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Identification of VanN-Type Vancomycin Resistance in an *Enterococcus faecium* Isolate from Chicken Meat in Japan

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Five VanN-type vancomycin-resistant *Enterococcus faecium* strains were isolated from a sample of domestic chicken meat in Japan. All isolates showed low-level resistance to vancomycin (MIC, 12 mg/liter) and had the same pulsed-field gel electrophoresis profile. The vancomycin resistance was encoded on a large plasmid (160 kbp) and was expressed constitutively. The VanN-type resistance operon was identical to the first resistance operon to be reported, with the exception of a 1-bp deletion in $vanT_N$ and a 1-bp substitution in $vanS_N$.

Since the first reports of vancomycin resistance in *Enterococcus faecium* in 1988 (12, 20), the glycopeptide-resistant enterococci (GRE) have become increasingly widespread throughout the world and are found as multiresistant opportunistic pathogens in hospitals and also in the environment (food animals). To date, nine types of operon structure conferring resistance to glycopeptides have been reported (5, 11). They are designated according to the characteristics of a key ligase gene that encodes either a D-alanyl–D-lactate or a D-alanyl–D-serine ligase (2). The D-alanyl–D-lactate ligase group includes the *vanA*, *vanB*, *vanD*, and *vanM* genes. The D-alanyl–D-serine group includes the *vanC1*, *vanC2*, *vanC3*, *vanE*, *vanG*, *vanL*, and *vanN* genes. Except for *vanC*-type resistance, which is intrinsic to *Enterococcus gallinarum* and *Enterococcus casseliflavus*, all resistance types are acquired externally. The D-Ala–D-Lac-type operons may be located on either plasmids or the chromosome. While the D-Ala–D-Ser-type *vanG*, *vanE*, and *vanL* operons have been detected only in the chromosome of *Enterococcus faecalis*, the location of VanN in *E. faecium* has not been clearly identified to date. VanG and VanN are transferable D-Ala–D-Ser resistance-type operons. The recently reported VanN type was identified in *Enterococcus faecium* isolated from a blood culture, showed a low level of vancomycin resistance (MIC, 16 mg/liter), and was susceptible to teicoplanin (0.5 mg/liter) (11). The *vanN* resistance operon was reported to be found on a transferable element. VanN-type resistance was detected in *E. faecium* strains isolated from a patient in France in 2008, which is the only report of VanN-type GRE to date. So far, there have not been any epidemiological data reported for VanN-type GRE strains obtained anywhere in the world and the significance of the acquisition of VanN-type resistance by enterococci is not clear.

In Japan, there is a lower incidence of GRE in humans and animals than in other countries (16). However, there are several reports showing the possible transmission of GRE or glycopeptide resistance between humans and food animals through food products such as chicken meat (8, 16). More than 10 years ago, we isolated GRE strains from both imported and domestic meats, including chicken meat (9). Since then, we have examined both imported and domestic meat samples as part of a surveillance program looking at GRE contamination (16, 19). While VanA- and VanB-type GRE strains, which show high-level resistance to glycopeptides, are occasionally detected in the samples, most of the GRE isolates from the meat samples had VanC-type resistance, which is carried naturally by enterococci that show low-level resistance to glycopeptides. We recently identified VanN-type GRE strains isolated from a sample of domestic chicken meat. Here we present the results of the analysis of those strains.

During the period from February to May 2011, a total of 322 meat and swab samples from meat destined for consumption in Japan were collected and investigated. The samples were obtained from two major national quarantine stations (Yokohama and Kobe) and from three meat inspection offices (Gunma, Kagoshima, and Miyazaki) in Japan. They included samples from 90 domestic chickens, 45 domestic pork meat samples, 85 imported chickens, and 102 imported pork meat samples. The country or region of origin of each sample is listed in Table 1. A hundred grams of each meat sample (mincemeat) was smashed and homogenized using an EXNIZER 400 (Organo, Japan) in 150 ml of buffered peptone water (Nissui, Japan). Eight milliliters of the supernatant from the homogenized meat sample was mixed with 32 ml of esculin saline buffer containing vancomycin at a concentration of 6 mg/liter and preincubated for 48 h at 37°C. After preincubation, 0.1 ml of each culture broth was spread on bile esculin azide agar (BEAA) plates containing vancomycin at a concentration of 6 mg/liter. The meat swab samples were preincubated for 48 h in 40 ml of brain heart infusion broth (Difco, Detroit, MI) containing vancomycin at a concentration of 6 mg/liter, and then 0.1 ml of each culture broth was spread on BEAA plates containing vancomycin at a concentration of 6 mg/liter. In the

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TABLE 1 GRE isolates from meat samples[a]

Country (prefecture) of origin	No. of chicken, pig samples	Corresponding no. of GRE strains/samples[b]		
		vanC1	*vanC2*	*vanN*
Japan (Gunma)	30, 15	62/21, 6/3	3/1, 3/1	0, 0
Japan (Miyazaki)	30, 15	77/27, 0	0, 0	5/1, 0
Japan (Kagoshima)	30, 15	15/5, 0	3/1, 0	0, 0
United States	3, 44	3/1, 0	0, 0	0, 0
Brazil	71, 0	173/59, 0	0, 0	0, 0
France	6, 0	0, 0	0, 0	0, 0
Philippines	5, 0	13/5, 0	0, 0	0, 0
Canada	0, 25	0, 0	0, 3/1	0, 0
Denmark	0, 19	0, 0	0, 2/1	0, 0
Mexico	0, 7	0, 0	3/1, 0	0, 0
Chile	0, 3	0, 0	0, 0,	0, 0
Spain	0, 2	0, 0	0, 0	0, 0
Hungary	0, 1	0, 0	0, 0	0, 0
Netherlands	0, 1	0, 0	0, 0	0, 0
Chicken, pig totals	175, 147	343/118, 6/3	9/3, 8/3	5/1, 0

[a] The vancomycin MICs of GRE isolates were more than 12 mg/liter.
[b] Neither VanA-type nor VanB-type GRE was detected in this study. VanC1-type and VanC2-type resistances are carried naturally by *E. gallinarum* and *E. casseliflavus*.

present study, enterococci showing a vancomycin MIC of >12 mg/liter were isolated and further analyzed as GRE, because the VanA-type GRE strain occasionally shows low-level resistance to vancomycin (18). Two colonies were picked at random from each GRE-positive sample giving multiple colonies on the selective plate. A total of 128 samples (40%) were positive for GRE, and 371 GRE isolates were obtained in total. To genotype the vancomycin resistance of the GRE isolates, multiplex PCR of the key ligase genes was performed (6). Three hundred forty-nine VanC1-type GRE strains, 17 VanC2-type GRE strains, and 5 unknown-type GRE strains were isolated (Table 1). It was considered that the VanC1-type GRE isolates would be *E. gallinarum* strains and the VanC2-type GRE isolates would be *E. casseliflavus* strains, as both species naturally exhibit low-level resistance to glycopeptides (5). At first, two unknown-type GRE strains were isolated from one domestic chicken that had been bred in Miyazaki Prefecture, Kyusyu Island, Japan. During this study, an additional three isolates of the unknown-type GRE strain were picked up from the master plate which produced the original two unknown-type GRE strains to give five isolates in total. These five isolates were then used to examine whether they arose from a single clone or from multiple clones. The five GRE isolates, named GU121-1, GU121-2, GU121-3, GU121-4, and GU121-5, were all identified as *E. faecium* strains on the basis of their *ddl* gene sequences (15). They showed low-level resistance to vancomycin (MIC, 12 mg/liter) and were susceptible to teicoplanin (MIC, 1.5 mg/liter). The five isolates were susceptible or showed intermediate resistance to other antibiotics (MICs [mg/liter]: ampicillin, ≤4; chloramphenicol, ≤4, ciprofloxacin, 1; erythromycin, 2; fosfomycin, 32; gentamicin, ≤125; kanamycin, ≤125; linezolid, ≤1; rifampin, 2; tetracycline, ≤2). Pulsed-field gel electrophoresis (PFGE) analysis confirmed that they were indistinguishable and were from a single GRE clone (see Fig. S1 in the supplemental material) (16). Of the five isolates, *E. faecium* GU121-1 was picked for further analysis as the representative strain in this study.

Multilocus sequence typing (MLST) analysis showed that one of seven genes, *purK*, was a new allele (number 58), and *E. faecium* isolate GU121-1 was categorized as a new sequence type (ST) designated ST669 (gene, allele number: AtpA, 9; Ddl, 8; Gdh, 14; PurK, 58; Gyd, 6; PstS, 27; Adk, 6). This new type showed the greatest similarity to previously reported ST329 (gene, allele number: AtpA, 9; Ddl, 8; Gdh, 14; PurK, 8; Gyd, 10; PstS, 27; Adk, 6) on the basis of information in the database (MLST database [http://efaecium.mlst.net/]). ST669 was a double-locus variant of ST329 (we found two nucleotide substitutions in *purK* and one in *gyd*). The VanA-type GRE strain categorized as ST329 was isolated from a blood sample from a patient hospitalized in the Netherlands in 1999. Both ST669 and ST329 do not belong to well-characterized clonal complex 17, which is found in the hospital-adapted and epidemic *E. faecium* strain cluster. ST669 was grouped as a satellite sequence type on the basis of the MLST analysis (10).

The specific PCR primer sets used to amplify the internal region of the reported resistance ligase genes (*vanA*, *vanB*, *vanC1*, *vanC2*, and *vanC3*) did not work, and no PCR product was obtained (6). Previously reported primers oligo V1 and oligo V2, which were designed on the basis of the ligase amino acid sequences, were then used for PCR amplification of the unknown ligase gene (7). The PCR product successfully amplified using this primer set was analyzed by direct DNA sequencing. The DNA sequence obtained for the ligase gene was homologous to the *vanL* ligase (around 70% identity at the base pair level) (4). On the basis of the DNA sequence obtained for the ligase gene, a pair of primers for inverse PCR was designed in order to examine the entire resistance gene cluster (operon) around the ligase gene. Several repeated inverse PCR amplifications with restriction enzymes HindIII, BamHI, and SalI were performed to determine the entire DNA sequence of the *vanL*-like resistance operon structure. A DNA region covering 12,344 bp that was located between a SalI site and an EcoRI site and included the predicted vancomycin resistance operon (including the *vanL*-like ligase gene) was determined in this study (Fig. 1). Analyses of the DNA sequence data showed that the unknown resistance operon structure containing the *vanL*-like gene was almost identical to the newly reported D-Ala–D-Ser VanN-type ligase and was composed of five genes designated *vanN*, $vanXY_N$, $vanT_N$, $vanR_N$, and $vanS_N$ (11). Compared to the previously reported VanN-type operon found in the *E. faecium* UCN71 strain, there was a 1-bp insertion and a 1-bp substitution in the VanN-type operon of *E. faecium* GU121-1. A thymidine residue was inserted at bp 1891 in the $vanT_N$ gene. The insertion resulted in a frameshift in the C-terminal region of $vanT_N$ and the production of an elongated $VanT_N$ peptide compared with the prototype protein (a length increase from 656 to 700 amino acids) (Fig. 1). A 1-bp substitution was located in $vanS_N$ gene at 466 bp. This caused an amino acid change of Ser to Pro at residue 156 of the $VanS_N$ protein.

Both the upstream and downstream regions of the *vanN* operon structure of *E. faecium* GU121-1 were examined in the present study. A DNA sequence of about 3,400 bp located upstream of the *vanN* ligase gene and a 3,400-bp DNA sequence located downstream of $vanS_N$ were determined (Fig. 1). Five open reading frames (ORFs) were identified in these regions; two ORFs were located upstream and three ORFs were downstream of the *van* resistance operon. These ORFs were transcribed in the opposite direction from the resistance genes. Homology analysis showed that most of the ORFs encoded unknown hypothetical

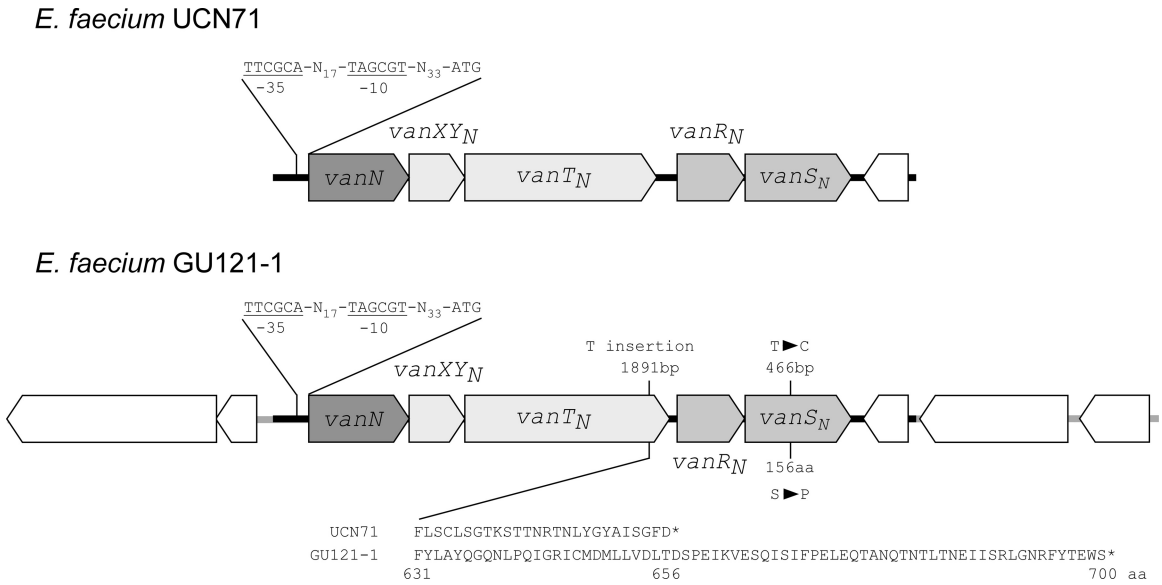

FIG 1 The VanN-type resistance operon structure and the predicted ORFs found around the resistance region of *E. faecium* GU121-1. The upper panel shows the operon structure located on the conjugative plasmid found in the first reported VanN-type vancomycin-resistant *E. faecium* UCN71 clinical isolate in France (GenBank accession number: JF802084). The lower panel shows the operon structure and the predicted ORFs located on the chromosomal DNA of *E. faecium* GU121-1 (AB701345). The horizontal open arrows indicate ORFs and their direction of transcription. The gray-colored ORFs show the VanN-type resistance gene clusters. The thick horizontal lines behind the ORFs show the DNA sequence regions determined. The thick black lines indicate regions corresponding to the published DNA sequence data of UCN71 (6,750 bp). The thick gray lines indicate the plasmid DNA sequence regions determined in this study (12,344 bp). The promoter regions (−35 and −10 sequences) for *vanN* genes are shown in detail.

proteins. It was obvious that there is no predicted gene related to mobile elements, including insertion sequence-related structures such as transposase and inverted repeat sequences. The ORF located downstream of the *vanN* operon was identical to that found in the reported DNA sequence, though its function is unknown (Fig. 1).

The first reported D-Ala–D-Ser VanN-type resistance was transferable between *E. faecium* strains at a low frequency (on the order of 10^{-10}) by filter mating experiments. We examined whether the VanN-type resistance could be transferred by conjugation *in vitro*. Vancomycin-resistant transconjugants could not be obtained from the five VanN-type GRE isolates by solid-surface mating experiments with *E. faecium* BM4105RF and *E. faecalis* FA2-2 as the recipient strains. Although no transconjugant was obtained in this study, we cannot dismiss the possibility of transferability of the VanN-type resistance of the GU121 isolates. If the resistances were transferable, the transfer frequencies were less than 4×10^{-8} per donor cell, which was the limit of detection of transconjugants in the present study. Further experimental studies and DNA sequence analysis are needed to clarify this point.

The plasmid DNAs of VanN-type strains were isolated by the alkaline-lysis method and analyzed (17). All five VanN-type *E. faecium* isolates showed the same plasmid profiles in agarose gel electrophoresis analysis (data not shown). The result of PFGE analyses using the S1 nuclease (Promega) showed that VanN-type strains harbor at least four plasmids (Fig. 2) (3). The conditions for electrophoresis were as follows: 19.5 h at 6 V/cm, 5.3 to 66.0 s nonlinear 21% (for 50 to 1,000 kbp), 0.5× Tris-borate-EDTA buffer, and 1% agarose gel. Four plasmids of approximately 160, 70, 60, and 40 kbp were identified. Standard PFGE analysis with the I-Ceu I endonuclease enzyme and Southern blotting hybridization using the specific probe for 23S rRNA confirmed that the four bands did not correspond to chromosomal DNA but were plasmid DNA (see Fig. S2 in the supplemental material) (13). The location of the VanN-type operon of the strains was also determined by Southern blotting hybridization using the *vanN* probe (Fig. 2; see Fig. S2 in the supplemental material). The 1,315-bp *vanN*-specific probe was constructed by PCR amplification using forward primer 5′-AGGAACATCACACTTCGAGG-3′ and reverse primer 5′-CGCATAGGTCGCTTGAACAA-3′. Hybridization analyses of PFGE showed that the *vanN* probe hybridized to the largest plasmid DNA band. The results clearly indicated that the *vanN*-type operon was located on the 160-kbp plasmid.

Gene expression in the VanN-type resistance operon was examined by the real-time PCR (RT-PCR) method to detect the transcription of the *vanN* ligase gene. An Applied Biosystems 7500 Fast Real-Time PCR System machine and SYBR premix EX *Taq* II (TaKaRa, Tokyo, Japan) were used for the RT-PCR experiment (1). The expression of *rrsA* was used as the endogenous control. The chromosomal D-Ala–D-Ala ligase gene of *E. faecium* BM4105S was also used as an internal control. The transcription level of *vanN* was measured in the presence of different concentrations of vancomycin in the culture (0, 1, 2, and 4 mg/liter). The transcription of *vanN* was detected without vancomycin in the medium, and the expression levels remained unchanged after the addition of vancomycin (see Fig. S3 in the supplemental material). These data indicated that the VanN-type vancomycin resistance gene was expressed constitutively and was not induced by the addition of glycopeptide to the culture medium. This observation is consistent with the report describing the VanN-type *E. faecium* UCN71 strain, in which the resistance operon is considered to be expressed constitutively and which has the same promoter sequence for the VanN-type operon (*vanN* ligase gene) (Fig. 1) (11).

In the present study, VanN-type vancomycin resistance en-

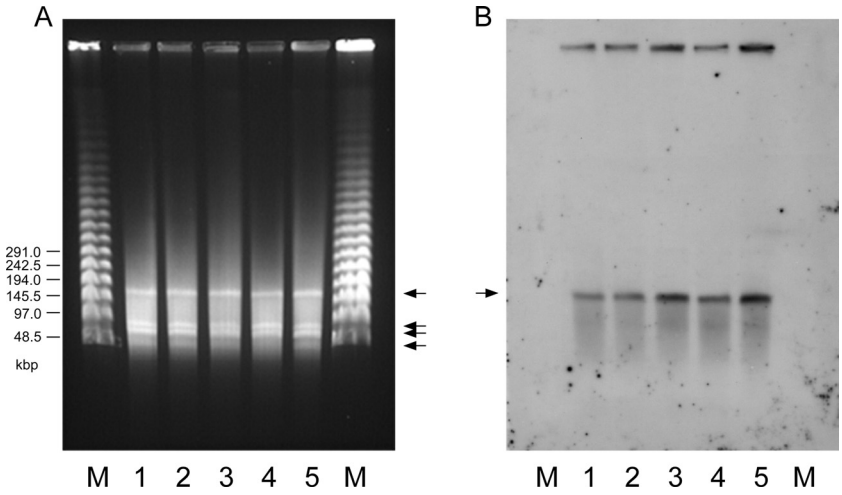

FIG 2 PFGE analysis of the plasmid DNAs of VanN-type GRE isolates using S1 nuclease enzyme and Southern hybridization with the *vanN* ligase gene probe. (A) PFGE analysis using S1 nuclease enzyme. At least four plasmid DNA bands were detected on the gel (arrows). (B) Southern hybridization of panel A blotted with the *vanN* ligase gene labeled using a nonisotopic digoxigenin system (Roche). Of the four plasmid bands, the largest, a 160-kbp plasmid DNA band, bound to the *vanN*-specific probe (arrow). Lanes M, lambda ladder PFGE molecular size marker (NEB); lanes 1, *E. faecium* GU121-1; lanes 2, GU121-2; lanes 3, GU121-3; lanes 4, GU121-4; lanes 5, GU121-5.

coded on a 160-kbp plasmid was identified in *E. faecium* isolates obtained from the Japanese environment (chicken meat). In our experiment, we were unable to demonstrate that the VanN-type vancomycin resistant plasmid is mobile; however, it might be acquired from another organism through mobilization by a self-transferable plasmid or by another mechanism (14). Our data indicate that VanN-type GRE strains have already spread within the environment.

Nucleotide sequence accession number. The sequence of the *vanL*-like resistance operon structure has been submitted to the DNA databases and was assigned accession number AB701345.

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