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			
Staphylococcus 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.

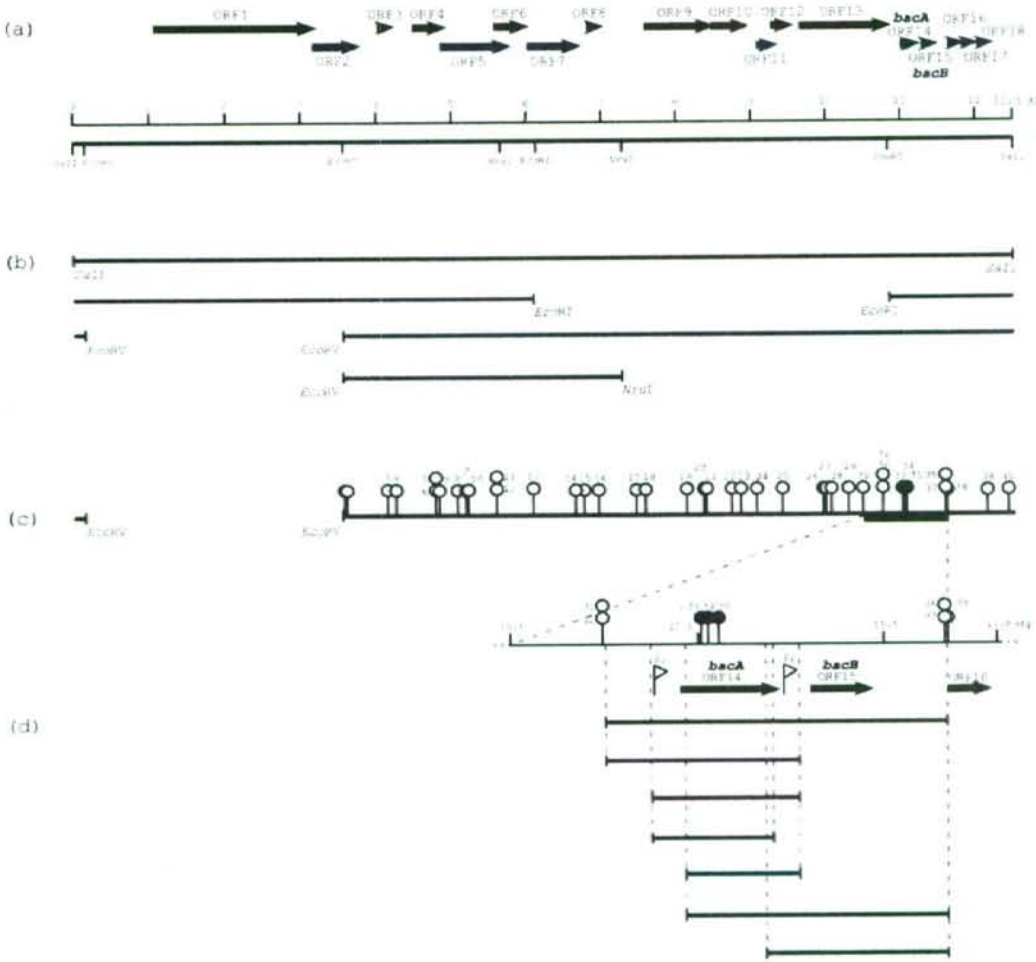
Cloning of the bacteriocin determinant of pTI1. The following recombinant plasmids conferred bacteriocin activity on the *E. faecalis* strain (Fig. 1b): a plasmid containing the SalI fragment (pMG401), which included all of pTI1 (12.5 kb); a plasmid containing EcoRI fragment A (pMG402), which is deleted 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.



	Bacteriocin expression in *E. faecalis* FA2-2	Bacteriocin expression in *E. hirae* ATCC9790	Immunity expression in *E. hirae* ATCC9790
pMG401	+	+	+
pMG402	+	+	+
pMG403	+	+	+
pMG404	−	−	−
pMG403	+	+	+
pMG405	+	+	+
pMG406	+	n.d.	n.d.
pMG407	+	n.d.	n.d.
pMG408	−	−	−
pMG409	−	−	−
pMG410	−	−	+
pMG411	−	−	+

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.

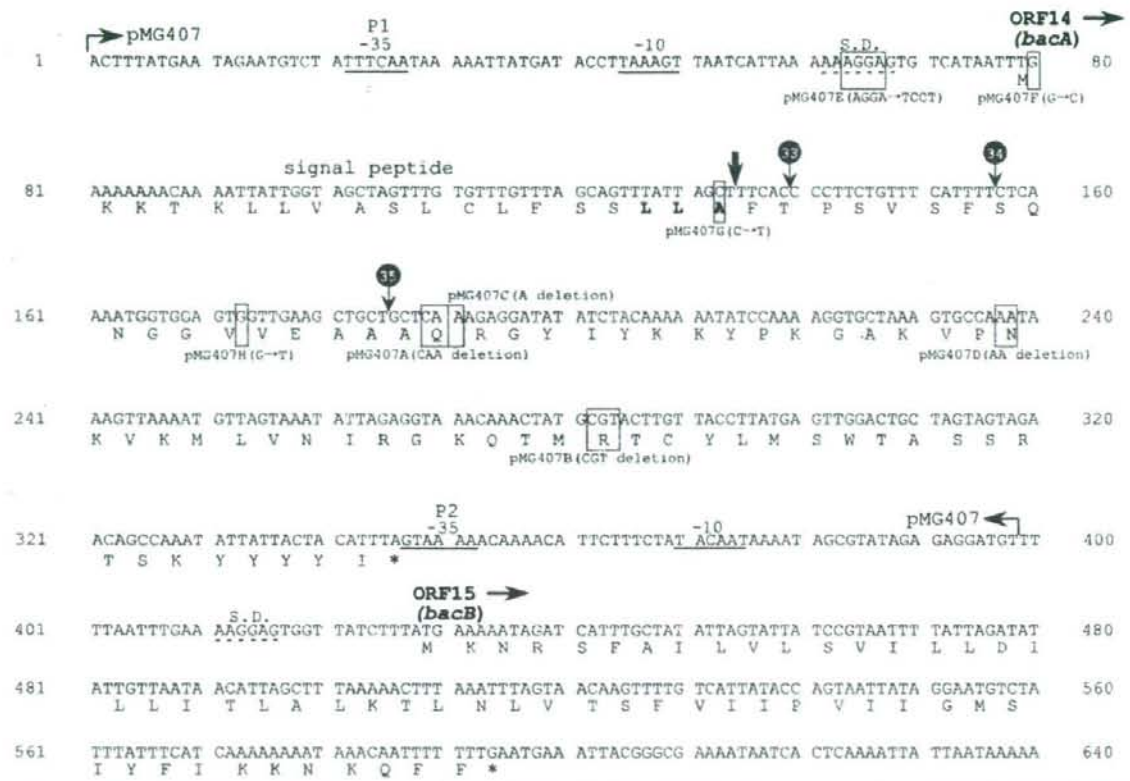


FIG. 2. Nucleotide sequence of *bacA* and *bacB* for bacteriocin 32 and deduced amino acid sequence. Potential promoters for P1 and P2 (−10 and −35) are underlined. S.D. ribosome-binding sequences are underlined with dashed lines. Boldface letters in the amino acid sequence, potential signal peptidase 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

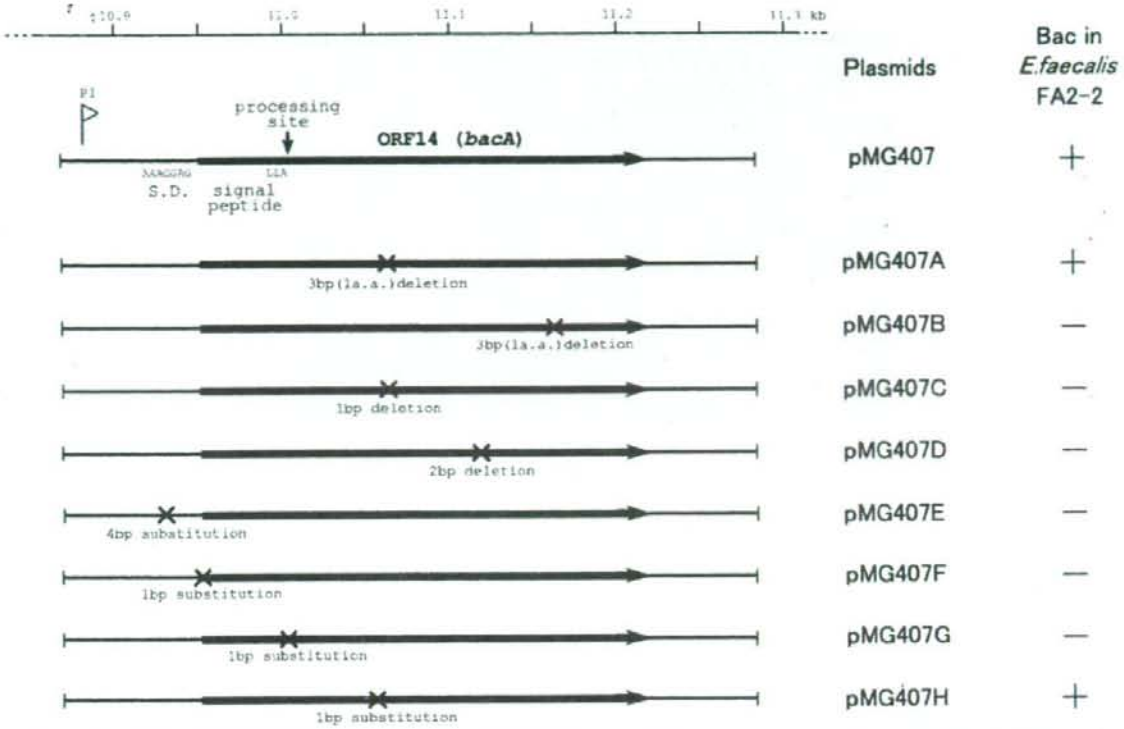


FIG. 3. Genetic analysis of bacteriocin 32 determinant. Mutants were generated by overlap extension PCR methods and bacteriocin expression. Precise regions of the mutations are shown in Fig. 2. Thick lines, the 398-bp PCR product contained in pMG407 that was cloned into pAM401; thick horizontal arrow, 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).

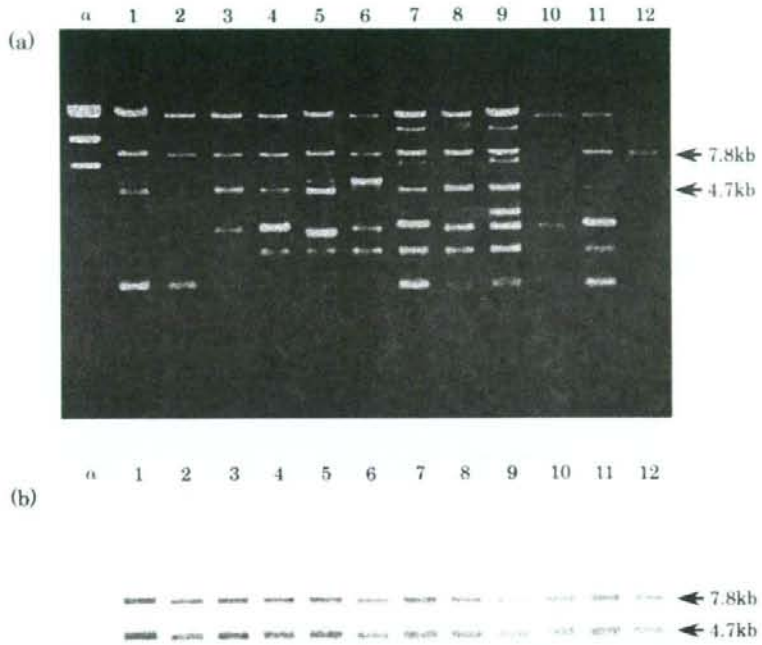


FIG. 4. Identification of pTI1-type plasmid in vancomycin-resistant *E. faecium* clinical strains that showed bacteriocin activity against *E. faecium*, *E. hirae*, and *E. durans*. (a) Agarose gel electrophoresis of EcoRI-digested plasmid DNAs isolated from the wild-type strains. (b) The gel was Southern 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.

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

The High Potency of a New Carbapenem Doripenem

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Abstract The potencies of the carbapenems; doripenem (DRPM), meropenem (MEPM) and imipenem (IPM) in preventing the emergence of carbapenem-resistant mutants were examined in *Pseudomonas aeruginosa* strains. The carbapenems predominantly selected carbapenem-resistant mutants or carbapenem mutants with reduced susceptibilities that were specifically resistant to carbapenems and had arisen as a result of the reduced level of expression of the outer membrane protein with a molecular weight of about 48,000 (OprD). The potency of carbapenems in preventing the growth of the mutants differed for DRPM, MEPM and IPM. The isolation frequency of the mutant was examined on agar plates containing each of the carbapenems at a concentration of 1/2 or 1/4 MIC of each carbapenem for that mutant. Mutants were not selected on agar containing DRPM at a frequency of greater than 10^{-9} per cell per generation, whereas mutants of each strain were selected on agar containing MEPM or IPM at frequencies of 10^{-7} to 10^{-9} per cell per generation. The drug concentrations and the drug concentration range for the selective increase of carbapenem resistant mutants in the broth culture containing each carbapenem differed for each carbapenem. DRPM exhibited both the lowest drug concentration and the narrowest range of drug concentration for selection of the carbapenem-resistant mutants. The results shown in this report indicated that DRPM exhibited the greatest ability to prevent the emergence of the mutant.

Keywords carbapenem, doripenem, carbapenem-resistant mutant, outer membrane protein, prevention of mutation

Introduction

Pseudomonas aeruginosa is an opportunistic pathogen for immunocompromised patients. The organism has both an intrinsic and acquired resistance to many antibiotics [1, 2], and treatment of infection by this organism is difficult. Carbapenems have a high potency against a broad spectrum of organisms and are one of the most active groups of β-lactam antibiotics against *P. aeruginosa*.

There is a group of carbapenem resistant *P. aeruginosa* strains that is specifically resistant to carbapenems and which does not show cross resistance to other β-lactams or other antibiotics. This type of imipenem (IPM)-resistant *P. aeruginosa* mutant was first isolated during clinical trials of IPM for the treatment of serious infections caused by *P. aeruginosa* [3]. The IPM-resistant mutants of *P. aeruginosa* were found to lack a 45 KD to 49 KD protein [3~6] in the outer membrane protein D2 (OprD), which forms a channel specific to IPM and its structural analogues [7].

In this study, we investigated the potency of doripenem (DRPM) [8~12], meropenem (MEPM) and IPM in preventing the emergence of carbapenem-resistant mutants under laboratory conditions.

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Materials and Methods

Bacteria, and Media

One hundred and forty-four clinical *P. aeruginosa* isolates were examined for carbapenem resistance. Of the 144 strains, 100 strains were isolated from Gunma University Hospital, 30 strains from Isesaki City Hospital, Isesaki City, Japan, and 14 strains from Gunma Chuo-hospital, Maebashi, Japan. Mueller-Hinton (MH) (Nissui, Tokyo, Japan) broth and MH agar or antibiotic medium 3 (AB3) (Difco, Detroit, Mich.) and AB3 agar were used to test the MICs for each antibiotic. Agar plates were prepared by adding 1.5% agar to broth medium. All bacterial strains were grown at 37°C. MICs obtained using MH medium and AB3 medium were not essentially different (data not shown). Mutation frequencies and the kinetics of bacterial growth were examined for each medium. As a result, AB3 medium was used throughout this study for its capacity to maintain a stable pH during course of bacterial growth.

Determination of MICs

MIC was determined by the agar dilution method. Overnight cultures of the strains grown in broth were diluted 100 times with fresh broth. One loopful of each dilution was plated on agar plates containing two-fold serially-diluted antibiotics. The plates were incubated for 18 hours at 37°C.

Antibiotics

Antibiotics used in this study were as follows: DRPM (a new carbapenem that had been developed by Shionogi Pharmaceuticals, Osaka) [8~12], MEPM (Sumitomo Pharmaceuticals, Osaka), IPM (Banyu Pharmaceuticals, Tokyo), piperacillin (PIPC, Toyama Chemicals, Tokyo), ceftazidime (CAZ, Tanabe Pharmaceuticals, Osaka) and gentamicin (GM, Schering-Plough K.K., Osaka) and ofloxacin (OFLX, Daiichi Seiyaku Co., Ltd., Tokyo).

Isolation Frequency of Mutant

The frequency of isolation of the mutant was estimated using the following equation for mutation frequency [13]: Mutation frequency per cell per generation=$[\Pi-\Pi 0/1n(P/P0)]$ where $\Pi 0$ and $P0$ are, respectively, the proportion of mutant cells and the total number of cells in the culture at the start of the experiment, and Π is the proportion of mutant cells after incubation when the number of total cells has risen to P.

Analysis of Outer Membrane Proteins (OMPs)

The OMPs of all strains were analyzed by SDS-PAGE of the sarkosyl insoluble fraction of membrane-preparation as described by Spratt [14]. Throughout this study, the OMPs of the carbapenem-resistant mutants of the representative strains obtained in each experiment were examined to confirm whether the mutants lacked, or had reduced amount of, OMP of molecular weight 45,000~49,000 (data not shown).

Results

Distribution of Carbapenem MICs among *P. aeruginosa* Isolates

The carbapenem resistance levels (MICs) of the 144 strains of *P. aeruginosa* were examined. There was a bi-modal distribution of the MICs for each drug in these strains (data not shown). However, the MICs showed a continuous distribution, and there was no complete break between the bi-modal distributions. The MICs of DRPM for these strains ranged from 0.1 to 1.6 μg/ml, and from 1.6 to 12.5 μg/ml. The MICs to MEPM ranged from 0.1 to 1.6 μg/ml, and from 1.6 to 50 μg/ml. The MICs of IPM for these strains ranged from 0.1 to 6.3, and from 6.3 to 100 μg/ml. The MIC_{90} values of DRPM, MEPM, and IPM were 3.2, 6.3, and 25 μg/ml, respectively. Ten carbapenem susceptible strains were chosen for further study. The susceptibilities to carbapenems and other antimicrobial agents by the ten strains are shown in Table 1.

Appearance of Colonies of Carbapenem-resistant Mutants within the Zone of Growth Inhibition

Figure 1 shows a typical result of the growth inhibition of the *P. aeruginosa* GP17 strain. The MICs of DRPM, MEPM and IPM for the parent strain GP17 were 0.1, 0.1 and 1.6 μg/ml, respectively.

In experiments with IPM and MEPM, there were several colonies within the clear zone of growth inhibition, or on the border between the clear zone of growth inhibition and the zone of cell growth. However, there were no colonies within the clear zone of growth inhibition when DRPM was used. The colonies that grew within the clear zone of growth inhibition in the MEPM or IPM experiments, or on the border in the MEPM experiment showed MIC values of 1.6 μg/ml to DRPM, 3.2 μg/ml to MEPM and 25 μg/ml to IPM. The MICs of PIPC, CAZ, GM and OFLX for the mutant strains were the same as those of parent strain. SDS-PAGE gel analysis of the OMPs of the representative mutant strain showed a marked reduction in a specific OMP with a molecular weight of about 48,000 (data not shown). These results indicated that the mutant resulted from the reduced expression of OprD and they exhibited reduced

Table 1 Drug susceptibilities of the *P. aeruginosa* strain and the carbapenem-resistant mutants

Strain No.	Strain[a]	MIC (μg/ml)						
		DRPM	MEPM	IPM/CS	PIPC	CAZ	GM	OFLX
GP 2	wild type	0.2	0.4	1.6	100	25	1.6	1.6
	carbapenem-resistant mutant	3.2	6.3	12.5	100	25	1.6	1.6
GP 3	wild type	0.2	0.4	1.6	12.5	3.2	3.2	1.6
	carbapenem-resistant mutant	3.2	6.3	25	12.5	3.2	3.2	1.6
GP 5	wild type	0.8	3.2	3.2	12.5	6.3	50	50
	carbapenem-resistant mutant	6.3	12.5	12.5	12.5	6.3	50	50
GP10	wild type	0.2	0.2	1.6	3.2	3.2	1.6	1.6
	carbapenem-resistant mutant	1.6	3.2	12.5	3.2	3.2	1.6	1.6
GP13	wild type	0.1	0.1	1.6	6.3	3.2	1.6	1.6
	carbapenem-resistant mutant	1.6	3.2	12.5	6.3	3.2	1.6	1.6
GP14	wild type	0.2	0.2	3.2	6.3	3.2	3.2	3.2
	carbapenem-resistant mutant	1.6	3.2	25	6.3	3.2	3.2	3.2
GP17	wild type	0.1	0.1	1.6	6.3	3.2	3.2	1.6
	carbapenem-resistant mutant	1.6	3.2	25	6.3	3.2	3.2	1.6
GP33	wild type	0.2	0.8	0.8	25	12.5	3.2	12.5
	carbapenem-resistant mutant	6.3	25	12.5	25	12.5	3.2	12.5
GP59	wild type	0.1	0.4	1.6	12.5	6.3	1.6	6.3
	carbapenem-resistant mutant	3.2	12.5	12.5	12.5	6.3	1.6	6.3
GP66	wild type	0.2	0.8	1.6	12.5	6.3	1.6	6.3
	carbapenem-resistant mutant	3.2	12.5	12.5	12.5	6.3	1.6	6.3

[a] Throughout this study, the drug susceptibilities of the mutant strains isolated in the presence of each selective drug of DRPM, MEPM and IPM were examined. The phenotype of drug susceptibilities of the mutants derived from a given strain were not essentially different in each experiment in this study. The drug susceptibilities of the representative mutant strains are shown in this table.

susceptibility or resistance to DRPM, MEPM, or IPM compared to the parent strain [3, 6, 7]. These results also suggested that both MEPM and IPM could specifically select for the carbapenem reduced susceptible mutants or carbapenem-resistant mutants of *P. aeruginosa* strains, and that DRPM could prevent growth of the mutants at a concentration that would inhibit cell growth. Throughout this study, the term "carbapenem-resistance" was used to where a mutant had reduced susceptibility to carbapenems compared to its parent strain, and not as any indication of absolute MIC.

Plating of Viable Cells of Each Strain on Selective Agar Plates Containing Different Concentrations of Each Carbapenem and the Carbapenem-resistance Level

To examine the presence of carbapenem-resistant mutants in the bacterial cell population that could arise during cell growth and to show quantitatively the results of Fig. 1, the number of cells of broth culture from each strain that grew

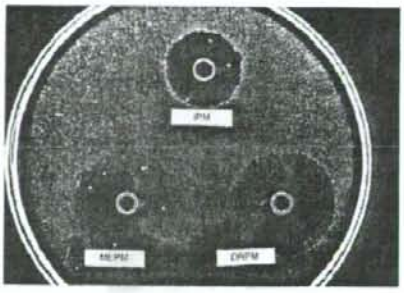

Fig. 1. Presence of carbapenem-resistant mutants within zones of growth inhibition of *P. aeruginosa* around cup containing carbapenem on agar plate.

0.1 ml of overnight culture of *P. aeruginosa* strain was spread on AB3 agar plate. Three cups were placed on the agar plate. 0.2 ml of 0.8 μg/ml of each carbapenem was put into each cup and the plate was incubated at 37°C for 18 hours. Colonies that grew in the clear zone of growth inhibition were purified on a drug free agar plate and resistance levels to carbapenems were examined by agar dilution methods.

on selective agar plates containing different concentrations of each carbapenem was determined (*i.e.*, population analysis) [15]. The results are shown as a survival curve in Fig. 2.

Almost all inoculated cells from each strain grew on the selective agar plates containing a drug concentration under the strain specific MIC of each carbapenem. The number of bacterial cells of each strain that grew on the selective agar plate containing the strain specific MIC of each carbapenem was around 10^2 to 10^4 cell per ml.

A number of colonies from each strain were grown on selective agar plates containing drug concentrations above the strain specific MIC of each drug. The drug concentration for MEPM ranging from the strain specific MIC to the drug concentration required for the complete inhibition of cell growth in each strain was greater than the drug concentration ranges of DRPM and IPM. The drug concentration range corresponded to the relatively wide zone of incomplete inhibition of cell growth by meropenem between the zone of complete inhibition and the zone of no inhibition of cell growth, as shown in Fig. 1.

Ten colonies of each strain grown on selective agar plates containing half of the concentration of each drug required for the complete inhibition of cell growth in each strain were examined for their drug resistance levels. One or two of the colonies that grew on the DRPM plates of GP2, GP33 and GP59 showed a carbapenem resistance (MIC) that was twice the DRPM concentration for the complete inhibition of cell growth, while the other colonies from each strain had the same level of carbapenem susceptibility as that of the parent strain. The ten colonies grown on the MEPM plates showed carbapenem resistance (MIC) that was the same level as the MEPM concentration for the complete inhibition of cell growth. The ten colonies grown on IPM showed carbapenem resistance (MIC) that was the same or twice the level of the IPM concentration for complete inhibition of cell growth.

The mutants selected with a given carbapenem showed resistance only to carbapenems and were susceptible to other antibiotics (Table 1). The SDS-PAGE gel of OMPs from a representative mutant from each strain showed a marked reduction in the specific OMP of around 48,000 molecular weight (data not shown).

Isolation Frequency of Carbapenem-resistant Mutants of *P. aeruginosa* Strains Grown in Broth without Carbapenem

The results of the population analysis shown in Fig. 2 implied that the isolation frequencies of carbapenem-resistant mutants would be different for the selective drugs DRPM, MEPM, and IPM when mutants are selected at a given concentration of each drug for each strain. The drug concentration for the selective agar plates was half the MIC of DRPM and MEPM, and 1/2 or 1/4 the MIC of IPM for the mutant of each strain.

Carbapenem-resistant mutants of the strains were not selected on selective agar plates containing DRPM, however, they were selected on selective agar plates containing MEPM or IPM at a frequency of around 10^{-7} to 10^{-9} per cell per generation (Table 2).

The carbapenem-resistant mutants tested from a given parent strain that had been selected with DRPM, MEPM, or IPM had the same resistance phenotype to carbapenems, and susceptibility to other antibiotics as the parent strain (Table 1).

OMPs of *P. aeruginosa* Strains

The OMPs of the representative strains and the carbapenem-resistant mutants shown in Table 2 were examined (Fig. 3). The OMPs of the GP3 and GP33 parent strains and the carbapenem-resistant mutants isolated from the selective agar plates containing either DRPM, MEPM, or IPM are shown in Figs. 3A and 3B, respectively. The OMPs of the representative mutant of each strain of the GP2, GP17 and GP66 strains are shown in Figs. 3C, D, and E, respectively. Marked reductions in the concentration of a specific outer membrane protein with a molecular weight of around 48,000 were observed for each of the mutants.

Increased Carbapenem-resistance Level (MIC) of *P. aeruginosa* Strain after Incubation with Carbapenem

If the carbapenem-resistant mutants arise during growth of the strain in broth culture, and the mutant strain can be increased selectively in the presence of carbapenem, the MIC of the carbapenem for the broth culture will increase beyond that seen for the parent strain.

The maximum resistance levels (MIC) of DRPM were lower than those of MEPM or IPM (the highest for the three carbapenems) for all strains tested (Fig. 4). The drug concentration ranges in broth cultures that gave rise to the maximum-resistance levels to each carbapenem in each strain were different for each of the carbapenems. The maximum resistance level to DRPM for each strain was obtained with broth cultures containing drug concentrations ranging from 1/4 or 1/2 MIC to MIC or twice MIC (Fig. 4). The maximum resistance level to MEPM for each strain was obtained with broth cultures containing drug concentrations ranging from 1/4 or 1/2 MIC to greater than MIC,with levels of four, eight or 16 times the MIC for each of the strains being observed (Fig. 4). The maximum-resistance level to IPM for each strain was obtained with

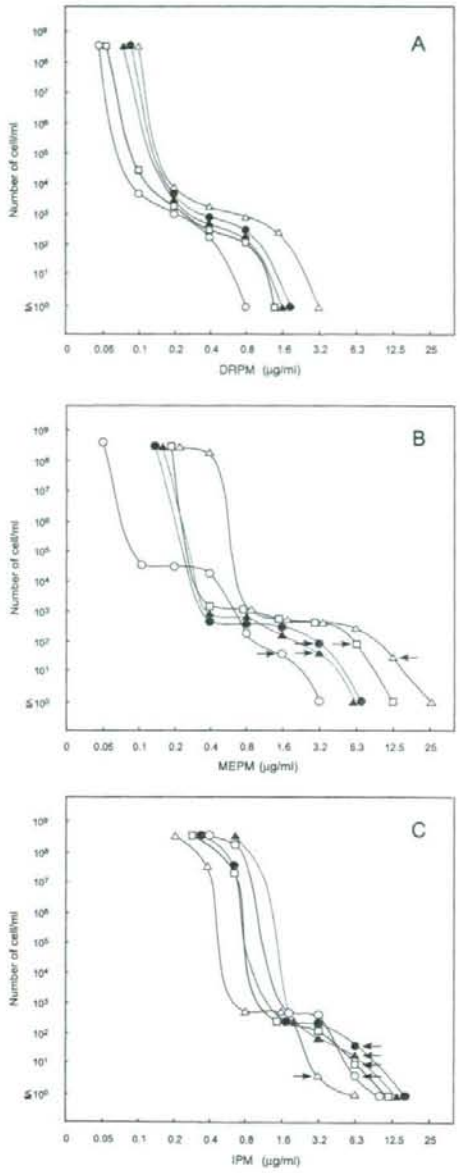

Fig. 2. Isolation of carbapenem-resistant mutant of *P. aeruginosa*.

Five representative strains are shown. Population analysis of each *P. aeruginosa* strain was carried out. Viable titers and antibiotic resistance levels (population analysis) of bacterial cells from each strain were determined by plating 0.1 ml diluted cultures on an AB3 agar plate containing different concentrations of each carbapenem. After overnight incubation of the plates at 37°C, the number of colonies was counted. Ten colonies from each strain that were picked from colonies grown on an agar plate containing one quarter or half of the drug concentration which completely inhibited cell growth of each strain were purified on drug free agar plates three times, and then examined for resistance levels to each carbapenem. The arrow shows that all colonies examined have stable resistance levels to each carbapenem. Viable cell number of each strain grown on selective agar plates containing different concentrations of DRPM (A), MEPM (B), and IPM (C), respectively, were counted. Symbols, ○, GP17; △, GP33; □, GP59; ●, GP2; ▲, GP3.

broth cultures containing drug concentrations ranging from 1/2 MIC to MIC, or two to four times the MIC for each of the strains. The drug concentrations of DRPM in the broth culture of each strain needed to select for carbapenem-resistant mutants showed a narrower drug concentration range than those of MEPM.

The range of DRPM concentrations required in broth culture to prevent growth of the mutants at the concentrations below the DPRM MIC for the carbapenem-resistant mutants of each strain were greater than those of MEPM, and equal to or greater than those of IPM.

Discussion

The mutants of the *P. aeruginosa* strain selected on agar

Table 2 Isolation frequency of carbapenem-resistant mutants and drug susceptibilities of the mutant strains

Strain No.	Drug	Carbapenem susceptibility of parent	Carbapenem susceptibility of mutant	Drug concentration in selective agar plate[a]	Isolation frequency of carbapenem-resistant mutant
		MIC (μg/ml)	MIC (μg/ml)	(μg/ml)	(per cell per generation)
GP2	DRPM	0.2	3.2	1.6	$<2\times10^{-9}$
	MEPM	0.4	6.3	3.2	1.74×10^{-8}
	IPM	1.6	12.5	6.3	4.81×10^{-8}
GP3	DRPM	0.2	3.2	1.6	$<2\times10^{-9}$
	MEPM	0.4	6.3	3.2	7.95×10^{-7}
	IPM	1.6	25	6.3	1.60×10^{-8}
GP5	DRPM	0.8	6.3	3.2	$<2\times10^{-9}$
	MEPM	3.2	12.5	6.3	4.57×10^{-8}
	IPM	3.2	12.5	6.3	1.45×10^{-8}
GP10	DRPM	0.2	1.6	0.8	$<2\times10^{-9}$
	MEPM	0.2	3.2	1.6	1.61×10^{-8}
	IPM	1.6	12.5	6.3	2.90×10^{-9}
GP13	DRPM	0.1	1.6	0.8	$<2\times10^{-9}$
	MEPM	0.1	3.2	1.6	1.09×10^{-7}
	IPM	1.6	12.5	6.3	4.63×10^{-8}
GP14	DRPM	0.2	1.6	0.8	$<2\times10^{-9}$
	MEPM	0.2	3.2	1.6	8.47×10^{-9}
	IPM	3.2	25	6.3	2.90×10^{-9}
GP17	DRPM	0.1	1.6	0.8	$<2\times10^{-9}$
	MEPM	0.1	3.2	1.6	8.36×10^{-8}
	IPM	1.6	25	6.3	3.07×10^{-8}
GP33	DRPM	0.2	6.3	3.2	$<2\times10^{-9}$
	MEPM	0.8	25	12.5	1.41×10^{-8}
	IPM	0.8	12.5	3.2	2.17×10^{-8}
GP59	DRPM	0.1	3.2	1.6	$<2\times10^{-9}$
	MEPM	0.4	12.5	6.3	2.57×10^{-8}
	IPM	1.6	12.5	6.3	1.22×10^{-8}
GP66	DRPM	0.2	3.2	1.6	$<2\times10^{-9}$
	MEPM	0.8	12.5	6.3	4.96×10^{-8}
	IPM	1.6	12.5	6.3	4.51×10^{-9}

[a] To determine the drug concentrations for selective agar plates, preliminary experiments were performed several times with different concentrations of each carbapenem for selective agar plates for each strain, and the isolation frequency of the mutant and the carbapenem-resistance levels were examined, and the most competent concentration of each carbapenem for each strain to specifically select the carbapenem-resistant mutant was determined. The data obtained in the experiment shown in Fig. 3 were also used to determine the drug concentration.

plates containing each of the carbapenems exhibited resistance or reduced susceptibility to carbapenems, and showed the same susceptibility to other antibiotics as the parent strain. The mutant strains exhibited a marked reduction in the concentration of a specific outer membrane protein with a molecular weight of about 48,000. These data indicated that the carbapenems predominantly selected carbapenem-resistant mutants that lacked or had a reduced expression of D2 porin (OprD) [3~7], although there was also a possibility that a mutant with increased expression of MexAB-OprM would be present in the culture [16~19].

The anti-pseudomonas activities of carbapenems are affected by carbapenem-specific porin OprD [3~7] and MexAB-OprM expression [16~22], which have influx and efflux functions, respectively. The loss or reduced expression of OprD of the *P. aeruginosa* strain increases the MICs of the carbapenems [3~7]. Previous studies suggested that the activities of MRPM and DRPM, but

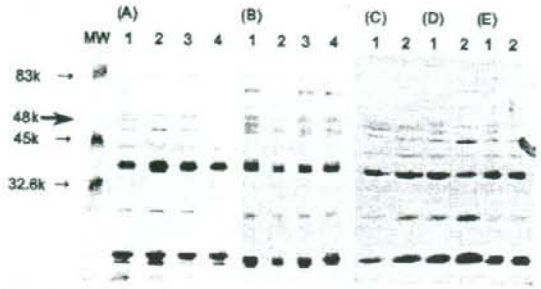

Fig. 3. SDS-PAGE of outer membrane proteins from carbapenem susceptible *P. aeruginosa* strains and the carbapenem-resistant mutants.

(A) *P. aeruginosa* GP3 and the mutants. lane 1, carbapenem susceptible GP3; lane 2 to lane 4, carbapenem-resistant mutants of GP3 isolated from selective agar plate containing DRPM, MEPM and IPM, respectively. (B) *P. aeruginosa* GP33 and the mutants. lane 1, carbapenem susceptible GP33; lane 2 to lane 4, carbapenem-resistant mutants of GP33 isolated from selective agar plate containing IPM, MEPM, and DRPM, respectively. (C) lane 1, carbapenem susceptible *P. aeruginosa* GP2; lane 2, carbapenem-resistant GP2 isolated from agar plate containing DRPM. (D) lane 1, carbapenem susceptible *P. aeruginosa* GP66; lane 2 carbapenem-resistant GP66 isolated from agar plate containing MEPM. (E) lane 1, carbapenem susceptible *P. aeruginosa* GP17; lane 2 carbapenem-resistant GP17 isolated from agar plate containing IPM; MW, molecular weight marker; bold arrow indicates outer membrane protein with molecular weight around 48,000. The carbapenem resistant mutant isolated from the selective agar plate containing doripenem was derived from the broth culture containing carbapenem (doripenem) to selectively increase the mutant strain as shown in following experiment described in Fig. 4.

not IPM, are also decreased by the increased production of the efflux protein MexAB-OprM [16, 17, 19, 21], although this mechanism rarely confers outright nor does it confer high level resistance. It confers almost the same level of resistance as is seen in the altered OprD-derivative [19, 21]. This type of mutant is predominantly selected by carbenicillin or CAZ [19]. A multiple resistance mechanism requiring both a lack of OprD for influx and increased expression of MexAB-OprM for efflux would be required to confer a relatively high resistance to MEPM and DRPM for *P. aeruginosa* [17, 19, 22~24]. This type of mutant has been isolated by selection of a MEPM-resistant mutant from a OprD deficient derivative on agar containing MEPM [23]. This type of double mutant would not be selected readily during carbapenem therapy [19, 21, 23, 24].

The MIC_{90} values of DRPM for *P. aeruginosa* strains were one dilution lower than those of MEPM and eight dilutions lower than IPM, indicating that the MICs of IPM were the highest of the three carbapenems [9, 12, 19]. DRPM resembled MEPM, and was more effective than IPM with respect to the MICs for *P. aeruginosa* strains. However, the potency of selection for the mutant cells or the potency of growth prevention of the mutant cells was different for DRPM, MEPM and IPM. A combination of population analysis to determine the presence of the carbapenem-resistant mutant in the culture of each strain (Fig. 1 and Fig. 2), the calculation of the isolation frequency of the carbapenem-resistant mutant on agar containing each carbapenem (Table 2), and experiments to selectively increase the carbapenem-resistant mutant in broth culture containing different concentrations of each carbapenem (Fig. 4) resulted in our drawing the following conclusions: IPM and MEPM selected the carbapenem-resistant mutant at the drug concentration that inhibited growth of the parent strain at a frequency of around 10^{-7}~10^{-8} per cell of the culture. However, DRPM could not specifically select the mutant strain at a drug concentration capable of inhibiting growth of the parent strain. In contrast to MEPM and IPM, the mutant could not be selected on agar at concentrations of four to 16 times the MIC of DRPM for the parent strain, which were 1/2 MIC for the mutant strains. The maximum drug concentrations of DRPM, MEPM, and IPM in the broth culture that allowed selective increase in carbapenem-resistant mutant in the broth culture were 1/8 to 1/16 MIC of DRPM, 1/2 to 1/4 MIC of MEPM and 1/2 to 1/16 MIC of IPM for the mutant strains, respectively. These data indicated that DRPM has the greatest potency in preventing the emergence of the carbapenem-resistant mutants. The present study suggested that the clinical evaluation of DRPM for the prevention of the emergence of the mutant strain may be worthwhile.

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Strain No.	Selective drug in broth culture	Drug susceptibility (MIC (μg/ml))		MIC for culture with selective drug / MIC for culture without selective drug	Range of drug concentration (ratio to MIC for wild type strain) which gave rise to the maximum resistance level
		Culture without selective drug (wild type strain)	Culture with selective drug[a]		1/4 1/2 1 2 4 8 16 32
GP3	Doripenem	0.2	3.2	16	
	Meropenem	0.4	6.3	16	
	Imipenem	1.6	25	16	
GP13	Doripenem	0.1	1.6	16	
	Meropenem	0.1	3.2	32	
	Imipenem	1.6	12.5	8	
GP14	Doripenem	0.2	1.6	8	
	Meropenem	0.2	3.2	16	
	Imipenem	3.2	25	8	
GP17	Doripenem	0.1	1.6	16	
	Meropenem	0.1	3.2	32	
	Imipenem	1.6	25	16	
GP33	Doripenem	0.2	6.3	32	
	Meropenem	0.8	25	32	
	Imipenem	0.8	12.5	16	
GP59	Doripenem	0.1	3.2	32	
	Meropenem	0.4	12.5	32	
	Imipenem	1.6	12.5	8	

Fig. 4. Increased carbapenem resistance levels of *P. aeruginosa* strains after incubation with carbapenem.

Overnight culture of each strain was diluted appropriately. Approximately 10^6 bacterial cells of each strain were inoculated into 1 ml of AB3 broth containing each different concentrations of each carbapenem and were incubated at 37°C for 18 hours with gentle shaking. After incubation, the resistance levels (MICs) to the same carbapenem used in broth culture were examined against each strain using agar dilution methods with around 10^4 inoculated cells. In this experiment, approximately 10^4 CFU or 10^6 CFU were inoculated onto each of a series of agar plates containing various antibiotic concentrations. The resistance levels of each strain in the broth culture containing each of the drugs were not essentially different from the different inoculum sizes on the drug plates. The results obtained by inoculating approximately 10^4 cells, are shown in here. [a] The MIC of each carbapenem in culture with the selective drug indicated the maximum level in the increased MIC for the cultures. Asterisk (*) in figure indicates drug resistance level (MIC) of each carbapenem for the strain with maximum resistance (*i.e.*, carbapenem-resistant mutant) where the MIC was indicated by ratio of MIC against a culture with selective drug to MIC against the culture without the selective drug (*i.e.*, MIC against the wild type strain).

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