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Cloning and Genetic Organization of the Bacteriocin 31 Determinant Encoded on the *Enterococcus faecalis* Pheromone-Responsive Conjugative Plasmid pYI17

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The conjugative plasmid pYI17 (57.5 kb) isolated from *Enterococcus faecalis* YI717 confers a pheromone response on the host and encodes the bacteriocin 31 gene. Bacteriocin 31 is active against *E. hirae* 9790, *E. faecium*, and *Listeria monocytogenes*. pYI17 was mapped physically by restriction enzyme analysis and the relational clone method. Deletion mutant and sequence analyses of the *Eco*RI fragment B cloned from pYI17 revealed that a 1.0-kb fragment contained the bacteriocin gene (*bacA*) and an immunity gene (*bacB*). This fragment induced bacteriocin activity in *E. faecalis* OG1X and *E. hirae* 9790. The *bacA* gene is located on the pYI17 physical map between 3.37 and 3.57 kb, and *bacB* is located between 3.59 kb and 3.87 kb, *bacA* encodes 67 amino acids, and *bacB* encodes 94 amino acids. The deduced amino acid sequence of the *bacA* protein contained a series of hydrophobic residues typical of a signal sequence at its amino terminus. The predicted mature *bacA* protein (43 amino acids) showed sequence homology with the membrane-active class II bacteriocins of lactic acid bacteria. Analysis of Tn*5* insertion mutants and the resulting transcripts indicated that these genes are transcribed as an operon composed of *bacA*, *bacB*, and an open reading frame located downstream of *bacB* designated ORF3.

Bacteriocins are produced by a wide variety of gram-positive and gram-negative bacteria. Bacteriocins are bacterial proteins which inhibit the growth of other bacteria closely related to the producer strain. They usually exhibit a relatively narrow spectrum of activity. Bacteriocins are thought to provide the producer strain with a selective advantage over other strains and are a factor in bacterial virulence. Research related to the gram-positive bacterial bacteriocins now appears to be centered on the bacteriocin activities of the lactic acid bacteria, and these bacteriocins were recently reviewed (24). Many of these strains are food grade organisms that are already widely used in the food industry, but recent research has indicated that these strains may find novel applications in food preservation. It is known that many *Enterococcus faecalis* strains produce bacteriocins (4, 7). The bacteriocin phenotype is frequently associated with the pheromone-responding conjugative plasmid of *E. faecalis*. These conjugative plasmids transfer at a high frequency in broth mating, a phenomenon related to their response to the specific peptide sex pheromones secreted by potential recipients (7–9, 14, 39, 40). The sex pheromone induces the synthesis of a surface aggregation substance that facilitates the formation of a mating aggregate (7–9, 14, 36).

To date, only a few of the *E. faecalis* bacteriocins have been genetically and biochemically characterized. These include the hemolysin/bacteriocin and the peptide antibiotic AS-48, which are encoded by the *E. faecalis* conjugative plasmids pAD1 (58 kb) (8, 9, 11, 20, 43) and pMB2 (33), respectively. pAD1 and pMB2 were originally derived from *E. faecalis* subsp. *zymogenes* DS16 (43) and *E. faecalis* subsp. *liquefaciens* S-48 (32), respectively.

E. faecalis subsp. *zymogenes* may be distinguished from other *E. faecalis* strains by its production of a cytotoxin capable of lysing human, rabbit, and horse erythrocytes. Strains producing this beta-hemolysin also produce a bacteriocin, which is encoded by the same genetic determinant (3, 23, 25). The hemolysin/bacteriocin has been associated with virulence in animal models (6, 26, 29). A significant number of *E. faecalis* clinical isolates produce hemolysin/bacteriocin (12, 21, 27). More than 50% of the *E. faecalis* clinical isolates studied carry transferable hemolysin/bacteriocin genes (21, 22, 27). More than 90% of these hemolysin/bacteriocin plasmids are identical to pAD1, respond to the pheromone cAD1, and belong to incompatibility group incHly (13, 22, 31). Thus, pAD1 is a typical *E. faecalis* hemolysin/bacteriocin plasmid. The hemolysin/bacteriocin gene is the only *E. faecalis* bacteriocin determinant to have been cloned and studied (19, 23, 38).

In this report, we describe the isolation of *E. faecalis* pheromone-responsive conjugative bacteriocin plasmids and the cloning and genetic organization of a new type of *E. faecalis* bacteriocin determinant (type 3) encoded on the plasmid.

MATERIALS AND METHODS

Bacteria, media, and regents. The strains and plasmids used in this study are listed in Table 1. Clinical isolates of *Streptococcus pyogenes* (group A), *S. agalactiae* (group B), *S. sanguis*, *S. pneumoniae*, *S. aureus*, or *S. epidermidis* were used as indicator strains for the bacteriocins of bacteriocinogenic clinical strains. *E. faecalis* strains were grown in Todd-Hewitt broth (THB) (Difco, Detroit, Mich.), antibiotic medium 3 (Difco), or N2GT broth (nutrient broth no. 2 [Oxoid Ltd., London, England] supplemented with 0.2% glucose and 0.1 M Tris–HCl [pH 7.5]). *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. Antibiotics were used at the following concentrations: ampicillin, 100 μg/ml; chloramphenicol, 20 μg/ml for *E. faecalis* and 50 μg/ml for *E. coli*; erythromycin, 12.5 μg/ml; rifampin, 25 μg/ml; fusidic acid, 25 μg/ml; streptomycin, 500 μg/ml; spectinomycin, 500 μg/ml; kanamycin, 40 μg/ml; tetracycline, 6 μg/ml.

Soft-agar assay for bacteriocin production and immunity. The bacteriocin production assay was performed as described previously (23). Fifty microliters of an overnight culture of the indicator strain or *E. hirae* ATCC 9790 grown in antibiotic medium 3 was added to 5 ml of molten soft agar (0.75%), which was then poured onto an antibiotic medium 3 plate. After solidification, each strain to be tested was inoculated into the soft agar with a toothpick. The test for

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TABLE 1. Bacterial strains and plasmids used in this study

Strains or plasmid	Relevant characteristic(s)	Reference or source
Strains		
Enterococcus faecalis		
FA2-2	Rif Fus; derivative of JH2	11
JH2SS	Str Spc; derivative of JH2	42
OG1-10	Str; derivative of OG1	14
OG1X	Str; proteinase negative derivative of OG1-10	24
OG1RF	Rif Fus; derivative of OG1	34
YI717	pYI17 (Bac); clinical isolate	This study
Enterococcus hirae ATCC 9790	Wild type, penicillin susceptible, PBP5 low producer	
Escherichia coli		
DH1	F^- *recA1 endA1 gyrA94 thi-1 relA1 hsdR17 supE44*	36
DH5α	*recA1 endA1 gyrA96 thi-1 relA1 hsdR17 supE44* φ80 *lacZ*ΔM15	Bethesda Research Laboratories
TH688	CSH57b *thr*::Tn*5*	41
Plasmids		
pYI17	Bac; conjugative plasmid (57.5 kb) from strain YI717	This study
pTV1Ts	Cat Erm; temperature-sensitive replicon; delivery vector for Tn*917*	46
pAM401	Cat Tet; *E. coli*-*E. faecalis* shuttle vector	44
pWM401	Cat Tet; *E. coli*-*E. faecalis* shuttle vector	45
pMW119	Amp *lacZ*; *E. coli* vector, low copy number	Nippon Gene Co.

immunity to the bacteriocin was performed essentially as described above, except that the indicator and bacteriocin-producing strains were replaced by *E. hirae* ATCC 9790 strains transformed by electrotransformation with the cloned plasmid and the wild-type bacteriocin-producing strain FA2-2 (pYI17), respectively.

Liquid bacteriocin assay. Bacteriocinogenic and nonbacteriocinogenic *E. faecalis* strains were grown in THB at 37°C with gentle shaking. During the late exponential phase, at an optical density of 110 Klett units (Klett-Summerson colorimeter no. 54 filter), the bacteria were removed by centrifugation at 43,000 × *g* for 30 min. Bacteriocin activities were measured for the resulting supernatant.

The bacteriocin indicator strain *E. hirae* 9790 was grown overnight in THB at 37°C. The overnight culture was diluted 10^4-fold with fresh THB. The diluted culture contained about 10^5 cells per ml. One volume (0.5 ml) of bacteriocin-containing supernatant was added to 10 volumes (5 ml) of the indicator strain. These cultures were incubated at 37°C with gentle shaking. Samples were removed at various times and appropriately diluted, and the dilution was plated out on the THB agar plate to determine cell survival.

Mating procedures and conjugative transfer of pYI17. Broth mating was performed as previously described (15, 16, 20) with a donor/recipient ratio of 1:10. Overnight cultures of 0.05 ml of donor and 0.45 ml of recipient were added to 4.5 ml of fresh N2GT broth. The mixtures were incubated at 37°C with gentle agitation for 6 h and then vortexed. The mixed culture was then plated on solid media containing appropriate antibiotics for selection. Colonies were counted after 48 h of incubation at 37°C. Pheromone induction and the mating experiments were performed as previously described (10). For experiments involving conjugative transfer of the bacteriocin plasmid pYI17, plasmids tagged with an erythromycin resistance marker were constructed by transforming pTV1ts::Tn*917* (Em^r) into OG1X(pYI17) and selecting for the transposition of Tn*917* from pTV1ts::Tn*917*, as described previously (16, 46).

Isolation and manipulation of plasmid DNA. Plasmid DNA was isolated by the alkaline lysis method (39). Plasmid DNA was treated with restriction enzymes and submitted 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) and were used in accordance with the supplier's specifications. Agarose was obtained from Wako Chemicals, Osaka, Japan. Gels with a 0.8% agarose concentration were used for size determination of large DNA fragments (greater than 0.5 kb), and 2.0% agarose gels were used to determine the sizes of smaller fragments (less than 0.5 kb) (18). A "glass milk" kit (Gene Clean II kit; Bio 101, Inc., La Jolla, Calif.) or low-melting-point agarose and β-agarase I (Nippon Gene) was used for the elution of the 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 (17). Transformants were selected on Luria-Bertani medium agar containing suitable antibiotics.

Determination of the pYI17 restriction map. Initially, pYI17 DNA was digested with *Eco*RI and the sizes of the *Eco*RI fragments were determined. The molecular sizes of *Eco*RI fragments A to J were 18.8, 10.9, 10.2, 6.7, 3.7, 3.5, 1.4, 1.4, 0.7, and 0.2 kb, respectively. To determine the order of the *Eco*RI fragments, pYI17 DNA was partially digested with a low concentration of *Eco*RI or completely digested with *Eco*RI, *Kpn*I, or *Sal*I and one of the other enzymes examined (double digestion). The digested DNAs were examined by agarose gel electrophoresis (data not shown), and the order of the fragments determined by these methods was A, C, B, F, D.

To determine the order of the other *Eco*RI fragments, a relational clone set was obtained by the methods previously described (18). After agarose gel electrophoresis of an *Eco*RI partial digest of pYI17 DNA, the fragments were eluted and used for cloning. The cloning vector and host strain were pWM401 and *E. coli* DH1, respectively.

Deletion mutant analysis. The cloned DNA was digested with *Bam*HI and *Sph*I and treated with exonuclease III for various periods of time, with mung bean nuclease, and then with Klenow fragment. The deleted DNAs were ligated and used to transform *E. coli* DH5α. The derivatives were tested for bacteriocin activity and immunity.

Generation of the transposon insertional mutants. Tn*5* (Km^r) insertion into the plasmid was performed as described elsewhere (40, 41). The plasmid was introduced into *E. coli* K-12 TH688 (with Tn*5* in the *thr* locus) (40) by chemical transformation. Transformants were streaked onto selective media containing 40 μg of kanamycin and 50 μg of chloramphenicol per ml, and the plates were left at room temperature for 10 days. The bacteria which grew on the selective plate were pooled; the plasmid DNA was then isolated and used to transform *E. coli* DH5α. The transformants were selected on plates containing kanamycin (40 μg/ml) and chloramphenicol (50 μg/ml) for the selection of Tn*5* kanamycin resistance and plasmid-borne chloramphenicol resistance, respectively. The transformants were purified and examined to determine the specific location of Tn*5* within the plasmid. The precise location of Tn*5* insertion was determined by nucleotide sequencing using a synthetic primer which hybridized to the end of Tn*5*.

DNA sequence analysis. Nucleotide sequence analysis was carried out as previously described (37). A deletion kit (Nippon Gene) was also utilized. To determine the sequence of the pYI17 bacteriocin determinant, a 7.9-kb *Eco*RI-*Nco*I fragment was cloned into the vector pMW119. The cloned DNA fragment was digested with *Bam*HI and *Sph*I and treated with exonuclease III for various periods of time, with mung bean nuclease, and with Klenow fragment. The deleted DNAs were ligated and used to transform *E. coli* DH5α. The resulting constructs were sequenced in both orientations with a 373A DNA sequencer (Applied Biosystems). The Taq Dye Primer and Taq Dye Terminator Cycle Sequencing kits (Applied Biosystems) were used in the sequence reaction. For homology analyses of the nucleotide and amino acid sequences, DNA Data Bank of Japan (DDBJ) (National Institute of Genetics, Mishima, Japan) was used.

Analysis of transcripts. Total RNA was extracted by a hot-phenol method similar to that previously described (40). Cells were harvested from overnight cultures (5-ml THB) of *E. faecalis* FA2-2 carrying the plasmid of interest and suspended in 1 ml of 25% sucrose in 50 mM Tris-HCl (pH 8.0). After the addition of 0.5 ml of 500 mM EDTA (pH 8.0) and 25 ml of lysozyme solution (20 mg/ml in water), the mixture was incubated at room temperature for 30 min and

the cells were pelleted and resuspended gently in 150 μl of lysis buffer (20 mM Tris-HCl [pH 8.0], 3 mM EDTA, 200 mM NaCl). An equal volume of lysis solution (1% sodium dodecyl sulfate in lysis buffer) was added, and the mixture was incubated at 95°C for 2 min with occasional mixing. Three hundred microliters of hot phenol saturated with lysis buffer (65°C) was added, and the mixture was incubated at 65°C for 3 min with occasional inversion of the tube. The sample was placed on ice for 5 min, and the aqueous phase was separated by centrifugation at 5,000 rpm for 5 min with Kubota 7820. The aqueous phase was reextracted with an equal volume of hot phenol, as described above, and then extracted three times with an equal volume of ether. RNA was precipitated by the addition of 0.1 volume of 3 M sodium acetate (pH 5.2) and 2 volumes of ethanol. The pellet was suspended in 10 μl of water, and 1.2 μl of 10× DNase I buffer was added (1 M sodium acetate [pH 5.2], 50 mM $MgCl_2$). The DNA was then digested with 5 U of DNase I (RNase free; Boehringer Mannheim Biochemicals) for 15 min at 25°C. The DNase I was then inactivated by heating at 80°C for 10 min. The RNA species were separated by electrophoresis in a 1.4% agarose gel containing 3-(*N*-morpholino)propanesulfonic acid. Northern (RNA) hybridization was carried out overnight at 42°C in the presence of 50% formamide (1). Two synthetic probes were obtained from Nippon Science Core Company Delivery Center. Probe 1 corresponded to the sequence 5′-GCTT TATTCCAGTCTACCCAACATTTTTGC-3′, which is located in the bacteriocin determinant (*bacA*). Probe 2 corresponded to the sequence 5′-AGTA AATCTAGTAATTCTTGTTGTTTATCC-3′, which is located in the immunity determinant (*bacB*) (see Fig. 3). The 5′ OH ends of these probes were labeled by [γ-^{32}P]ATP (4,500 Ci/mmol; ICN Biomedicals Inc.) with T4 polynucleotide kinase (Bethesda Research Laboratories). RNA marker molecules were obtained from Bethesda Research Laboratories.

Determination of the initiation site for transcription. Primer extension was conducted essentially as described elsewhere (1). Probe 1 was used as the oligonucleotide primer (see Fig. 3). Primer DNA (0.5 μg) was treated with 15 U of T4 polynucleotide kinase in the presence of 250 μCi of [γ-^{32}P]ATP (4,500 Ci/mmol; ICN Biomedicals Inc.). The RNA prepared from a 10-ml THB culture of FA2-2(pYI17) was hybridized with 50 ng of kinase-treated primer overnight at 30°C in 30 μl of hybridization solution (166 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid] [pH 7.5], 1.0 M NaCl, 0.33 mM EDTA). Primer extension was conducted with 50 U of Moloney murine leukemia virus reverse transcriptase (New England Biolabs, Inc.) in 25 μl of a reaction mixture (0.6 mM of each deoxynucleotide triphosphate, 50 mM Tris-HCl [pH 8.3], 8 mM $MgCl_2$, 30 mM KCl, 10 mM dithiothreitol, 50 U of ribonuclease inhibitor [TOYOBO, Inc., Ltd.]). The reaction mixture was incubated at 42°C for 90 min. The samples were run on a sequencing gel after pancreatic RNase I treatment. The gel was then dried and autoradiographed. The related sequencing reaction was carried out by the chain termination DNA sequencing method with a Sequenase version 2.0 DNA sequencing kit (Amersham).

Nucleotide sequence accession number. The nucleotide sequence data reported in this article will be available from the DDBJ, EMBL, and GenBank nucleotide sequence databases under accession number D78257.

RESULTS

Bacteriocinogenic strains of *E. faecalis*. A total of 270 *E. faecalis* strains were tested for bacteriocin production against the indicator strains *E. faecalis* OG1-10 (14) and *E. hirae* 9790 and other bacterial species such as *S. aureus*. One hundred and forty-seven (54%) of the 270 strains were bacteriocinogenic. Of these 147 strains, 100 strains produced a hemolysin/bacteriocin. Of the other 47 strains, 27 strains were omitted from this study because the strains exhibited phage activity (data not shown).

The remaining 20 bacteriocinogenic strains were classified into four groups on the basis of their bacteriocin activity. One strain showed activity against a broad spectrum of bacteria, including *E. faecalis*, other *Streptococcus* spp., and *S. aureus*. Four strains were active against *E. faecalis*. Twelve strains were active against *E. hirae*. Three strains were active against *E. faecalis* and *E. hirae.*

Isolation of conjugative plasmids conferred bacteriocin activity against *E. hirae*. The three strains which were active against *E. faecalis* and *E. hirae* were designated YI717, YI718, and YI719; they each harbored a single plasmid of 57.5 kb, and the plasmids were designated pYI17, pYI18, and pYI19, respectively. pYI17, pYI18, and pYI19 exhibited similar *Eco*RI restriction profiles (data not shown). To examine whether the plasmid conferred bacteriocinogenic activity on its host, each of the strains was used as a donor in mating experiments with a plasmid-free recipient strain. Broth matings were performed between each of the strains and the recipient strain *E. faecalis* FA2-2 (Rif^r Fus^r). After a 4-h incubation of the broth mating cultures, the appropriately diluted mixtures were plated on an agar plate selective for the recipient strains. After overnight incubation of the plates, a total of about 500 *E. faecalis* FA2-2 colonies were obtained from each mating, these were examined for bacteriocin production. Approximately one in 500 cells obtained from the mating experiments using YI717, YI718, and YI719 expressed bacteriocin activity against *E. hirae* 9790. The bacteriocinogenic transconjugant harbored a plasmid, which had an *Eco*RI restriction profile similar to that of the plasmid in the donor strain. This indicated that the plasmids conferred bacteriocin activity against *E. hirae*. Plasmid pYI17, isolated from YI717, was used as a representative of these plasmids.

Bacteriocin activity and the thermostability of the bacteriocin conferred by pYI17. *E. faecalis* FA2-2 carrying pYI17 exhibits bacteriocin activity against *E. hirae* 9790. *E. faecalis* FA2-2 and OG1X carrying pYI17 were examined for bacteriocin activity against the enterococci and other gram-positive bacteria, including *Listeria monocytogenes*, as described in Materials and Methods. The plasmid-carrying strains had bacteriocinogenic activity against *E. hirae* 9790, *L. monocytogenes*, and two of five *E. faecium* clinical isolates (data not shown). *E. hirae* 9790 was used routinely as an indicator strain for the bacteriocin activity of pYI17.

The thermostability of bacteriocin was examined in the culture supernatant of FA2-2(pYI17). The culture supernatant of FA2-2(pYI17) was prepared as described in Materials and Methods. Then, the culture supernatant was autoclaved for 10 min, and the bacteriocin activity of the heat-treated culture supernatant was examined as described in Materials and Methods. Cell survival of the indicator strain was reduced by a factor of 1/10,000 after a 1-h incubation in heat-treated culture supernatant (data not shown). On the other hand, the indicator strain grew normally in heat-treated nonbacteriocinogenic FA2-2 culture supernatant (data not shown). These results indicate that the bacteriocin was heat stable.

Conjugative transfer of pYI17. pYI17 was tagged with Tn*917* (Em^r), as described in Materials and Methods. pMG114 is a derivative of pYI17::Tn*917* and was used in the transfer experiments. Conjugative transfer of the plasmid from *E. faecalis* OG1-10 to OG1RF was examined. pMG114 underwent normal transfer, which usually requires about 60 min before a significant level of transfer occurs, and was shown to give rise to an increased number of transconjugants between 2 and 3 h after the start of the mating experiment. Pheromone induction and a mating experiment were performed as described in Materials and Methods to examine the pheromone response of pMG114. The donor cells of OG1-10(pMG114) were exposed (120 min) to an FA2-2 culture filtrate (pheromone) to induce aggregation-mating functions before a short (10-min) mating period. The short mating was carried out between the induced or uninduced donor cells and the uninduced recipient cells. The transfer frequency of the induced plasmid was 10^{-2} per donor cell and that of the uninduced plasmid was less than 10^{-5} per donor cell, indicating that plasmid pYI17 conferred the pheromone response.

Restriction map of pYI17. Mapping was carried out as described in Materials and Methods. pYI17 consisted of *Eco*RI fragments A through J. The order of the fragments determined by partial or double digestion with restriction enzymes was A, C, B, F, D. Seven clones containing different *Eco*RI fragments were obtained by the relational clone methods (18). The clones contained *Eco*RI fragments D and G; D, G, and E; D, G, E, I,

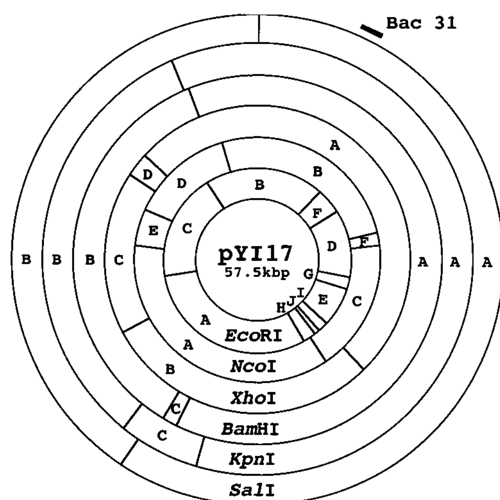

FIG. 1. Physical map of pYI17. The location of the bacteriocin 31 (Bac 31) determinant is indicated.

and J; E and I; E, I, J, and H; I, J, and H; and H and A; the order of these fragments was D, G, E, I, J, H, A. Together with the results of the partial and double digestions, these results indicated that the order of the *Eco*RI fragments was A, C, B, F, D, G, E, I, J, H in a clockwise orientation (Fig. 1). The cleavage sites for *Nco*I, *Xho*I, *Bam*HI, *Kpn*I, and *Sal*I are also shown in Fig. 1. *Eco*RI fragments G and H are both 1.4 kb. There is an *Nco*I site in *Eco*RI fragment H, but no site for any of the restriction enzymes examined in *Eco*RI fragment G.

Cloning of a DNA fragment carrying the bacteriocin determinant and sequencing of the DNA fragment. No relational clone containing *Eco*RI fragment B, C, or F was obtained. *Eco*RI fragments B, C, and F were cloned into pWM401. Bacteriocin activity was examined in bacteria containing each of the relational clone set and the cloned *Eco*RI fragments B, C, and F. Bacteriocin activity was not expressed by any *E. coli* DH1 clone. Each cloned DNA was introduced into *E. faecalis* OG1X by electrotransformation (17), and the transformants were examined for bacteriocin activity. A plasmid, designated pMG108, containing *Eco*RI fragment B expressed bacteriocin activity. *E. hirae* 9790 was transformed with pMG108 DNA, and the transformant expressed bacteriocin activity. The 10.9-kb *Eco*RI fragment B has one *Nco*I site located 3 kb from an *Eco*RI site. pWM401 contains an *Nco*I site 0.3 kb from an *Eco*RI site. pMG108 DNA was digested with *Nco*I and self-ligated. The ligated DNA was used to transform *E. coli* DH5α. The clone, designated pMG109, contained the *Eco*RI-*Nco*I (7.9-kb) fragment originating from *Eco*RI fragment B. *E. faecalis* OG1X or *E. hirae* 9790 was transformed with pMG109 DNA. The transformants expressed bacteriocin activity.

pMG109 (pWM401::*Eco*RI-*Nco*I fragment [7.9 kb]) was digested with *Eco*RI and *Nco*I. The *Eco*RI-*Nco*I fragment (7.9 kb) was treated with Klenow fragment to produce blunt ends and was then cloned into the *Sma*I site of pMW119. Two clones which possessed the 7.9-kb insert in opposite orientations were digested with *Bam*HI and *Sph*I and trimmed by a nuclease, as described in Materials and Methods for DNA sequence analysis. The resulting constructs were sequenced as described in Materials and Methods. Computer analysis revealed the presence of several open reading frames (ORFs) in the region spanning map positions 56 to 4.9 kb in the clockwise orientation of the pYI17 (57.5 kb) map. Figure 2 shows the ORFs which had a good ribosome binding site in a 20-base region upstream of the predicted start codon.

Generation of deletion mutants and Tn*5* insertion mutants. pMG109 (pWM401::*Eco*RI-*Nco*I fragment [7.9 kb]) was digested with *Eco*RI and *Nco*I and treated with Klenow fragment to blunt end the 7.9-kb fragment. The 7.9-kb fragment was cloned into the *Eco*RV site of pAM401. Clones pMG110 and pMG120 carried the 7.9-kb insert in opposite orientations, and both were used in further studies (Fig. 2).

A nested deletion kit (Nippon Gene, Inc.) was used to produce deletion mutants. The deletion mutants were examined for bacteriocin activity and immunity, as described in Materials and Methods. Deletion mutants pMG111 and pMG112 were derived from pMG110 and possessed deletions between positions 3.9 and 4.9 kb and between 3.4 and 4.9 kb, respectively (Fig. 2a). Strains containing pMG111 expressed both bacteriocin activity and immunity, while the strain containing pMG112 did not express either bacteriocin activity or immunity. The deletion mutants pMG121 and pMG122, derived from pMG120, possessed deletions located between 54.5 and 2.7 kb and between 54.5 and 3.0 kb of the physical map, respectively (Fig. 2a). Strains containing either pMG121 or pMG122 expressed bacteriocin activity and immunity. These results indicated that the region spanning 3.0 to 3.9 kb of the physical map was required for the expression of bacteriocin 31 (bacteriocin activity and immunity).

Deletion mutant pMG122 contains the bacteriocin 31 determinant. To locate the region involved in bacteriocin expression, direct subcloning of restriction fragments was used to construct pMG122 deletion derivatives (Fig. 2b). Subclone pMG123, which possesses a fragment located between 3.0 and 4.0 kb of the physical map, conferred bacteriocin activity on *E. faecalis* OG1X and *E. hirae* 9790 and encoded both ORF1 and ORF2. This confirmed that the determinant was located between 3.0 and 3.9 kb of the physical map. Subclone pMG124 possessed a 0.57-kb *Dra*I fragment located between 3.0 and 3.6 kb of the map and encoded ORF1. This fragment conferred bacteriocin activity on *E. faecalis* OG1X but did not transform *E. hirae* 9790. Subclone pMG125 possessed ORF2 and a deletion located between 3.0 and 3.5 kb, corresponding to part of ORF1, and did not confer bacteriocin activity or immunity on either *E. faecalis* OG1X or *E. hirae* 9790.

Mutants with altered bacteriocin expression were isolated by Tn*5* insertion into pMG122, as described in Materials and Methods. The precise locations of Tn*5* insertion into the ORFs were determined by DNA sequence analysis, and the results are shown in Fig. 3. Two of the six Tn*5* inserts exhibited altered bacteriocin expression (Fig. 2c). The pMG122-1 insert was mapped to the middle of ORF1. This resulted in defective bacteriocin activity and immunity in *E. faecalis* OG1X and *E. hirae* 9790. The pMG122-2 insert was mapped to the C-terminal region of the deduced amino acid sequence of ORF1. When an inoculum of 10^8 or more OGIX(pMG122-2) cells was used in the soft-agar assay, the strain exhibited bacteriocin activity; however, this activity was not observed when the inoculum was less than 10^8 cells. These results implied that OGIX(pMG122-2) required a large bacterial inoculum size for bacteriocin activity to be detected and that this activity was leaky. pMG122-2 did not transform *E. hirae* 9790, suggesting that pMG122-2 was defective in immunity. Strains containing other insertion derivatives did not exhibit any altered phenotype for bacteriocin expression.

DNA sequence analysis of ORFs. DNA sequence analysis of ORF1 and ORF2, which are essential for bacteriocin expression, was performed (Fig. 3). DNA sequence analysis of ORF3,

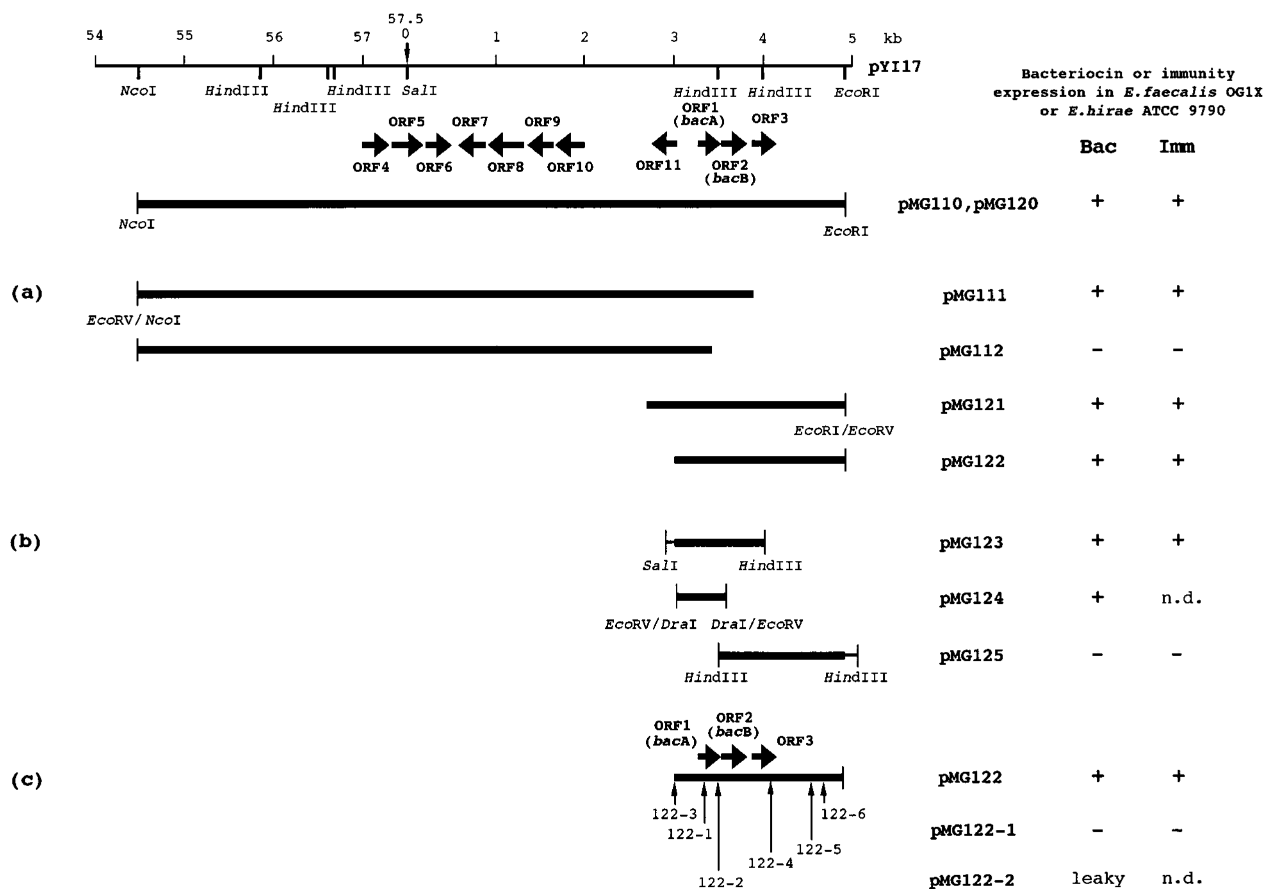

FIG. 2. Physical maps of the *Eco*RI-*Nco*I fragment (7.9 kb) of pYI17 (which is carried on pMG110 or pMG120 in opposite orientations), subclones, and Tn*5* insertions into the subclone. Thick horizontal arrows indicate ORFs on the pYI17 physical map and the direction of ORF transcription. Thick lines represent the cloned pYI17 DNA fragment. Thin lines represent vector DNA. The vertical bars at the ends of the designated plasmid segments indicate the restriction sites used for construction; the absence of a bar indicates that the end was generated by a nested deletion. (a) Deletion mutants of pMG110 and pMG120; (b) subclones obtained by direct subcloning of pMG122 restriction fragments; (c) map of Tn*5* insertions into pMG122. The arrows indicate the point of each Tn*5* insertion and the identification number of the related pMG plasmid. pMG122-1 and pMG122-2 are Tn*5* insertions with altered bacteriocin expression. The insertions from pMG122-3 to pMG122-6 did not affect bacteriocin expression. Bac, bacteriocin expression on *E. faecalis* OG1X or *E. hirae* 9790; Imm, immunity or resistance against bacteriocin 31 on *E. hirae* 9790. n.d., the plasmid did not transform *E. hirae* 9790. leaky, when an inoculum of 10^8 or more OG1X(pMG122-2) cells was used in the soft-agar assay, the strain exhibited bacteriocin activity; however, this activity was not observed when the inoculum was less than 10^8 cells. This is described in the text.

which is in the same proximity, was also performed (Fig. 3). ORF1, which was designated *bacA*, encoded a 67-amino-acid protein with a molecular mass of 7.5 kDa. The ATG start codon was preceded by a putative potential ribosome binding site (G A G G T G A) 6 bp upstream. The deduced BacA protein had a span of hydrophobic residues typical of a signal sequence at its amino terminus, and a potential signal peptidase processing site corresponding to the V-E-A sequence was located at positions 22 to 24 (Fig. 4A). Comparison of the primary structure of the deduced amino acid sequences of the BacA mature protein showed 35 to 37% homology of the identical residues with the mature proteins of sakacin A, sakacin P, sakacin 674, curvacin A, carnobacteriocin BM1, pediocin PA-1, and leucocin A, which are class II bacteriocins produced by lactic acid bacteria (Fig. 4B). As with class II bacteriocins (2, 5, 28, 30), the peptide had a hydrophilic N-terminal region, contained the consensus sequence Tyr-Gly-Asn-Gly-Val-X-Lys-, and had a relatively hydrophobic C-terminal region. It also had a large number of residues identical to those in class II bacteriocins (Fig. 4B). The putative signal sequence (24 amino acid residues) did not show any significant homology with any other reported protein or leader peptides.

ORF2, designated *bacB*, encoded a 94-amino-acid protein with a molecular mass of 11.0 kDa. The ATG start codon was preceded by a potential ribosome binding site (A A G G A) 10 bp upstream. There was no obvious promoter sequence upstream of the start codon. Comparison of the amino acid sequence of *bacB* did not show significant homology with any other reported protein.

ORF3 encoded 90 amino acid residues with a molecular mass of 10.3 kDa. The start codon was preceded by a potential ribosome binding site (A G G A G A) 5 bp upstream. There was no obvious promoter sequence upstream of the start codon. A putative transcription termination signal for ORF3, consisting of 16-base inverted repeats separated by 10 bases, was identified downstream of ORF3. This structure has an estimated free energy of -13.1 kcal (1 cal = 4.184 J)/mol and may serve as the transcription termination signal. Comparison of the primary structure of the deduced amino acid sequence of ORF3 showed a high degree of homology of the identical residues (60%) with the deduced amino acid sequence (88 amino acid) of ORF-α2, which is located immediately downstream of ORF-α1 in carnobacteriocin BM1 (35). The carnobacteriocin BM1 is produced by *Carnobacterium piscicola* LV17B and is a class II bacteriocin. The function of the product of ORF3 or ORF-α2 (30) remains unknown.

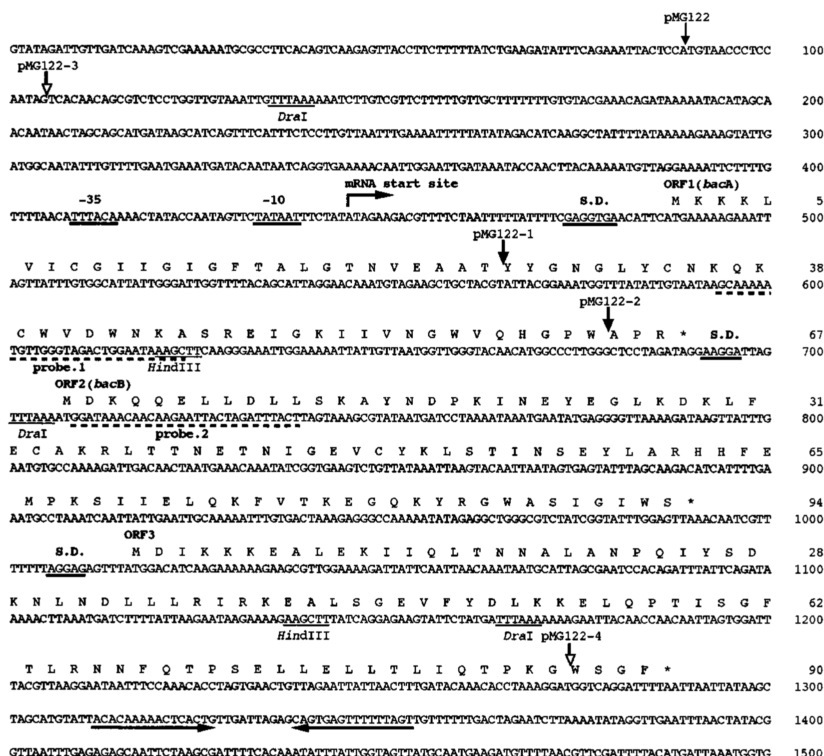

FIG. 3. Nucleotide sequence of the bacteriocin region of plasmid pYI17 and the deduced amino acid sequence. The potential promoter (−10 and −35) and Shine-Dalgarno (S.D.) ribosome binding sequence are underlined. Inverted-repeat sequences are indicated by horizontal arrows and are described in the text. The vertical arrows of pMG122-1 and pMG122-2 mark the locations of Tn*5* insertions that gave rise to altered bacteriocin expression. The vertical open arrows of pMG122-3 and pMG122-4 mark the locations of the Tn*5* insertions that gave rise to normal bacteriocin expression. The vertical arrow of pMG122 indicates the end of plasmid pMG122, which was generated by a nested-deletion process (Fig. 2). The sequences of synthetic oligonucleotides used for Northern blot analyses and primer extension analysis are underlined with dashed lines (probes 1 and 2). An mRNA start site was determined by primer extension. Accession number, D78257.

Analysis of transcripts of *bacA*, *bacB*, and ORF3. DNA sequence analysis revealed that *bacA*, *bacB*, and ORF3 were transcribed in the same (left-to-right) direction. The potential promoter sequence was identified upstream of the start codon of *bacA*, and a putative transcription terminator signal was identified downstream of ORF3. There was no obvious promoter sequence or terminator between *bacA* and *bacB* or between *bacB* and ORF3. The transcripts of these genes were analyzed by Northern hybridization and primer extension analysis, as described in Materials and Methods. The results of Northern analyses are shown in Fig. 5. Probe 1, which corresponded to a 30-base sequence in *bacA* (Fig. 5a), detected one transcript (about 900 bases). Probe 2, which corresponded to a 30-base sequence in *bacB*, detected a band similar in size to the band detected with probe 1 (Fig. 5b).

Primer extension was used to determine the transcription start sites. As the results of Northern analysis show, transcription appeared to be initiated at a promoter upstream of *bacA*. Figure 6 shows the results of primer extension analysis performed on RNA prepared by hybridization with probe 1. Transcription was found to start with an A base located 7 bases downstream of the −10 box. These results indicated that transcription initiated from a promoter upstream of *bacA*, continued through *bacB* and ORF3, and terminated at the terminator downstream of ORF3. Thus, the bacteriocin 31 determinant consisted of an operon construct containing the *bacA*, *bacB*, and ORF3 genes.

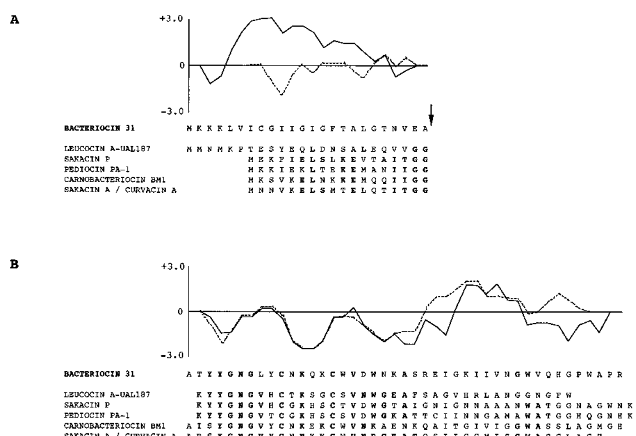

FIG. 4. Comparison of the amino acid sequence of the predicted BacA protein of bacteriocin 31 with the amino acid sequence of bacteriocins containing the consensus sequence Tyr-Gly-Asn-Gly-Val (Y G N G V) of the class II lactic acid bacterium bacteriocins and their hydropathy profiles. (A) Protein hydropathy profiles of the predicted signal peptide of BacA and the leader peptide of sakacin A, which is representative of class II bacteriocins, are shown at the top. Solid line, BacA; dashed line, sakacin A. Amino acid sequences of the predicted signal peptide of the BacA N-terminal extension and leader peptides with the GG motif of lactic acid bacterium bacteriocins are shown below. The vertical arrow indicates the cleavage site in the prebacteriocins. (B) Protein hydropathy profiles of the mature protein of BacA and sakacin A, which is representative of class II bacteriocins, is shown at the top. Solid line, BacA; dashed line, sakacin A. Amino acid sequences of the predicted mature protein of BacA and class II bacteriocins are shown below.

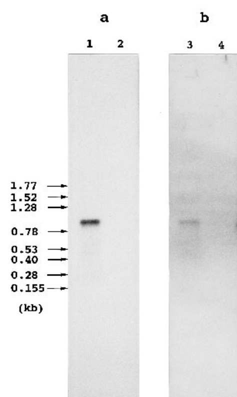

FIG. 5. Northern blot analyses of the *bacA* and *bacB* genes. Probe 1 and probe 2 are shown in Fig. 3. Total mRNA prepared from *E. faecalis* FA2-2 cells carrying pYI17 was tested with probe 1 (a) and probe 2 (b). Lanes 1 and 3, cells harboring pYI17; lanes 2 and 4, plasmid-free cells. The positions of the RNA markers are noted on the left.

DISCUSSION

The bacteriocin determinant for bacteriocin 31, which consists of two genes, was isolated from the *E. faecalis* pheromone-responsive conjugative plasmid pYI17. Deletion mutant analysis showed that a region of 0.9 kb spanning map positions 3.0 to 3.9 kb was required for bacteriocin expression. This plasmid carried the *bacA* and *bacB* genes in this order in the regions spanning map positions 3.37 to 3.57 kb and 3.59 to 3.87 kb, respectively. Transformation with a plasmid carrying the two genes conferred bacteriocin activity on *E. faecalis* OG1X and *E. hirae* 9790. The deletion mutant pMG124, which contains only *bacA*, expressed bacteriocin activity in *E. faecalis* OG1X but did not transform *E. hirae* 9790, indicating that *bacA* encoded the bacteriolysin of the bacteriocin determinant and implying that *bacB* determined immunity.

A characteristic of bacteriocin-producing strains is the ability to resist the action of their own bacteriocin through a specific immunity mechanism. Bacteriocin 31 was active against *E. hirae* 9770 and *E. faecium* but showed no activity against *E. faecalis. E. hirae* 9790 was unable to support a derivative with the bacteriocin determinant impaired in immunity. Clones containing the two genes transformed *E. hirae* 9790 at an efficiency of 10^4 to $10^3/\mu g$ of DNA. The efficiency of transformation was sufficient for the introduction of DNA into *E. hirae* 9790; however, as mentioned above, the deletion mutant (pMG124) containing only *bacA* did not transform *E. hirae* 9790. These results indicated that *E. hirae* 9790 was unable to harbor a derivative defective in *bacB* and that *bacB* determined the immunity associated with bacteriocin 31.

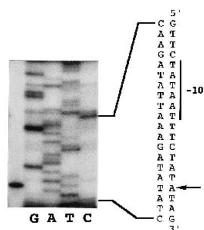

FIG. 6. Primer extension analysis. The primer used is probe 1, which is located within the *bacA* gene (Fig. 3), and total mRNA was prepared from FA2-2(pYI17). The results are shown on the left, alongside the DNA sequence determined by using the same primer and a DNA template prepared from *E. coli* DH1(pMG122), which represents this region. The site of the first nucleotide is indicated by the arrow and is also shown in Fig. 3.

The deletion of the *bacA* coding region or the insertion of Tn*5* into the *bacA* coding region of the cloned fragment containing the *bacA* and *bacB* genes resulted in defective expression of the bacteriocin and immunity. These results suggested that the defective immunity resulted from a polar effect by the deletion of, or insertion into, *bacA*. Northern hybridization and primer extension analysis of the bacteriocin coding region indicated that transcription initiated from the *bacA* promoter, read through *bacB* and ORF3, and terminated at a termination signal downstream of ORF3.

Bacteriocin 31 showed a relatively narrow spectrum of activity and, of the enterococci examined, was active against *E. hirae* and *E. faecium* but not against *E. faecalis*. Genetic and sequence analysis showed that the BacA gene encoded a 67-amino-acid prepeptide protein, with a putative signal sequence of 24 residues at the N terminus, which is predicted to give rise to a 43-amino-acid mature protein. The deduced amino acid sequence of the mature protein of BacA showed a high degree of homology of the identical residues with the class II bacteriocin of lactic acid bacteria. These bacteriocins are heat-stable, nonlanthionine, membrane-active peptides characterized by a Gly (−2)–Gly (−1)–X(+1) processing site in the bacteriocin precursor. The N-terminal region of these peptides is hydrophilic (Fig. 4B), contains the consensus sequence Tyr-Gly-Asn-Gly-Val-Xaa-Lys- (Fig. 4B), shares a large number of identical residues, and contains cysteins that may participate in the formation of a disulfide bridge. These similarities indicate that the N terminus is essential to the activity and specificity of these bacteriocins. The most hydrophobic region of these peptides is the C-terminal region (Fig. 4B). Although the precise mode of bacteriocin action remains unknown, they are considered to be membrane-active peptides that destroy cell integrity via the formation of membrane channels in which amphiphilic α-helices or β-sheets form two faces, one hydrophilic and one hydrophobic (5, 28, 30). These structural motifs have been predicted for a number of bacteriocins (28, 30), and it has been suggested that pore formation occurs through the barrel stave mechanism (28, 30).

The putative signal sequence of BacA did not show sequence homology with the bacteriocins of lactic acid bacteria (Fig. 4A). The putative signal peptidase recognition site of the putative signal sequence of BacA was predicted to be V-E-A. The hydropathy analysis profile of BacA indicated that the leader sequence was strongly hydrophobic. Comparison of the N-terminal extensions of the lactic acid bacterium bacteriocins reveals a high degree of homology in the amino acid sequences and a conserved Gly-Gly motif located at positions −1 and −2 of the suspected proteolytic processing site (28, 30). Analysis of the hydropathy profiles of the leader sequences of lactic acid bacterium bacteriocins indicates that they are relatively hydrophilic (28, 30).

These results suggest that the consensus sequence, the number of identical residues in the N-terminal region, and the hydrophobicity of the C-terminal region are features shared by membrane-active peptides originating from *E. faecalis* or lactic acid bacteria.

Bacteriocin 31 is the third type (type 3) of bacteriocin to be analyzed either genetically or biochemically in *E. faecalis*. All three types of determinants are located on pheromone-responding conjugative plasmids (11, 23, 32, 33). The other two bacteriocins are hemolysin/bacteriocin (type 1) (4) and AS-48 (type 2), which are encoded by plasmids pAD1 (59 kb) (11, 23) and pMB2 (56 kb) (32, 33), respectively. The genes of the hemolysin/bacteriocin are located in an approximately 7-kb

region and consist of two regions for the production of the L (lysin) and A (activator) components (23). Component A activates component L, and the activated L possesses the hemolysin/bacteriocin activity. Component A also plays a role in immunity to the bacteriocin produced (23). Recent reports showed that four genes (i.e., $cylL_L$, $cylL_S$, *cylM*, and *cylB*) required for expression of the cytolysin precursors of $CylL_L$ and $CylL_S$ (19) and the A component (cytolysin A) share physical and biochemical features with serine protease (38). AS-48 is a peptide antibiotic and is active against a wide variety of gram-positive and gram-negative bacteria (32, 33). This determinant has not yet been cloned, but biochemical analysis of AS-48 and sequence analysis of the determinant for peptide AS-48 has been reported. The mature AS-48 protein consists of 70 amino acid residues and is believed to be a cyclic molecule produced by posttranscriptional modification (32).

Research is now under way to determine whether the features characteristic of bacteriocin 31 are common to other *E. faecalis* bacteriocins and to define the function of ORF3.

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