$, *vanD* and $vanX_D$). In the downstream region of $vanX_D$ gene, a putative integrase-like protein gene (*intD*) was located.

Figure 6. Nucleotide sequence alignment of *ddl* gene encoded by AA620, AA622 and AA624 compared with BM4524

The genetic information for enterococci was obtained from the genome database in NCBI (http://www.ncbi.nlm.nih.gov/). Alignments of *ddl* gene sequence of AA620, AA622 and AA624 with respect to typical *E. faecium* strain BM4524 (accession no.; AF550665.1) were carried out using ClustalW.

Figure 7. Amino acid sequence alignment of $VanS_D$ encoded by AA620, AA622, AA624 and the other VanD-type VRE.

The genetic information for VanD-type vancomycin resistant enterococci was obtained from the genome database in NCBI (http://www.ncbi.nlm.nih.gov/). Alignments of $VanS_D$ amino acid sequence of AA620/622/624 with respect to typical VanD-type vancomycin resistant enterococci such as BM4339 (AF130997.1), N97-330 (accession no.; AF175293.1), 10/96A (accession no.; AY082011.1), A902 (accession no.;EU999036.1), GV5 (accession no.;AB242319.1) and MRY12-252 (unpublished) were carried out using ClustalW. Solid lines indicated the deletion of AA620 or the

insertion of AA622/624, respectively. Dot lines indicated the functional domain of $VanS_D$ protein.

Table 1. Bacterial strains used in this study.

Strain/plasmid	Species	VRE genotype	MIC (mg/L)[a]	
			VAN	TEC
AA620	*E. faecium*	*vanD*	64	2
AA622	*E. faecium*	*vanD*	16	1
AA624	*E. faecium*	*vanD*	16	1

[a]VAN, vancomycin; TEC, teicoplanin

Table 2. MLST analysis of the strains used in this study.

	atpA	*ddl*	*gdh*	*purK*	*gyd*	*pstS*	*adk*	ST
AA620	1 or 15	(1[a])	1	1	1	1	1	17 or 78
AA622	1 or 15	1	1	1	1	1	1	17 or 78
AA624	1 or 15	1	1	1	1	1	1	17 or 78

[a]12bp deletions were identified in the sequence of ddl gene of AA620.

Table 3. Nucleotides and amino acid identities of *vanD* gene cluster.

	Amino acid identities (%)[b]									
Strain[a]	AA620	BM4339	CCRI-16110	N97-330	A902	N03-0072	GV5	N15-508	N04-0414	10/96A
AA620	-	98.74	98.01	96.52	91.66	85.31	80.46	72.73	72.69	56.55
BM4339	99.25	-	97.69	96.19	91.7	85.15	80.7	72.93	72.65	56.83
CCRI-1611	98.8	98.67	-	97.2	91.54	85.51	80.54	72.81	72.73	57.07
N97-330	97.56	97.48	98.03	-	90.61	85.59	80.98	72.81	72.73	57.07
A902	95.16	95.23	95.29	94.63	-	89.84	85.43	73.34	73.13	61.52
N03-0072	91.93	91.94	92.15	92.22	94.2	-	89.56	71.48	71.72	65.65
GV5	87.26	87.46	87.23	87.72	89.92	92.43	-	72.08	72.61	75.72
N15-508	66.34	66.47	66.3	66.22	66.4	65.58	64.66	-	85.72	47.93
N04-0414	65.83	65.89	65.88	65.81	65.72	65.38	64.79	91.55	-	48.54
10/96A	63.09	63.28	63.06	63.54	65.75	68.25	75.1	53.46	53.73	-
	Nucleotide identities (%)[c]									

[a]The genetic information for VanD-type vancomycin resistant enterococci was obtained from the genome database in NCBI (http://www.ncbi.nlm.nih.gov/). BM4339 (accession no.;AF130997.1), CCRI-16110 (accession no.;EF508033.1), N97-330 (accession no.;AF175293.1), A902 (accession no.;EU999036.1), N03-0072 (accession no.;AY489045.1), GV5 (accession no.;AB242319.1), N15-508 (accession no.; KT825491.1), N04-0414 (accession no.; DQ172830.1), 10/96A (accession no.; AY082011.1).

[b,c]Identities were calculated by ClustalW.