

Elucidation of host diversity of the VanD-carrying genomic islands in enterococci and anaerobes

Yusuke Hashimoto¹, Junzo Hisatsune^{2,3,4}, Masato Suzuki ⁴, Jun Kurushima¹, Takahiro Nomura¹, Hidetada Hirakawa¹, Naoko Kojima⁵, Yuichi Ono⁵, Yutaka Hasegawa⁵, Koichi Tanimoto⁶, Motoyuki Sugai^{2,3,4} and Haruyoshi Tomita^{1,6*}

¹Department of Bacteriology, Gunma University Graduate School of Medicine, 3-39-22 Showa-machi, Maebashi, Gunma 371-8511, Japan; ²Department of Antimicrobial Resistance, Hiroshima University Graduate School of Biomedical and Health Sciences, 1-2-3 Kasumi, Minami-ku, Hiroshima 734-8551, Japan; ³Project Research Center for Nosocomial Infectious Diseases, Hiroshima University, 1-2-3 Kasumi, Minami-ku, Hiroshima 734-8551, Japan; ⁴Antimicrobial Resistance Research Center, National Institute of Infectious Diseases, 4-2-1 Aoba-cho, Higashimurayama, Tokyo 189-0002, Japan; ⁵Infection Control Team, Gunma Prefectural Cardiovascular Center, 3-12 Ko Kameizumi-machi, Maebashi, Gunma 371-0004, Japan; ⁶Laboratory of Bacterial Drug Resistance, Gunma University Graduate School of Medicine, 3-39-22 Showa-machi, Maebashi, Gunma 371-8511, Japan

*Corresponding author. E-mail: tomitaha@gunma-u.ac.jp

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Background: VanD is a rare type of vancomycin resistance worldwide. However, the host diversity of the *vanD* gene cluster and the structural similarity of their genomic islands are not well understood.

Methods: Three VanD-type *Enterococcus faecium* strains (AA620, AA622 and AA624) isolated from a Japanese patient who underwent vancomycin treatment in 2017 were analysed. This study utilized WGS analysis to characterize the three VanD-type *E. faecium* strains and describes the diversity of hosts possessing VanD-carrying genomic islands.

Results: The three isolates exhibited variable MICs of vancomycin. In the relatively vancomycin-resistant AA620, mutations were identified in *vanS_D* and *ddl*. The strains AA622 and AA624 had intact *ddl* and harboured two *vanD* gene clusters. qRT-PCR results revealed the *ddl* mutation to be a factor affecting the high vancomycin resistance range of AA620. WGS data showed the 155 kb and 185 kb genomic islands harbouring the *vanD* gene cluster inserted in the coding region of the *lysS* gene, located in the chromosome in AA620 and AA622/624, respectively. Comparing the VanD-carrying genomic islands to available sequences of other enterococci and enteric anaerobes revealed how the genomic islands of these organisms isolated worldwide shared similar core genes and backbones. These anaerobes belonged to various genera within the order Eubacteriales. The phylogenetic cluster of the genomic island core genome alignment did not correlate with the host-species lineage, indicating horizontal gene transfer in the gut microbiota.

Conclusions: By horizontal gene transfer, various bacteria forming the gut microbiota maintain VanD-carrying genomic islands.

Introduction

Among the vancomycin-resistant types, the relatively rare VanD-type *Enterococcus faecium* was first isolated in 1991.¹ The *vanD* gene cluster is exclusively found within the chromosome and characterized by constitutive expression due to various *vanS_D* mutations.² Additionally, most VanD-type isolates have a mutation in *ddl*.³ It is speculated that anaerobic bacteria in the bowel flora serve as reservoirs for *vanD*.^{4,5} A detailed genome analysis by Top *et al.*⁶ describes how the *vanD* gene cluster is present in

enterococcal genomic islands of various sizes. More recently, structural similarities in the genomic islands of *E. faecium* and *Blautia coccoides* were identified.⁷ However, information on the VanD-carrying genomic islands in gut microbiota remains largely unknown.

Here we report three VanD-type *E. faecium* strains isolated from a patient who underwent vancomycin treatment. Additionally, our WGS analysis delineates the diversity of hosts possessing VanD-carrying genomic islands.

Materials and methods

Bacterial strains and antibiotic susceptibility testing

The bacterial strains used in this study are listed in Table S1 (available as [Supplementary data](#) at JAC-AMR Online). MICs were determined using the agar dilution method as recommended by the CLSI guidelines (<http://clsi.org/>).

PFGE

Enterococcal DNA embedded in an agarose block was digested overnight at 25 °C using SmaI (Roche, Basel, Switzerland) and subjected to PFGE using a CHEF-Mapper (Bio-Rad, CA, USA).

qRT-PCR analysis

RNA extraction and cDNA synthesis were conducted as described in the [Supplementary data](#). qRT-PCR was performed using the Luna Universal qPCR Master Mix (New England BioLabs, MA, USA) on an ABI 7500 Fast RT-PCR system (ABI, CA, USA).

WGS and bioinformatics analyses

WGS was conducted as described in the [Supplementary data](#). The assembly methods and software used for WGS analysis are described as well.

Accession numbers

The nucleotide sequences of the genomes of AA620 and AA622 were deposited in the DNA Data Bank of Japan database (<https://www.ddbj.nig.ac.jp/>) under the accession numbers SAMD00324535 and SAMD00324536, respectively.

Results and discussion

Three strains of VanD-type *E. faecium* obtained from the same patient with different vancomycin MIC values

In 2017, three VanD-type *E. faecium* strains were isolated from urine (AA620) and stool (AA622 and AA624) specimens of an 82-year-old surgical patient with abdominal aortic aneurysm. Cefmetazole was used during the perioperative period. The patient then developed sepsis/acute respiratory distress syndrome (ARDS) with unknown pathogens and was treated with tazobactam/piperacillin, doripenem, vancomycin and levofloxacin. The three strains isolated from one patient in a short period exhibited variable MICs of vancomycin. AA620 was moderately resistant to vancomycin (MIC = 64 mg/L), compared with AA622 and AA624 (MIC = 8 mg/L) (Table S1).

WGS analysis of three VanD-type *E. faecium* strains

We performed WGS analysis on these three strains to obtain detailed genomic insight. Consistent with the PFGE results, SNP analysis showed only 15 SNPs in AA622 and 13 SNPs in AA624 compared with the AA620 genome, suggesting a clonal relationship among these three strains (Figure S1 and Table S2).⁸ However, when the hybrid assembly was performed and compared with the genome of AA620, the genome of AA622 contained an overlapping tandem region of about 30 kb, suggesting the presence of two *vanD* gene clusters in AA622 (Figure 1a). The *vanD* nucleotide

sequence showed the highest similarity with VanD₁ in *E. faecium* BM4339 (98.8%) (Table S3).¹

Our investigations showed how a 1 bp deletion in AA620, *vanS_D* led to a frameshift mutation (503delT, Leu168fs) (Figure 1b and Figure S2). On the other hand, AA622 harboured two *vanD* gene clusters (Figure 1a and b). The *vanS_D*₁ of AA622 was relatively similar to that of BM4339. In contrast, 3 bp insertions in *vanS_D*₂ were observed (714_715insGAA, Leu238_Glu239insGlu) (Figure 1b and Figure S2).

qRT-PCR analyses indicated constitutive expression of the *vanD* gene cluster (Figure S3). Moreover, contrary to the susceptibility test result, the expression levels of *vanX_D* were markedly decreased in AA620 compared with AA622. We hypothesized that the loss of the four conserved domains of VanS_D in AA620 and the gene dose effect of AA622 would be responsible for the difference in the transcript levels (Figure S3).

Whereas AA622 possessed an intact *ddl* encoding Ddl ligase producing _D-Ala⁴-_D-Ala⁵ for the ends of the peptidoglycan precursors, we identified a 12 bp deletion in the *ddl* of AA620 adjacent to a region critical for enzymatic activity (Figure S4).⁹ Most ends of the peptidoglycan precursors in AA620 could be _D-Ala⁴-_D-Lac⁵ produced by the acquired VanD ligase instead of the defective intrinsic Ddl ligase, which led to the phenotype with a higher resistance level than in AA622.³

The structure of the VanD-carrying genomic island of AA620

The assembled AA620 WGS data revealed that the 155 kb genomic island harbouring the *vanD* gene cluster was inserted in the chromosome, disrupting the *lysS* gene (Figure 1c). The 11 bp direct repeated sequences, the presumed attachment sites (*att*) of the integrative and conjugative element (ICE), were located at both ends of the genomic island (Figure 1c).⁶ The G + C content of the genomic island was 44.2%, which was higher than that of the genome of *E. faecium* (38.1%) (Table S4), suggesting an exogenous acquisition of this genomic island. On the genomic island of AA620, genes related to VirB4 and Type 4 coupling protein were found, which are predicted to be conjugative systems. As for cargo genes, we identified the *vanD* gene cluster and multiple ABC transporters predicted to confer resistance to antibiotic peptides (Figure 1c and Table S4).¹⁰ In particular, in AA622, the 30 kb overlapping region contains the ABC transporter as well as the *vanD* gene cluster, which may provide a predominant influence on the host with respect to niche competitions in the gut microbiota.

Conjugation experiments with *E. faecalis* and *E. faecium* as recipients by filter mating were performed in this study to confirm transferability potential, although the genomic island transfer was not confirmed.^{11,12} Since the only structural difference between AA622 and AA620 genomic islands is in the duplicated region, only AA620 was used for further analyses.

Host diversity of VanD-carrying genomic islands

We performed a blast search to gain insight into this genomic island and retrieved seven enterococcal genomic islands and five non-enterococcal genomic islands with a structure similar to AA620.^{2,6,7,13–19}

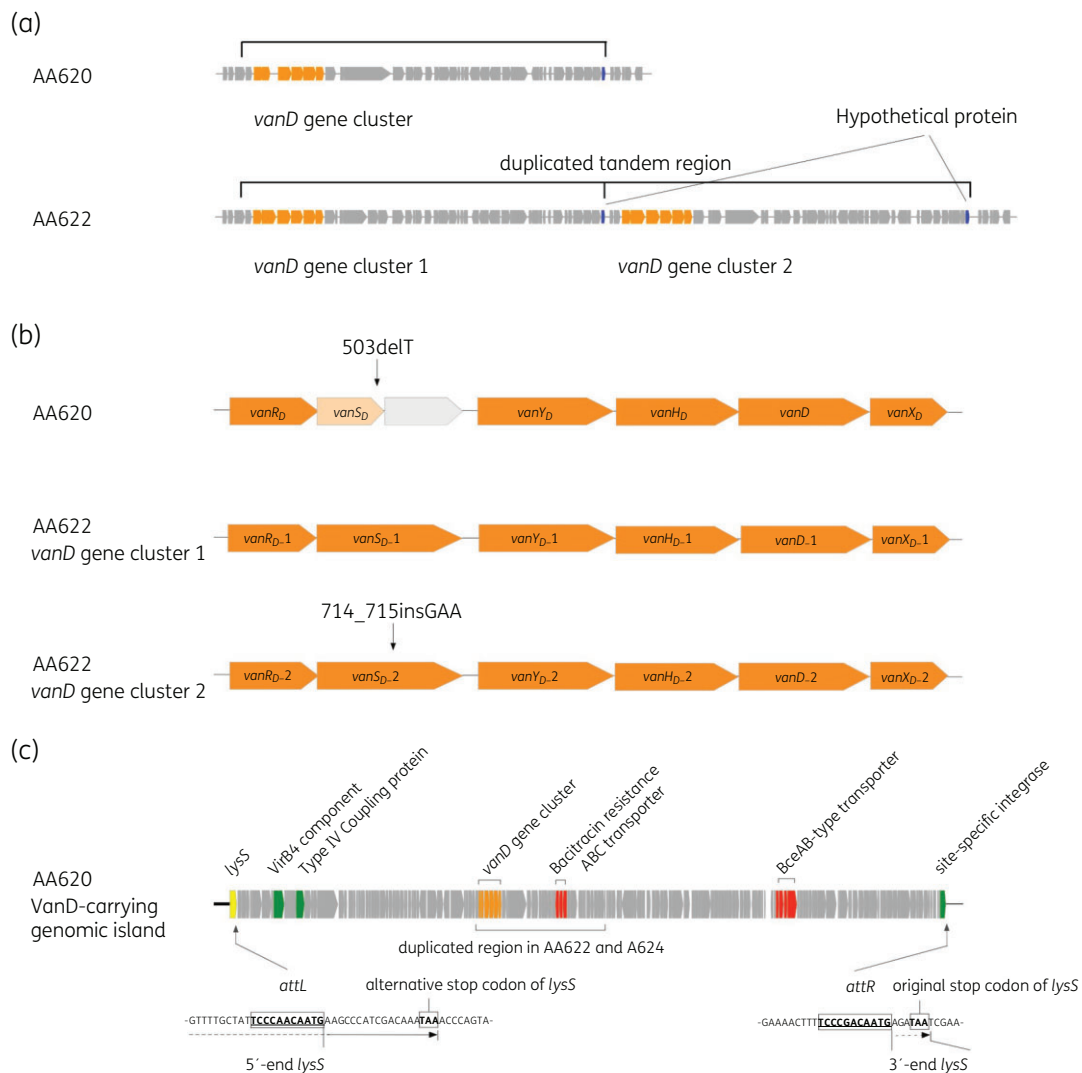


Figure 1. Genetic structure of (a) duplicated tandem regions, (b) *vanD* gene clusters and (c) VanD-carrying genomic island of AA620. (a) The duplicated tandem region of AA622 is shown in comparison to AA620. The orange panels represent the *vanD* gene cluster and the blue panels represent homologous hypothetical proteins. (b) The panels show the genetic structure of the *vanD* gene cluster of AA620 and AA622. The dark orange panels represent the *vanD* gene cluster genes, and the pale orange panel represents the truncated *vanD* gene cluster gene. The arrows indicate the key mutation sites compared with the *vanD* gene cluster genes of BM4339. (c) The panels show the genetic structure of the VanD-carrying genomic island of AA620. Nucleotide sequences corresponding to putative *att* sites (*attL*, *attR*) are circled by squares. The orange panels represent the *vanD* gene cluster, the yellow panel represents the *lysS* gene, the green panels represent putative conjugation and transfer-related genes and the red panels represent ABC transporters. The visualization of the duplicated tandem regions, *vanD* gene clusters and the VanD-carrying genomic island was constructed using GenomeMatcher (3.02).

Five non-enterococci with similar genomic islands belonged to the phylum Firmicutes, class Clostridia and order Eubacteriales. But they belonged to two families: the four strains YL58 (*Blautia coccoides*), SCSK (*Blautia producta*), M18-1 and Marseille-P5551 (*Luxibacter massiliensis*) belonged to family Lachnospiraceae, while strain 668 (ASJ35, *Ruthenibacterium lactatiformans*) belonged to family Oscillospiraceae (Table S4).^{15,16,18,19} All of these five strains were anaerobic bacteria that form the gut microbiota. The sizes of the genomic islands varied from about 119 kb to 200 kb. The integration site was assumed to be the *lysS*, except for 668 and SCSK, with a similar sequence of the putative *att* site (Table S4). In comparison with the G + C content of the genomes

of these anaerobic bacteria, a significant difference was also observed for 668 (*R. lactatiformans*), suggesting its exogenous integration into the chromosomal genome by horizontal gene transfer (HGT), as in enterococci. Relative synonymous codon usage analysis showed similar results (Figure S5). Examination of the cargo genes showed that only YL58 lacked any antimicrobial resistance gene in the genomic island, but all other strains possessed the *vanD* gene cluster (Table S4). In addition, only NEF1 possessed *tet(O)*.

Thirteen genomic islands, including AA620, were subjected to further analysis based on the core genes (Table S5). The distribution of core genes showed that the left end region possessing T4SS

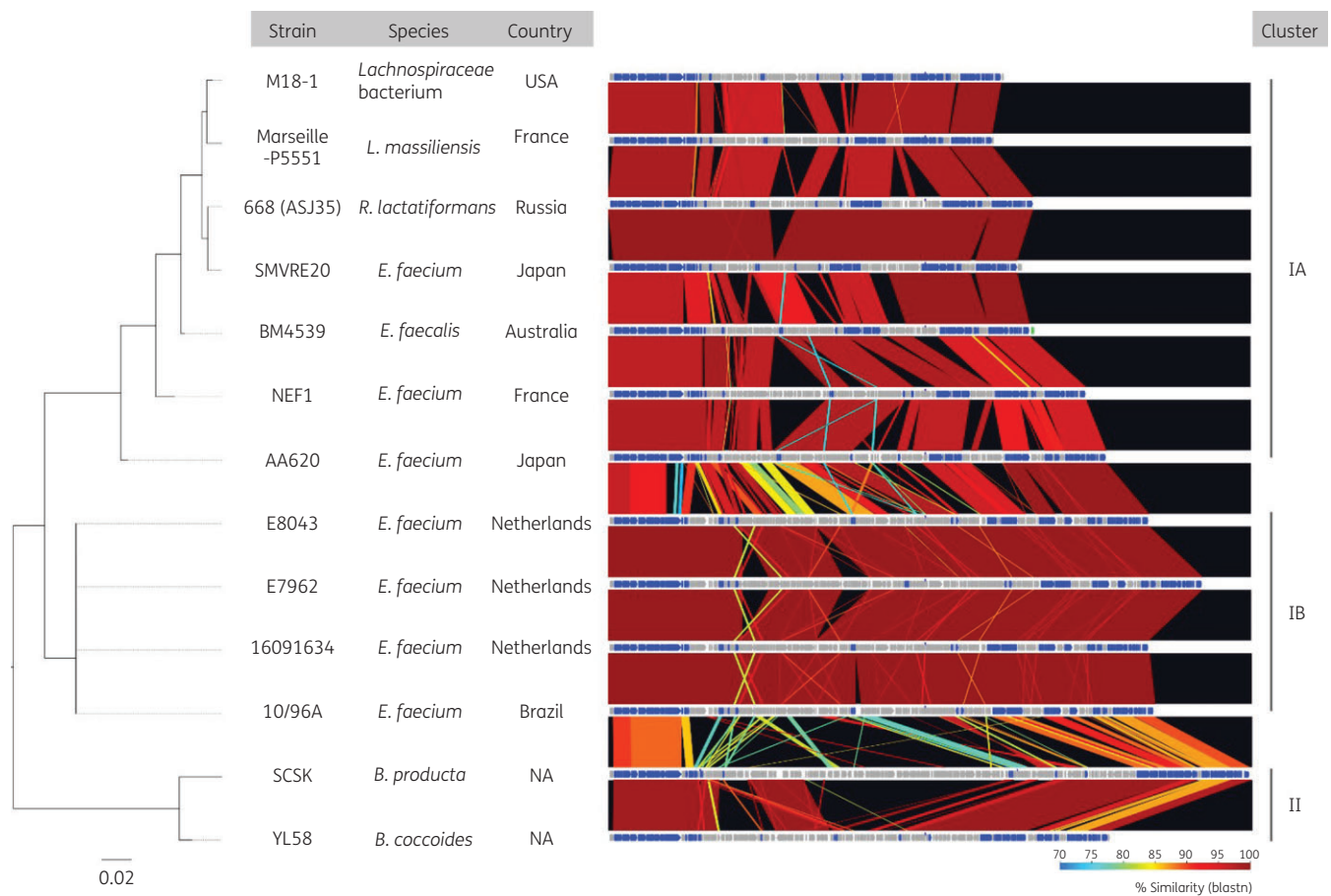


Figure 2. Comparison of genetic structures of 13 genomic islands. Core genes were identified using Roary (3.13.0) from 13 genomic islands (Table S5). The best-scoring maximum likelihood tree from RAxML (v8.2.4) was midpoint-rooted and visualized in FigTree software (v1.4.4). The blue panels represent 72 core genes, and multiple alignments of the genomic islands were constructed using GenomeMatcher (3.02). The genetic information for the genomic islands was obtained from the genome database in NCBI (<http://www.ncbi.nlm.nih.gov/>); M18-1 (accession number: SAMN01730992), Marseille-P5551 (accession number: SAMEA4979949), 668 (accession number: SAMN04224338), SMVRE20 (accession number: SAMD00156897), BM4539 (accession number: SAMN02333866), NEF1 (accession number: SAMN02333863), AA620 (accession number: LC467712), E8043 (accession number: SAMEA4885557), E7962 (accession number: SAMEA4885495), 16091634 (accession number: SAMN09532480), 10/96A (accession number: SAMN02333862), SCSK (accession number: SAMN14679094) and YL58 (accession number: SAMN03854117). NA indicates not applicable.

and the right end region possessing integrase were highly conserved in all strains (Figure 2). The study of the core genome alignment tree for all genomic islands was divided into two major clusters (Cluster I and Cluster II), and Cluster I was further divided in two (Cluster IA and Cluster IB). The genomic islands of strains M18-1, Marseille-P5551 and 668 showed high sequence similarity to those of enterococci (Cluster IA). The genomic island of AA620 belonged to this cluster along with SMVRE20, BM4539 and NEF1. In Cluster IB, four enterococcal strains shared high nucleotide sequence similarity in the total length of their genomic islands. Unlike Cluster I, Cluster II contained two strains belonging to the genus *Blautia* (SCSK and YL58), which were significantly distinct from the genomic islands of enterococci and other enteric anaerobes.

Based on the core genome alignment, the phylogenetic tree analysis of genomic islands failed to match the host species lineage. These results collectively suggested that the VanD-carrying genomic islands were independently transmitted and spread by HGT to multiple genera of gut microbiota-forming bacteria.

Furthermore, the fact that this type of VanD-carrying genomic island is identified in geographically diverse regions and different species of bacteria suggests a ubiquitous existence of that genomic island.

Unfortunately, no non-enterococcal strains harbouring the VanD-carrying genomic islands were isolated from the same patient in this study. The number of genomic islands that could be analysed was also limited. Although the exact origin of the *vanD* gene cluster is unknown, the present study suggests that gut anaerobes of various genera extensively retain VanD-carrying genomic islands, which may act as reservoirs. Further *in vivo* and *in vitro* studies are required on the HGT of the VanD-carrying genomic island and the effects of this genomic island on the host.

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Transparency declarations

None to declare.

Supplementary data

[Supplementary data](#), including Figures [S1](#) to [S6](#) and Tables [S1](#) to [S5](#), are available at [JAC-AMR Online](#).

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