TABLE 3. Drug resistance patterns of bacteriocinogenic strains and transferabilities of bacteriocin activity with drug resistance

Strain[a]	Drug resistance pattern[b]	Transfer frequency[c] (% of bacteriocinogenic transconjugants)	
		Vam[r]	Gen[r]
VRE74	Apc Gen Kan Tei Van	2×10^{-8} (50)	$<1 \times 10^{-8}$
VRE78	Apc Gen Kan Tei Van	2×10^{-6} (100)	$<1 \times 10^{-8}$
VRE82	Apc Gen Kan Tei Van	2×10^{-8} (90)	$<1 \times 10^{-8}$
VRE83	Apc Gen Kan Tei Van	2×10^{-7} (80)	$<1 \times 10^{-8}$
VRE94	Apc Gen Kan Tei Van	$<1 \times 10^{-8}$	$<1 \times 10^{-8}$
VRE252	Apc Gen Kan Tei Van	$<1 \times 10^{-8}$	4×10^{-8} (75)
VRE272	Apc Gen Kan Str Tei Van	$<1 \times 10^{-8}$	4×10^{-8} (100)
VRE278	Apc Gen Kan Str Tei Van	2×10^{-7} (75)	4×10^{-8} (5)
VRE319	Apc Gen Kan Str Tei Van	6×10^{-8} (100)	4×10^{-8} (35)
VRE330	Apc Gen Kan Str Tei Van	$<1 \times 10^{-8}$	$<1 \times 10^{-8}$
VRE351	Apc Gen Kan Str Tei Van	2×10^{-7} (75)	9×10^{-5} (8)
VRE367	Apc Kan Str Tei Van	1×10^{-7} (100)	NT[d]
VRE418	Apc Kan Str Tei Van	$<1 \times 10^{-8}$	NT
VRE419	Apc Gen Kan Str Tei Van	$<1 \times 10^{-8}$	$<1 \times 10^{-8}$
VRE424	Apc Gen Kan Str Tei Van	$<1 \times 10^{-8}$	3×10^{-8} (50)
VRE437	Apc Gen Kan Str Tei Van	$<1 \times 10^{-8}$	$<1 \times 10^{-8}$
VRE455	Apc Gen Kan Str Tei Van	$<1 \times 10^{-8}$	$<1 \times 10^{-8}$
VRE477	Apc Gen Kan Str Tei Van	$<1 \times 10^{-8}$	2×10^{-8} (75)
VRE506	Apc Kan Mino Tet Tei Van	$<1 \times 10^{-8}$	NT
VRE576	Apc Gen Kan Tet Tei Van	$<1 \times 10^{-8}$	$<1 \times 10^{-8}$
VRE595	Apc Gen Kan Tet Tei Van	$<1 \times 10^{-8}$	$<1 \times 10^{-8}$

[a] The strains exhibited bacteriocin activity against *E. faecalis*, *E. faecium*, *E. durans*, *L. monocytogenes*, and *L. denitrificans*.
[b] Abbreviations: Apc, ampicillin; Gen, gentamicin; Kan, kanamycin; Mino, minocycline; Str, streptomycin; Tet, tetracycline; Tei, teicoplanin; Van, vancomycin.
[c] The frequency was calculated as the number of selected transconjugants per donor cell.
[d] NT, not tested.

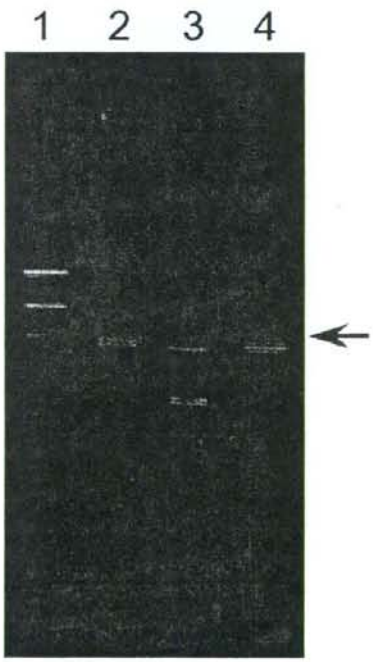

FIG. 1. Agarose gel electrophoresis of EcoRI-digested plasmid DNAs of bacteriocinogenic strain VRE82 and transconjugants. Lanes: 1, HindIII-digested lambda DNA; 2, *E. faecium* VRE82 (wild-type VRE strain); 3, nonbacteriocinogenic VRE BM4105RF transconjugant; 4, bacteriocinogenic VRE BM4105RF transconjugant. Arrow, 6.2-kb band.

kbp EcoRI fragment was designated pMG501. *E. faecalis* FA2-2 and *E. hirae* ATCC 9790 were transformed with pMG501. The transformants expressed bacteriocin activity identical to that of wild-type strain VRE82. These results implied that bacteriocinogenic VRE82 harbored a 6.2-kbp plasmid that conferred bacteriocin activity and had one EcoRI site. The 6.2-kbp plasmid was designated pDT1, and the bacteriocin encoded by pDT1 was designated Bac 43.

DNA sequence of pDT1. The DNA sequence of pDT1 was determined with plasmid pMG501. There was a possibility that another small EcoRI fragment lay in the gap formed by the single EcoRI site of pDT1, but this was too small to detect by agarose gel electrophoresis. PCR was also performed with the plasmid DNAs of VRE82 and the J1 and J2 primers (Table 2), which lie on either side of the single EcoRI site of pDT1 (Fig. 2). Sequence analysis of the PCR products confirmed that there was no other fragment lying in the gap formed by the EcoRI site of the 6.2-kbp plasmid. pDT1 was found to be 6,173 bp in length. Computer analysis revealed the presence of eight ORFs (ORF1 to ORF8) in pDT1, and all were oriented in the same direction. Figure 2a shows the ORFs that had a good ribosome binding site within a 20-base region upstream of the predicted start codon. Homology analysis of each ORF was performed with the DDBJ data bank. Each of the predicted proteins encoded by ORF5, ORF6, ORF8, and ORF9 showed no significant homology to the reported proteins. The ORF7 protein showed significant homology to the replication proteins of the plasmid found in gram-positive bacteria and designated the *repA* gene of pTD1. The analyses of the remaining four ORFs (ORF1 to ORF4) are described later.

Generation of Tn*5* insertion mutants. Tn*5* insertion mutant forms of the pMG501 clone containing pDT1 were generated.

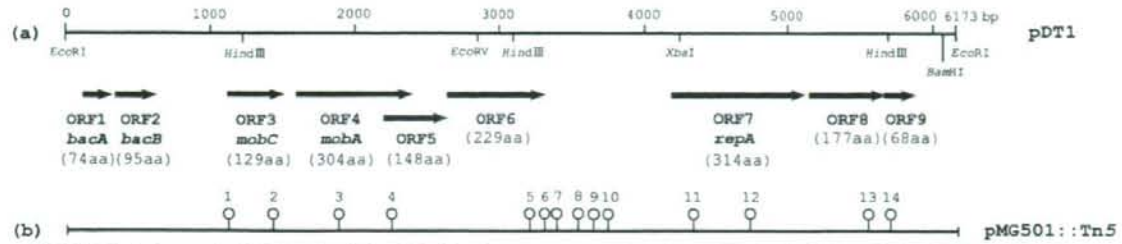

FIG. 2. Physical map of pDT1 showing deduced ORFs and transposon insertions into pDT1 of pMG501 (pAM401::pDT1). (a) Physical map of pDT1 (6.2 kbp) and deduced ORFs. Thick horizontal arrows indicate ORFs on pDT1 and the direction of transcription. (b) Map of Tn5 insertions into pDT1 of pAM401::pDT1. Open circles indicate Tn5 insertion mutants. Numbers beside symbols are mutant identification numbers. aa, amino acids.

Fourteen insertions in pDT1 were obtained (Fig. 2b). Inserts were obtained in each of the ORFs, except ORF1 and ORF2. All of the insertion mutants expressed bacteriocin activity and immunity at the same level as wild-type pMG501 in an *E. faecium* BM4105RF background with respect to the bacteriocin activity obtained by soft-agar assay. The result implied that seven ORFs (ORF3 to ORF9) were not related to the expression of Bac 43. Although we could not exclude any potential polar effects on the adjacent gene(s) by transposon insertion, it was probable that ORF1 and ORF2 were the bacteriocin determinant.

Cloning of PCR products that confer bacteriocin production. The PCR products that corresponded to the 0- to 700-bp region of the map position and contained ORF1 and ORF2 were cloned into pAM401. Transformation of *E. faecalis* FA2-2 was performed with pAM401 carrying the PCR products. The transformants were selected on a selective agar plate containing chloramphenicol for selection of pAM401 and examined for bacteriocin activity. The results are shown in Fig. 3. pMG502 carried a 737-bp fragment and contained both ORF1 and ORF2. *E. faecalis* FA2-2 containing pMG502 expressed bacteriocin activity and immunity. pMG503 contains ORF1 and the N-terminal region of ORF2. pMG503 could not transform *E. faecalis* FA2-2. pMG504 had a deletion in the N-terminal region of ORF1 and contained the C-terminal region of ORF1 and all of ORF2. *E. faecalis* FA2-2 containing pMG504 did not express bacteriocin activity but expressed immunity. pMG505 had a deletion in the N-terminal region of ORF1 and the C-terminal region of ORF2 and contained the C-terminal region of ORF1 and the N-terminal region of ORF2. *E. faecalis* FA2-2 containing pMG505 expressed neither bacteriocin activity nor immunity. These results indicated that the fragment containing both ORF1 and ORF2 conferred bacteriocin activity and immunity on the *E. faecalis* strain.

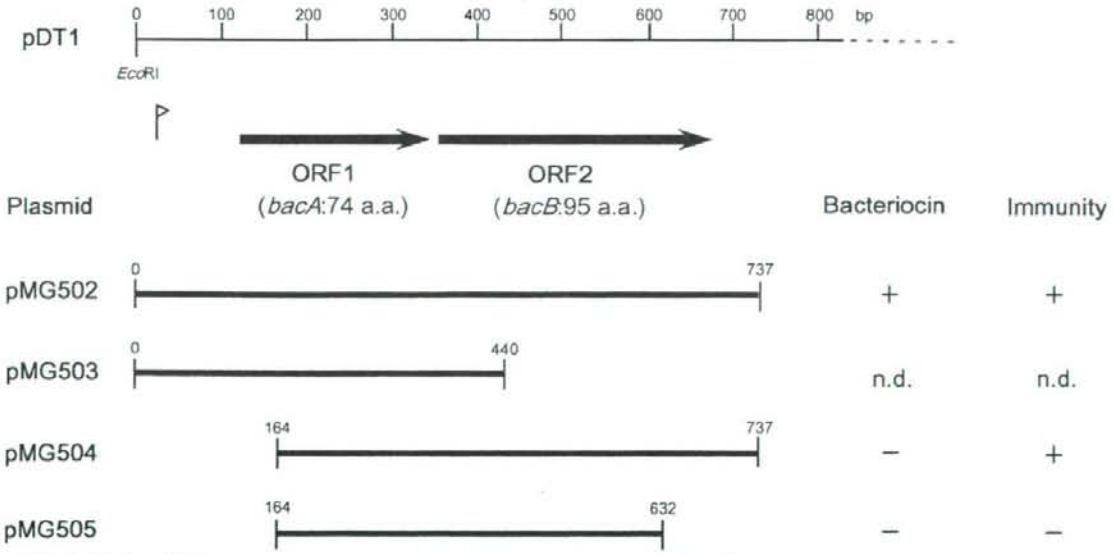

FIG. 3. Cloning of PCR products from the region of the bacteriocin determinant of pDT1. Thick lines represent the cloned PCR product. The numbers at the ends of the thick lines represent the 5′ and 3′ ends of the segment on the map (base pairs). The vertical bar with an arrowhead is the potential promoter. a.a., amino acids; n.d., the pasmid did not transform *E. faecalis* FA2-2.

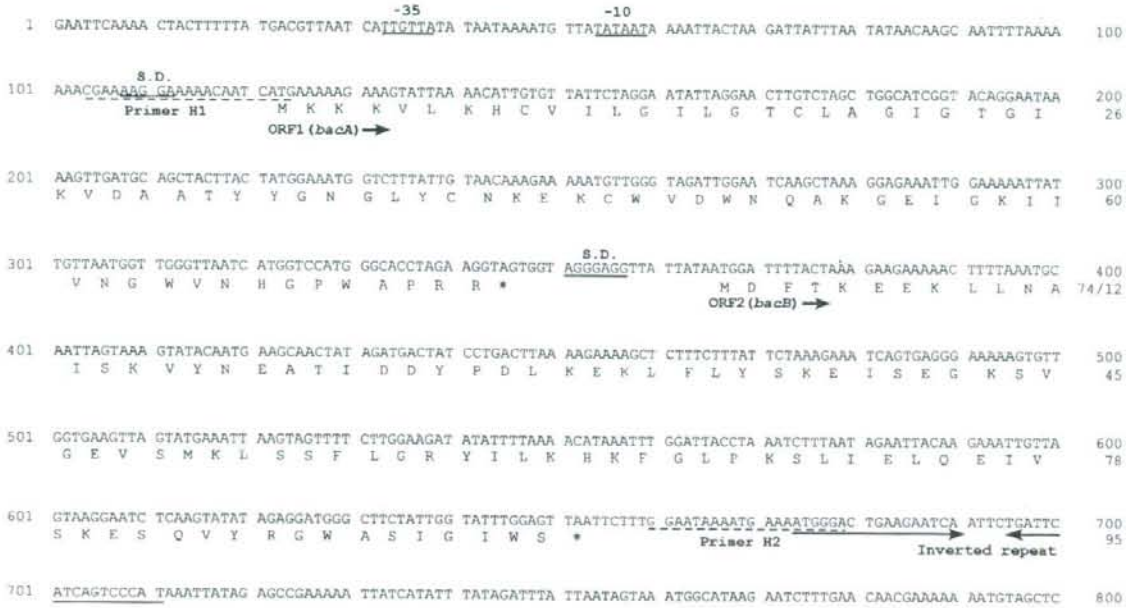

FIG. 4. Nucleotide sequence of *bacA* and *bacB* of bacteriocin 43 and deduced amino acid sequence. Potential promoters (−10 and −35) and S.D. ribosome binding sequences are underlined. The inverted repeat sequence is indicated by horizontal arrows. Primers H1 and H2, which were used for PCR analysis of the bac43 determinant in clinical isolates, are indicated by dashed lines. The accession number is AB178871.

ORF1 encoded the bacteriocin, and ORF2 encoded immunity against this bacteriocin. ORF1 and ORF2 were designated *bacA* and *bacB*, respectively.

DNA sequence analysis of ORF1 (*bacA*) and ORF2 (*bacB*). A homology search of *bacA* and *bacB* was performed against the DDBJ protein database. *bacA* encoded a 74-amino-acid protein. The ATG start codon was preceded by a potential Shine-Dalgarno (S.D.) ribosome binding site (AAGGAA) at a location 9 bp upstream (Fig. 4). The deduced BacA protein had a span of hydrophobic residues typical of a signal sequence in its N-terminal region, and a potential signal peptidase processing site corresponding to the V-D-A sequence was located at positions 28 to 30 (Fig. 5). Comparison of the primary structure of the deduced amino acid sequence of the mature BacA protein, which was composed of 44 amino acid residues, showed 50 to 86% homology with the mature Bac 31 (42), enterocin SE-K4 (13), enterocin P (4), divercin V41 (30), and listerocin 743A (26) proteins, which belong to the class IIa bacteriocins produced by LAB (14, 22), and 98% homology with Bac RC714 (43 amino acids), which corresponds to a protein lacking the last amino acid residue (44th Arg) of Bac 43 (10). As with the class IIa bacteriocins, the BacA protein had a hydrophobic N-terminal region, contained the consensus sequence Tyr-Gly-Asn-Gly-Lys(Val) (YGNGL[V]), and had a relatively hydrophilic C-terminal region (Fig. 5). The putative signal sequences (30-amino-acid sequences) did not show any significant homology with any other reported proteins or leader peptides.

bacB encoded a 95-amino-acid deduced protein without a putative signal sequence. The ATG start codon was preceded by a potential S.D. ribosome binding site (AGGGAG) at a location 9 bp upstream (Fig. 4). Comparison of the primary structure of the deduced amino acid sequence of the BacB protein showed 50% and 25% homology with the immunity proteins of bacteriocin 31 and enterocin SE-K4, respectively.

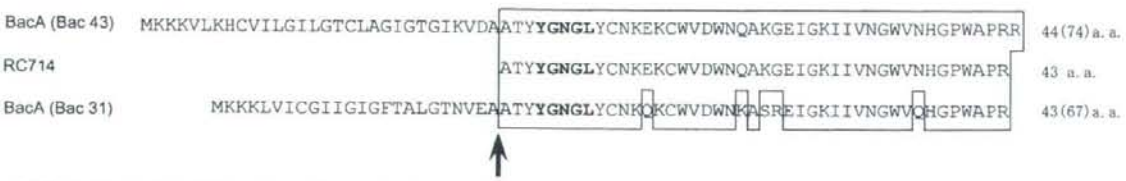

FIG. 5. Comparison of the amino acid sequence of the predicted BacA protein of bacteriocin 43 with the amino acid sequences of homologous bacteriocins. The sequences of the predicted BacA protein and other class IIa bacteriocins are shown. The consensus sequence Tyr-Gly-Asn-Gly-Lys (Val) (YGNGL[V]) of class IIa bacteriocins is indicated in boldfaced letters. The vertical arrow and dashed line indicate the cleavage site in the prebacteriocins. Identical amino acids (a.a.) are boxed.

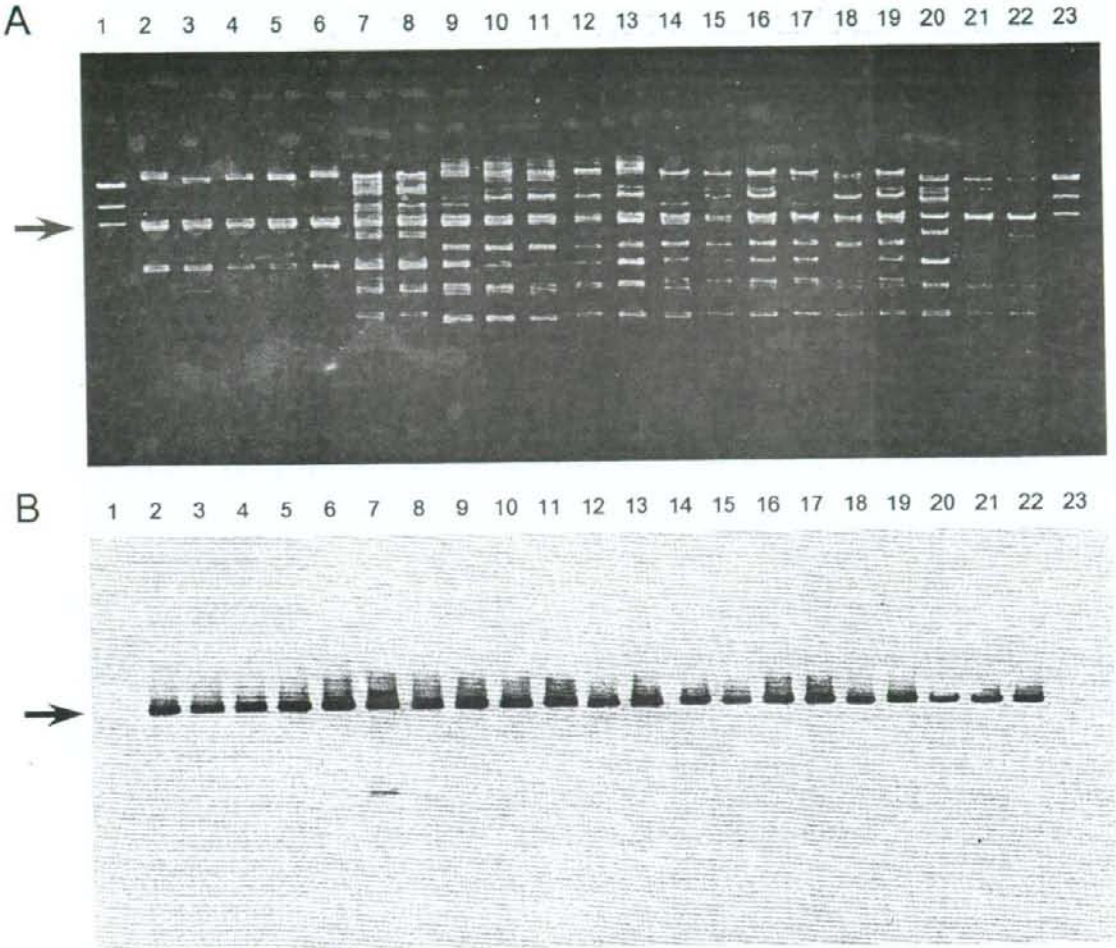

FIG. 6. EcoRI-digested plasmid DNAs isolated from 21 VRE strains that showed bacteriocin activity against *E. faecalis*, *E. faecium*, *E. hirae*, *E. durans*, and *L. monocytogenes*. (A) Agarose gel electrophoresis of EcoRI-digested plasmid DNAs. (B) The gel was Southern blotted and hybridized with the *bacA* probe. Lanes: 1 and 23, HindIII-digested lambda DNA; 2 to 22, strains 74, 78, 82, 83, 94, 252, 272, 278, 319, 330, 351, 367, 418, 419, 424, 437, 455, 477, 506, 576, and 595, respectively. Arrows, 6.2-kb bands.

Identification of a Bac 43 determinant in VRE strains producing the same bacteriocin spectrum as Bac 43. Plasmid DNAs isolated from each of the 21 VRE strains that showed the same bacteriocin activity as that of Bac 43 were examined for the presence of the bac43 determinant by PCR analysis with specific primers H1 and H2 for the *bacA* and *bacB* genes of bac43, respectively. The PCR primers are shown in Table 2 and Fig. 4. The 21 strains gave rise to the expected 576-bp product of *bacA* and *bacB* by PCR analyses (data not shown). Each of the PCR products specific for the *bacA* and *bacB* genes was sequenced. The nucleotide sequences of the genes from the strains were identical to those of the genes carried by pDT1 (data not shown). These indicated that the Bac 43-type bacteriocinogenic 21 strains carried *bacA* and *bacB* of bacteriocin 43 on the plasmid. Plasmid DNAs isolated from each of the 21 VRE strains were also examined for the presence of the bac43 determinant by Southern analysis with a specific probe (Table 2). EcoRI fragments of plasmid DNAs from each of the 21 strains were separated by agarose gel electrophoresis (Fig. 6A). The gel was Southern blotted and hybridized with the bac43 determinant (Fig. 6B). The probe hybridized to a specific EcoRI fragment with a molecular size of 6.2 kbp in each of the 21 strains that had been confirmed to carry the bac43 determinant by PCR analysis and DNA sequencing.

The banding patterns obtained by pulsed-field gel electrophoresis of SmaI-digested genomic DNA(s) were used to compare the 21 strains, which showed 12 different patterns. These data suggested that the Bac 43-type bacteriocin of each of the 21 strains was encoded on a pDT1-type plasmid and that the

TABLE 4. Mobilization of Tn*5* insertion mutant forms of pDT1 (pMG501::Tn*5*) by pHTβ[a]

# or Strain no.[b]	Plasmid	Location of Tn*5* insertion	Position of insertion (bp) on pDT1 map	Transfer frequency[c]	
				Cm^r	Van^r
Vector	pAM401			$<1.0 \times 10^{-6}$	6×10^{-2}
Wild type	pMG501 (pAM401::pDT1)			9.5×10^{-4}	5×10^{-2}
1	pMG501-01	*mobC* (ORF3)	1106	$<1.0 \times 10^{-6}$	3×10^{-2}
2	pMG501-02	*mobC* (ORF3)	1420	$<1.0 \times 10^{-6}$	2×10^{-2}
3	pMG501-03	*mobA* (ORF4)	1884	$<1.0 \times 10^{-6}$	4×10^{-2}
4	pMG501-04	*mobA* (ORF4) and ORF5	2228	$<1.0 \times 10^{-6}$	6×10^{-2}
5	pMG501-05	ORF6	3197	1.2×10^{-4}	5×10^{-2}
6	pMG501-06	ORF6	3296	4.1×10^{-5}	6×10^{-2}
7	pMG501-07	Downstream of ORF6	3338	2.2×10^{-4}	8×10^{-2}
8	pMG501-08	Between ORF6 and ORF7	3535	1.1×10^{-3}	5×10^{-2}
9	pMG501-09	Between ORF6 and ORF7	3641	3.4×10^{-4}	3×10^{-2}
10	pMG501-10	Between ORF6 and ORF7	3725	2.1×10^{-4}	4×10^{-2}
11	pMG501-11	ORF7	4329	1.0×10^{-4}	4×10^{-2}
12	pMG501-12	ORF7	4730	1.2×10^{-4}	6×10^{-2}
13	pMG501-13	ORF8	5546	2.0×10^{-4}	5×10^{-2}
14	pMG501-14	ORF9	5703	4.0×10^{-4}	5×10^{-2}

[a] Mating experiments were performed with *E. faecalis* FA2-2 carrying plasmids pMG501::Tn*5* and pHTβ as the donor strain and *E. faecalis* JH2SS as the recipient strain. The donor strain harbored both pHTβ (Van^r) as a mobilizer plasmid and each of the pAM401 derivatives (Cm^r) containing a Tn*5* insertion mutant form of pMG501 as the tester plasmid.

[b] The pMG501 derivative numbers correspond to the insertion mutant numbers in Fig. 2b.

[c] The frequency was calculated as the number of selected transconjugants per donor cell.

pDT1-type plasmid had been disseminated among different *E. faecium* VRE strains in the clinical environment.

Identification of the mobilization determinant. To examine the determinant for the mobilization of pDT1, each of the Tn*5* insertion mutant forms of pMG501 in *E. faecalis* FA2-2 shown in Fig. 2b was tested for the ability to be mobilized by the coresident vancomycin resistance-encoding conjugative plasmid pHTβ (63.7 kb) (45) (Table 4). *E. faecalis* JH2SS was used as the recipient strain. Each insertion mutant ORF, with the exceptions of ORF3 and ORF4, was mobilized by the pHTβ plasmid (Table 4). These results implied that ORF3 and ORF4 conferred the ability to mobilize the pDT1 plasmid.

DNA sequence analysis of ORF3 and ORF4 was performed by DDBJ against the protein database. ORF3 encoded a 129-amino-acid protein. The GTG start codon was preceded by a potential S.D. ribosome binding site (AGGA) at a location 13 bp upstream. ORF4 encoded a 304-amino-acid protein. The ATG start codon was preceded by an S.D. ribosome binding site (AAGGAG) at a location 12 bp upstream. Comparison of the primary structures of the deduced amino acid sequences of the ORF3 and ORF4 proteins showed 55% homology with the MobC protein encoded by *S. aureus* plasmid pRJ9 (35) and 45% homology with the MobA protein encoded by *E. faecalis* plasmid pEF1071 (2), respectively. The reported MobC and MobA proteins were the relaxosome and nickase for plasmid DNA, respectively (2, 35). ORF3 and ORF4 were designated *mobC* and *mobA*, respectively.

Identification of the mobilization determinant in 21 VRE strains producing the same bacteriocin spectrum as Bac 43. Plasmid DNAs isolated from each of the 21 VRE strains that showed the same bacteriocin activity as Bac 43 were examined for the presence of the mobilization determinant by PCR analysis with primers M1 and M2, which are specific for *mobC* and *mobA*, respectively. The PCR primers are shown in Table 2. The 21 strains gave rise to the expected 1,274-bp product by PCR analysis (not shown). This suggested that all of the 21 strains producing the same bacteriocin spectrum as Bac 43 possessed the *mobC* and *mobA* genes on a pDT1-type plasmid.

Analysis of the Bac 43 determinant in vancomycin-sensitive *E. faecium* and *E. faecalis* isolates. The plasmid DNAs of 149 vancomycin-sensitive *E. faecium* and *E. faecalis* isolates were examined for the presence of the bac43 determinant by PCR analysis with primers specific for the *bacA* and *bacB* genes of bac43. Of the 149 isolates tested, 46 *E. faecium* isolates were isolated from healthy Japanese medical students between 2002 and 2003 and 56 *E. faecium* isolates and 47 *E. faecalis* isolates were isolated at Gunma University Hospital, Japan, between 1990 and 1993. One *E. faecium* strain from a student gave rise to the expected 576-bp product and produced a bacteriocin with the same spectrum as Bac 43. The bac43 determinant was not identified in other strains.

DISCUSSION

Bac 43 was identified in the VanA-type VRE strain designated VRE82. Bac 43 was active against *E. faecalis*, *E. faecium*, *E. hirae*, *E. durans*, and *L. monocytogenes* strains and was carried by plasmid pDT1 (6.2 kbp), which was efficiently mobilized to the recipient *E. faecalis* or *E. faecium* strain at a frequency of 10^{-5} to 10^{-7} per donor cell with the coresident conjugative vancomycin resistance plasmid. The Bac 43 determinant consisted of the bacteriocin structural gene *bacA* and the immunity gene *bacB*.

The deduced mature BacA protein showed 86% homology with the mature Bac 31 protein isolated from an *E. faecalis* strain (42) and 98% homology with the mature Bac RC714 protein isolated from VRE RC714 (10). RC714 is a 43-amino-acid protein and is identical to the mature BacA protein but lacks the last residue (44th Arg) at the C-terminal region. There was no homology between the deduced amino acid sequence of the leader peptides of BacA of Bac 43 and Bac 31 (Fig. 5). The deduced BacB protein of Bac 43 showed 50%

homology with the BacB protein of Bac 31. Bac 31 is active against *E. faecium*, *E. hirae*, *E. durans*, and *L. monocytogenes* but is not active against *E. faecalis* (42). This implied that the six-amino-acid difference in the bacteriocin proteins of Bac 31 and Bac 43 resulted in the different bacteriocin activity spectra, as well as differences in the immunity proteins, as an adaptation in their bacteriocin activities.

Bacteriocinogenic *E. faecium* strain RC714 has been isolated from a VanA-type resistant *E. faecium* VRE clinical isolate (10). Mature Bac RC714 has been purified and characterized (10). As described above, the deduced BacA protein of Bac 43 showed 98% homology with Bac RC714 and was almost identical to RC714. Bac RC714 has been isolated only from one *E. faecium* VRE clinical isolate, and Bac 43 was also isolated only from VRE isolates, with the exception of one isolate from a healthy student. These data suggested that there would be a tendency for Bac RC714 or the Bac 43-type bacteriocin to be isolated in VRE clinical isolates than in vancomycin-sensitive isolates.

Two main types of bacteriocins were identified in the 277 (44%) bacteriocinogenic strains of the 636 VRE strains that were tested, and they were classified according to their bacteriocin activities (25). Bac 32 and Bac 32-type bacteriocins, which are active against *E. faecium*, *E. hirae*, and *E. durans* and are determined by bac32, were identified in 193 (70%) of the 277 bacteriocinogenic VRE strains (25). The other type of bacteriocin that was identified is active against *E. faecalis*, *E. faecium*, *E. hirae*, *E. durans*, and *L. monocytogenes* and was detected in 21 (3.3%) of the 277 bacteriocinogenic VRE strains (25). In this study, we showed that Bac 43 was representative of the Bac 43-type bacteriocins produced by the 21 bacteriocinogenic VRE isolates. The Bac 43 or Bac 43-type bacteriocinogenic VRE strains were the second most prevalent isolates after the Bac 32 or Bac32-type bacteriocinogenic VRE strains. However, the isolation frequency of Bac 43 or Bac 43-type bacteriocinogenic strains was far lower than that of the Bac 32 and Bac 32-type bacteriocinogenic strains. Both bacteriocins are carried by mobilizable plasmids and could be efficiently transferred to another strain by conjugative plasmids harbored by the VRE strains. The bacteriocinogenic VRE strains showed multiple-drug resistance. These characteristics indicated that Bac 32- and Bac 43-type bacteriocinogenic strains might have the same selective advantage in a clinical environment. The only difference in bacteriocin activity between Bac 43 and Bac 32 was that Bac 43 was active against *E. faecalis* and *L. monocytogenes*, whereas Bac 32 was not (25).

The well-characterized *E. faecium* bacteriocins (i.e., enterocins) are produced by food grade organisms that have been isolated from fermented foods (1, 3–6, 15, 19). Bacteriocinogenic food grade organisms are characteristically active against *L. monocytogenes* (34), which is a frequent cause of food-borne listeriosis (20). These food grade bacteriocinogenic *E. faecium* strains might have a selective advantage in their particular ecological niche. Bac 32 is not active against *L. monocytogenes* and is prevalent among the bacteriocins in *E. faecium* clinical isolates (25). The present study supports the previous hypothesis that the dominant type of bacteriocin in *E. faecium* clinical isolates might differ from the dominant type of bacteriocin found in food grade *E. faecium* isolates, which are active against *L. monocytogenes* (25).

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Pheromone-Responsive Conjugative Vancomycin Resistance Plasmids in *Enterococcus faecalis* Isolates from Humans and Chicken Feces

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The drug resistances and plasmid contents of a total of 85 vancomycin-resistant enterococcus (VRE) strains that had been isolated in Korea were examined. Fifty-four of the strains originated from samples of chicken feces, and 31 were isolated from hospital patients in Korea. *Enterococcus faecalis* KV1 and KV2, which had been isolated from a patient and a sample of chicken feces, respectively, were found to carry the plasmids pSL1 and pSL2, respectively. The plasmids transferred resistances to vancomycin, gentamicin, kanamycin, streptomycin, and erythromycin to *E. faecalis* strains at a high frequency of about 10^{-3} per donor cell during 4 hours of broth mating. *E. faecalis* strains containing each of the pSL plasmids formed clumps after 2 hours of incubation in broth containing *E. faecalis* FA2-2 culture filtrate (i.e., the *E. faecalis* sex pheromone), and the plasmid subsequently transferred to the recipient strain in a 10-min short mating in broth, indicating that the plasmids are responsive to *E. faecalis* pheromones. The pSL plasmids did not respond to any of synthetic pheromones for the previously characterized plasmids. The pheromone specific for pSL plasmids has been designated cSL1. Southern hybridization analysis showed that specific FspI fragments from each of the pSL plasmids hybridized with the aggregation substance gene (*asa1*) of the pheromone-responsive plasmid pAD1, indicating that the plasmids had a gene homologous to *asa1*. The restriction maps of the plasmids were identical, and the size of the plasmids was estimated to be 128.1 kb. The plasmids carried five drug resistance determinants for *vanA*, *ermB*, *aph(3')*, *aph(6')*, and *aac(6')/aph(2')*, which encode resistance to vancomycin, erythromycin, kanamycin, streptomycin, and gentamicin/kanamycin, respectively. Nucleotide sequence analyses of the drug resistance determinants and their flanking regions are described in this report. The results described provide evidence for the exchange of genetic information between human and animal (chicken) VRE reservoirs and suggest the potential for horizontal transmission of multiple drug resistance, including vancomycin resistance, between farm animals and humans via a pheromone-responsive conjugative plasmid.

Multiple-drug-resistant enterococci, and vancomycin-resistant enterococci (VRE) in particular, are a major cause of nosocomial infections. The acquired glycopeptide resistance of VanA has been predominantly identified in *Enterococcus faecium* isolates (4, 12, 13). In the United States, there is a high incidence of VanA-type *E. faecium* among human clinical isolates obtained from health care environments (46). Avoparcin has not been approved for use in animal feeds in the United States, and VRE have not been isolated outside health care environments from sources such as healthy human fecal samples or animals (12, 35). In Europe, VanA-type *E. faecium* isolates are frequently isolated outside the health care environment from materials such as sewage, food, animals, and healthy human fecal samples (4, 35); however, there is a low incidence of VRE among clinical isolates obtained from within the health care environment (35). A major factor that has contributed to the dissemination of VRE in the United States and Europe is now evident. In the United States, it is likely that the excessive use of glycopeptide antibiotics in the health care environment has resulted in the selective increase of VRE in the human intestine (25, 34), which has subsequently been spread by nosocomial transmission. In Europe, it is strongly suggested that the use of avoparcin as a growth promoter in animal feed has resulted in the selective increase of VRE in the human community (27, 49, 52). In both cases, the direct selective pressure of glycopeptides is the largest contributing factor in the selective increase of VRE in the different habitats. Korea, like many European countries, has used avoparcin for 13 years, from 1984 to 1996, as a growth promoter in food animals, including chickens, and VRE have been found in both the health care environment and chicken feces (41). In Korea, vancomycin injections have been used for treatment of infection by β-lactam-resistant gram-positive bacteria, including methicillin-resistant *Staphylococcus aureus*, since 1992, and VRE are also frequently isolated as nosocomial pathogens from hospitalized patients (42).

Vancomycin resistance can be disseminated both by the clonal spread of resistant enterococci and by the horizontal transmission of the resistance genes. Horizontal transmission of vancomycin resistance can be explained by the fact that the VanA-type determinant is encoded on transposon Tn*1546* or a Tn*1546*-like transposon (2) that frequently resides on a conjugative plasmid in VanA-type *E. faecium* and that the plasmid is able to transfer by mating on solid surfaces (30). The pheromone-independent pMG1-like conjugative plasmids, which transfer highly efficiently between enterococcal strains during

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Genotype or phenotype	Description	Reference
Strains			
E. faecalis FA2-2	*rif fus*	Derivative of JH2	11
E. faecalis JH2SS	*str spc*	Derivative of JH2	44
E. faecalis OG1S	*str*	Derivative of OG1	9
E. faecium BM4105RF	*rif fus*	Derivative of plasmid-free *E. faecium* BM4105	5
Plasmids			
pAD1	*hly/bac uvr*	59.6-kb pheromone-responsive plasmid from *E. faecalis* DS16	11
pMG1	Gm^r	65.1-kb conjugative plasmid from *E. faecium* strain	28
pAM714	*hly/bac erm*	pAD1::Tn*917*; wild-type transfer	26

broth mating (28, 47), are commonly found in *E. faecium* (28, 46). The Tn*1546*-like transposon is also present on the pMG1-like conjugative *E. faecium* plasmid (45, 47).

Most pheromone-responsive plasmids are also found in *Enterococcus faecalis* (10, 15). These plasmids exhibit a narrow host range and transfer between *E. faecalis* strains at a high frequency (10^0 to 10^{-2} per donor cell) within a few hours during broth matings. The plasmids confer a mating response to a small peptide (i.e., a sex pheromone) secreted by potential recipient cells. This mating signal induces the synthesis of a surface aggregation substance that facilitates the formation of mating aggregates. Plasmid-free recipients secrete multiple sex pheromones, each specific for a donor harboring a related pheromone-responsive plasmid. Once a plasmid is acquired by the recipient, secretion of the related pheromone ceases, whereas other unrelated pheromones continue to be produced. Determinants encoded on pheromone-responsive plasmids include those for hemolysin, bacteriocin, and resistance to UV light and antibiotics, including VanA-type resistance (10, 22).

In this report, we show that identical pheromone-responsive plasmids that encode multiple drug resistance, including VanA-type resistance, were isolated from both a hospital patient and a sample of chicken feces.

MATERIALS AND METHODS

Bacteria, plasmids, media, and antibiotics. Eighty-five VRE isolates were used in the present study, with 31 (*E. faecium*, 25; *E. faecalis*, 6) isolated from hospital patients in Korea between 1998 and 2000 and 54 (*E. faecium*, 50; *E. faecalis*, 2; *Enterococcus durans*, 2) isolated from samples of chicken feces in Korea in 1998. The laboratory strains and plasmids used in the current study are listed in Table 1. The *E. faecalis* and *E. faecium* strains were grown in Todd-Hewitt Broth (Difco Laboratories, Detroit, MI) or N2GT broth (nutrient broth no. 2 [Oxoid Ltd., London, United Kingdom] supplemented with 0.2% glucose and 100 mM Tris-HCl [pH 7.5]). The N2GT broth was also used in the sex pheromone experiments. Mueller-Hinton (MH) broth and MH agar were used for the sensitivity disk agar-N (Nissui, Tokyo, Japan) assay to test the MICs of the antibiotics. The agar plates were prepared by adding 1.5% agar to the broth medium.

The antibiotics used in this study were as follows: ampicillin (Ap), chloramphenicol (Cm), erythromycin (Em), fusidic acid, gentamicin (Gm), kanamycin (Km), rifampin, spectinomycin, streptomycin (Sm), tetracycline (Tc), teicoplanin (Tei), and vancomycin (Vcm). To select for transconjugants in the mating experiments, antibiotics were used at the following concentrations: Em, 10 μg/ml; fusidic acid, 12.5 μg/ml; Gm, 125 μg/ml; Km, 500 μg/ml; rifampin, 12.5 μg/ml; spectinomycin, 125 μg/ml; Sm, 500 μg/ml; Tei, 16 μg/ml; and Vcm, 16 μg/ml.

MIC determination. The MICs of the antibiotics were determined by the agar dilution method according to the CLSI (formerly NCCLS) criteria using MH agar (36).

Mating procedures. The broth matings were performed as previously described with a donor/recipient ratio of 1:9 (28). Unless otherwise described, the broth mating was carried out for 4 h.

Solid-surface mating was performed on agar plates as described previously (23). In both mating experiments, transconjugants were counted after 48 h of incubation at 37°C. *E. faecium* BM4105RF and *E. faecalis* FA2-2 were used as recipient strains in mating experiments with VRE isolates from human and chicken feces.

Pheromone induction. The detection of aggregation (clumping) was performed as previously described (7, 15, 16). A culture filtrate of plasmid-free FA2-2 was used as the pheromone. Generally, 0.5 ml of pheromone was mixed with 0.5 ml of fresh N2GT broth and 20 μl of the overnight-cultured cells that were to be tested for the ability to respond. The mixtures were cultured for 2 to 4 h at 37°C with shaking and were examined for clumping.

Short mating induced by the pheromone was performed as follows (28). After induction, 0.1 ml of each donor strain was mixed with 0.9 ml of the recipient strain and the mixture was incubated for 10 min at 37°C. The mixture was then plated on selective plates containing the appropriate antibiotics.

Synthetic pheromone (final concentration, 100 ng/ml) in N2GT was used to replace the natural pheromone (culture filtrate of strain FA2-2) in some experiments. The synthetic pheromones were purchased from SAWADY Technology Co., Ltd. (Tokyo, Japan).

Isolation and manipulation of plasmid DNA. Plasmid DNA was isolated from 5 ml of overnight culture by the alkali lysis method (39). Lysozyme treatment (4 mg/ml for 30 min at 37°C) was performed before alkali lysis. DNA manipulation and analysis of plasmid DNA were carried out by standard protocols (3, 39).

Pulsed-field gel electrophoresis (PFGE). Lysis of cells in agarose plugs was performed according to the standard protocols (3, 39), except that the cells were treated with lysozyme at a concentration of 20 mg/ml. The reaction mixture for SmaI digestion of whole chromosomal DNA was incubated at 25°C overnight. The gels were electrophoresed with a clamped homogeneous electric field (6V/cm at 15°C for 24 h; Switch times were ramped from 1 to 25 seconds [CHEF-DR II; Bio-Rad Laboratories, Richmond, CA]) and then stained with ethidium bromide and photographed with a UV light source.

Southern hybridization. After agarose gel electrophoresis, DNAs were transferred to a nylon membrane by capillary transfer. Southern hybridization was performed using the digoxigenin-based nonradioisotope system (Roche Diagnostics GmbH, Germany), and all procedures were based on the manufacturer's manual and standard protocols (3, 39). Hybridization was performed overnight at 42°C in the presence of 50% formamide. The probes for *asa1* and the drug resistance genes were generated by PCR amplification. The nucleotide sequences of the primer pairs are shown in Table 2. PCR products were separated by agarose gel electrophoresis and purified from the agarose gel blocks with Wizard SV Gel and the PCR Clean-Up System (Promega, Madison, WI). Probes were labeled using a digoxigenin labeling kit (Roche Diagnostics GmbH), and signals were detected using a digoxigenin chemiluminescence detection kit (Roche Diagnostics GmbH).

PCR and specific primers. The sets of specific primers used in PCR amplification are listed in Table 2. A TaKaRa *Taq* (TAKARA BIO Inc., Shiga, Japan) and a Thermal Cycler Model 9600 (Perkin-Elmer, Wellesley, MA) were used for the PCRs unless otherwise stated. Long PCR was performed using an Expand Long Template PCR System (Roche Diagnostics GmbH) and a Thermal Cycler Model 9600 (Perkin-Elmer).

TABLE 2. Nucleotide sequences of PCR primers

Gene	Primer(s)	Sequence (5′-3′)	Position[a] (5′-3′)	Product size (bp)	Reference
aac(6′)-Ii	AAC6Ii/F	TGGCCGGAAGAATATGGAGA	73–92	410	32
	AAC6Ii/B	TTTGGTAAGACACCTACG	482–462		
ant(4′)-Ia	ANT4Ia/F	GGAAGCAGAGTTCAGCCATG	180–199	266	32
	ANT4Ia/B	TGCCTGCATATTCAAACAGC	445–426		
ant(9′)-Ia	ANT9Ia/F	GGTTCAGCAGTAAATGGTGGT	103–123	476	32
	ANT9Ia/B	TGCCACATTCGAGCTAGGGTT	578–557		
aph(2″)-Ic	APH2Ic/F	ATACAATCCGTCGAGTCGCT	61–80	837	32
	APH2Ic/B	GTTGGCCTTATCCTCTTCCA	897–878		
aac(6′)-aph(2″)	AAC6APH2/F	TGATGATTTTCCTTTGATGT	45–64	1,395	This study
	AAC6APH2/B	CAATCTTTATAAGTCCTTTT	1439–1420		
aph(3′)-IIIa	APH3/F	GCCGATGTGGATTGCGAAAA	454–473	292	48
	APH3/B	GCTTGATCCCCAGTAAGTCA	745–726		
ant(6′)-Ia	ANT6/F	ACTGGCTTAATCAATTTGGG	179–208	597	48
	ANT6/B	GCCTTTCCGCCACCTCACCG	775–756		
ermB	ermB-1/F	CGAAATTGGAACAGGTAAAG	102–121	546	This study
	ermB-2/B	TTCATTGCTTGATGAAACTG	647–631		
asa1	asa1-5/F	GGTGTGTTAGGAGTTGTAGG	85–104	1,115	This study
	asa/B	ATTCCATAGACAATTGTGGC	1199–1180		
vanA (Tn*1546*)	VanA-1	GCATGGCAAGTCAGGTG	7272–7288[b]	1,114	This study
	X-2	GATCAATGGCACTGCCGCG	8385–8367[b]		
ORF1 (Tn*1546*)	ORF1B1	CGTCCTGCCGACTATGATTATTT	1913–1891[b]		23
vanX (Tn*1546*)	X-1	GTAGGGACATACGAGTTGGC	8136–8155[b]		This study
vanY (Tn*1546*)	Y-REV1	CCATATATTCCTCGAGAACG	9769–9750[b]		This study
orfx (Tn*5405*)	ORFX-6/F	ATGGTAGATAATATTATTAAATCAGTAG	1–28		This study
δ (pMD101)	uk-14/F	CGCTTGGCGTTGGTACAG	502–485		This study
γ (pMD101)	orf1-4/B	ATGAGTACAGTTATTTTAGCTG	1–22		This study

[a] The positions given are from the first base of the coding sequences of the genes.
[b] The positions given are from the first base of the left inverted repeat of Tn*1546*.

DNA sequencing and computer analysis. The DNA fragments to be sequenced were amplified by long PCR, separated by agarose gel electrophoresis, and purified from the agarose gel block as described above. The PCR products purified from the agarose gel blocks were sequenced directly by the primer-walking method (33). The sequencing reactions were conducted by PCR using a Dye Terminator Cycle Sequencing FS Ready Reaction Kit (Perkin-Elmer), and the sequences were determined using an ABI Prism 310 sequencer (Perkin-Elmer). For sequencing of the region containing the drug resistance determinants, two fragments containing *ant6*, *aph3*, *ermB*, and part of open reading frame 1 (ORF1) of Tn*1546* were amplified using two sets of primers (ANT6/F and ermB-2/B; ermB-1/F and ORF1B1). The DNA sequences of the two fragments were determined as described above. Based on the sequences obtained and the sequences of homologous genes found in the database, which were expected to lie in the flanking region, three sets of primers (ORFX-6/F and ANT6/B; uk-14/F and Y-REV1; X-1 and orf1-4/B) were designed and used for the amplification of the flanking regions by long PCR. The PCR products were purified, and the DNA sequences were determined by primer walking (33). A homology search using BLAST was performed through the NCBI website (http://www.ncbi.nlm.nih.gov/Tools/index. html).

Nucleotide sequence accession number. The nucleotide sequence data reported here have been deposited in the DDBJ, EMBL, and GenBank nucleotide sequence databases under accession number AB247327.

RESULTS

Drug resistance VRE isolates. Eighty-five VRE isolates, 54 of which had been obtained from samples of chicken feces and 31 from hospital patients in Korea, were examined for drug resistance. All of the isolates were resistant to more than four drugs, except for one *E. faecalis* isolate that showed resistance only to vancomycin (data not shown). Of the 85 VRE isolates, the numbers and percentages of 83 strains of *E. faecium* and *E. faecalis* resistant to the drugs tested are shown in Table 3. The isolation frequencies of gentamicin- and kanamycin-resistant *E. faecium* strains from chicken fecal samples were lower than those of *E. faecium* and *E. faecalis* strains from other sources. The two *E. faecalis* strains from chicken feces showed multiple resistances to Cm, Em, Gm, Km, Sm, Tei, and Vcm, suggesting that the resistances might be encoded on a plasmid.

Plasmids of VRE isolates. Plasmid DNA was prepared from each of the 85 VRE isolates and digested by EcoRI. The digested plasmid DNAs were analyzed by agarose gel electrophoresis, and the restriction fragment patterns were compared. Of these VRE isolates, *E. faecalis* KV1 and *E. faecalis* KV2, which were isolated from a patient and from a sample of

TABLE 3. Antimicrobial drug resistances of VRE

Drug	No. of drug-resistant isolates (%)[a]			
	E. faecium		*E. faecalis*	
	From chicken (n[b] = 50)	From human (n = 25)	From chicken (n = 2)	From human (n = 6)
Ap	26 (52)	24 (96)	0 (0)	0 (0)
Cm	3 (6)	7 (28)	2 (100)	1 (17)
Em	43 (86)	24 (96)	2 (100)	5 (83)
Gm	0 (0)	20 (80)	2 (100)	4 (67)
Km	1 (2)	21 (84)	2 (100)	4 (67)
Sm	50 (100)	23 (92)	2 (100)	1 (17)
Tc	50 (100)	14 (56)	1 (50)	3 (50)
Tei	50 (100)	16 (64)	2 (100)	3 (50)
Vcm	50 (100)	25 (100)	2 (100)	6 (100)

[a] The numbers in parentheses indicate the percentages of resistant isolates. The drug resistance levels (MICs) of ampicillin, chloramphenicol, erythromycin, gentamicin, kanamycin, streptomycin, tetracycline, teicoplanin, and vancomycin were equal to or greater than 8, 32, 16, 64, 1,024, 512, 8, 16, and 64 μg/ml, respectively.
[b] n, number of strains tested.

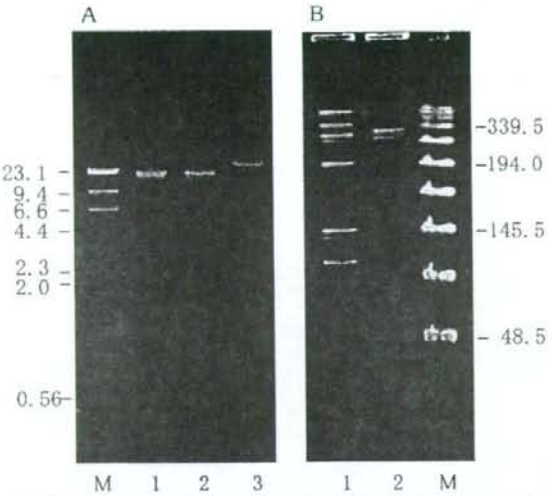

FIG. 1. Agarose gel electrophoresis of EcoRI-digested plasmid DNAs and PFGE of SmaI-digested total DNA from KV strains. (A) Agarose gel electrophoresis of EcoRI-digested plasmid DNA isolated from KV strains. Lanes: M, HindIII-digested λ DNA; 1, KV1; 2, KV2; 3, pMG1. (B) PFGE of SmaI-digested total DNAs from KV strains. Lanes: 1, KV1; 2, KV2; M, Lambda ladder PFG Marker (New England Biolabs, Beverly, MA). The numbers on the right and left indicate the sizes of molecular markers in kilobase pairs.

chicken feces, respectively, were found to harbor similar (indistinguishable and apparently identical) plasmids with respect to the EcoRI restriction profile (Fig. 1A). The two strains showed the same drug resistance levels (MICs) to vancomycin (1,024 μg/ml), teicoplanin (128 μg/ml), chloramphenicol (>512 μg/ml), gentamicin (>512 μg/ml), streptomycin (>512 μg/ml), kanamycin (>512 μg/ml), and erythromycin (64 μg/ml), but KV1 also showed resistance to tetracycline (128 μg/ml).

Pulsed-field gel electrophoresis was performed on the SmaI-digested total DNAs. As shown in Fig. 1B, the SmaI-digested patterns for the total DNAs were different, suggesting the presence of the same plasmid in different host strains.

Conjugal transfer of drug resistance. The transferability of vancomycin resistance from each of the two strains to *E. faecalis* FA2-2 or *E. faecium* BM4105RF was examined by broth mating for 4 hours. The transconjugants were selected on agar plates containing 16 μg/ml of vancomycin for the selection of the transconjugant and 12.5 μg/ml of rifampin and 12.5 μg/ml of fusidic acid for counterselection of the donor strain. The vancomycin resistance was transferred to *E. faecalis* FA2-2 at a frequency of about 10^{-3} per donor cell and was not transferred to *E. faecium* BM4105RF at a detectable frequency (less than 10^{-8} per donor cell) (Table 4). The transconjugants exhibited resistance to vancomycin, teicoplanin, gentamicin, streptomycin, kanamycin, and erythromycin. Repeated transfer experiments were performed between *E. faecalis* FA2-2 and *E. faecalis* JH2SS (Table 4). The vancomycin resistance was transferred at a frequency of about 10^{-3} per donor cell between these strains, and the transconjugants obtained in each experiment also exhibited resistance to vancomycin, teicoplanin, gentamicin, streptomycin, kanamycin, and erythromycin, suggesting that the conjugative plasmids conferred these drug resistances.

Plasmid DNA was isolated from each of the wild-type strains and the *E. faecalis* FA2-2 transconjugant. The plasmid DNA was digested with EcoRI and examined by agarose gel electrophoresis. The plasmid DNAs isolated from each of the strains were identical with respect to the EcoRI restriction profiles obtained by agarose gel electrophoresis analysis. These data suggested that each of the wild-type strains harbored a single drug resistance plasmid and that the plasmid had transferred to the recipient strain. The plasmid DNAs isolated from the transconjugant, which was derived from each of the wild-type strains, *E. faecalis* KV1 and KV2, were designated pSL1 and pSL2, respectively. The restriction map of the pSL1/pSL2 plasmid was determined by agarose gel electrophoresis of the restriction fragments. Each of the plasmid DNAs was digested with XhoI, EagI, or FspI or double digested with XhoI and EagI, XhoI and FspI, or EagI and FspI. Agarose gel electrophoresis analysis of the digested DNAs was performed to determine the cleavage sites within the plasmid. The physical maps of pSL1 and pSL2 were identical, and the molecular size of each of the plasmids was 128.1 kb (Fig. 2).

Pheromone responses of pSL1 and pSL2. The mating mixture of the donor strain, *E. faecalis* JH2SS harboring pSL1 or pSL2, and the recipient strain, *E. faecalis* FA2-2, formed a mating aggregate, and vancomycin resistance transferred efficiently to the recipient strain at a frequency of about 10^{-3} per donor cell during 4 h of broth mating (Table 4). Pheromone inductions and mating experiments were performed as described in Materials and Methods to examine the *E. faecalis* pheromone responses of pSL1/pSL2. The donor cells of *E. faecalis* JH2SS carrying pSL1 or pSL2 and *E. faecalis* OG1S carrying pSL1 or pSL2 were exposed for 2 hours to an FA2-2 culture filtrate (i.e., pheromone) to induce aggregation-mating functions before a short (10-min) mating period. The short mating was carried out between the induced or uninduced donor cells and the plasmid-free recipient *E. faecalis* FA2-2. Transconjugants were selected on agar plates containing van-

TABLE 4. Transfer frequencies of pSL plasmids in *Enterococcus*

Donor	Recipient	Transfer frequency (no. of transconjugants per donor cell)[a]
KV1	*E. faecium* BM4105RF	$<10^{-8}$
KV1	*E. faecalis* FA2-2	7×10^{-3}
KV2	*E. faecium* BM4105RF	$<10^{-8}$
KV2	*E. faecalis* FA2-2	4×10^{-3}
E. faecalis FA2-2 (pSL1)	*E. faecalis* JH2SS	1×10^{-3}
E. faecalis FA2-2 (pSL2)	*E. faecalis* JH2SS	2×10^{-3}
E. faecalis JH2SS (pSL1)	*E. faecalis* FA2-2	8×10^{-4}
E. faecalis JH2SS (pSL2)	*E. faecalis* FA2-2	7×10^{-4}

[a] Overnight cultures of 0.05 ml of donor and 0.45 ml of recipient were added to 4.5 ml of fresh N2GT, and the mixtures were incubated at 37°C with gentle agitation for 4 h. Portions of the mixed cultures were then plated on solid media with appropriate selective antibiotics. Colonies were counted after incubation for 48 h at 37°C.

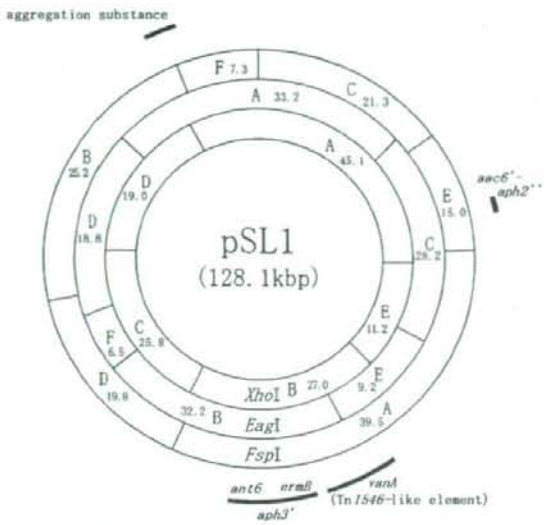

FIG. 2. Physical map of pSL plasmids. The fragments produced by the restriction endonuclease digestion of pSL plasmid DNA are denoted by letters. The numbers indicate the sizes of the fragments in kilobase pairs. The arcs indicate the approximate regions of the antibiotic resistance determinants and aggregation substance gene.

comycin, rifampin, and fusidic acid. The transfer frequency of vancomycin resistance from the induced donor cells was about 10^{-4} per donor cell, and that from the uninduced donor cells was less than 10^{-8} per donor cell, indicating that plasmids pSL1 and pSL2 conferred a pheromone response (Table 5).

The pheromone induction and mating experiments were performed with the synthetic pheromones cAD1, cPD1, cCF10, cOB1, and cAM373 to determine the specific pheromone for plasmids pSL1 and pSL2. Plasmids pSL1 and pSL2 did not respond to any of the synthetic pheromones, suggesting that pSL1 and pSL2 differed from the pheromone-responsive plasmids pAD1, pPD1, pCF10, pOB1, and pAM373 with respect to their pheromone responses (Table 5). The pheromone specific for pSL plasmids has been designated cSL1.

DNA-DNA hybridization. Of the pheromone-responsive plasmids that have been studied, the pheromone-related conjugative systems for pAD1 (8, 19), pCF10 (17, 18), and pPD1 (20, 43) have been well characterized. The genes involved in the regulation of the pheromone response have been identified and are known to be clustered in a 7-kb region on each plasmid (20, 31), and there is gene homology between the plasmids (20, 24). There is also homology between the genes for the aggregation substance, which are located downstream of the regulatory region (20, 21). The 7-kb regulatory region contains genes for surface receptor binding to exogenous pheromone; a positive regulator for the expression of *tra* genes, including aggregation substance; and a negative regulator that represses the expression of the positive regulator in the absence of pheromone and derepresses it in the presence of the imported exogenous pheromone. The N-terminal region of the aggregation substance gene *asa1* of pAD1 was amplified by PCR using specific primers (Table 2), and the amplified fragment was used as a probe for Southern hybridization with pSL1 or pSL2 DNA. The DNA fragment hybridized to specific restriction fragments from pSL1 and pSL2 (Fig. 3). These results indicate that pSL1 and pSL2 contain sequences that are homologous with the consensus sequence found in the aggregation substance gene of the pheromone-responsive plasmids.

The pSL plasmids were studied for homology with the pheromone-responsive plasmids pAD1 and pPD1 and the pheromone-independent plasmid pMG1 (28) by Southern hybridization. Both the pSL1 and pSL2 DNA probes hybridized to a restriction fragment of pAD1 and pPD1 DNA (data not shown), whereas the plasmid DNA probe did not hybridize with any restriction fragments of pMG1 DNA (data not shown). These findings indicated that the pSL plasmids contained sequences homologous with those of the pheromone-responsive plasmids and that they did not contain any sequences homologous with the pheromone-independent plasmid pMG1.

Drug resistance determinant. The drug resistance determinants carried on the plasmids pSL1 and pSL2 were examined by PCR analysis. Specific PCR primers for the drug resistance determinants for vancomycin, gentamicin, streptomycin, kanamycin, and erythromycin were designed based on database sequences (Table 2). The plasmids pSL1 and pSL2 conferred high levels of resistance to vancomycin and teicoplanin (MICs, 1,024 μg/ml and 128 μg/ml) and gave rise to the expected 1,114-bp PCR product with the primer specific for the *vanA* gene, indicating that pSL1 and pSL2 encoded a VanA-type determinant.

TABLE 5. Transferability of pSL plasmids during 10-min mating after exposure to the *E. faecalis* pheromone[a]

Donor	Recipient	Exposure to pheromone[b]	Transfer frequency in 10-min mating (no. of transconjugants per donor cell)
JH2SS (pSL1)	FA2-2	+[c]	7×10^{-5}
		−	$<10^{-8}$
JH2SS (pSL2)	FA2-2	+[c]	2×10^{-4}
		−	$<10^{-8}$
JH2SS (pAM714)	FA2-2	+[c]	4×10^{-4}
		−	$<10^{-8}$
JH2SS (pSL1)	FA2-2	cAD1[d]	$<10^{-8}$
		cPD1[d]	$<10^{-8}$
		cCF10[d]	$<10^{-8}$
		cOB1[d]	$<10^{-8}$
		cAM373[d]	$<10^{-8}$
JH2SS (pSL2)	FA2-2	cAD1[d]	$<10^{-8}$
		cPD1[d]	$<10^{-8}$
		cCF10[d]	$<10^{-8}$
		cOB1[d]	$<10^{-8}$
		cAM373[d]	$<10^{-8}$

[a] For induction with the pheromone, 0.1 ml of an overnight culture of the donor strain was diluted with 0.9 ml of a 1:1 mixture of pheromone and fresh N2GT broth. The overnight culture was similarly diluted with N2GT broth without induction as a control. Each culture was incubated for 2 hours with gentle agitation at 37°C. After induction, 0.1 ml of each donor strain was mixed with 0.9 ml of the recipient strain, and the mixture was incubated for 10 min at 37°C. The mixture was plated on selective plates.

[b] +, exposed; −, not exposed.

[c] Culture filtrate of FA2-2 was used as the pheromone.

[d] N2GT with a synthetic pheromone was used as the pheromone. The final concentration of synthetic pheromone was 100 ng/ml.

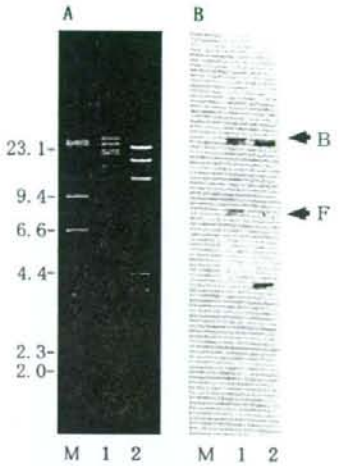

FIG. 3. Agarose gel electrophoresis of pSL plasmid digested with restriction endonuclease and hybridization with *asa1* probe. (A) Agarose gel electrophoresis of endonuclease-digested plasmid DNAs. (B) The gel was Southern blotted and hybridized with the *asa1* probe. Lanes: 1, FspI-digested pSL1 DNA; 2, EcoRI-digested pAD1 DNA. Arrows B and F on the right indicate the FspI fragments B and F of pSL1, respectively, which hybridized to the *asa1* probe. The numbers on the left indicate the positions and sizes in kilobase pairs of the λ HindIII molecular size markers.

Primers specific for the aminoglycoside modification enzyme genes were used to identify the aminoglycoside determinant (Table 2). Plasmids pSL1 and pSL2 gave rise to the expected PCR products with the primers specific for *aac(6')-aph(2")*, *ant(6)-Ia*, and *aph(3')-IIIa*, which encode gentamicin/kanamycin, streptomycin, and kanamycin resistance, respectively. Plasmids pSL1 and pSL2 gave rise to the expected PCR product with the primer specific for *ermB*, which encodes erythromycin resistance. The most commonly acquired macrolide resistance mechanism among the enterococci is the production of methylases for an adenine residue in the 23S ribosome RNA of the 50S ribosomal subunit, which is encoded by the *ermB* gene (37, 40).

The PCR products amplified with the specific primers for each resistance gene were purified from the agarose gel and used as Southern hybridization probes to examine their approximate locations on the restriction map. The results are shown in Fig. 2 and 4. The *aac(6')-aph(2")* genes hybridized to the FspI E fragment, EagI C fragment, and XhoI A fragment; the *vanA* gene hybridized to the FspI A fragment, EagI B and E fragment, and XhoI B fragment; and the *ant(6)-Ia* gene, *aph(3')-IIIa* gene, and *ermB* gene hybridized to the FspI A fragment, EagI B fragment, and XhoI B fragment (Fig. 4). These data implied that *vanA*, *ant(6)-Ia*, *aph(3')-IIIa*, and *ermB*, which encode VanA, streptomycin, and kanamycin, and erythromycin resistance, respectively, are located between 53 kb (an XhoI site between XhoI fragments B and E) and 74 kb (an FspI site between FspI fragments A and D) of the pSL maps (Fig. 2). *aac(6')-aph(2")*, which encodes gentamicin/kanamycin resistance, was located in the FspI E fragment of the pSL plasmids.

DNA sequence analysis of drug resistance determinants and their flanking regions. A region of approximately 22 kb containing the drug resistance determinants for streptomycin, kanamycin, erythromycin, and vancomycin and their flanking regions was amplified by long PCR using the primers specific for the drug resistance determinants and primers that were designed based on the sequences of homologous genes listed in the database (Fig. 5). The PCR products were sequenced. Computer analysis revealed the presence of several ORFs in this region (Fig. 5). Figure 5 shows the ORFs that had a good ribosome binding site in the 20-base region upstream of the predicted start codon. A homology search of ORFs carried on the 22-kb region of the pSL1 and pSL2 plasmids was per-

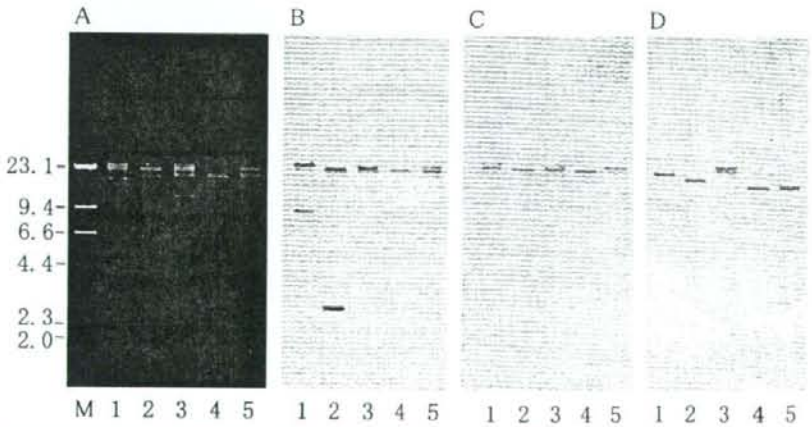

FIG. 4. Agarose gel electrophoresis of restriction endonuclease-digested pSL plasmid DNAs and Southern hybridization with drug resistance genes. (A) Agarose gel electrophoresis of endonuclease-digested pSL plasmid DNAs. Lanes: 1, EagI digestion; 2, EagI/XhoI double digestion; 3, XhoI digestion; 4, XhoI/FspI double digestion; 5, FspI digestion. The gels were run in triplicate and then Southern blotted and hybridized with a *vanA* probe (B), *aph3* probe (C), or *aac6/aph2* probe (D). The numbers on the left indicate the positions and sizes in kilobase pairs of the λ HindIII molecular size markers.

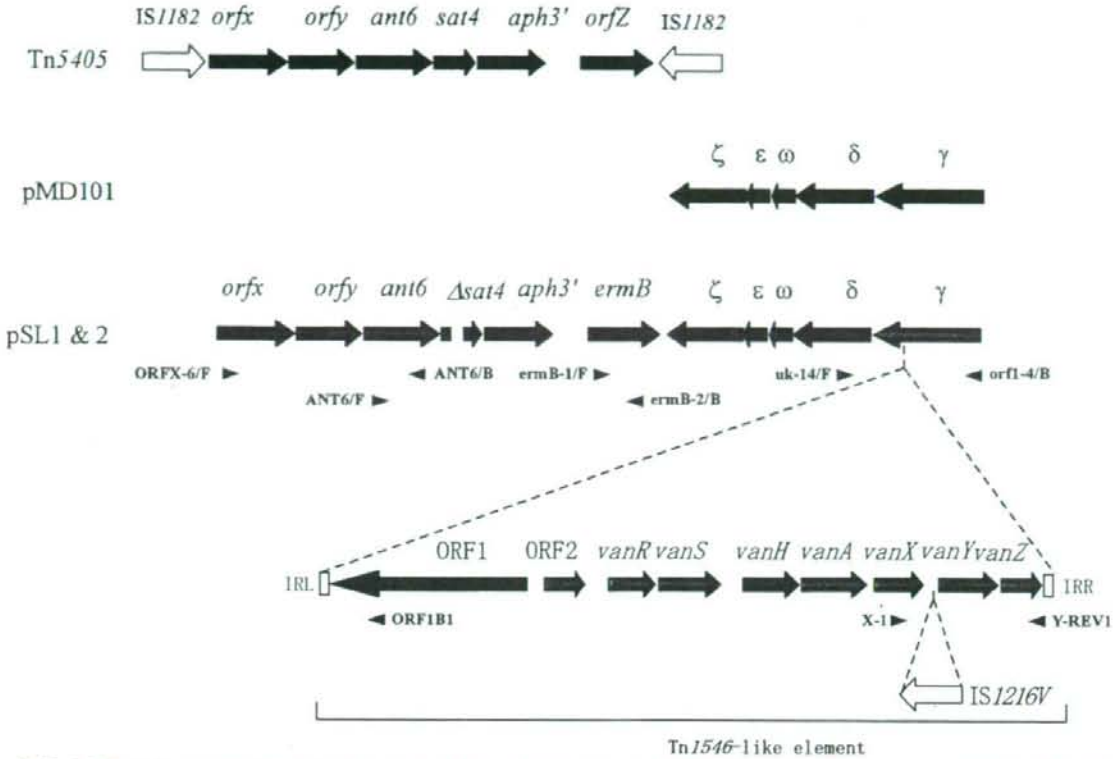

FIG. 5. Schematic representation of ORFs in the multidrug resistance region in the pSL plasmid. The filled arrows represent the ORFs and their directions of transcription. The open arrows represent the IS elements. The open boxes labeled IRL and IRR indicate the left and the right inverted repeats of the Tn*1546*-like element, respectively. The dotted lines above the Tn*1546*-like element indicate the insertion of the Tn*1546*-like element in the γ gene. The space in Δ*sat4* ORF indicates the deletion of 62 bp. The small arrowheads indicate the approximate locations and directions of the primers used for PCR to amplify the template DNAs to be sequenced.

formed by BLAST against the protein databases, and the results are shown in Table 6. The deduced amino acid sequences of the ORFs showed a high level of amino acid identity (100 to 98%) with ORFs carried on other plasmids or transposons (Table 6). Each of the ORFs that was designated an ORF or gene corresponded to a reported ORF or gene. Genes corresponding to *ant6*, *aph(3')-IIIa*, and *ermB*, which encode streptomycin, kanamycin, and erythromycin resistance, were identified in the 22-kb region of the pSL plasmids. *sat4* encodes streptothicin resistance (50). The Δ*sat4* carried on the pSL plasmids had a 62-bp deletion from nucleotides 230 to 291 within the 543-bp nucleotide sequence of the *sat4* gene. The *ant6*, Δ*sat4*, *aph(3')-IIIa*, and *ermB* genes formed a cluster and were located in that order. *orfx* and *orfy*, which are carried on Tn*5405* of *Staphylococcus aureus*, were upstream of *ant(6)*. Tn*5405* is flanked by IS*1182*, and it contains the genes *orfx*, *orfy*, *ant(6)*, *sat(4)*, *aph(3')-IIIa*, and *orfz* in that order (Fig. 5) (14). The pSL plasmids carried all of the genes corresponding to the VanA-type vancomycin resistance genes carried on Tn*1546* (Fig. 5). The Tn*1546*-like transposon carried on the pSL plasmids carried IS*1216V* (809 bp) in a noncoding region between *vanX* and *vanY* (Fig. 5). ORFγ, -δ, -ω, -ε, and -ζ, which corresponded to the reported ORFs on pMD101, were found within this region of the pSL plasmids (Fig. 5) (6). A Tn*1546*-like transposon was present as an insert in ORFγ (Fig. 5). The N-terminal position of ORFγ was located downstream of *vanZ*, which is contained within the Tn*1546*-like transposon (Fig. 5). The C-terminal portions of ORFγ, -δ, -ω, -ε, and -ζ were located between the 3′ end of *ermB* and the 3′ end of ORF1, which is contained within the Tn*1546*-like transposon (Fig. 5).

DISCUSSION

E. faecium was the most prevalent of the vancomycin-resistant enterococci examined (i.e., 92% and 81% of isolates from chickens and patients, respectively), followed by *E. faecalis* (i.e., 4% and 19% of isolates from chickens and patients, respectively). The VRE isolates from both chickens and patients showed multiple drug resistance. However, the isolation frequencies of gentamicin and kanamycin resistances were significantly lower in *E. faecium* isolates obtained from chickens than in the isolates obtained from patients, suggesting that the use of different antimicrobial agents in the farming and hospi-

TABLE 6. ORFs and IS identified in multidrug resistance region

ORF and IS	5′/3′ ends of segment on map (bp)[a]	Size (no. of amino acids)	Identification	Amino acid identity (%)	Reference(s)
orfx	1/870	289	Tn*5405*	100	14
orfy	851/1585	244	Tn*5405*	100	
ant6	1618/2526	302	Tn*5405*	100	
Δ*sat4*	2523/3003[b]	84[d]	Tn*5405*	92[e]	
aph3	3096/3890	264	Tn*5405*	100	
ermB leader peptide	4433/4516	27	*E. faecalis* 373	100	37
ermB	4641/5378	245	*E. faecalis* 373	100	
ζ	6504/5641	287	pMD101	99	6
ε	6778/6506	90	pMD101	99	
ω	7010/6795	71	pMD101	99	
δ	7998/7102	298	pMD101	98	
γ	8256/8101[c]	51	pMD101	99[f]	
ORF1	11297/8331	988	Tn*1546*	100	2
ORF2	11443/12018	191	Tn*1546*	100	
vanR	12232/12927	231	Tn*1546*	100	
vanS	12905/14059	389	Tn*1546*	100	
vanH	14274/15242	322	Tn*1546*	100	
vanA	15235/16266	343	Tn*1546*	100	
vanX	16272/16880	202	Tn*1546*	100	
IS*1216V*	17283/18091		IS*1216V*	100	2, 29
vanY	18125/19036	303	Tn*1546*	100	2
vanZ	19189/19674	161	Tn*1546*	100	
γ	21918/19930[c]	663	pMD101	99[f]	6

[a] The positions given are from the first base of the sequence in the database (accession no. AB247327).
[b] Δ*sat4* had a 62-bp deletion between 2751 and 2752 compared with wild-type *sat4*.
[c] ORF was interrupted by the insertion of a Tn*1546*-like element between 8101 and 8102.
[d] Δ*sat4* produces the protein prematurely terminated after amino acid 84, because of the 62-bp deletion. Wild-type *sat4* produces a full-length protein of 180 amino acids.
[e] The first 84 amino acids of Δ*sat4* and wild-type *sat4* were compared. The DNA sequences of both genes were identical, except for the 62-bp deletion in Δ*sat4*.
[f] Amino acid sequence deduced from the DNA sequence without the insertion of the Tn*1546*-like element was compared with that of wild-type γ of pMD101.

tal environments selected for *E. faecium* strains that differed in their drug resistance profiles.

Based on the physical map and sequence data of the regions of drug resistance determinants, the indistinguishable conjugative and pheromone-responsive plasmids pSL1 and pSL2 were identified from VanA-type *E. faecalis* strains KV1 and KV2, which were isolated from a human clinical sample and a chicken fecal sample, respectively. Plasmids pSL1 and pSL2 did not respond to any of the synthetic pheromones of cAD1, cPD1, cCF10, cOB1, and cAM373, which are pheromones for the previously characterized plasmids pAD1, pPD1, pCF10, pOB1, and pAM373, respectively (10). The pheromone specific to pSL plasmid has not been previously characterized and has been designated cSL1.

The two strains showed identical multiple drug resistance patterns, with the exception that KV2 was sensitive to tetracycline, but they showed different PFGE patterns with SmaI-digested chromosomal DNAs. The plasmids encoded multiple drug resistances to vancomycin, erythromycin, gentamicin, kanamycin, streptomycin, and teicoplanin. The linkage of the multiple drug resistance determinants on the plasmid may enable the multiple-drug-resistant *E. faecalis* strains to be selected in each of the two different environments.

The well-analyzed prototype pheromone-responsive plasmids pAD1 (8, 19), pCF10 (17, 18), and pPD1 (20, 43) have molecular sizes of around 60 kb and carry the genes for Hly/Bac and UV, Tc, and Bac21 resistances, respectively. The pheromone-responsive vancomycin resistance plasmid pHKK100 has a molecular size of 55 kb and encodes Hly/Bac and VanA resistance (22). The molecular size of the pSL1 and pSL2 plasmids was estimated to be 128.1 kb, which is relatively large in comparison with the prototype pheromone-responsive plasmids. Nucleotide sequence analysis revealed the *orfx*, *orfy*, *ant6*, Δ*sat4*, *aph3′*, and *ermB* gene clusters, which, with the exception of *ermB*, correspond to the gene clusters found in Tn*5405* of *S. aureus*. The ORFs corresponding to the γ, δ, ω, ε, and ζ ORFs that are carried on the *Streptococcus pyogenes* plasmid pMD101 were located at the 3′ end relative to *ermB*. The VanA resistance transposon, which is a Tn*1546*-like element, was inserted into ORFγ. These results implied that the relatively large pheromone-responsive pSL plasmids may be the result either of the transposition of different transposons or of recombination between a plasmid and an *E. faecalis* pheromone-responsive plasmid and that the resulting multiple drug resistance plasmid could be selected for in an environment where antibiotics are used.

In Europe, VanA-type VRE are widespread among food animals and foods of animal origin (4, 27, 49, 52). There are a number of routes by which these VREs of animal origin may be transmitted to humans. The food chain has been implicated

as a possible route for the transmission of VanA-type VRE to humans. There has been one report that indistinguishable VRE and *vanA*-containing elements were found in a turkey sample and a turkey farmer, suggesting that the transmission of a VRE strain from a turkey to a human had occurred (49). The strains of VRE isolated from human and nonhuman sources show variations in pulsed-field gel electrophoresis of SmaI fragments of the chromosomal DNA (1, 38), suggesting that the transmission of the VanA resistance element has contributed significantly to the dissemination of VanA resistance (2). The heterogeneity of the VanA resistance determinant has been described as a result of structural changes. The structural changes of the VanA resistance determinant result from the insertion of IS*1216V*- (809 bp) or IS*1216V*- and IS*3* (2,268 bp)-like elements into the noncoding region of the VanA determinant (51). In these studies, an identical Tn*1546* type could be found in isolates obtained from both humans and farm animals (49, 51). We have shown identical substitutions of three amino acids in the *vanS* gene of the VanA-type determinant in isolates from humans and imported chickens (23, 38). These results suggest that the horizontal transmission of the vancomycin resistance transposon from farm animals to humans is possible (23, 38, 49, 51).

Our results provide evidence for genetic exchange between human and animal (chicken) VRE reservoirs and imply that humans would acquire VRE probably through the ingestion of food (chicken); the pheromone-responsive plasmid would then have transferred to the human-adapted *E. faecalis* strain from animal VRE temporarily colonizing the human intestine.

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Letters to the Editor

First VanD-Type Vancomycin-Resistant *Enterococcus raffinosus* Isolate[▽]

The vancomycin-resistant *Enterococcus raffinosus* strain GV5 was resistant to vancomycin (MIC, 1,024 μg/ml) and teicoplanin (MIC, 256 μg/ml). The species of strain GV5 was determined by sequencing of a specific PCR product for the 16S rRNA gene of *E. raffinosus*. GV5 was isolated from a stool specimen and from a bedsore on the necrotic inferior limb of a diabetic 73-year-old man in Japan.

DNA sequence analysis of the *vanD* operon was performed by sequencing the PCR products with primers specific for each gene in the *vanD4* operon of *E. faecium* 10/96A (8); it showed that GV5 encodes a 5,654-bp *vanD* gene cluster consisting of $vanR_DS_DY_DH_DDX_D$, which is homologous to the corresponding genes in the reported VanD-type strains and is located on the chromosome (accession no. AB242319) (3, 6, 7, 9). The *vanD* gene cluster was compared with that of the corresponding genes of the *vanD4* gene cluster of *E. faecium* 10/96A (Fig. 1) (8). $vanR_D$ and $vanX_D$ were completely identical to the equivalent genes in 10/96A. There was one amino acid substitution in both $VanH_D$ and VanD, where Ile_{169} was converted to Phe and Gly_{121} was converted to Val, respectively. The reported $VanS_D$ contains five blocks of the conserved sequences H, N, G1, F, and G2 (2, 4, 8), which are contained in phosphate transmitters of two-component regulator systems (1, 11). Block H sequences consist of the residues L_{164}AHDL KTPLS$_{173}$, including a putative autophosphorylation site, His_{166} (14). The Thr_{170} residue in the block H sequence has been replaced by Ile in $VanS_D$ of GV5, suggesting that this mutation might result in the constitutive expression of resistance due to impaired $VanS_D$ function to dephosphorylate phosphorylated $VanR_D$. $vanY_D$ of GV5, which has a molecular size of 1,068 bp, is completely identical to that of 10/96A with the exception of an additional adenosine insertion in $vanY_D$ of 10/96A (8). The nucleotide sequence from position 346 to position 354 of GV5 $vanY_D$ is C_{346}AAAAAAAC$_{354}$, and the

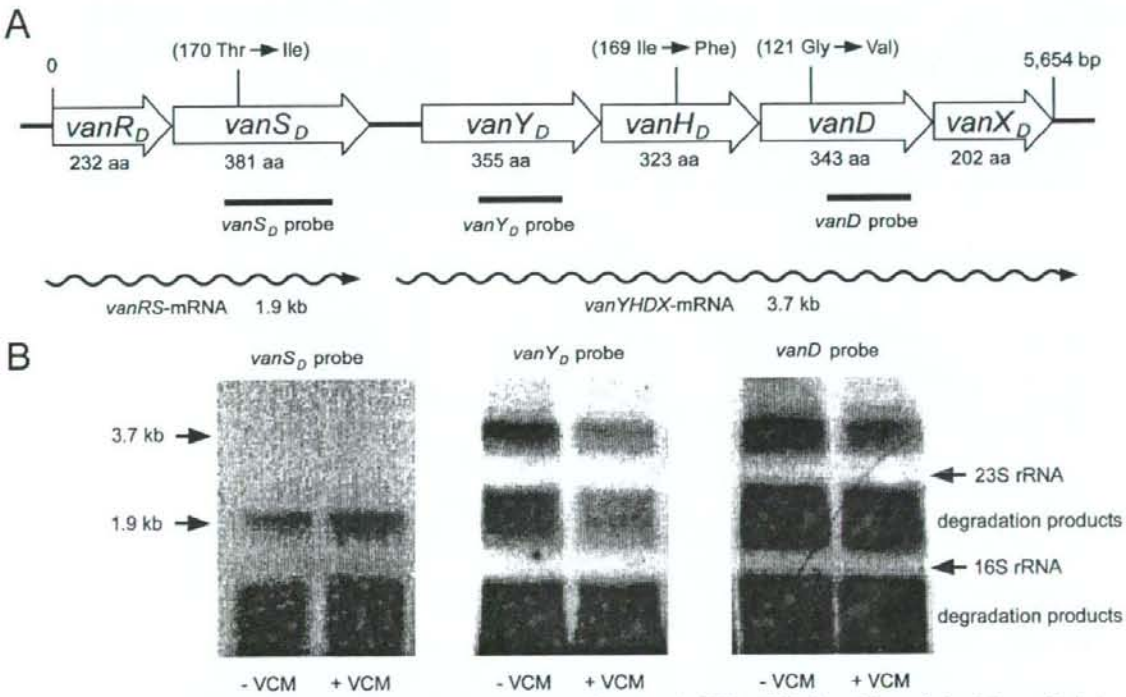

FIG. 1. Schematic representation of the *vanD* gene cluster from *E. raffinosus* strain GV5 and Northern blot analysis of the *vanD* cluster. (A) Open arrows represent coding sequences and indicate the direction of transcription. The PCR fragments internal to the $vanS_D$, $vanY_D$, and *vanD* genes used in the hybridization experiments are indicated below the corresponding regions. Amino acids with arrows within parentheses indicate substitutions compared with the reported sequence of the *vanD4* operon of *E. faecium* 10/96A (8). (B) Northern hybridization was performed according to a protocol described previously (13). RNAs were prepared from strains cultured with 6 μg/ml of vancomycin (+VCM) or without vancomycin (−VCM) for 2 h. Thirty micrograms of RNA was used in each lane. The sizes of the RNAs were determined by using the sizes of RNA molecular weight markers (Invitrogen, Inc.), and the arrows and the numbers on the left indicate the positions and sizes of the largest bands in each experiment.

sequence from position 346 to position 355 of 10/96A $vanY_D$ is $C_{346}AAAAAAAAC_{355}$. If an adenosine residue were inserted within the seven adenosines located between nucleotides 346 and 354 of GV5 $vanY_D$, the codon sequence at positions 415 to 417 of the resulting gene would become the TGA translation stop codon as a result of the frameshift mutation, and translation would be terminated prematurely after amino acid 138, as in the $VanY_D$ protein of 10/96A (8).

In Northern hybridizations with $vanY_D$ and *vanD* probes, identical bands of about 3.7 kb in size, which correspond to the transcript of $vanY_DH_DDX_D$ (5), were observed in both the absence and the presence of vancomycin (Fig. 1). The $vanS_D$ probe detected an approximately 1.9-kb band, which corresponds to the size of the transcript of $vanR_DS_D$, in the absence and presence of vancomycin (Fig. 1). These results indicate that the *vanD4* cluster in GV5 is expressed constitutively (2, 3, 6, 9, 12).

Analysis of the D-Ala:D-Ala ligase gene (*ddl*) on the chromosome of strain GV5 (accession no. AB242318) revealed that there are two amino acid substitutions—Asn_{271} is converted to Asp, and Gly_{319} is converted to Asp—compared to the wild-type DDL of *E. raffinosus* JCM8733 (accession no. AB242317), which implies that the amino acid substitutions might result in impaired function of GV5 DDL (10).

Several VanD-type vancomycin-resistant enterococci have been identified among *E. faecium* and *E. faecalis* (3, 6, 7, 9). We have described the first VanD-type *E. raffinosus* strain and showed evidence that there is species divergence in enterococci that encode VanD resistance as well as nucleotide divergence between the VanD determinants (3, 6, 7, 9).

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Bac 32, a Novel Bacteriocin Widely Disseminated among Clinical Isolates of *Enterococcus faecium*

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A total of 636 vancomycin-resistant *Enterococcus faecium* (VRE) isolates that had been obtained between 1994 and 1999 from the Medical School Hospital of the University of Michigan, Ann Arbor, were tested for bacteriocin production. Two hundred seventy-seven (44%) of the strains were bacteriocinogenic; and 193 of these exhibited activity against *Enterococcus faecium*, *Enterococcus hirae*, and *Enterococcus durans*. Strain VRE200 harbors the highly efficient conjugative gentamicin resistance plasmid pG200 (70 kb) and bacteriocin plasmid pT11 (12.5 kb). The bacteriocin encoded on pT11 was designated bacteriocin 32 (Bac 32). Bacteriocin 32 was active against *E. faecium*, *E. hirae*, and *E. durans* but showed no activity against *Listeria monocytogenes*. The Bac 32 genetic locus consists of a bacteriocin gene (*bacA*) and an immunity gene (*bacB*). Neither of these genes showed significant homology to any known bacteriocin determinants. The deduced *bacA* product is 89 amino acids in length, with a putative signal peptide of 19 amino acids at the N terminus. The *bacB* gene encodes a deduced 55-amino-acid protein without a signal sequence. One hundred eighty-nine strains (97.9%) of the 193 strains with activity against the 3 test enterococcal strains gave rise to the expected specific PCR product with a primer specific for *bacA*, indicating that there is a high incidence of Bac 32 production among VRE clinical isolates. Data from Southern analyses of plasmid DNA from 189 of the Bac 32-producing strains with a plasmid pT11-specific probe suggested that 137 (72.5%) of the strains harbored a pT11-type plasmid. Bac 32 or Bac 32-type bacteriocin activity and the determinant genes were also identified in 22 (39.3%) of a total of 56 vancomycin-sensitive *E. faecium* clinical isolates, which suggests that this bacteriocin is widely disseminated among *E. faecium* strains.

Multiple-drug-resistant enterococci and vancomycin-resistant enterococci (VRE) in particular are major causes of nosocomial infection. The acquired glycopeptide resistance provided by VanA has predominantly been identified in *Enterococcus faecium* isolates (23, 31, 46). Multiple resistance to commonly used antibiotics provide these organisms with a selective advantage within environments, such as the health care environment for humans (23, 34) or the food animal production environment (2, 29, 39, 47), where antibiotics are used. The direct selective pressure of antimicrobial agents is the largest contributing factor in the selective increase of multiple-drug-resistant enterococci in different habits. However, the ecological factors influencing the proliferation of the organisms involved in these epidemiological scenarios have not yet been elucidated.

Bacteriocins are produced by a wide variety of gram-positive and gram-negative bacteria. They are bacterial proteins which inhibit the growth of other bacteria that are closely related to the producer strain, and they usually exhibit a relatively narrow spectrum of activity. Bacteriocins are thought to provide the producer strain with an ecological or selective advantage over other strains. Bacteriocin production has been described for several genera of lactic acid bacteria (LAB) (13). LAB bacteriocins can be divided into two main classes (36): class I consists of modified bacteriocins (the lantibiotics); and class II consists of the small heat-stable nonlantibiotics. Class II bacteriocins are further divided into subgroup IIa, which are pediocin-like bacteriocins with strong antilisteria effects, and subgroup IIb, which are non-pediocin-like bacteriocins and those with two peptides that require the complementary actions of both peptides for full antimicrobial activity. *Enterococcus faecalis* and *E. faecium* bacteriocins have been genetically and biochemically well characterized in the genus *Enterococcus*. *E. faecalis* bacteriocins include the β-hemolysin/bacteriocin (cytolysin) (9, 11, 18, 20, 25, 26), the peptide antibiotic AS-48 (33), bacteriocin 21 (Bac 21) (44), and bacteriocin 31 (43). These *E. faecalis* bacteriocins have been identified in clinical isolates (9, 32, 43, 44). The well-characterized *E. faecium* bacteriocins have been identified from food-grade organisms (8). These include enterocins A (1), B (3), P (7), I (15), and L50A and L50B (6). These bacteriocins belong to LAB class II bacteriocins and are active against *Listeria monocytogenes* (36). Enterocins A and P are pediocin-like bacteriocins (36).

In contrast to the bacteriocins obtained from *E. faecalis* clinical isolates, little is known about the bacteriocins present in *E. faecium* clinical isolates. In this report, we describe the high level of incidence of a specific bacteriocin in both vancomycin-resistant and vancomycin-sensitive *E. faecium* clinical isolates.

MATERIALS AND METHODS

Bacteria, media, and reagents. The laboratory strains and plasmids used in this study are listed in Table 1. A total of 640 vancomycin-resistant *Enterococcus faecium* (VRE) clinical isolates were obtained from different patients who had been admitted to the University of Michigan Medical School Hospital, Ann

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