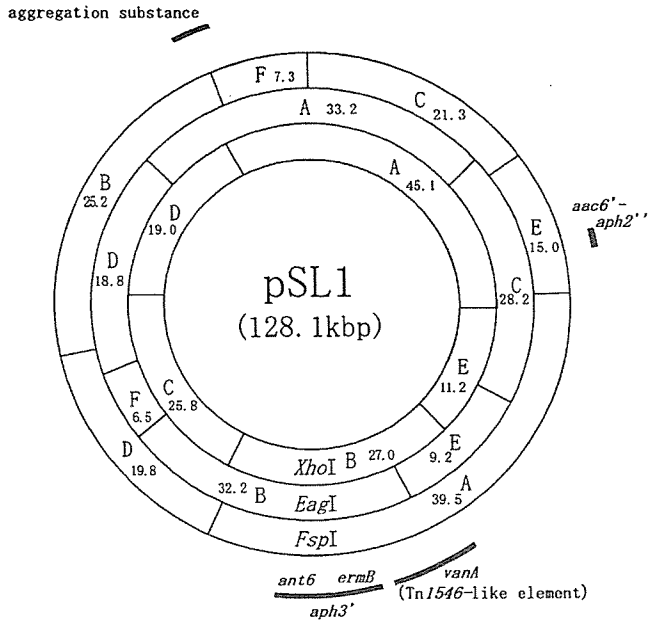

FIG. 2. Physical map of pSL plasmids. The fragments produced by the restriction endonuclease digestion of pSL plasmid DNA are denoted by letters. The numbers indicate the sizes of the fragments in kilobase pairs. The arcs indicate the approximate regions of the antibiotic resistance determinants and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

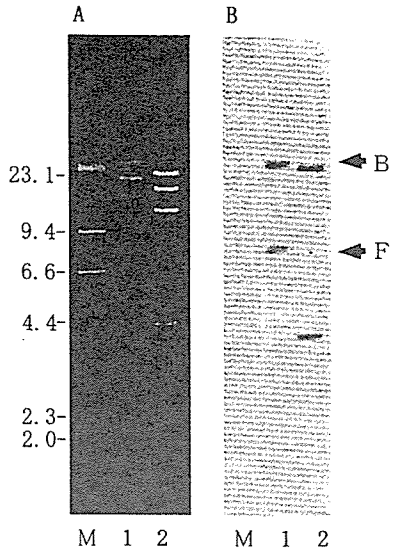

FIG. 3. Agarose gel electrophoresis of pSL plasmid digested with restriction endonuclease and hybridization with *asa1* probe. (A) Agarose gel electrophoresis of endonuclease-digested plasmid DNAs. (B) The gel was Southern blotted and hybridized with the *asa1* probe. Lanes: 1, FspI-digested pSL1 DNA; 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-

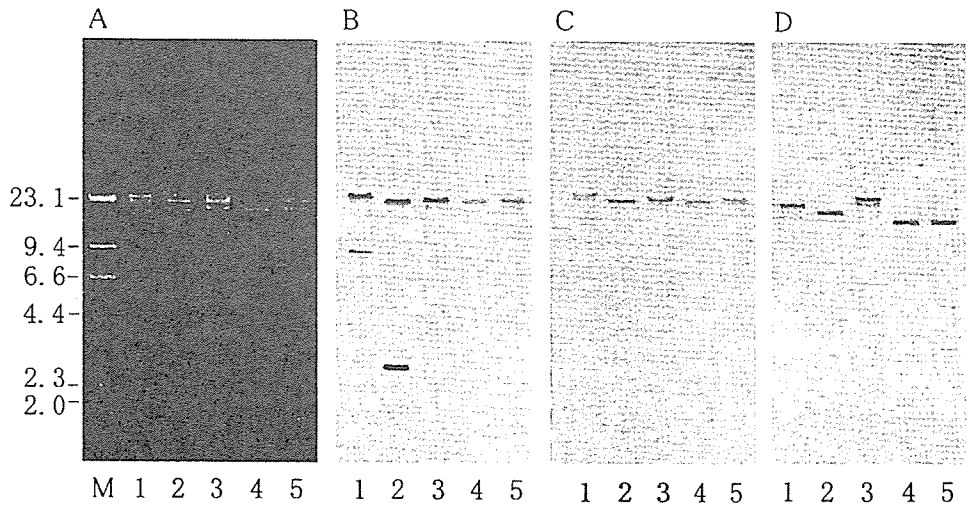

FIG. 4. Agarose gel electrophoresis of restriction endonuclease-digested pSL plasmid DNAs and Southern hybridization with drug resistance genes. (A) Agarose gel electrophoresis of endonuclease-digested pSL plasmid DNAs. Lanes: 1, EagI digestion; 2, EagI/XhoI double digestion; 3, XhoI digestion; 4, XhoI/FspI 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.

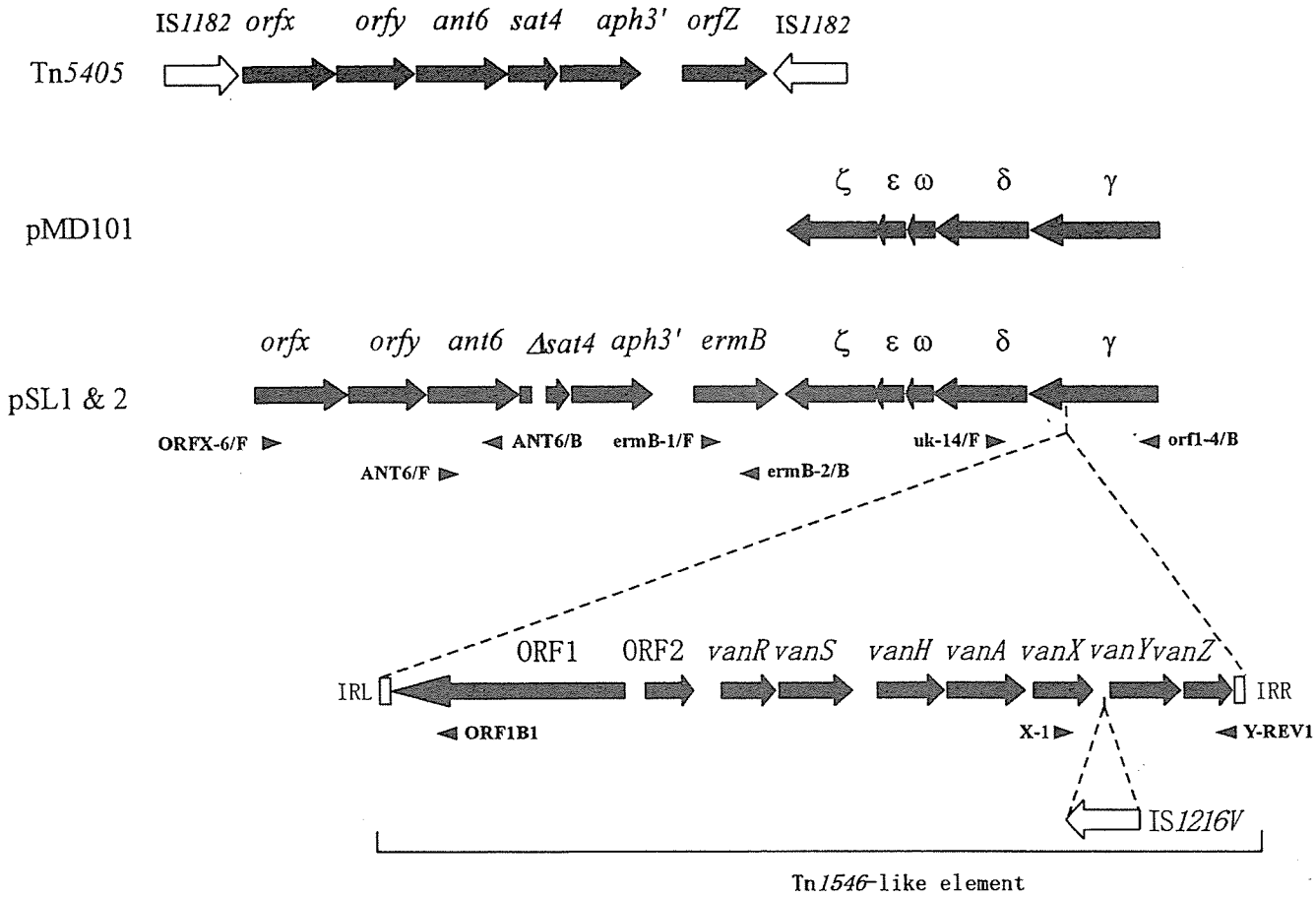

FIG. 5. Schematic representation of ORFs in the multidrug resistance region in the pSL plasmid. The filled arrows represent the ORFs and their directions of transcription. The open arrows represent the IS elements. The open boxes labeled IRL and IRR indicate the left and the right inverted repeats of 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.

ACKNOWLEDGMENTS

This work was supported by grants from the Japanese Ministry of Education, Culture, Sports, Science and Technology [Tokuteiryoiki (C) and Kiban (B) and (C)] and the Japanese Ministry of Health, Labor and Welfare (H15-Shinko-9).

We thank Yong Ho Park and Yeonhee Lee for providing VRE strains and helpful advice, Elizabeth Kamei for helpful advice, and Takahiro Nomura for technical assistance.

REFERENCES

1. **Aarestrup, F. M., P. Butaye, and W. Witte.** 2002. Nonhuman reservoirs of enterococci, p. 55–99. *In* M. S. Gilmore (ed.), The enterococci: pathogenesis, molecular biology, and antibiotic resistance. American Society for Microbiology, Washington, D.C.
2. **Arthur, M., C. Molinas, F. Depardieu, and P. Courvalin.** 1993. Characterization of Tn*1546*, Tn*3*-related transposon conferring glycopeptide resistance by synthesis of depsipeptide peptidoglycan precursors in *Enterococcus faecium* BM4147. J. Bacteriol. **175**:117–127.
3. **Ausubel, F. M., R. Brent, R. W. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl.** 1987. Current protocols in molecular biology. John Wiley & Sons, Inc., New York, N.Y.
4. **Bates, J., J. Z. Jordens, and D. T. Griffiths.** 1994. Farm animals as a putative reservoir for vancomycin-resistant enterococcal infection in man. J. Antimicrob. Chemother. **34**:507–514.
5. **Carlier, C., and P. Courvalin.** 1990. Emergence of 4′,4″-aminoglycoside nucleotidyltransferase in *Enterococcus*. Antimicrob. Agents Chemother. **34**: 1565–1569.
6. **Ceglowski, P., and J. C. Alonso.** 1994. Gene organization of the *Streptococcus pyogenes* plasmid pDB101: sequence analysis of the *orfη-copS* region. Gene **145**:33–39.
7. **Clewell, D. B.** 1981. Plasmids, drug resistance, and gene transfer in the genus *Streptococcus*. Microbiol. Rev. **45**:409–436.
8. **Clewell, D. B.** 1993. Sex pheromones and the plasmid-encoded mating response in *Enterococcus faecalis*, p. 349–367. *In* D. B. Clewell (ed.), Bacterial conjugation. Plenum Press, New York, N.Y.
9. **Clewell, D. B., and B. L. Brown.** 1980. Sex pheromone cAD1 in *Streptococcus faecalis*: induction of a function related to plasmid transfer. J. Bacteriol. **143**:1063–1065.
10. **Clewell, D. B., and G. M. Dunny.** 2002. Conjugation and genetic exchange in enterococci, p. 265–300. *In* M. S. Gilmore (ed.), The enterococci: pathogenesis, molecular biology, and antibiotic resistance. American Society for Microbiology, Washington, D.C.
11. **Clewell, D. B., P. K. Tomich, M. C. Gawron-Burke, A. E. Franke, Y. Yagi, and F. Y. An.** 1982. Mapping of *Streptococcus faecalis* plasmids pAD1 and pAD2 and studies relating to transposition of Tn*917*. J. Bacteriol. **152**:1220–1230.
12. **Coque, T. M., J. F. Tomayko, S. C. Ricke, P. C. Okhyusen, and B. E. Murray.** 1996. Vancomycin-resistant enterococci from nosocomial, community, and animal sources in the United States. Antimicrob. Agents Chemother. **40**: 2605–2609.
13. **DeLisle, S., and T. M. Perl.** 2003. Vancomycin-resistant enterococci: a road map on how to prevent the emergence and transmission of antimicrobial resistance. Chest **123**:504S–518S.
14. **Derbise. A., K. G. Dyke, and N. el Solh.** 1996. Characterization of a *Staphylococcus aureus* transposon, Tn*5405*, located within Tn*5404* and carrying the aminoglycoside resistance genes, *aphA-3* and *aadE*. Plasmid **35**:174–188.
15. **Dunny, G. M., B. L. Brown, and D. B. Clewell.** 1978. Induced cell aggregation and mating in *Streptococcus faecalis*; evidence for a bacterial sex pheromone. Proc. Natl. Acad. Sci. USA **75**:3479–3483.
16. **Dunny, G. M., R. A. Craig, R. L. Carron, and D. B. Clewell.** 1979. Plasmid transfer in *Streptococcus faecalis*: production of multiple sex pheromones by recipients. Plasmid **2**:454–465.
17. **Dunny, G. M., and B. A. Leonard.** 1997. Cell-cell communication in gram-positive bacteria. Annu. Rev. Microbiol. **51**:527–564.
18. **Dunny, G. M., B. A. Leonard, and P. J. Hedberg.** 1995. Pheromone-inducible conjugation in *Enterococcus faecalis*: interbacterial and host-parasite chemical communication. J. Bacteriol. **177**:871–876.
19. **Francia, M. V., W. Haas, R. Wirth, E. Samberger, A. Muscholl-Silberhorn, M. S. Gilmore, Y. Ike, K. E. Weaver, F. Y. An, and D. B. Clewell.** 2001. Completion of the nucleotide sequence of the *Enterococcus faecalis* conjugative virulence plasmid pAD1 and identification of a second transfer origin. Plasmid **46**:117–127.
20. **Fujimoto, S., H. Tomita, E. Wakamatsu, K. Tanimoto, and Y. Ike.** 1995. Physical mapping of the conjugative bacteriocin plasmid pPD1 of *Enterococcus faecalis* and identification of the determinant related to the pheromone response. J. Bacteriol. **177**:5574–5581.
21. **Galli, D., A. Friesenegger, and R. Wirth.** 1992. Transcriptional control of sex-pheromone-inducible genes on plasmid pAD1 of *Enterococcus faecalis* and sequence analysis of a third structural gene for (pPD1-encoded) aggregation substance. Mol. Microbiol. **6**:1297–1308.
22. **Handwerger, S., M. J. Pucci, and A. Kolokathis.** 1990. Vancomycin resistance is encoded on a pheromone response plasmid in *Enterococcus faecium* 228. Antimicrob. Agents Chemother. **34**:358–360.
23. **Hashimoto, Y., K. Tanimoto, Y. Ozawa, T. Murata, and Y. Ike.** 2000. Amino acid substitutions in the VanS sensor of the VanA-type vancomycin-resistant enterococcus strains result in high-level vancomycin resistance and low-level teicoplanin resistance. FEMS Microbiol. Lett. **185**:247–254.
24. **Hirt, H., R. Wirth, and A. Muscholl.** 1996. Comparative analysis of 18 sex pheromone plasmids from *Enterococcus faecalis*: detection of a new insertion element on pPD1 and implications for the evolution of this plasmid family. Mol. Gen. Genet. **252**:640–647.
25. **Huycke, M. M., D. F. Sahm, and M. S. Gilmore.** 1998. Multiple-drug resistant enterococci: the nature of the problem and an agenda for the future. Emerg. Infect. Dis. **4**:239–249.
26. **Ike, Y., and D. B. Clewell.** 1984. Genetic analysis of the pAD1 pheromone response in *Streptococcus faecalis*, using transposon Tn*917* as an insertional mutagen. J. Bacteriol. **158**:777–783.
27. **Ike, Y., K. Tanimoto, Y. Ozawa, T. Nomura, S. Fujimoto, and H. Tomita.** 1999. Vancomycin-resistant enterococci in imported chickens in Japan. Lancet **353**:1854.
28. **Ike, Y., K. Tanimoto, H. Tomita, K. Takeuchi, and S. Fujimoto.** 1998. Efficient transfer of the pheromone-independent *Enterococcus faecium* plasmid pMG1 (Gm^r) (65.1 kb) to *Enterococcus* strains during broth mating. J. Bacteriol. **180**:4886–4892.
29. **Jensen, L. B.** 1998. Internal size variations in Tn*1546*-like elements due to the presence of IS*1216V*. FEMS Microbiol. Lett. **169**:349–354.
30. **Kak, V., and J. W. Chow.** 2002. Acquired antibiotic resistances in enterococci, p. 355–383. *In* M. S. Gilmore (ed.), The enterococci: pathogenesis, molecular biology, and antibiotic resistance. American Society for Microbiology, Washington, D.C.
31. **Kao, S. M., S. B. Olmsted, A. S. Viksnins, J. C. Gallo, and G. M. Dunny.** 1991. Molecular and genetic analysis of a region of plasmid pCF10 containing positive control genes and structural genes encoding surface proteins involved in pheromone-inducible conjugation in *Enterococcus faecalis*. J. Bacteriol. **173**:7650–7664.
32. **Kobayashi, N., M. Alam, Y. Nishimoto, S. Urasawa, N. Uehara, and N. Watanabe.** 2001. Distribution of aminoglycoside resistance genes in recent clinical isolates of *Enterococcus faecalis*, *Enterococcus faecium* and *Enterococcus avium*. Epidemiol. Infect. **126**:197–204.
33. **Machida, R. J., M. U. Miya, M. Nishida, and S. Nishida.** 2002. Complete

mitochondrial DNA sequence of *Tigriopus japonicus* (*Crustacea:Copepoda*). Mar. Biotechnol. **4**:406–417.
34. **Martone, W. J.** 1998. Spread of vancomycin-resistant enterococci: why did it happen in the United States? Infect. Control. Hosp. Epidemiol. **19**:539–545.
35. **McDonald, L. C., M. J. Kuehnert, F. C. Tenover, and W. R. Jarvis.** 1997. Vancomycin-resistant enterococci outside the health-care setting: prevalence, sources, and public health implications. Emerg. Infect. Dis. **3**:311–317.
36. **National Committee for Clinical Laboratory Standards.** 2000. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically, 5th ed. Approved standard M7-A4. National Committee for Clinical Laboratory Standards, Wayne, Pa.
37. **Oh, T. G., A. R. Kwon, and E. C. Choi.** 1998. Induction of *ermAMR* from a clinical strain of *Enterococcus faecalis* by 16-membered-ring macrolide antibiotics. J. Bacteriol. **180**:5788–5791.
38. **Ozawa, Y., K. Tanimoto, T. Nomura, M. Yoshinaga, Y. Arakawa, and Y. Ike.** 2002. Vancomycin resistant enterococci (VRE) in humans and imported chickens in Japan. Appl. Environ. Microbiol. **68**:6457–6461.
39. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
40. **Schmitz, F. J., R. Sadurski, A. Kray, M. Boos, R. Geisel, K. Kohrer, J. Verhoef, and A. C. Fluit.** 2000. Prevalence of macrolide-resistance genes in *Staphylococcus aureus* and *Enterococcus faecium* isolates from 24 European university hospitals. J. Antimicrob. Chemother. **45**:891–894.
41. **Seo, K. S., J. Y. Lim, H. S. Yoo, W. K. Bae, and Y. H. Park.** 2005. Comparison of vancomycin-resistant enterococci isolates from human, poultry and pigs in Korea. Vet. Microbiol. **106**:225–233.
42. **Shin, J. W., D. Yong, M. S. Kim, K. H. Chang, K. Lee, J. M. Kim, and Y. Chong.** 2003. Sudden increase of vancomycin-resistant enterococcal infections in a Korean tertiary care hospital: possible consequences of increased use of oral vancomycin. J. Infect. Chemother. **9**:62–67.
43. **Tanimoto, K., H. Tomita, and Y. Ike.** 1996. The *traA* gene of the *Enterococcus faecalis* conjugative plasmid pPD1 encodes a negative regulator for the pheromone response. Plasmid **36**:55–61.
44. **Tomich, P. K., F. Y. An, and D. B. Clewell.** 1980. Properties of erythromycin-inducible transposon Tn*917* in *Streptococcus faecalis*. J. Bacteriol. **141**:1366–1374.
45. **Tomita, H., and Y. Ike.** 2005. Genetic analysis of transfer-related regions of the vancomycin resistance *Enterococcus* conjugative plasmid pHTβ: identification of *oriT* and a putative relaxase gene. J. Bacteriol. **187**:7727–7737.
46. **Tomita, H., C. Pierson, S. K. Lim, D. B. Clewell, and Y. Ike.** 2002. Possible connection between a widely disseminated conjugative gentamicin resistance (pMG1-like) plasmid and the emergence of vancomycin resistance in *Enterococcus faecium*. J. Clin. Microbiol. **40**:3326–3333.
47. **Tomita, H., K. Tanimoto, S. Hayakawa, K. Morinaga, K. Ezaki, H. Oshima, and Y. Ike.** 2003. Highly conjugative pMG1-like plasmids carrying Tn*1546*-like transposons that encode vancomycin resistance in *Enterococcus faecium*. J. Bacteriol. **185**:7024–7028.
48. **Udo, E. E., N. Al-Sweih, P. John, and T. D. Chugh.** 2002. Antibiotic resistance of enterococci isolated at a teaching hospital in Kuwait. Diagn. Microbiol. Infect. Dis. **43**:233–238.
49. **van den Bogaard, A. E., L. B. Jensen, and E. E. Stobberingh.** 1997. Vancomycin-resistant enterococci in turkeys and farmers. N. Engl. J. Med. **337**: 1558–1559.
50. **Werner, G., B. Hildebrandt, and W. Witte.** 2001. Aminoglycoside-streptothricin resistance gene cluster *aadE-sat4-aphA-3* disseminated among multiresistant isolates of *Enterococcus faecium*. Antimicrob Agents Chemother. **45**:3267–3269.
51. **Willems, R. J., J. Top, N. van den Braak, A. van Belkum, D. J. Mevius, G. Hendriks, M. van Santen-Verheuvel, and J. D. van Embden.** 1999. Molecular diversity and evolutionary relationships of Tn*1546*-like elements in enterococci from humans and animals. Antimicrob. Agents Chemother. **43**:483–491.
52. **Witte, W.** 1998. Medical consequences of antibiotic use in agriculture. Science **279**:996–997.

ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Nov. 2006, p. 3966–3967 Vol. 50, No. 11
0066-4804/06/$08.00+0 doi:10.1128/AAC.00607-06

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

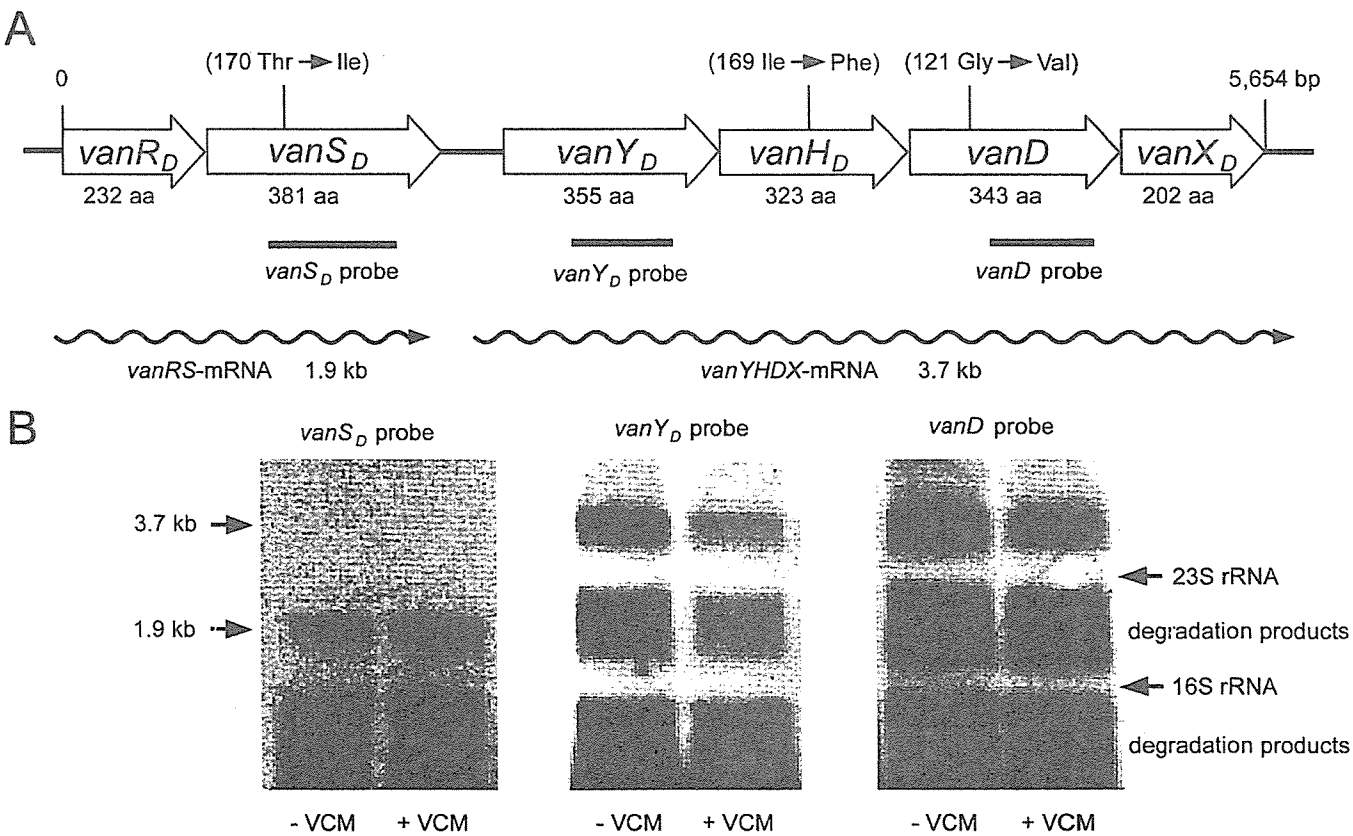

FIG. 1. Schematic representation of the *vanD* gene cluster from *E. raffinosus* strain GV5 and Northern blot analysis of the *vanD* cluster. (A) Open arrows represent coding sequences and indicate the direction of transcription. The PCR fragments internal to the $vanS_D$, $vanY_D$, and *vanD* genes 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).

This work was supported by grants from the Japanese Ministry of Education, Culture, Sports, Science, and Technology [Tokuteiryoiki (2) (16017216), Kiban (C) (17590384)] and from the Japanese Ministry of Health, Labor, and Welfare (H15-Shinko-9, H18-Shinko-11).

We thank Yoshiyuki Ozawa and Elizabeth Kamei for helpful advice.

REFERENCES

1. **Baptista, M., P. Rodrigues, F. Depardieu, P. Courvalin, and M. Arthur.** 1999. Single-cell analysis of glycopeptide resistance gene expression in teicoplanin-resistant mutants of a VanB-type *Enterococcus faecalis*. Mol. Microbiol. **32**:17–28.
2. **Boyd, D. A., J. Conly, H. Dedier, G. Peters, L. Robertson, E. Slater, and M. R. Mulvey.** 2000. Molecular characterization of the *vanD* gene cluster and a novel insertion element in a vancomycin-resistant enterococcus isolated in Canada. J. Clin. Microbiol. **38**:2392–2394.
3. **Boyd, D. A., P. Kibsey, D. Roscoe, and M. R. Mulvey.** 2004. *Enterococcus faecium* N03-0072 carries a new VanD-type vancomycin resistance determinant: characterization of the VanD5 operon. J. Antimicrob. Chemother. **54**:680–683.
4. **Casadewall, B., and P. Courvalin.** 1999. Characterization of the *vanD* glycopeptide resistance gene cluster from *Enterococcus faecium* BM4339. J. Bacteriol. **181**:3644–3648.
5. **Casadewall, B., P. E., Reynolds, O. H. Ambur, and P. Courvalin.** 2001. Regulation of expression of the *vanD* glycopeptide resistance gene cluster from *Enterococcus faecium* BM4339. J. Bacteriol. **183**:3436–3446.
6. **Dalla Costa, L. M., P. E. Reynolds, H. A. Souza, D. C. Souza, M. F. Palepou, and N. Woodford.** 2000. Characterization of a divergent VanD-type resistance element from the first glycopeptide-resistant strain of *Enterococcus faecium* isolated in Brazil. Antimicrob. Agents Chemother. **44**:3444–3446.
7. **Depardieu, F., M. Kolbert, H. Pruul, J. Bell, and P. Courvalin.** 2004. VanD-type vancomycin-resistant *Enterococcus faecium* and *Enterococcus faecalis*. Antimicrob. Agents Chemother. **48**:3892–3904.
8. **Depardieu, F., P. E. Reynolds, and P. Courvalin.** 2003. VanD-type vancomycin-resistant *Enterococcus faecium* 10/96A. Antimicrob. Agents Chemother. **47**:7–18.
9. **Ostrowsky, B. E., N. C. Clark, C. Thauvin-Eliopoulos, L. Venkataraman, M. H. Samore, F. C. Tenover, G. M. Eliopoulos, R. C. Moellering, Jr., and H. S. Gold.** 1999. A cluster of VanD vancomycin-resistant *Enterococcus faecium*: molecular characterization and clinical epidemiology. J. Infect. Dis. **180**:1177–1185.
10. **Ozawa, Y., P. Courvalin, and M. Galimand.** 2000. Identification of enterococci at the species level by sequencing of the genes for D-alanine:D-alanine ligases. Syst. Appl. Microbiol. **23**:230–237.
11. **Parkinson, J. S., and E. C. Kofoid.** 1992. Communication modules in bacterial signaling proteins. Annu. Rev. Genet. **26**:71–112.
12. **Perichon, B., P. Reynolds, and P. Courvalin.** 1997. VanD-type glycopeptide-resistant *Enterococcus faecium* BM4339. Antimicrob. Agents Chemother. **41**:2016–2018.
13. **Tanimoto, K., and Y. Ike.** 2002. Analysis of the conjugal transfer system of the pheromone-independent highly transferable enterococcus plasmid pMG1: identification of a *tra* gene (*traA*) up-regulated during conjugation. J. Bacteriol. **184**:5800–5804.
14. **Wright, G., T. Holman, and C. Walsh.** 1993. Purification and characterization of VanR and the cytosolic domain of VanS: a two-component regulatory system required for vancomycin resistance in *Enterococcus faecium* BM4147. Biochemistry **32**:5057–5063.

Koichi Tanimoto
Laboratory of Bacterial Drug Resistance
Gunma University Graduate School of Medicine

Takahiro Nomura
Department of Bacteriology and Bacterial Infection Control
Gunma University Graduate School of Medicine

Hideyuki Maruyama†
Ichihara Hospital
Teikyo University School of Medicine
Ichihara, Chiba 299-0111, Japan

Haruyoshi Tomita
Department of Bacteriology and Bacterial Infection Control
Gunma University Graduate School of Medicine

Naohiro Shibata
Yoshichika Arakawa
Department of Bacterial Pathogenesis and Infection Control
National Institute of Infectious Diseases
Gakuen, Musashi-Murayama
Tokyo 208-0011, Japan

Yasuyoshi Ike*
Department of Bacteriology and Bacterial Infection Control
and Laboratory of Bacterial Drug Resistance
Gunma University Graduate School of Medicine
Maebashi, Gunma 371-8511, Japan

*Phone: 81-27-220-7990
Fax: 81-227-220-7996
E-mail: yasuike@med.gunma-u.ac.jp

† Present address: Saiseikai Narashino Hospital, Narashino, Chiba, Japan.

▽ Published ahead of print on 25 September 2006.

ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Apr. 2006, p. 1202–1212 Vol. 50, No. 4
0066-4804/06/$08.00+0 doi:10.1128/AAC.50.4.1202–1212.2006

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

Takako Inoue,[1] Haruyoshi Tomita,[1] and Yasuyoshi Ike[1,2]*

Department of Bacteriology and Bacterial Infection Control[1] and Laboratory of Bacterial Drug Resistance,[2] Gunma University Graduate School of Medicine, Maebashi, Gunma 371-8511, Japan

Received 11 July 2005/Returned for modification 19 August 2005/Accepted 5 January 2006

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 pTI1 (12.5 kb). The bacteriocin encoded on pTI1 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 pTI1-specific probe suggested that 137 (72.5%) of the strains harbored a pTI1-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

* Corresponding author: Mailing address: Department of Bacteriology and Bacterial Infection Control, Gunma University Graduate School of Medicine, Maebashi, Gunma 371-8511, Japan. Phone: 81-27-220-7992. Fax: 81-27-220-7996. E-mail: yasuike@med.gunma-u.ac.jp.

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype or phenotype	Description	Reference or source
Strains			
Enterococcus faecalis			
FA2-2	Rif^r Fus^r	Derivative of JH2	42
JH2SS	Str^r Spc^r	Derivative of JH2	42
OG1-10	Str^r	Derivative of OG1	14
Enterococcus faecium			
BM4105RF	Rif^r Fus^r	Clinical isolate	
VRE200	Van^r pTI1 (Bac) p200G (Gm^r)	Clinical isolate	This study
Enterococcus hirae ATCC 9790			
Enterococcus durans ATCC 49135			
Enterococcus raffinosus JCM8733			
Enterococcus gallinarium BM4174			
Staphyrococcus aureus FDA209P			
Escherichia coli			
DH5α	*recA1 endA1 gyrA96 thi-1 relA1 hsdR17 supE44 lacZ*ΔM15		Bethesda Research Laboratories
TH688	CSH57b *thr*::Tn*5*		
Plasmids			
pTI1	Bac	Mobilizable plasmid (12.5 kb)	This study
pG200	Gm^r	Conjugative plasmid (70 kb)	45; this study
pAM401	Cm^r Tc^r	*E. coli-E. faecalis* shuttle vector	48
pBluescript-SK(+)	Ap^r *lacZ*	*E. coli* vector	Stratagene
pMG110		pAM401 containing 7.9-kb EcoRI-NcoI fragment from pYI17	43

Arbor, between 1994 and 1999 (45). Of the 640 VRE clinical isolates, 636 isolates were used in this study. Fifty-six vancomycin-sensitive *E. faecium* clinical isolates and 56 vancomycin-sensitive *E. faecalis* clinical isolates were included in the study. These strains were obtained from different patients at the Gunma University School of Medicine Hospital, Maebashi, Gunma, Japan, between 1990 and 1993. Forty-six *E. faecium* nonclinical isolates were obtained from the feces of healthy students attending Gunma University School of Medicine, Maebashi, Japan, in 2002 and 2003. Enterococcal strains were grown in Todd-Hewitt broth (THB; Difco, Detroit, MI) or antibiotic medium 3 (Difco). *Escherichia coli* strains were grown in Luria-Bertani medium. Solid and soft media were prepared by the addition of 1.5 or 0.75% (wt/vol) agar, respectively. All cultures were grown at 37°C. The following antibiotics were used at the indicated concentrations: ampicillin, 100 μg/ml; chloramphenicol, 20 μg/ml for enterococci and 50 μg/ml for *E. coli*; rifampin, 25 μg/ml; fusidic acid, 25 μg/ml; streptomycin, 500 μg/ml; spectinomycin, 500 μg/ml for enterococci and 50 μg/ml for *E. coli*; kanamycin, 40 μg/ml; tetracycline, 12.5 μg/ml; vancomycin, 5 μg/ml; and gentamicin, 200 μg/ml.

Soft-agar assay for bacteriocin production and immunity. The bacteriocin production assay was performed as described previously (26). The test for immunity to the bacteriocin was performed essentially as described previously (26).

Conjugative transfer and mobilization experiments. Broth mating was performed as described previously (14, 25), with a donor/recipient ratio of 1:10. Colonies were counted after 48 h of incubation at 37°C. Solid-surface mating was performed on agar plates. The mating mixture of donor and recipient was made as described above for the broth matings, and 10 μl of the mixed culture was spotted onto THB agar without antibiotics. The plates were then incubated overnight at 37°C. After incubation, the bacteria that had grown on the agar plates were scraped off and transferred into 1 ml of fresh THB, and then 0.1 ml of the mixture was spread onto appropriate selective agar plates. The colonies were counted after 48 h of incubation at 37°C. The conjugative transfer frequency was calculated as the ratio of the number of transconjugants to the number of donors. The mobilized transfer frequency was calculated as the ratio of the number of transconjugants which showed bacteriocin activity to the number of donors.

Isolation and manipulation of plasmid DNA. Plasmid DNA was isolated by the alkaline lysis method (37). Plasmid DNA was treated with restriction enzymes and subjected to agarose gel electrophoresis for analysis of DNA fragments, etc. Restriction enzymes were obtained from Nippon Gene (Toyama, Japan); New England Biolabs, Inc.; and Takara (Tokyo, Japan). They were used in accordance with the suppliers ' specifications. Agarose was obtained from Wako Chemicals, Osaka, Japan. Electrophoresis on 0.8% agarose gels was used to determine the size of large DNA fragments (those larger than 0.5 kb), and 2.0% agarose gels were used for analysis of small fragments (those smaller than 0.5 kb) (17). A "glass milk" kit (Gene Clean II kit; Bio 101, Inc., La Jolla, CA) was used for the elution of DNA fragments from agarose gels. The eluted fragments were ligated to dephosphorylated, restriction enzyme-digested vector DNA with T4 DNA ligase and were then introduced into *E. coli* by electrotransformation (16). Transformants were selected on Luria-Bertani medium agar containing the appropriate antibiotics.

DNA sequence analysis. Nucleotide sequences were obtained as described previously (38). A deletion kit (Nippon Gene) was also used. To determine the sequence of plasmid pTI1, HindIII-B and -D fragments were cloned into the vector pBluescript-SK(+). Each of the cloned DNA fragments was digested with BamHI and SacI or KpnI and XhoI and then treated with exonuclease III for various periods of time, followed by treatment with mung bean nuclease and then the Klenow fragment. The deleted DNAs were self-ligated with T4 DNA ligase. The ligation product was used to transform *E. coli* DH5α. The resulting constructs were sequenced in both orientations by using a model 377 DNA sequencer (Applied Biosystems) with *Taq* Dye Primer and *Taq* BigDye Terminator cycle sequencing kits (Applied Biosystems). The remaining regions and gapped regions were sequenced directly by using the appropriate primers. A database search was performed by using the BLASTn and tBLASTx programs of the National Center for Biotechnology Information, Bethesda, MD (http://www.ncbi.nlm.nih.gov).

TABLE 2. Oligonucleotides used in this study

Primer	Sequence and restriction sites	Description
Tn*5*	5′-TTAGGAGGTCACATGG-3′	Analysis of Tn*5* insertion mutant
10509	5′-GGGCCGGATCCGTTCCTGTTAGAGAATCCG-3′	Analysis of *bac* determinant
10760	5′-GGGCCGGATCCTAGTCAAGGTGGTTCTGCC-3′	
10872	5′-GGGCCGGATCCACTTTATGAATAGAATGTC-3′	
10965	5′-GGGCCGGATCCTATTGGTAGCTAGTTTGTG-3′	
11179	5′-GGGCCGGATCCTGCTAGTAGTAGAACAGCC-3′	
11197′	5′-AAATTGGATCCGGCTGTTCTACTACTAGC-3′	
11269′	5′-AAATTGGATCCACATCCTCTCTATACGC-3′	
11667′	5′-AAATTGGATCCTACATGGGTAGTACGTGGG-3′	
4-A	5′-GTTGAAGCTGCTGCTAGAGGATATATCTAC-3′	Analysis of *bac* determinant (mutagenesis by overlap extension PCR)
4-A′	5′-GTAGATATATCCTCTAGCAGCAGCTTCAAC-3′	
4-B	5′-GGTAAACAAACTATGACTTGTTACCTTATG-3′	
4-B′	5′-CATAAGGTAACAAGTCATAGTTTGTTTACC-3′	
4-C	5′-GGTTGAAGCTGCTGCTCAAGAGGATATATC-3′	
4-C′	5′-GATATATCCTCTTGAGCAGCAGCTTCAACC-3′	
4-D	5′-GGTGCTAAAGTGCCATAAAGTTAAAATG-3′	
4-D′	5′-CATTTTAACTTTATGGCACTTTAGCACC-3′	
P1	5′-GAATAGAATGTCTATGTCCATAAAAATTATG-3′	
P1′	5′-CATAATTTTTATGGACATAGACATTCTATTC-3′	
P2	5′-CAAAACATTCTTTCTAGACACTAAAATAGC-3′	
P2′	5′-GCTATTTTAGTGTCTAGAAAGAATGTTTTG-3′	
S. D.	5′-GTTAATCATTAAAAATCCTGTGTCATAATTTG-3′	
S. D.′	5′-CAAATTATGACACAGGATTTTTAATGATTAAC-3′	
start	5′-GGAGTGTCATAATTTCAAAAAAACAAAATTATTGG-3′	
start′	5′-CCAATAATTTTGTTTTTTTGAAATTATGACACTCC-3′	
SP	5′-GTTTAGCAGTTTATTAGTTTTCACCCCTTC-3′	
SP′	5′-GAAGGGGTGAAAACTAATAAACTGCTAAAC-3′	
V~V	5′-CTCAAAATGGTGGAGTTGTTGAAGCTGCTG-3′	
V~V′	5′-CAGCAGCTTCAACAACTCCACCATTTTGAG-3′	

Cloning of pTI1 *bacA* and *bacB*. To determine the bacteriocin determinant (*bacA*) and the immunity determinant (*bacB*) of the bacteriocin, a set of pAM401-based clones containing various DNA fragments of pTI1 was constructed. Each of the EcoRI fragments and the EcoRV fragments of pTI1 was separated by agarose gel electrophoresis and cloned into the EcoRI and EcoRV sites of pAM401, respectively. A clone harboring the EcoRV-NruI fragment, which mapped between 3.6 kb and 7.3 kb, was constructed as follows. The clone containing EcoRV fragment A was partially digested with a low concentration of NruI and then self-ligated. Each cloned plasmid DNA was prepared from *E. coli* and used to transform *E. faecalis* FA2-2 to test for bacteriocin activity or *Enterococcus hirae* ATCC 9790 to test for immunity to the bacteriocin. A variety of amplified PCR products were also cloned for further detailed study.

Generation of transposon (Tn*5*) insertion mutants. Tn*5* (Km^r) insertion into the cloned plasmid DNA was performed as described elsewhere (41). Target plasmid pMG403 (plasmid pAM401 containing EcoRV fragment A) was introduced into *E. coli* K-12 TH688 (with Tn*5* in the *thr* locus) by electrotransformation. Transformants were spread onto selective plates containing kanamycin and chloramphenicol, and the plates were left at room temperature for 10 days. The bacteria that grew on the selective plates were pooled, and the plasmid DNA was then isolated and used to transform *E. coli* DH5α. The transformants were selected on plates containing kanamycin and chloramphenicol for the selection of Tn*5*-mediated kanamycin resistance and plasmid-mediated chloramphenicol resistance, respectively. The transformants were purified and examined to determine the location of Tn*5* within the plasmid. The precise locations of Tn*5* insertion were determined by DNA sequence analysis by using a synthetic primer that hybridized to the end of Tn*5*.

PCR amplification and overlap extention PCR techniques. PCR amplification was performed by using the thermostable DNA polymerase TaKaRa *Taq* (Takara Bio Inc.) and a Perkin-Elmer 9600 thermal cycler. The PCR conditions varied according to the primers used and the size of the anticipated product. The custom primers used in this study were obtained from Invitrogen (Tokyo, Japan) and are listed in Table 2. All primers were designed to incorporate a restriction enzyme recognition site at the 5′ end to facilitate cloning of the PCR products into the identical site within the vector plasmids. All clones were sequenced in both orientations to confirm that they conformed to the desired structures. Site-directed mutagenesis was performed by overlap extension PCR. The procedures used were based on those described previously (21). Complementary oligonucleotide primers incorporating nucleotide changes were prepared to generate two DNA fragments with overlapping ends in the first round of PCR amplification. Specific mutations could be introduced by the subsequent fusion of the two fragments during the second round of PCR amplification. The resulting fusion product was used for cloning.

Pulsed-field gel electrophoresis. A gel block containing genomic DNA was digested with 10 U of SmaI overnight. Electrophoresis was then carried out in a 1% agarose gel with 45 mM Tris-borate and 1 mM EDTA, and the following settings were applied: 1 to 21 s, 6 V/cm^2, and 20 h. The gel was stained with ethidium bromide for UV observation.

DNA-DNA hybridization. Southern hybridization was performed with the digoxigenin-based nonradioisotope system of Boehringer GmbH (Mannheim, Germany), and all procedures were based on the manufacturer's manual and standard protocols (37). The plasmid DNA was isolated and digested with EcoRI. Hybridization was performed overnight at 42°C in the presence of 50% formamide. To make a probe for the detection of whole pTI1 plasmid DNA, a mixture of the purified DNAs of EcoRI fragments A and B of pTI1 from the deletion mutants was used. The probes were generated by using a DIG probe synthesis kit (Roche Diagnostics GmbH, Mannheim, Germany). The signals were detected with nitroblue tetrazolium–5-bromo-4-chloro-3-indolylphosphate stock solution (Roche Diagnostics GmbH).

Nucleotide sequence accession number. The nucleotide sequence data reported in this article are available from nucleotide sequence databases under accession number AB205024.

RESULTS

Bacteriocinogenic strains of vancomycin-resistant *Enterococcus faecium* clinical isolates. A total of 636 vancomycin-resistant *Enterococcus faecium* strains were tested for bacteriocin production against the indicator strains *Staphylococcus aureus* FDA209P, *Enterococcus faecalis* FA2-2 (42) and OG1-10 (14), *Enterococcus faecium* BM4105RF, *Enterococcus hirae* ATCC 9790, *Enterococcus durans* ATCC 49135, *Enterococcus raffino-*

sus JCM8733, *Enterococcus gallinarium* BM4174, *Streptococcus agalactiae*, *Streptococcus pyogenes*, *Listeria monocytogenes*, and *Listeria denitrificans*. Two hundred seventy-seven (44%) of the 636 strains tested were bacteriocinogenic and were classified into three groups on the basis of their activity spectra. Of the 277 bacteriocin producers tested, 21 strains (3.3%) showed bacteriocin activity against *E. faecalis*, *E. faecium*, *E. hirae*, *E. durans*, *L. monocytogenes*, and *L. denitrificans*; 193 strains (69.7%) showed activity against *E. faecium*, *E. hirae*, and *E. durans*; and 4 strains (0.6%) showed activity against *E. faecalis*. The remaining 57 bacteriocinogenic strains showed a small zone of bacteriolysis against *E. hirae*. Bacteriocinogenic strains that were active against *E. faecium*, *E. hirae*, and *E. durans* were the most frequently isolated, accounting for 30.3% of all the VRE strains tested and 69.7% of the bacteriocinogenic strains.

Identification of a plasmid-coded bacteriocin active against *E. faecium*, *E. hirae*, and *E. durans*. Of the VRE strains which showed bacteriocin activity against *E. faecium*, *E. hirae*, and *E. durans*, the *E. faecium* VRE200 strain was chosen for further analysis. Broth matings were performed between VRE200 and the recipient strain *E. faecium* BM4105RF (Rif^r Fus^r) or *E. faecalis* FA2-2 (Rif^r Fus^r) in order to examine whether bacteriocin production was reliant on the presence of a plasmid. Gentamicin resistance (Gm^r) was used as a selective marker for the transconjugants since VRE200 also harbored the pMG1-like conjugative plasmid pG200 (Gm^r; 70 kbp) (45), and rifampin and fusidic acid were used for counterselection against the donor strain. Gentamicin-resistant transconjugants were tested for bacteriocin activity against *E. faecium*, *E. hirae*, and *E. durans*. About 40% of the transconjugants showed bacteriocin activity identical to that of donor strain VRE200 with respect to the size of the bacteriolysis zone, and the remaining transconjugants showed no bacteriocin activity. Plasmid DNA was isolated from each of the transconjugants, treated with restriction enzymes, and analyzed by agarose gel electrophoresis. All of the nonbacteriocinogenic gentamicin-resistant transconjugants harbored only the pG200 plasmid. However, all bacteriocinogenic gentamicin-resistant transconjugants harbored an additional plasmid DNA with a molecular size of 12.5 kbp, which was designated pTI1. These results implied that pTI1 (12.5 kbp) conferred on its host strain the ability to produce bacteriocin. pTI1 was mobilized in strains of *E. faecium*, *E. hirae*, and *E. faecalis* by the pG200 plasmid at a high frequency of about 10^{-3} in broth mating and conferred the ability to produce bacteriocin on these enterococcal strains. Repeated experiments involving broth and solid-surface matings, as well as curing and electrotransformation techniques, failed to produce isolates carrying only pTI1.

E. faecium and *E. faecalis* strains containing pTI1 expressed bacteriocin activity against *E. faecium*, *E. hirae*, and *E. durans*, unlike bacteriocin 31, which is encoded on the *E. faecalis* bacteriocin plasmid, pYI17, and is active against *E. faecium*, *E. hirae*, *E. durans*, and *L. monocytogenes* (43). Plasmid pMG110 is pAM401 carrying a 7.9-kbp fragment of pYI17 that contains the Bac 31 determinant. *E. hirae* ATCC 9790(pTI1) showed bacteriocin activity against *E. hirae* ATCC 9790(pMG110) and vice versa (data not shown). These results suggested that the bacteriocin encoded on pTI1 differs from bacteriocin 31 based on its bacteriocin spectrum and the immunity against its own bacteriocin. The bacteriocin encoded on pTI1 was designated bacteriocin 32.

DNA sequence of bacteriocin plasmid pTI1. The DNA sequence of pTI1 was determined and was found to be 12,463 bp in length. Computer analysis revealed the presence of 18 open reading frames (ORFs) in pTI1, and all were oriented in the same direction. Figure 1a shows the ORFs which had a good ribosome binding site within a 20-base region upstream of the predicted start codon.

Comparison of the amino acid sequences and the nucleotide sequences of the pTI1 ORFs did not reveal homology to known bacteriocin-related or immunity-related proteins.

Cloning of the bacteriocin determinant of pTI1. The following recombinant plasmids conferred bacteriocin activity on the *E. faecalis* strain (Fig. 1b): a plasmid containing the SalI fragment (pMG401), which included all of pTI1 (12.5 kb); a plasmid containing EcoRI fragment A (pMG402), which is deleted between 6.1 kb and 10.9 kb of pTI1; or a plasmid containing EcoRV fragment A (pMG403), which is deleted between 0.2 kb and 3.6 kb of pTI1. The clone of an EcoRI-NruI fragment (pMG404) that carries the region between 3.6 kb and 7.3 kb of pTI1 did not confer bacteriocin activity. These results implied that the determinant for bacteriocin 32 is not located between 0.2 kb and 10.9 kb of the pTI1 map but could be between the EcoRI site at 10.8 kb and the SalI site at 12.5 kb on the map (Fig. 1b).

Analysis of Tn*5* insertion mutants. For detailed analysis of the determinant for bacteriocin 32, Tn*5* insertion mutants of clone pMG403 containing EcoRV fragment A were generated. A total of 40 insertions in the cloned segment were obtained (Fig. 1c), and three of these resulted in the loss of bacteriocin activity, while activity was retained by the remaining 37. The three nonbacteriocinogenic mutants, designated pMG403-33, pMG403-34, and pMG403-35, were mapped at positions 11,009 bp, 11,027 bp, and 11,056 bp, respectively, within ORF14 (Fig. 1c). pMG403-33, pMG403-34, and pMG403-35 were introduced into *E. hirae* ATCC 9790 to test for immunity to the bacteriocin.

VRE200 did not show bacteriocin activity against ATCC 9790(pMG403-33), ATCC 9790(pMG403-34), or ATCC 9790 (pMG403-35), indicating that the mutants expressed immunity.

Mutants pMG403-31 and pMG403-32 or mutants pMG403-36 and pMG403-37, which expressed bacteriocin activity, were located the closest to the nonbacteriocinogenic mutants and mapped to positions 10,745 bp and 11,659 bp, respectively, on either side of the nonbacteriocinogenic mutants. These results indicated that the 0.9-kb span of DNA segment that mapped between 10,745 bp and 11,659 bp of pTI1 was required to express bacteriocin 32 (Fig. 1c). The 0.9-kb DNA segment contained ORF14 and ORF15.

Cloning of PCR products conferring bacteriocin production. To determine the precise region that conferred bacteriocin production, PCR products incorporating segments of the 0.9-kb region of pTI1 encoding ORFs 14 and 15 were used. These recombinant plasmids were used to transform *E. faecalis* FA2-2, which is not sensitive to Bac 32, or *E. hirae* ATCC 9790, which is sensitive to Bac 32, followed by selection for resistance to chloramphenicol (encoded by pAM401). Chloramphenicol-resistant transformants were then examined for bacteriocin activity. The results are shown in Fig. 1d.

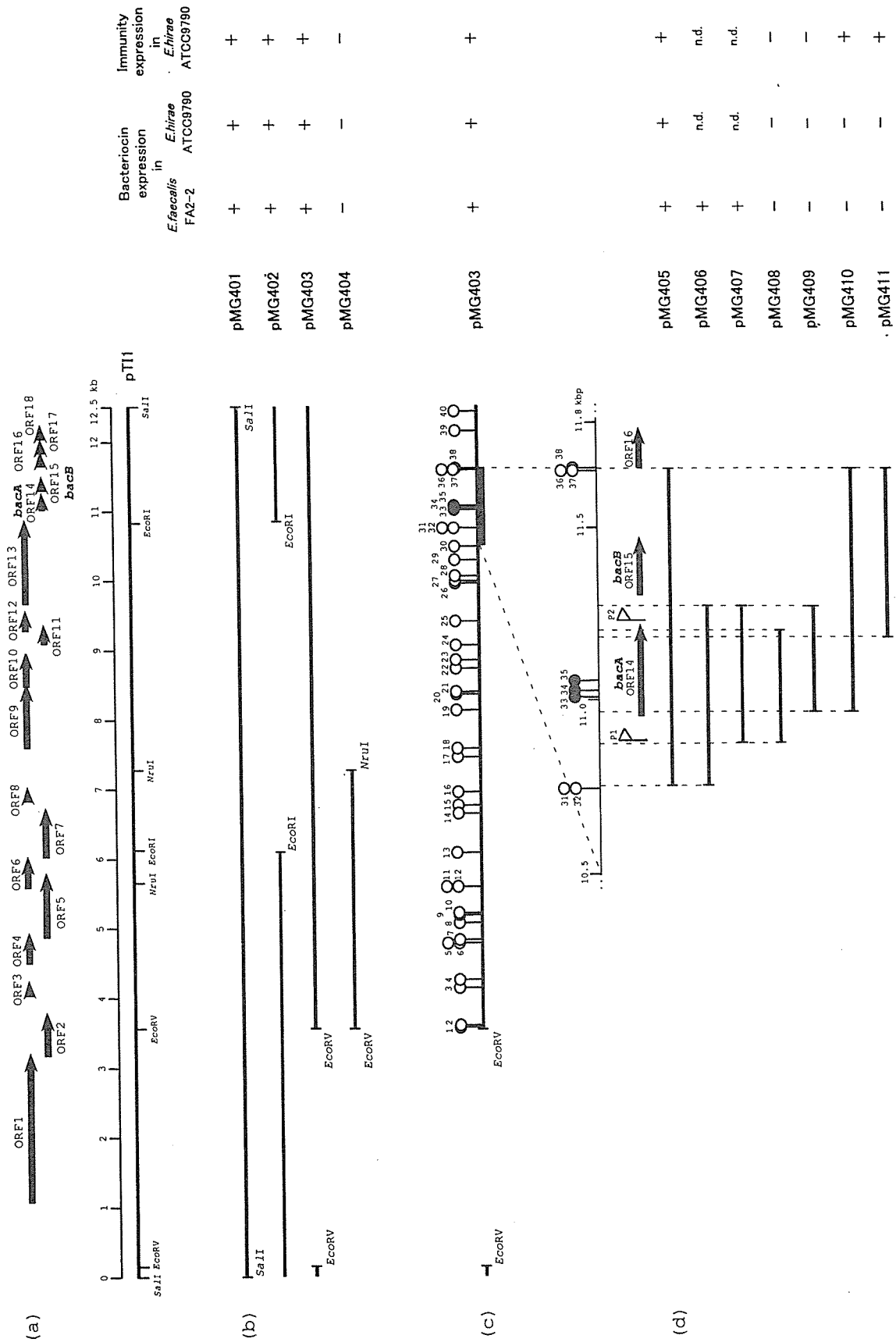

FIG. 1. Physical map of pTI1 (12.5 kbp) showing the deduced ORFs, subclones, transposon insertions into the subclone, and cloning of the PCR products from the region of the bacteriocin 32 determinant. (a) Physical map of pTI1 (12.5 kbp) and deduced ORFs. Thick horizontal arrows, the deduced ORFs encoded on pTI1 and the direction of transcription. (b) Deletion mutants of pTI1. (c) Map of Tn*5* insertions into pMG403. Vertical bars with circles, the point of Tn*5* insertion; solid circles, mutants that did not express bacteriocin but that expressed immunity; open circles, bacteriocinogenic mutants. The numbers beside the symbols indicate the mutant identification numbers. (d) Cloning of PCR products from the region of the bacteriocin determinant of pTI1. Thick lines, the cloned PCR product; vertical bars with arrowheads, potential promoters of P1 and P2. n.d., the plasmid did not transform *E. hirae* 9790.

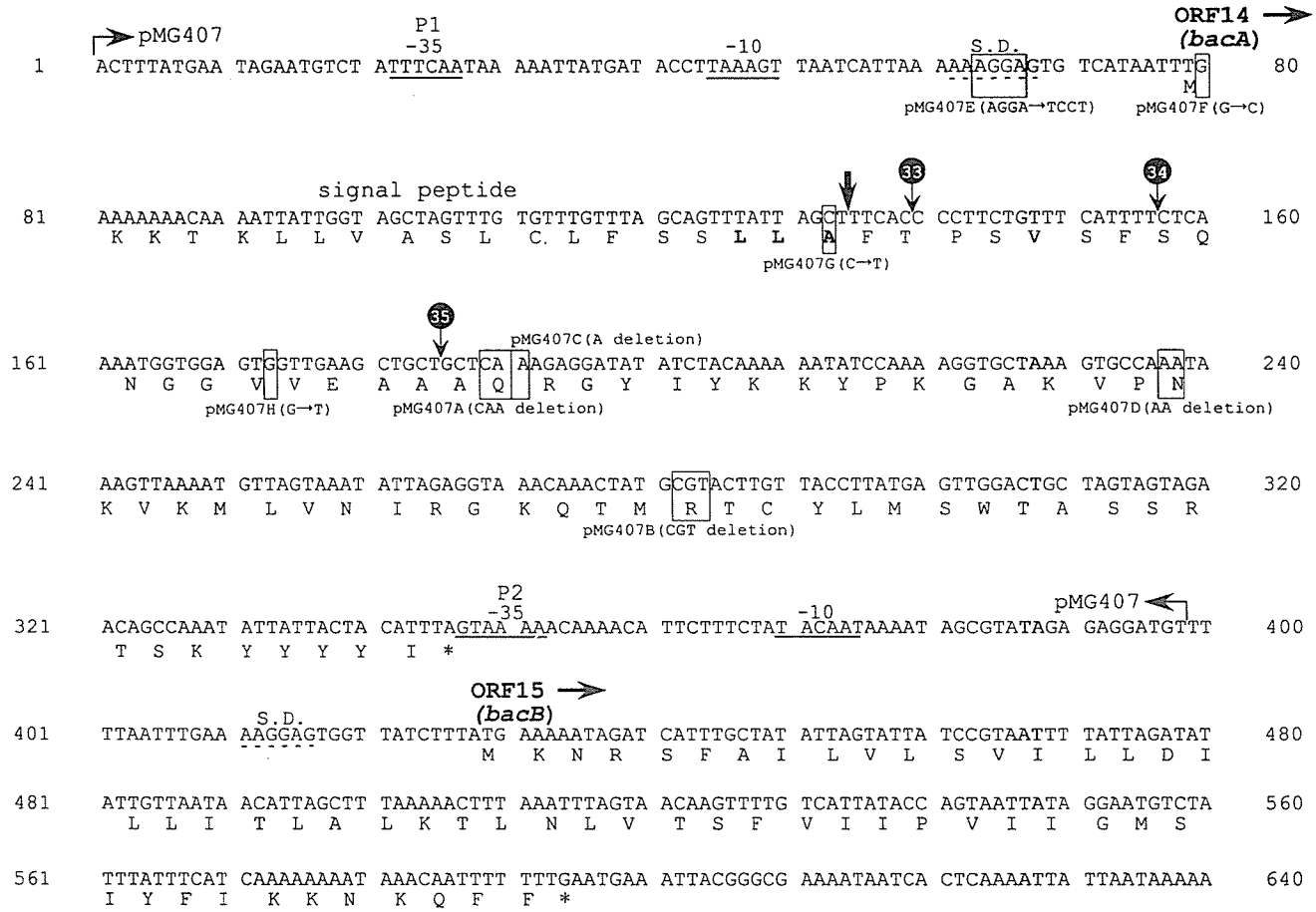

FIG. 2. Nucleotide sequence of *bacA* and *bacB* for bacteriocin 32 and deduced amino acid sequence. Potential promoters for P1 and P2 (−10 and −35) are underlined. S.D. ribosome-binding sequences are underlined with dashed lines. Boldface letters in the amino acid sequence, potential signal peptidase processing site; arrows with the identification number for pMG407 at nucleotide positions 1 and 398, the respective left and right end nucleotides of the 398-bp fragment contained in plasmid pMG407, which was cloned into pAM401. pMG407 was used to construct mutants by overlap extension PCR. Arrow with a numbered shaded circle, the point of Tn*5* insertion that abolished bacteriocin expression; nucleotide(s) shown in boxes beside the identification number for the plasmids, substitutions or deletions of the nucleotide(s); nucleotides and arrows within parentheses, nucleotide substitution.

pMG405 carries a 908-bp fragment that contains both ORF14 and ORF15. *E. faecalis* FA2-2 and *E. hirae* ATCC 9790 strains containing pMG405 expressed bacteriocin activity and immunity. pMG406 and pMG407 contain only ORF14. *E. faecalis* FA2-2 was transformed with pMG406 and pMG407, and the transformants expressed bacteriocin activity. *E. hirae* ATCC 9790 could not be transformed with pMG406 and pMG407, suggesting that these plasmids did not confer immunity. The insert in pMG408 corresponds to a fragment that has an 18-bp deletion in the C-terminal region of ORF14 of pMG407. The insert in pMG409 corresponds to the fragment with a deletion of 16 bp in the N-terminal region of ORF14 of pMG407. *E. faecalis* FA2-2 and *E. hirae* ATCC 9790 strains carrying pMG408 or pMG409 did not express bacteriocin activity, and the *E. hirae* ATCC 9790 strain did not express immunity to the bacteriocin activity of *E. faecalis* FA2-2(pMG401).

The 489-bp insert fragment of pMG411 contains the 37 bp C-terminal region of ORF14 and the complete ORF15. The 703-bp insert fragment of pMG410 has a 16-bp deletion in the N-terminal region of ORF14 and contains the complete ORF15. *E. faecalis* FA2-2 and *E. hirae* ATCC 9790 strains containing pMG410 or pMG411 did not express bacteriocin activity, but the *E. hirae* ATCC 9790 strains expressed immunity to the bacteriocin activity of *E. faecalis* FA2-2(pMG401).

These results indicate that ORF14 encodes the bacteriocin and that ORF15 encodes immunity against this bacteriocin. The fragment containing both ORF14 and ORF15 conferred bacteriocin activity and immunity on the *E. hirae* strain. ORF14 and ORF15 were designated *bacA* and *bacB*, respectively. The bacteriocin was active against *E. hirae*, *E. faecium*, and *E. durans* but not *E. faecalis*.

bacA encodes an 89-amino-acid protein. The TTG start codon was preceded by a potential Shine-Dalgarno (S.D.) ribosome binding site (AAAGGAG) at a location 10 bp upstream. There was no homology between *bacA* and any reported bacteriocin genes. Computer analysis suggested that the deduced *bacA* protein had a signal peptide sequence, and a potential signal peptidase processing site corresponding to the L-L-A sequence was located at positions 17 to 19 (Fig. 2). *bacB* encoded a 55-amino-acid protein. The ATG start codon

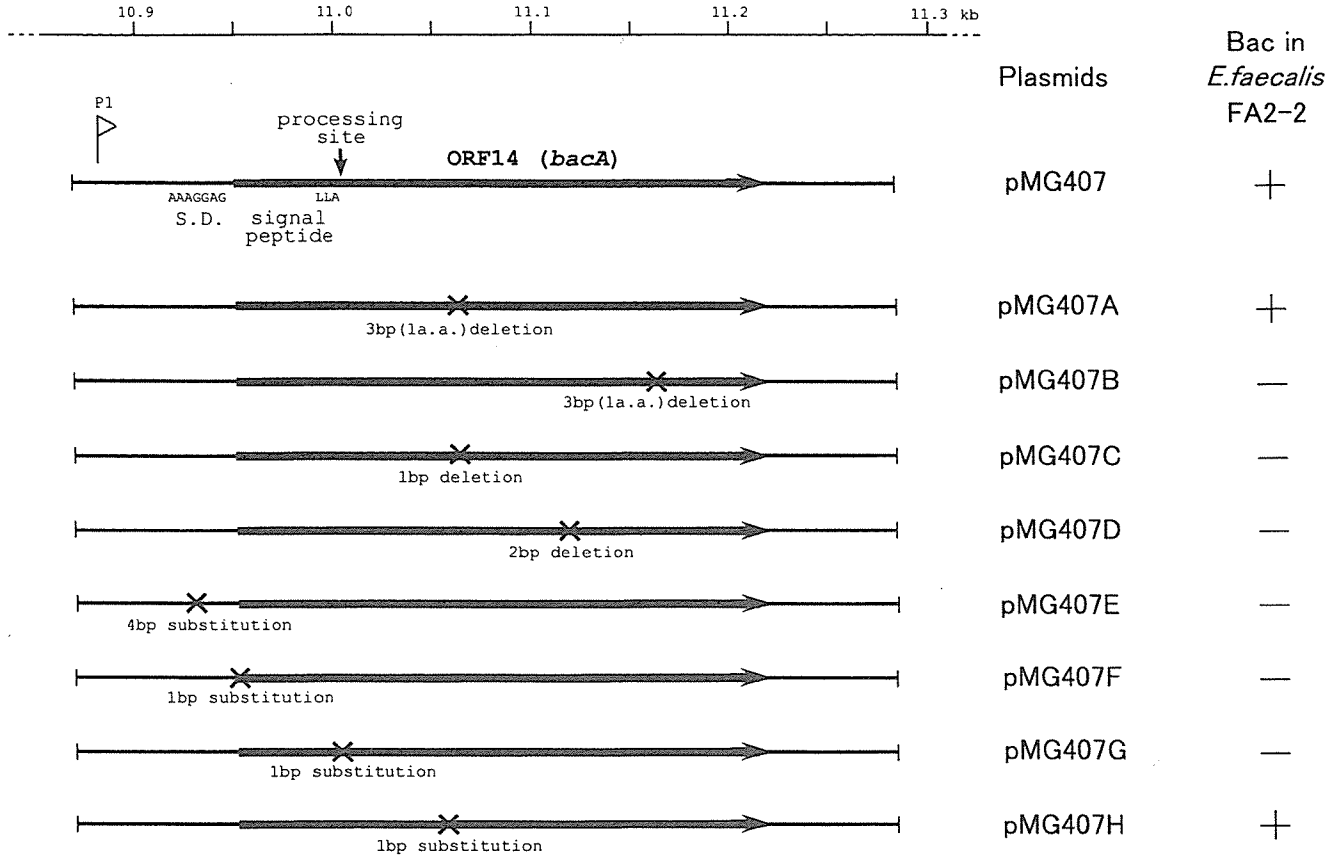

FIG. 3. Genetic analysis of bacteriocin 32 determinant. Mutants were generated by overlap extension PCR methods and bacteriocin expression. Precise regions of the mutations are shown in Fig. 2. Thick lines, the 398-bp PCR product contained in pMG407 that was cloned into pAM401; thick horizontal arrow, ORF14 (*bacA*) in the 398-bp fragment and the direction of transcription; vertical arrow, potential signal peptidase processing site; vertical bar with an arrowhead beside P1, potential sequence upstream of the *bacA* promoter; Bac, bacteriocin expression against *E. hirae* ATCC 9790; crosses on the thick horizontal arrow, the locations where mutations were introduced. The S.D. ribosome-binding sequence is also indicated. a.a., amino acid.

was preceded by a potential ribosome binding site (AAGGAG) located 11 bp upstream. There was no homology with any known immunity genes for bacteriocins or any other reported genes.

Mutational analysis of ORF14 (*bacA*) by overlap extension PCR method. To confirm that *bacA* determined bacteriocin expression, mutants of *bacA* were constructed by the overlap extension PCR method. The mutants were constructed by using pMG407, and the mutant plasmids were then used to transform *E. faecalis* FA2-2 to examine bacteriocin activity. The results are shown in Fig. 2 and 3. pMG407A had a 3-bp deletion of CAA in *bacA* that resulted in the deletion of Gln −38. This mutant maintained the ability to produce the bacteriocin. pMG407B had a 3-bp deletion of CGT in *bacA* that resulted in the in-frame deletion of Arg −69. pMG407C had a 1-bp deletion (A of *bacA*) that caused a shift in the reading frame, while pMG407D had a 2-bp deletion (AA of *bacA*) that caused a shift in the reading frame. The last three mutants were unable to produce bacteriocin.

pMG407E had a substitution of 4 bp in the potential S.D. ribosome binding sequence of *bacA* that changed the AAA GGAG sequence to AATCCTG, resulting in the functional disruption of the S.D. ribosome binding sequence. pMG407F had a 1-bp G-to-C substitution in the putative start codon of *bacA*, which changed the start Met codon (TTG) to Phe (TTC). pMG407G had a 1-bp C-to-T substitution in the putative signal peptide processing site of *bacA* that converted the signal peptide processing site from L-L-A to L-L-V. Computer analysis showed that the L-L-V sequence could not be recognized as a signal peptide processing site. pMG407H had a 1-bp G-to-T substitution in another putative *bacA* start codon, but this substitution caused no change in the amino acid sequence. The GTG codon coding for Val could be translated as a start codon (Met), whereas a GTT codon coding for Val could not be translated as a start codon. The first three mutants were unable to produce bacteriocin. The last mutant expressed bacteriocin activity.

These results imply that *bacA*, which encodes 89 amino acids, starts from a TTG start codon and encodes a putative signal sequence (19 amino acid residues). The *bacA* protein did not show any significant homology with any other reported protein or leader peptide. These data clearly show that *bacA* is the determinant or structural gene for bacteriocin 32 (Fig. 2 and 3).

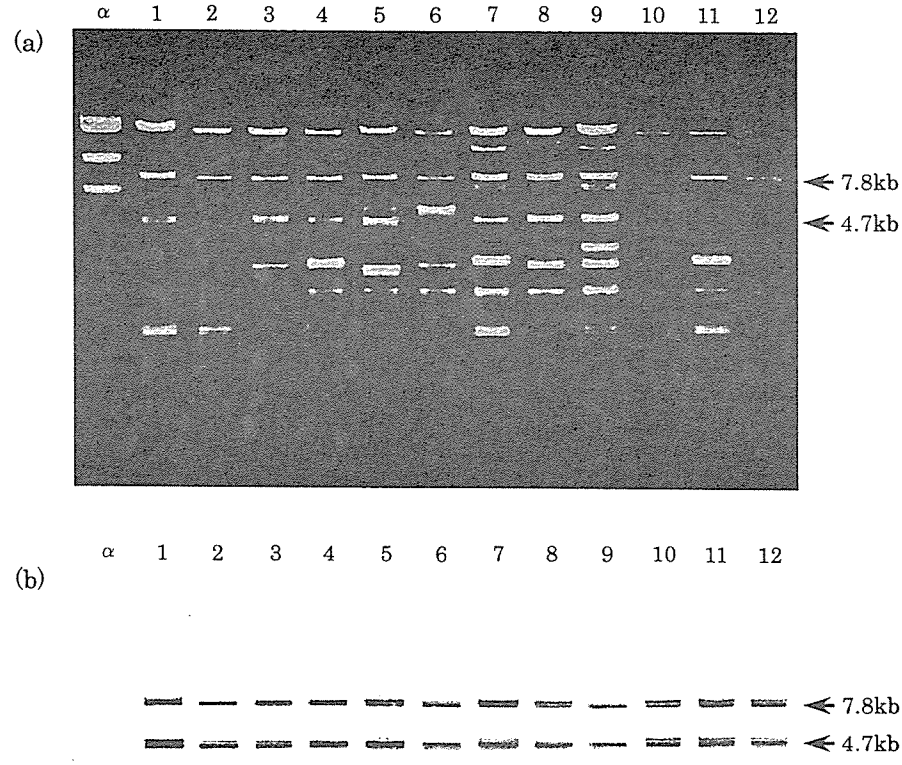

FIG. 4. Identification of pTI1-type plasmid in vancomycin-resistant *E. faecium* clinical strains that showed bacteriocin activity against *E. faecium*, *E. hirae*, and *E. durans*. (a) Agarose gel electrophoresis of EcoRI-digested plasmid DNAs isolated from the wild-type strains. (b) The gel was Southern blotted and hybridized to plasmid pTI1. Lanes: α, HindIII-digested bacteriophage lambda DNA; lanes 1 to 12, results for strains 6, 9, 92, 123, 163, 187, 354, 412, 474, 520, 200, and *E. faecium* BM4105RF(pTI1 + p200G), respectively.

Identification of the Bac 32 determinant and pTI1 type plasmid in bacteriocinogenic strains of VRE isolates. Plasmid DNAs isolated from each of the 636 VRE strains were examined for the presence of the gene encoding Bac 32 by PCR analysis with a primer specific for *bacA*. Of the 636 VRE strains tested, 238 (37.4%) strains gave rise to the expected 398-bp product with the *bacA*-specific primer. Of the 238 positive strains, 189 showed bacteriocin activity against *E. faecium*, *E. hirae*, and *E. durans* identical to that of Bac 32; 18 strains showed bacteriocin activities different from that of Bac 32 and a spectrum of activities wider than that of Bac 32, suggesting that these strains might encode several bacteriocins, including Bac 32; and 31 strains showed no bacteriocin activity. As described above, of the total of 277 bacteriocinogenic strains, 193 (69.7%) strains showed activity against *E. faecium*, *E. hirae*, and *E. durans*. The results shown here suggest that the majority (i.e., 189 [98%]) of the 193 bacteriocinogenic strains that had activity against *E. faecium*, *E. hirae*, and *E. durans* encoded Bac 32.

Plasmid DNAs from each of the 189 strains were examined for the presence of the pTI1 type plasmid by Southern analysis with a pTI1 plasmid probe (Fig. 4). EcoRI fragments of plasmid DNA(s) from each of the 189 strains were separated by agarose gel electrophoresis (Fig. 4a), tested by Southern blotting, and hybridized with pTI1 plasmid DNA (Fig. 4b). The pTI1 plasmid DNA hybridized to EcoRI fragments of 7.8 kbp and 4.7 kbp, which correspond to the EcoRI-A and -B fragments of pTI1, respectively (Fig. 4a and b). The EcoRI restriction profiles of plasmid DNAs from 137 (72.5%) of the 189 positive strains showed a restriction pattern identical to that of plasmid pTI1 with respect to the EcoRI fragments that hybridized to plasmid pTI1, suggesting that the majority of strains which encoded the Bac 32 bacteriocin harbored a pTI1-type plasmid.

Transferability of the pTI1-type plasmid of Bac 32 bacteriocinogenic isolates. Of the 137 strains which encoded Bac 32 and harbored a pTI1-type plasmid, 56 gentamicin-resistant strains were randomly selected to examine the transferability of the bacteriocin activity encoded on the pTI1-type plasmid by cotransfer with Gm^r. Broth mating was performed between each of the 56 strains and the recipient strain *E. faecium* BM4105RF for 2 h at 37°C. Gentamicin-resistant transconjugants were obtained from 36 (64%) of these 56 strains at frequencies of 10^{-5} to 10^{-7} per donor cell on a selective agar plate containing gentamicin, rifampin, and fusidic acid. Approximately 100 transconjugants from each strain were examined for bacteriocin activity by the soft-agar assay method described in Materials and Methods. From 10 to 50% of the gentamicin-resistant transconjugants obtained from 29 (80%) of the 36 strains exhibited bacteriocin activity.

The gentamicin-resistant bacteriocinogenic transconjugants that were isolated from each of the 29 strains were shown to harbor both the gentamicin-resistant pMG1-like plasmid (45) and the pTI1-type plasmid by agarose gel electrophoresis analysis of EcoRI fragments produced by restriction digestion of the plasmid DNA(s) obtained from the transconjugants and

Southern hybridization analysis with the pMG1 probe or pTI1 probe (data not shown).

Plasmid DNA from the bacteriocinogenic and gentamicin-resistant transconjugants was examined for the presence of the bacteriocin 32 determinant by PCR analysis with the *bacA*-specific primer. The strains gave rise to the expected 398-bp product specific for the *bacA* gene (data not shown). On the other hand, plasmid DNA from transconjugants that showed only Gm^r did not give rise to the specific PCR product (data not shown). These results imply that the pTI1-type plasmids that were identified in the Bac 32-type bacteriocinogenic strains encode the Bac 32 determinant and are efficiently cotransferred or mobilized by the conjugative transfer of the gentamicin-resistant transferable plasmid (45).

The banding patterns obtained by pulsed-field gel electrophoresis of SmaI-digested genomic DNA(s) were used to compare the 29 strains that were identified as containing the pTI1-type plasmid. The 29 strains showed 14 different banding patterns (data not shown). These data indicate that the pTI1-type plasmids have disseminated among different *E. faecium* strains in the clinical environment.

Identification of Bac 32 in vancomycin-sensitive *E. faecium* isolates. Fifty-six vancomycin-sensitive *E. faecium* clinical isolates that had been obtained from patients in a Japanese hospital were examined for drug resistance and Bac 32 bacteriocin activity. Forty-five (80%) isolates were drug resistant, and approximately 30% of the isolates were resistant to more than three drugs (data not shown). The number and percentage of strains resistant to the drugs tested are as follows: ampicillin, 32 strains (57.1%); chloramphenicol, 2 strains (3.6%); erythromycin, 24 strains (42.9%); gentamicin, 9 strains (16.1%); kanamycin, 19 strains (33.9%); streptomycin, 6 strains (10.7%); and tetracycline, 39 strains (69.6%). Tetracycline-resistant strains were isolated at relatively high frequencies in comparison with the frequencies of isolation of strains resistant to other drugs, but no vancomycin-resistant strain was isolated. Plasmid DNAs isolated from each of the 56 isolates were examined for the presence of the Bac 32 gene by PCR analysis with a primer specific for *bacA* of Bac 32. A total of 22 (39.3%) of the strains tested gave rise to the expected 398-bp product with the *bacA*-specific primer. These 22 strains showed bacteriocin activity against *E. faecalis*, *E. hirae*, and *E. durans* identical to that produced by Bac 32.

Of the bacteriocinogenic strains, seven strains were randomly selected to examine the transferability of bacteriocin activity by cotransfer with drug resistance. The seven strains were confirmed to be different strains on the basis of pulsed-field gel electrophoresis analysis of SmaI-digested genomic DNAs of these strains (data not shown). The drug resistance patterns of the seven strains were as follows: ampicillin, erythromycin, gentamicin, kanamycin, and tetracycline; ampicillin, erythromycin, kanamycin, streptomycin, and tetracycline; ampicillin, erythromycin, kanamycin, and tetracycline; ampicillin, gentamicin, kanamycin, and tetracycline; ampicillin, kanamycin, and tetracycline; ampicillin and tetracycline; and ampicillin and tetracycline, respectively. Filter mating was performed between each of the seven strains and recipient strain *E. faecium* BM4105RF overnight at 37°C. The drug resistance characteristics of each strain were used as a selective marker for the transconjugants. Tetracycline-resistant transconjugants were obtained from each of the seven strains at frequencies of 10^{-4} to 10^{-6} per donor cell on a selective agar plate containing tetracycline, rifampin, and fusidic acid. No transconjugants resistant to any of the other antibiotics to which the donors exhibited resistance were obtained. Tetracycline-resistant transconjugants (approximately 100 derived from each donor strain) were examined for bacteriocin activity. Between 40 and 60% of the tetracycline-resistant transconjugants from each of the seven vancomycin-sensitive donor strains exhibited bacteriocin activity. PCR analysis of the plasmid DNA from the bacteriocinogenic and tetracycline-resistant transconjugants of each of the seven strains with the *bacA*-specific primer gave rise to the expected 398-bp product specific for the *bacA* gene (data not shown). Plasmid DNA from transconjugants that only showed tetracycline resistance did not give rise to the *bacA*-specific PCR product (data not shown). Each of the PCR products specific for the *bacA* gene was sequenced. The nucleotide sequences of the *bacA* genes from two of the seven test strains were identical to that of the *bacA* gene encoded by pTI1. The nucleotide sequences and the deduced amino acid residues of the *bacA* genes of the remaining five test strains revealed that a C230-to-A nucleotide substitution had occurred compared to the sequence of *bacA* of pTI1. This nucleotide substitution resulted in a Thr77-to-Asn amino acid substitution in the N-terminal region of BacA. A total of 46 *E. faecium* isolates isolated in 2002 and 2003 from healthy students were examined for Bac 32-type bacteriocin activity. Forty-one of the isolates were drug sensitive, and the remaining five strains were resistant to one or a few drugs (data not shown). Of the 46 strains, plasmid DNA isolated from one strain that was drug sensitive gave rise to the *bacA*-specific PCR product. The remaining 45 strains did not give rise to the PCR product.

Fifty-six *E. faecalis* clinical isolates were also examined by PCR analysis for the presence of the Bac 32 gene. Plasmid DNA from each of these strains did not give rise to the *bacA*-specific PCR product.

These results indicate that Bac 32 or a Bac 32-like bacteriocin has been specifically disseminated among VRE, as well as vancomycin-sensitive *E. faecium* clinical isolates.

DISCUSSION

A new bacteriocin, which was designated Bac 32, was identified in a VanA-type vancomycin-resistant *E. faecium* VRE200 strain. Bac 32 exhibited a relatively narrow spectrum of activity and was active against *E. faecium*, *E. hirae*, and *E. durans* strains. Bac 32 is encoded on plasmid pTI1 (12.5 kbp), which was efficiently mobilized for transfer to a recipient *E. faecalis* or *E. faecium* strain at a frequency of 10^{-5} to 10^{-7} per donor cell with the coresident conjugative gentamicin resistance plasmid (45). The Bac 32 determinant consisted of the bacteriocin structural gene, *bacA*, and the immunity gene, *bacB*. The *bacA* gene encoded a deduced 89-amino-acid protein with a putative signal sequence of 19 amino acid residues at the N terminus that was predicted to give rise to a 70-amino-acid mature protein. The *bacB* gene encoded a deduced 55-amino-acid protein without a putative signal sequence. The deduced BacA and BacB proteins had no homology to known bacteriocins, indicating that Bac 32 is new type of bacteriocin from *E. faecium*.

Epidemiological studies showed that Bac 32 is representative of the Bac 32-type bacteriocins produced by a variety of VRE isolates. Many of the VRE isolates (i.e., 44% of the 636 isolates tested) were bacteriocinogenic; and a majority of these (70%) exhibited bacteriocin activity against *E. faecium*, *E. hirae*, and *E. durans*, which is identical to the bacteriocinogenic spectrum of Bac 32. The Bac 32 structural gene, *bacA*, was identified in 189 (98%) of the 193 bacteriocinogenic isolates and 189 (30%) of the 636 VanA-type VRE isolates; indicating that there is a high incidence of Bac 32 production among the VRE clinical isolates. Analysis of the plasmid DNAs from the Bac 32-producing strains suggested that the majority of the strains (i.e., 137 [72.5%] of the 189 strains) harbored a pTI1-type plasmid and that this plasmid could cotransfer to a recipient strain along with gentamicin resistance.

Previously, we showed that many of the VRE isolates (i.e., 492 [77%] of the 640 isolates) that were used in this study exhibit a high level of resistance to gentamicin and that about 50% of the gentamicin-resistant strains harbor the gentamicin resistance pMG1-like conjugative plasmid that transfers highly efficiently to a recipient strain by broth mating (45). These data imply that the transfer of bacteriocin production encoded on the pTI1-type plasmid might result from the mobilization of the pTI1-type plasmid by the gentamicin resistance-conferring conjugative plasmid.

Bac 32 or Bac 32-like activity and the *bacA* gene were also identified in a high proportion (40%) of vancomycin-sensitive *E. faecium* clinical isolates that had been obtained from a Japanese hospital. Among these *E. faecium* isolates, the frequency of isolation of gentamicin-resistant strains was lower than that of isolates resistant to other drugs, and none of the isolates were VRE. Plasmid-encoded Bac 32-like activity was cotransferred with tetracycline resistance. The mechanism of the transfer has yet to be investigated.

E. faecalis and *E. faecium* are commonly isolated from the clinical environment (28, 35, 40), but the frequency of isolation of *E. faecalis* is greater than that of *E. faecium* (28). In the case of VanA- or VanB-type VRE, *E. faecium* is predominantly isolated from the health care environment (4). It is known that many *E. faecalis* clinical isolates produce bacteriocin (9, 43, 44) and that the bacteriocin phenotype is frequently associated with the pheromone-responding conjugative plasmid of *E. faecalis* (9, 10, 17, 25, 32, 43, 44). Several of the *E. faecalis* bacteriocins that have been identified from clinical isolates have been well characterized. Of these bacteriocins, β-hemolysin/bacteriocin (cytolysin) is predominantly found in *E. faecalis* clinical isolates (9, 22, 24, 28, 43). The β-hemolysin/bacteriocin (cytolysin) production by *E. faecalis* clinical isolates is frequently encoded on the *E. faecalis* pheromone-responsive conjugative pAD1-type plasmid (10, 25). β-Hemolysin/bacteriocin (cytolysin) is associated with the virulence of *E. faecalis* in animal models (5, 27, 30). An *E. faecalis* strain that produces β-hemolysin/bacteriocin (cytolysin) might have an ecological advantage over other strains in colonizing human tissue, and the pAD1-type plasmid that encodes β-hemolysin/bacteriocin (cytolysin) might contribute to the dissemination of the β-hemolysin/bacteriocin (cytolysin) determinant among *E. faecalis* strains. *E. faecalis* clinical isolates usually show multiple-drug resistance (9, 35, 40), which provides the organism with a selective advantage within a clinical setting. These factors could result in the prevalence of β-hemolysin/bacteriocin (cytolysin) in *E. faecalis* isolates within the clinical environment.

In contrast to the *E. faecalis* bacteriocins, the well-characterized *E. faecium* bacteriocins (i.e., enterocins) are produced by food-grade organisms that have been isolated from fermented foods. These include enterocin A (1), B (3), P (7), I (15), and L50A and L50B (6). It is characteristic of the bacteriocinogenic food-grade organisms that they are active against *Listeria monocytogenes* (36), which is a frequent cause of foodborne listeriosis (19). These food-grade bacteriocinogenic *E. faecium* strains might have a selective advantage in their particular ecological niche, and their ability to inhibit the growth of *L. monocytogenes* in food plays an important role in the microbial safety of the final product.

To our knowledge, only one bacteriocinogenic *E. faecium* strain, strain RC714, has been isolated from a VanA-type resistant *E. faecium* VRE clinical isolate and has been analyzed for bacteriocin activity (12). The amino acid sequence of bacteriocin RC714 of *E. faecium* RC714 shows a high level of identity (88%) with bacteriocin 31 encoded on conjugative plasmid pYI17 of *E. faecalis* YI717, which is a pediocin-like bacteriocin (43). The RC714 bacteriocin is active against *E. faecalis*, *E. faecium*, and *L. monocytogenes* (12). It is not known if bacteriocin RC714-producing *E. faecium* strains are disseminated widely in the clinical environment.

In this study, we identified a novel bacteriocin, designated bacteriocin 32, that showed a high incidence of dissemination in vancomycin-sensitive *E. faecium* clinical isolates obtained from a Japanese hospital and VanA-type vancomycin-resistant *E. faecium* isolates obtained from a hospital in the United States. VRE are usually multiply resistant to commonly used antibiotics (4, 45), as are vancomycin-sensitive *E. faecium* clinical isolates, compared to the numbers of antibiotics to which isolates from healthy students are resistant. Multiple drug resistance provides *E. faecium* with a selective advantage in the clinical environment, and the ability of these strains to produce bacteriocin might confer an additional advantage to the organism in this particular ecological environment. In the case of VRE, the pTI1-type plasmid is efficiently mobilized by the Gm^r conjugative pMG1-type plasmid. In the case of vancomycin-sensitive *E. faecium* isolates, Bac 32 production could be cotransferred along with tetracycline resistance. These factors could contribute to the dissemination of bacteriocin 32-producing *E. faecium* strains within the clinical environment of the hospital and may be responsible for their emergence as the dominant strain.

Bacteriocin 32 was not active against *L. monocytogenes*, which differs from the activities of the well-characterized enterocins isolated from food-grade *E. faecium* strains. This finding suggests 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 and that the type of bacteriocin that becomes dominant within any environment is influenced by the surrounding ecology.

ACKNOWLEDGMENTS

This work was supported by grants from the Japanese Ministry of Education, Culture, Sports, Science and Technology [Tokuteiryoiki

(C), Kiban (B), (C)] and the Japanese Ministry of Health, Labor and Welfare (H15-Shinko-9).

REFERENCES

1. **Aymerich, T., H. Holo, L. S. Havarstein, M. Hugas, M. Garriga, and I. F. Nes.** 1996. Biochemical and genetic characterization of enterocin A from *Enterococcus faecium*, a new antilisterial bacteriocin in the pediocin family of bacteriocins. Appl. Environ. Microbiol. **62**:1676–1682.
2. **Bates, J., Z. Jordens, and J. B. Selkon.** 1993. Evidence for an animal origin of vancomycin-resistant enterococci. Lancet **342**:490–491.
3. **Casaus, P., T. Nilsen, L. M. Cintas, I. F. Nes, P. E. Hernandez, and H. Holo.** 1997. Enterocin B, a new bacteriocin from *Enterococcus faecium* T136 which can act synergistically with enterocin A. Microbiology **143**:2287–2294.
4. **Cetinkaya, Y., P. Falk, and C. G. Mayhall.** 2000. Vancomycin-resistant enterococci. Clin. Microbiol. Rev. **13**:686–707.
5. **Chow, J. W., L. A. Thal, M. B. Perri, J. A. Vazquez, S. M. Donabedian, D. B. Clewell, and M. J. Zervos.** 1993. Plasmid-associated hemolysin and aggregation substance production contribute to virulence in experimental enterococcal endocarditis. Antimicrob. Agents Chemother. **37**:2474–2477.
6. **Cintas, L. M., P. Casaus, H. Holo, P. E. Hernandez, I. F. Nes, and L. S. Havarstein.** 1998. Enterocins L50A and L50B, two novel bacteriocins from *Enterococcus faecium* L50, are related to staphylococcal hemolysins. J. Bacteriol. **180**:1988–1994.
7. **Cintas, L. M., P. Casaus, L. S. Havarstein, P. E. Hernandez, and I. F. Nes.** 1997. Biochemical and genetic characterization of enterocin P, a novel *sec*-dependent bacteriocin from *Enterococcus faecium* P13 with a broad antimicrobial spectrum. Appl. Environ. Microbiol. **63**:4321–4330.
8. **Cleveland, J., T. J. Montville, I. F. Nes, and M. L. Chikindas.** 2001. Bacteriocins: safe, natural antimicrobials for food preservation. Int. J. Food Microbiol. **71**:1–20.
9. **Clewell, D. B.** 1981. Plasmids, drug resistance, and gene transfer in the genus *Streptococcus*. Microbiol. Rev. **45**:409–436.
10. **Clewell, D. B., P. K. Tomich, M. C. Gawron-Burke, A. E. Franke, Y. Yagi, and F. Y. An.** 1982. Mapping of *Streptococcus faecalis* plasmids pAD1 and pAD2 and studies relating to transposition of Tn*917*. J. Bacteriol. **152**:1220–1230.
11. **Cox, C. R., P. S. Coburn, and M. S. Gilmore.** 2005. Enterococcal cytolysin: a novel two component peptide system that serves as a bacterial defense against eukaryotic and prokaryotic cells. Curr. Protein Pept. Sci. **6**:77–84.
12. **del Campo, R., C. Tenorio, R. Jimenez-Diaz, C. Rubio, R. Gomez-Lus, F. Baquero, and C. Torres.** 2001. Bacteriocin production in vancomycin-resistant and vancomycin-susceptible *Enterococcus* isolates of different origins. Antimicrob. Agents Chemother. **45**:905–912.
13. **De Vuyst, L., and E. J. Vandamme.** 1994. Antimicrobial potential of lactic acid bacteria, p. 91–142. *In* L. De Vuyst and E. J. Vandamme (ed.), Bacteriocins of lactic acid bacteria: microbiology, genetics and applications. Blackie Academic & Professional, London, United Kingdom.
14. **Dunny, G. M., B. L. Brown, and D. B. Clewell.** 1978. Induced cell aggregation and mating in *Streptococcus faecalis*: evidence for a bacterial sex pheromone. Proc. Natl. Acad. Sci. USA **75**:3479–3483.
15. **Floriano, B., J. L. Ruiz-Barba, and R. Jimenez-Diaz.** 1998. Purification and genetic characterization of enterocin I from *Enterococcus faecium* 6T1a, a novel antilisterial plasmid-encoded bacteriocin which does not belong to the pediocin family of bacteriocins. Appl. Environ. Microbiol. **64**:4883–4890.
16. **Fujimoto, S., H. Hashimoto, and Y. Ike.** 1991. Low cost device for electrotransformation and its application to the highly efficient transformation of *Escherichia coli* and *Enterococcus faecalis*. Plasmid **20**:131–135.
17. **Fujimoto, S., H. Tomita, E. Wakamatsu, K. Tanimoto, and Y. Ike.** 1995. Physical mapping of the conjugative bacteriocin plasmid pPD1 of *Enterococcus faecalis* and identification of the determinant related to the pheromone response. J. Bacteriol. **177**:5574–5581.
18. **Gilmore, M. S., R. A. Segarra, M. C. Booth, C. P. Bogie, L. R. Hall, and D. B. Clewell.** 1994. Genetic structure of the *Enterococcus faecalis* plasmid pAD1-encoded cytolytic toxin system and its relationship to lantibiotic determinants. J. Bacteriol. **176**:7335–7344.
19. **Giraffa, G.** 2003. Functionality of enterococci in dairy products. Int. J. Food Microbiol. **88**:215–222.
20. **Haas, W., B. D. Shepard, and M. S. Gilmore.** 2002. Two-component regulator of *Enterococcus faecalis* cytolysin responds to quorum-sensing autoinduction. Nature **415**:84–87.
21. **Ho, S. N., H. D. Hunt, R. M. Horton, J. K. Pullen, and L. R. Pease.** 1989. Site-directed mutagenesis by overlap extension using the polymerase chain reaction. Gene **77**:51–59.
22. **Huycke, M. M., and M. S. Gilmore.** 1995. Frequency of aggregation substance and cytolysin genes among enterococcal endocarditis isolates. Plasmid **34**:152–156.
23. **Huycke, M. M., D. F. Sahm, and M. S. Gilmore.** 1998. Multiple-drug resistant enterococci: the nature of the problem and an agenda for the future. Emerg. Infect. Dis. **4**:239–249.
24. **Huycke, M. M., C. A. Spiegel, and M. S. Gilmore.** 1991. Bacteremia caused by hemolytic, high-level gentamicin-resistant *Enterococcus faecalis*. Antimicrob. Agents Chemother. **35**:1626–1634.
25. **Ike, Y., and D. B. Clewell.** 1984. Genetic analysis of the pAD1 pheromone response in *Streptococcus faecalis*, using transposon Tn*917* as an insertional mutagen. J. Bacteriol. **158**:777–783.
26. **Ike, Y., D. B. Clewell, R. A. Segarra, and M. S. Gilmore.** 1990. Genetic analysis of the pAD1 hemolysin/bacteriocin determinant in *Enterococcus faecalis*: Tn*917* insertional mutagenesis and cloning. J. Bacteriol. **172**:155–163.
27. **Ike, Y., H. Hashimoto, and D. B. Clewell.** 1984. Hemolysin of *Streptococcus faecalis* subspecies *zymogenes* contributes to virulence in mice. Infect. Immun. **45**:528–530.
28. **Ike, Y., H. Hashimoto, and D. B. Clewell.** 1987. High incidence of hemolysin production by *Enterococcus* (*Streptococcus*) *faecalis* strains associated with human parenteral infections. J. Clin. Microbiol. **25**:1524–1528.
29. **Ike, Y., K. Tanimoto, Y. Ozawa, T. Nomura, S. Fujimoto, and H. Tomita.** 1999. Vancomycin-resistant enterococci in imported chickens in Japan. Lancet **353**:1854.
30. **Jett, B. D., H. G. Jensen, R. E. Nordquist, and M. S. Gilmore.** 1992. Contribution of the pAD1-encoded cytolysin to the severity of experimental *Enterococcus faecalis* endophthalmitis. Infect. Immun. **60**:2445–2452.
31. **Leclercq, R., E. Derlot, J. Duval, and P. Courvalin.** 1988. Plasmid-mediated resistance to vancomycin and teicoplanin in *Enterococcus faecium*. N. Engl. J. Med. **319**:157–161.
32. **Martinez-Bueno, M., A. Galvez, E. Valdivia, and M. Maqueda.** 1990. A transferable plasmid associated with AS-48 production in *Enterococcus faecalis*. J. Bacteriol. **172**:2817–2818.
33. **Martinez-Bueno, M., M. Maqueda, A. Galvez, B. Samyn, J. V. Beeumen, J. Coyette, and E. Valdivia.** 1994. Determination of the gene sequence and the molecular structure of the enterococcal peptide antibiotic AS-48. J. Bacteriol. **176**:6334–6339.
34. **Martone, W. J.** 1998. Spread of vancomycin resistant enterococci: why did it happen in the United States? Infect. Control Hosp. Epidemiol. **19**:539–545.
35. **Ma, X., M. Kudo, A. Takahashi, K. Tanimoto, and Y. Ike.** 1998. Evidence of nosocomial infection in Japan caused by high-level gentamicin-resistant *Enterococcus faecalis* and identification of the pheromone-responsive conjugative plasmid encoding gentamicin resistance. J. Clin. Microbiol. **36**:2460–2464.
36. **Nes, I. F., and H. Holo.** 2000. Class II antimicrobial peptides from lactic acid bacteria. Biopolymers **55**:50–61.
37. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
38. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**:5463–5467.
39. **Schouten, M. A., A. Voss, and J. A. Hoogkamp-Korstanje.** 1997. VRE and meat. Lancet **349**:1258.
40. **Shiojima, M., H. Tomita, K. Tanimoto, S. Fujimoto, and Y. Ike.** 1997. High-level plasmid-mediated gentamicin resistance and pheromone response of plasmids present in clinical isolates of *Enterococcus faecalis*. Antimicrob. Agents Chemother. **41**:702–705.
41. **Tanimoto, K., and D. B. Clewell.** 1993. Regulation of the pAD1-encoded sex pheromone response in *Enterococcus faecalis*: expression of the positive regulator TraE1. J. Bacteriol. **175**:1008–1018.
42. **Tomich, P. K., F. Y. An, S. P. Damle, and D. B. Clewell.** 1979. Plasmid-related transmissibility and multiple-drug resistance in *Streptococcus faecalis* subsp. *zymogenes* DS16. Antimicrob. Agents Chemother. **15**:828–830.
43. **Tomita, H., S. Fujimoto, K. Tanimoto, and Y. Ike.** 1996. Cloning and genetic organization of the bacteriocin 31 determinant encoded on the *Enterococcus faecalis* pheromone-responsive conjugative plasmid pYI17. J. Bacteriol. **178**:3585–3593.
44. **Tomita, H., S. Fujimoto, K. Tanimoto, and Y. Ike.** 1997. Cloning and genetic and sequence analyses of the bacteriocin 21 determinant encoded on the *Enterococcus faecalis* pheromone-responsive conjugative plasmid pPD1. J. Bacteriol. **179**:7843–7855.
45. **Tomita, H., C. Pierson, S. K. Lim, D. B. Clewell, and Y. Ike.** 2002. Possible connection between a widely disseminated conjugative gentamicin resistance (pMG1-like) plasmid and the emergence of vancomycin resistance in *Enterococcus faecium*. J. Clin. Microbiol. **40**:3326–3333.
46. **Uttley, A. H., C. H. Collins, J. Naidoo, and R. C. George.** 1988. Vancomycin-resistant enterococci. Lancet **i**:57–58.
47. **van den Bogaard, A. E., L. B. Jensen, and E. E. Stobberingh.** 1997. Vancomycin-resistant enterococci in turkeys and farmers. N. Engl. J. Med. **337**:1558–1559.
48. **Wirth, R., F. Y. An, and D. B. Clewell.** 1986. Highly efficient protoplast transformation system for *Streptococcus faecalis* and a new *Escherichia coli-S. faecalis* shuttle vector. J. Bacteriol. **165**:831–836.

J. Antibiot. 59(4): 220–228, 2006

ORIGINAL ARTICLE

THE JOURNAL OF ANTIBIOTICS

Potency of Carbapenems for the Prevention of Carbapenem-Resistant Mutants of *Pseudomonas aeruginosa*

The High Potency of a New Carbapenem Doripenem

Shihomi Sakyo, Haruyoshi Tomita, Koichi Tanimoto, Shuhei Fujimoto, Yasuyoshi Ike

JAPAN ANTIBIOTICS RESEARCH ASSOCIATION