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Cloning and Genetic and Sequence Analyses of the Bacteriocin 21 Determinant Encoded on the *Enterococcus faecalis* Pheromone-Responsive Conjugative Plasmid pPD1

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The pheromone-responsive conjugative plasmid pPD1 (59 kb) of *Enterococcus faecalis* encodes the bacteriocin 21 (*bac21*) determinant. Cloning, transposon insertion mutagenesis and sequence analysis of the *bac21* determinant showed that an 8.5-kb fragment lying between kb 27.1 and 35.6 of the pPD1 map is required for complete expression of the bacteriocin. The 8.5-kb fragment contained nine open reading frames (ORFs), *bacA* to *bacI*, which were oriented in the same (upstream-to-downstream) direction. Transposon insertions into the *bacA* to *bacE* ORFs, which are located in the proximal half of *bac21*, resulted in defective bacteriocin expression. Insertions into the *bacF* to *bacI* ORFs, which are located in the distal half of *bac21*, resulted in reduced bacteriocin expression. Deletion mutant analysis of the cloned 8.5-kb fragment revealed that the deletion of segments between kb 31.6 and 35.6 of the pPD1 map, which contained the distal region of the determinant encoding *bacF* to *bacI*, resulted in reduced bacteriocin expression. The smallest fragment (4.5 kb) retaining some degree of bacteriocin expression contained the *bacA* to *bacE* sequences located in the proximal half of the determinant. The cloned fragment encoding the 4.5-kb proximal region and a Tn*916* insertion mutant into pPD1 *bacB* trans-complemented intracellularly to give complete expression of the bacteriocin. *bacA* encoded a 105-residue sequence with a molecular mass of 11.1 kDa. The deduced BacA protein showed 100% homology to the broad-spectrum antibiotic peptide AS-48, which is encoded on the *E. faecalis* conjugative plasmid pMB2 (58 kb). *bacH* encoded a 195-residue sequence with a molecular mass of 21.9 kDa. The deduced amino acid sequence showed significant homology to the C-terminal region of HlyB (31.1% identical residues), a protein located in the *Escherichia coli* alpha-hemolysin operon that is a representative bacterial ATP-binding cassette export protein.

It is known that many *Enterococcus faecalis* strains produce bacteriocins (3, 6). Bacteriocins are bacterial proteins or peptides which inhibit the growth of other bacteria that are closely related to the producer strain. They usually exhibit a relatively narrow spectrum of activity. However, it has become evident that many of the *E. faecalis* bacteriocins studied to date have a somewhat broader spectrum of activity, affecting more distantly related species (3, 19, 36). Bacteriocins are thought to provide the producer strain with a selective advantage over other strains and are a factor in bacterial virulence. The *E. faecalis* 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 specific peptide sex pheromones secreted by potential recipients (6, 7, 12, 13, 26, 45, 46). The sex pheromone induces the formation of a mating aggregate (6, 7, 12).

To date, three types of *E. faecalis* bacteriocins have been genetically and biochemically characterized. These include hemolysin/bacteriocin (type 1) (3), the peptide antibiotic AS-48 (type 2) (37), and bacteriocin 31 (type 3) (51), which are encoded by the *E. faecalis* conjugative plasmid pAD1 (58 kb) (7, 9, 26, 50), pMB2 (58 kb) (36), and pYI17 (57.5 kb) (51), respectively. Of these plasmids, pAD1 and pYI17 are pheromone-responsive plasmids. pAD1, pMB2, and pYI17 were originally isolated from *E. faecalis* subsp. *zymogenes* DS16 (50), *E. faecalis* subsp. *liquefaciens* S-48 (36), and *E. faecalis* YI717 (51), respectively.

The hemolysin/bacteriocin of pAD1 has been associated with virulence in animal models (4, 31, 33). A significant number of *E. faecalis* clinical isolates produce hemolysin/bacteriocin (10, 32). More than 50% of the hemolytic clinical isolates carry transferable hemolysin determinants (32). pAD1 is a typical *E. faecalis* hemolysin/bacteriocin plasmid (27, 35). The mechanism of hemolysin/bacteriocin production in *E. faecalis* has been studied in detail with the hemolysin/bacteriocin determinant of pAD1 (23, 24, 28, 42). The hemolysin/bacteriocin determinant is encoded on the 7-kb region of pAD1 and consists of an L region and A region (28). The L region is composed of four open reading frames (ORFs), $cylL_L$, $cylL_S$, $cylL_M$, and $cylL_B$ (24), and the A region contains *cylA* (42). The CylA protein has physical and biochemical features in common with serine protease (42). Component A also plays a role in immunity to the bacteriocin produced (24, 28, 30, 42). The $CylL_L$ and $CylL_S$ proteins are posttranslationally modified by $CylL_M$ (2, 31), and the modified $CylL_L$ and $CylL_S$ are secreted via $CylL_B$, which is the ATP-binding exporter (23). The extracellular cytolysin precursors, $CylL_L$ and $CylL_S$, are activated to the active cytolysin by CylA (2, 31). AS-48 (type 2) is a peptide antibiotic which is active against a wide variety of gram-positive and gram-negative bacteria (21, 36, 37). This determinant has not yet been cloned, but biochemical analysis of AS-48 and sequence analysis of the determinant for peptide AS-48 have 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 (21, 37). Bacteriocin 31 (type 3) is active against *Enterococcus hirae* 9790, *Enterococcus*

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant genotype or phenotype	Comment(s)	Reference or source
Strains			
E. faecalis			
FA2-2	Rif^r Fus^r	Derivative of JH2	9
JH2SS	Str^r Spc^r	Derivative of JH2	49
OG1X	Str^r	Protease-negative derivative of OG1-10	29
UV202	Rif^r Fus^r	UV-sensitive mutant of JH2-2	54
E. coli			
DH1	F *recA1 endA1 gyrA94 thi-1 relA1 hsdR17 supE44*		40
DH5α	*recA1 endA1 gyrA96 thi-1 relA1 hsdR17 supE44* Δ*lacU169* ϕ80 *lacZ*ΔM15	Bethesda Research Laboratories	
TH688	CSH57b *thr*::Tn*5*		47
S. aureus FDA209P			
Plasmids			
pPD1	Bac	59-kb conjugative plasmid from strain 39-5	54
pAM401	Cat^r Tet^r	*E. coli-E. faecalis* shuttle vector	53
pMW119	Amp^r, *lacZ*	*E. coli* vector, low copy number	Nippon Gene Co., Ltd.
pE/BDFJ	Bac Cat^r	*Eco*RI-relational clone of pPD1; pAM401 containing the *Eco*RI B, D, F, and J fragments	19
pHT1	Bac Cat^r	Deleted subclone derived from pE/BDFJ	This study
pMBS221	Amp^r	Subclone for DNA sequencing analysis; pMW119 containing the 19.7-kb *Bam*HI-*Sal*I fragment of pPIT7022	19

faecium, and *Listeria monocytogenes* and exhibits a relatively narrow spectrum of activity (51). The bacteriocin 31 determinant consists of the bacteriocin gene (*bacA*) and an immunity gene (*bacB*). The predicted mature *bacA* protein (43 amino acids) showed sequence homology to the membrane-active class II bacteriocins of lactic acid bacteria.

pPD1 (59 kb) encodes the response to the sex pheromone cPD1 and bacteriocin production (6, 55). It is another well-studied plasmid in the pheromone-related conjugation system of *E. faecalis* (19, 20, 39, 48, 54). Recently, the pPD1 (58-kb) physical map (19) and the sequence and genetic analysis of the determinant related to the pheromone response on pPD1 have been reported (19, 39, 48). We have also shown that the bacteriocin activity encoded on pPD1 is lethal to a wide variety of gram-positive bacteria including *Staphylococcus aureus* and that the determinant is located between kb 27.1 and 35.6 of the pPD1 map (19). In this report, we describe the cloning and genetic and sequence analyses of the bacteriocin 21 (Bac21) determinant encoded on the *E. faecalis* pheromone-responsive conjugative plasmid pPD1.

MATERIALS AND METHODS

Bacteria, media, and regents. The strains and plasmid used in this study are listed in Table 1. The Tn*917* and Tn*916* insertion mutants of the pPD1 bacteriocin determinant, which were isolated in previous studies (19), are listed in Table 2. *E. faecalis* strains were grown in Todd-Hewitt broth (Difco, Detroit, Mich.), antibiotic medium 3 (Difco), or N2GT (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 0.5% (wt/vol) agar, respectively. All cultures were grown at 37°C. Antibiotics were used at the following concentrations: 100 μg of ampicillin per ml, 20 μg of chloramphenicol per ml for *E. faecalis* and 50 μg/ml for *E. coli*, 12.5 μg of erythromycin per ml, 25 μg of rifampin per ml, 25 μg of fusidic acid per ml, 500 μg of streptomycin per ml, 500 μg of spectinomycin per ml, 40 μg of kanamycin per ml, and 6 μg of tetracycline per ml.

Isolation and manipulation of plasmid DNA. Plasmid DNA was isolated by the alkali lysis method (1, 40, 43). It was treated with restriction enzymes and subjected to agarose gel electrophoresis for analysis of DNA fragments. Restriction enzymes were obtained from Nippon Gene (Toyama, Japan), New England Biolabs, Inc., and Takara (Tokyo, Japan) and were used as specified by the suppliers. Agarose was obtained from Wako Chemicals (Osaka, Japan). Gels with a 0.8% agarose concentration were used for the size determination of DNA fragments. A "glass milk" kit (Gene Clean II kit [Bio 101, Inc., La Jolla, Calif.]) or low-melting-point agarose and β-agarose I (Nippon Gene) was used for the elution of the DNA fragments from agarase 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 (18). Transformants were selected on Luria-Bertani medium agar containing suitable antibiotics.

Soft-agar assay for bacteriocin production. The bacteriocin production assay was performed as described previously (28). A 5-μl sample of an overnight culture of the indicator strain, *S. aureus* FDA209P, grown in antibiotic medium 3 was added to 5 ml of molten soft agar (0.5%), 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.

Mating procedures. Broth matings were performed as described previously (6, 12, 26). OG1X, JH2SS, FA2-2, and UV202 were used as host strains in the mating experiments (Table 2).

Generation of the transposon (Tn*5*) insertional mutants. Tn*5* (Km^r) insertion into the cloned bacteriocin 21 plasmid was performed as described elsewhere (46, 47). The target plasmid was introduced into *E. coli* K-12 TH688 (with Tn*5* in the *thr* locus) (47) by chemical transformation (40). Transformants were streaked onto selective media containing 40 μg of kanamycin per ml and 50 μg of chloramphenicol per ml, and the plates were left at room temperature for 10 days. Bacteria which grew on the selective plates were pooled; the plasmid DNA was then isolated and used to transform *E. coli* DH5α (Bethesda Research Laboratories). 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 bacteriocin activity was examined.

DNA sequence analysis. Nucleotide sequence analysis was carried out as previously described (41). A deletion kit (Nippon Gene) was used for the generation of a nested deletional clone set. Plasmid pMBS221 (19), which carried the bacteriocin 21 determinant, had been constructed previously from pPIT7022 (pPD1::Tn*917*) and was used to determine the sequence of the bacteriocin 21 determinant. pMBS221 was constructed by inserting a fragment of approximately 18 kb isolated from between kb 20.05 and 38.05 of pPD1 into the vector pMW119. pMBS221 was digested with *Sal*I and *Sph*I and treated with exonuclease III for various periods. The plasmid was then treated with mung bean nuclease and Klenow fragment. The deleted DNAs were ligated and used to

TABLE 2. Transposon insertion mutants of pPD1 and bacteriocin expression

Location in Fig. 1c	Plasmid(s)[a]	Transposon	Map position (kb)[b]	Location of insert	Bac[c]
	pPD1				++
	pHT1				++
1	pPIT7011	Tn*917*	26.8	Upstream of *bacA*	++
2	pPIT7148	Tn*917*	27.1	Upstream of *bacA*	++
3	pMG301–pMG304	Tn*5*	27.3	*bacA*	−
4	pMG305	Tn*5*	27.4	*bacA*	−
5	pMG306	Tn*5*	27.5	*bacA*	−
6	pMG307	Tn*5*	27.5	*bacA*	−
7	pMG101	Tn*916*	27.6	Between *bacA* and *bacB*	+
8	pMG102, pMG111	Tn*916*	27.6	Between *bacA* and *bacB*	−
9	pMG128, pMG129	Tn*916*	28.3	*bacB*	−
10	pMG126, pMG127	Tn*916*	28.3	*bacB*	−
11	pMG308	Tn*5*	28.5	*bacB*	−
12	pPIT7421	Tn*917*	29.0	*bacB*	−
13	pMG309	Tn*5*	29.0	*bacB*	−
14	pMG116–pMG118	Tn*916*	29.3	*bacB*	−
15	pMG119–pMG122	Tn*916*	29.3	*bacB*	−
16	pMG310	Tn*5*	29.8	*bacC*	−
17	pPIT7431	Tn*917*	30.2	Between *bacC* and *bacD*	+
18	pMG123–pMG125	Tn*916*	30.3	Promoter of *bacD*	−
19	pMG112–pMG115	Tn*916*	30.5	*bacD*	−
20	pMG311	Tn*5*	30.9	*bacE*	−
21	pMG312	Tn*5*	31.0	*bacE*	−
22	pPIT7433	Tn*917*	31.9	*bacF*	+
23	pMG313	Tn*5*	32.5	*bacG*	+
24	pMG314	Tn*5*	32.6	*bacG*	+
25	pMG315, pMG316	Tn*5*	33.0	*bacG*	+
26	pMG317–pMG319	Tn*5*	34.0	*bacH*	+
27	pMG320	Tn*5*	34.1	*bacH*	+
28	pMG321, pMG322	Tn*5*	34.3	*bacI*	+
29	pPIT7214, pPIT7414, pPIT7434	Tn*917*	34.5	*bacI*	+
30	pMG323, pMG324	Tn*5*	34.9	*bacI*	+
31	pMG325	Tn*5*	35.2	*bacI*	+
32	pPIT7006	Tn*917*	36.3	Downstream of *bacI*	++

[a] The host strain of the derivative was *E. faecalis* OG1X. Tn*917* and Tn*916* insertion mutants were isolated in a previous study (19). Tn*5* insertion mutants were isolated in this study.

[b] The map position is the distance from the 0 point of pPD1 (19).

[c] Bac, bacteriocin expression. Symbols: ++, bacteriolysis halo produced by wild-type strain (Fig. 1); +, diameter of wild-type bacteriolysis halo about half that of the wild type (Fig. 1); −, no bacteriolysis.

transform *E. coli* DH5α. The resulting constructs were sequenced 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. Two independent fragments of pE/BDFJ were inserted into vector pMW119 to determine the sequence of the complementary strand of bacteriocin 21. The bacteriocin 21 determinant was located between a *Hin*dIII site at kb 26.4 of pPD1 and an *Eco*RI site at kb 35.8. One subclone contained a 1.6-kb *Hin*dIII fragment originating from the region located between kb 26.4 and 28.0 of the pPD1 map. The other subclone contained a 7.9-kb fragment originating from the kb 27.7-to-35.6 region of the pPD1 map, which contained the *Eco*RI D and F fragments. Sets of nested deletion clones were constructed from these subclones, and the resulting clones were sequenced as described above. For homology analyses of the nucleotide and amino acid sequences, the DNA Data Bank of Japan (DDBJ) (National Institute of Genetics, Mishima, Japan) was used.

Mapping of insertions. The precise locations of the insertions were determined by nucleotide sequencing with appropriate synthetic primers which hybridized to the ends of the transposons (8, 42). Primers for Tn*917* and Tn*5* corresponded to the sequences 5′-GGG AGC ATA TCA CTT TTC TTG GAG-3′ and 5′-CAG ATT TAG CCC AGT CGG-3′, respectively (43, 51). Primers for Tn*916* corresponded to the sequences 5′-AGG ATA AAT CGT CGT ATC AAA GCTC-3′ and 5′-ATG AGG AAA TAT GCA AAG AAA CGTG-3′, respectively, which are located on either side of Tn*916* (8).

Deletion mutant analysis. The cloned pHT1 DNA was digested with *Nco*I, and a *Kpn*I linker was ligated into the *Nco*I site. A nested deletion kit (Nippon Gene) was used to produce deletion mutants. The pHT1 DNA with the new *Kpn*I site was digested with *Kpn*I and *Aat*II and treated with exonuclease III for various periods, followed by incubation with mung bean nuclease and then with Klenow fragment. The deleted DNAs were ligated and used to transform *E. coli* DH5α. The resulting constructs were sequenced with a primer corresponding to the sequence 5′-AAA ATT ACG CCC CGC CCT GC-3′, which is located in the vector DNA, and the sequences of the deleted ends were determined. The deleted DNA was isolated from each *E. coli* DH5α transformant and used to transform *E. faecalis* UV202. The *E. faecalis* UV202 derivatives were tested for bacteriocin activity.

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 no. D85752.

RESULTS

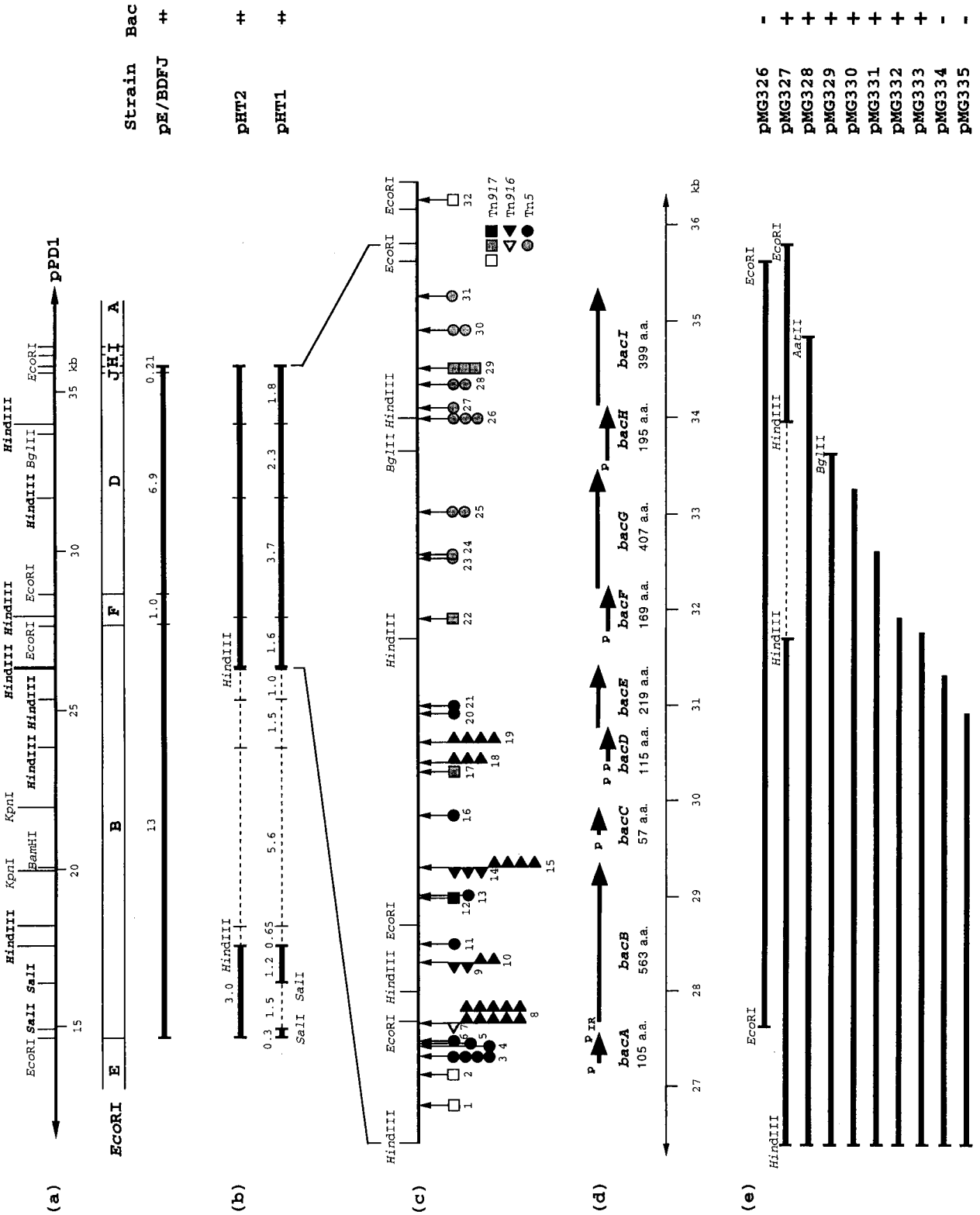
Cloning of the pPD1 bacteriocin 21 determinant. The physical map and the location of the bacteriocin 21 determinant on the pheromone-responsive conjugative bacteriocin plasmid pPD1 have been described previously (19). pPD1 consists of 10 *Eco*RI fragments designated A to J with molecular sizes of 22.5, 13.0, 9.0, 6.9, 5.0, 1.0, 0.6, 0.36, 0.28, and 0.23 kb, respectively. The order of the *Eco*RI fragments is AGCEBFDJHI in a clockwise orientation (19). Tn*917* or Tn*916* transposon mutagenesis of pPD1 shows that a minimum of 6.9 kb is required for bacteriocin 21 expression (19). The region involved lies between kb 27.1 and 35.6 of the pPD1 map (19) and includes *Eco*RI fragments D and F and part of fragment B (19). Plasmid pE/BDFJ, which contains *Eco*RI fragments B, D, F, and J in pAM401, confers bacteriocin 21 expression on *E. faecalis* (19).

In this study, the first step was cloning of the bacteriocin 21 determinant. To locate the precise region involved in bacteriocin 21 expression carried on pE/BDFJ, subcloning was used to construct pE/BDFJ deletion derivatives (Fig. 1). Since pE/BDFJ contains eight *Hin*dIII sites, it was partially digested with this enzyme, and the digested DNAs were self-ligated and transformed into *E. coli* DH1. The plasmid DNAs were isolated from the *E. coli* transformants and were used for transformation of *E. faecalis* OG1X. The *E. faecalis* OG1X transformants were examined for bacteriocin activity. The subclone pHT2 contained the smallest fragment conferring bacteriocin activity (Fig. 1). pHT2 possessed a deletion of a fragment containing four *Hin*dIII fragments located between kb 17.6 and 26.4 of the pPD1 map (Fig. 1a). pHT2 was then digested with *Sal*I to delete a 1.5-kb *Sal*I fragment located between kb 14.9 and 16.4 of the pPD1 map (19) and was self-ligated with T4 DNA ligase. The resulting subclone, designated pHT1 (Fig. 1a), encoded bacteriocin 21 expression. pHT1 contained a region originating from the sequence between kb 26.4 and 35.8 of the pPD1 map. These results were consistent with results showing that Tn*917* or Tn*916* insertions between kb 27.6 and 34.5 of the pPD1 map affected the bacteriocin phenotype (19).

DNA sequence analysis of the cloned bacteriocin 21 determinant. DNA sequence analysis of the pHT1 9.4-kb region was performed as described in Materials and Methods. Computer analysis revealed the presence of nine ORFs in the region, and all were oriented in the same direction (Fig. 1d).

Figure 2 shows the nucleotide sequences of the ORFs preceded by a consensus ribosome-binding site (44) within 20 bases of the predicted start codon and the deduced amino acid sequence. The ORFs were named *bacA* to *bacI* in order starting with the ORF located furthest upstream. The sizes of all the putative gene products are shown in Fig. 1d.

The deduced amino acid sequences of the nine ORFs were


(a)
pPD1
EcoRI SalI SalI HindIII KpnI BamHI KpnI HindIII HindIII HindIII EcoRI EcoRI HindIII HindIII BglII HindIII EcoRI
15 20 25 30 35 kb
EcoRI
E B F D JHI A
13 1.0 6.9 0.21
Strain Bac
pE/BDFJ +
pHT2 +
pHT1 +
(b)
3.0 HindIII
0.3 1.5 1.2 0.65 5.6 1.5 1.0 1.6 3.7 2.3 1.8
SalI SalI
HindIII
(c)
HindIII EcoRI HindIII EcoRI HindIII BglII HindIII EcoRI EcoRI
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32
Tn917
Tn916
Tn5
(d)
P P IR
bacA 105 a.a.
bacB 563 a.a.
bacC 57 a.a.
P P
bacD 115 a.a.
bacE 219 a.a.
P
bacF 169 a.a.
bacG 407 a.a.
P
bacH 195 a.a.
bacI 399 a.a.
27 28 29 30 31 32 33 34 35 36 kb
(e)
EcoRI
HindIII
EcoRI
HindIII
AatII
BglII
HindIII
pMG326 -
pMG327 +
pMG328 +
pMG329 +
pMG330 +
pMG331 +
pMG332 +
pMG333 +
pMG334 -
pMG335 -

compared with those of other known proteins by using the DDBJ service. *bacA* encoded a 105-residue peptide with a molecular mass of 11.1 kDa. A putative transcription termination site for *bacA*, consisting of a 17-base inverted repeat separated by 9 bases, was identified downstream of *bacA*. The deduced protein, BacA, showed 100% homology to the antibiotic peptide AS-48 (37) (Fig. 3). The nucleotide sequence of the promoter region preceding the 5′ end of *bacA* and the downstream region of the TAA stop codon of *bacA* also showed complete homology to the AS-48 sequence. Two nucleotides in the *bacA* sequence differed from the AS-48 sequence (Fig. 3).

bacH encoded a 195-residue peptide with a molecular mass of 21.9 kDa. The deduced amino acid sequence of the BacH protein showed significant homology to the C-terminal region of the ATP-binding export proteins (11, 15, 16, 23, 25, 52). Figure 4 shows a comparison between the deduced amino acid sequence and the amino acid sequence of the C-terminal region of representative ATP-binding export proteins. There was about 26 to 33% homology between the deduced amino acid sequence of the BacH protein and the C-terminal region of the ATP-binding export proteins (Fig. 4). The BacH protein showed the greatest homology in the amino acid sequence to the C-terminal 191 amino acids of the deduced amino acid sequence of the HlyB protein encoded on the *E. coli* alpha-hemolysin determinant. The largest clusters of homologous residues in BacH and the HlyB protein lie between BacH amino acids 6 to 15 and amino acids 111 to 142, which includes a stretch of 9 of 10 and 23 of 32 identical residues, respectively (Fig. 4). The consensus amino acid sequence for the putative ATP-binding site, the sequence GXGKST, was found to lie between amino acids 9 and 14 of the BacH protein.

Comparison of the amino acid sequences encoded by other ORFs did not show significant homology to those of other reported proteins.

Bacteriocin expression and the precise locations of Tn*917* and Tn*916* insertion mutants. Throughout this paper, the term "bacteriocin expression" is used to indicate a phenotype associated with external bacteriocin activity and is unrelated to actual gene expression. When bacteriocin activity was examined by the soft agar method as described in Materials and Methods, the Tn*917* or Tn*916* insertion mutants were observed to exhibit altered bacteriocin expression (Fig. 5). One type of mutant produced no bacterial lysis zone, indicating that the mutants were completely defective for bacteriocin activity. The other mutants produced bacterial lysis zones with diameters approximately half that of the wild-type strain, indicating that the mutants expressed the reduced bacteriocin activity. A typical bacteriolytic zone produced by each type of mutant is shown in Fig. 5.

The precise locations of the insertions in 5 Tn*917* and 29 Tn*916* insertion mutants were determined by sequence analysis as described in Materials and Methods. The location of the insertion of each transposon and the level of bacteriocin expression shown by the mutant strains are shown in Fig. 1c, Table 2, and Fig. 2. Tn*917* insertion into *bacB* resulted in defective bacteriocin activity. Insertion into the noncoding region between *bacC* and *bacD* or into *bacF* and *bacI* resulted in reduced bacteriocin expression. For the Tn*916* insertion mutant, the pMG101 insert was mapped to the noncoding region 18 bp upstream of *bacB*, and this resulted in reduced bacteriocin expression. The other Tn*916* insertion mutants resulted in the complete disruption of bacteriocin expression. The series of pMG102 to pMG111 inserts were mapped to the noncoding region 18 bp upstream of *bacB*; these also resulted in the complete disruption of bacteriocin expression. In this case, Tn*916* was inserted in the orientation opposite that of the pMG101 insert.

Tn*5* insertion mutant analysis of pHT1 plasmid. For detailed analysis of the bacteriocin determinant, mutants with altered bacteriocin expression were isolated by Tn*5* insertion into the pHT1 plasmid, as described in Materials and Methods. A total of 24 Tn*5* insertional mutants were obtained. Of these mutants, 12 exhibited defective bacteriocin activity while the other 12 exhibited reduced bacteriocin expression. The precise locations of the Tn*5* insertions into the ORFs were determined by DNA sequence analysis, and the results are shown in Fig. 2. Insertions into *bacA*, *bacB*, *bacC*, and *bacE* resulted in disruption of bacteriocin expression. Insertion into *bacF*, *bacG*, *bacH*, and *bacI* resulted in reduced bacteriocin expression.

Deletion mutant analysis of pHT1. A combination of the direct subcloning of restriction fragments and nested deletions was used to produce deletion mutants of plasmid pHT1 (Fig. 1e). Deletion mutants which possessed deletions of segments that lie between kb 31.6 and 35.8 of the pPD1 map produced a reduced level of bacteriocin activity. The region between kb 31.6 and 35.8 contained *bacF*, *bacG*, *bacH*, and *bacI*, which are involved in the distal half of the determinant. Deletion mutants which possessed deletions in the proximal half of the determinant did not express the bacteriocin activity (Fig. 1e). The deletion mutant pMG326, which possessed a deletion in the *bacA* gene, also did not express the bacteriocin activity.

Complementation studies. Deletion mutant analysis showed that the deletion of a region between kb 31.6 and 35.8 of the pPD1 map resulted in reduced bacteriocin expression. The smallest fragment having any degree of bacteriocin expression contained the *bacA*-to-*bacE* region of the proximal half of the determinant and was cloned as a 5.2-kb fragment in pMG333. This region was located between kb 26.4 and 31.6 of the pPD1 map. These results indicate that a region spanning map positions kb 31.6 to 35.8, which contains the distal half of the determinant, was required for complete bacteriocin expression. To examine whether the distal half of the determinant from an insertion mutant with a disrupted proximal region of the pPD1 determinant could restore full bacteriocin expression, complementation studies were performed. The insertion mutant pMG116, which has a Tn*916* insertion into *bacB*, and pPIT7214, which has a Tn*916* insertion into *bacI*, were trans-

FIG. 1. Physical map of the 21.11-kb fragment of pPD1 (which is carried on pE/BDFJ), subclones, and transposon insertions into the subclone. Heavy lines represent the cloned pPD1 DNA fragment. Dotted lines represent deletions in pPD1. Bac ++, complete bacteriocin expression; +, reduced bacteriocin expression; −, no bacteriocin expression. (a) Physical map of the 21.11-kb fragment of pPD1 (19). (b) Deletion mutants of pE/BDFJ. (c) Map of transposon insertions. The arrows indicate the points of transposon insertion. The number of each transposon insertion is shown in Table 2. Symbols below the arrows: square; Tn*917* insertions; horizontal arrowheads, Tn*916* insertions (the direction of each arrowhead shows the orientation of the Tn*916* insertion; i.e., the direction of the arrowhead corresponds to the right end of Tn*916*); circle, Tn*5* insertions; solid symbols, no bacteriocin expression; hatched symbols, reduced bacteriocin expression; open symbols, normal bacteriocin expression. Numbers below the symbols indicate the location numbers of the insertions. (d) Horizontal arrows show deduced ORFs in the bacteriocin 21 determinant and the direction of ORF transcription. The number under each ORF shows the number of deduced amino acid (a.a.) residues encoded on the ORF. P and IR indicate the potential promoter sequence (44) upstream of the ORF and the inverted-repeat sequences identified downstream of the ORF, respectively (Fig. 2). (e) Deletion mutants of pHT1. pMG326 was obtained by direct subcloning of the *Eco*RI fragment of pHT1. Deletion mutants pMG327 through pMG335 were obtained by a nested deletion as described in Materials and Methods. The deleted ends were determined by DNA sequencing.

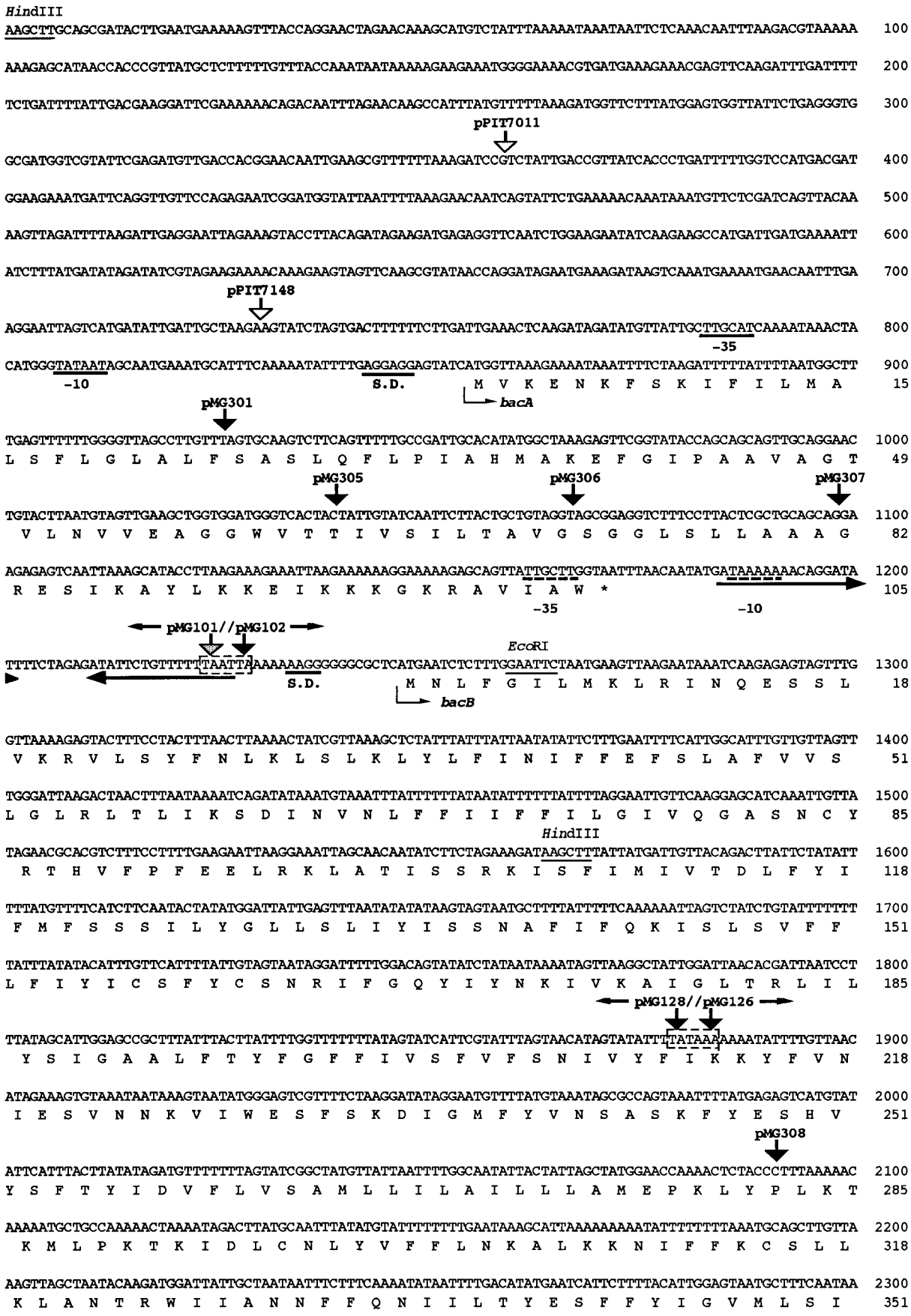

HindIII
pPIT7011
pPIT7148
-35
-10
S.D.
bacA
pMG301
pMG305
pMG306
pMG307
pMG101//pMG102
EcoRI
bacB
pMG128//pMG126
pMG308

FIG. 2

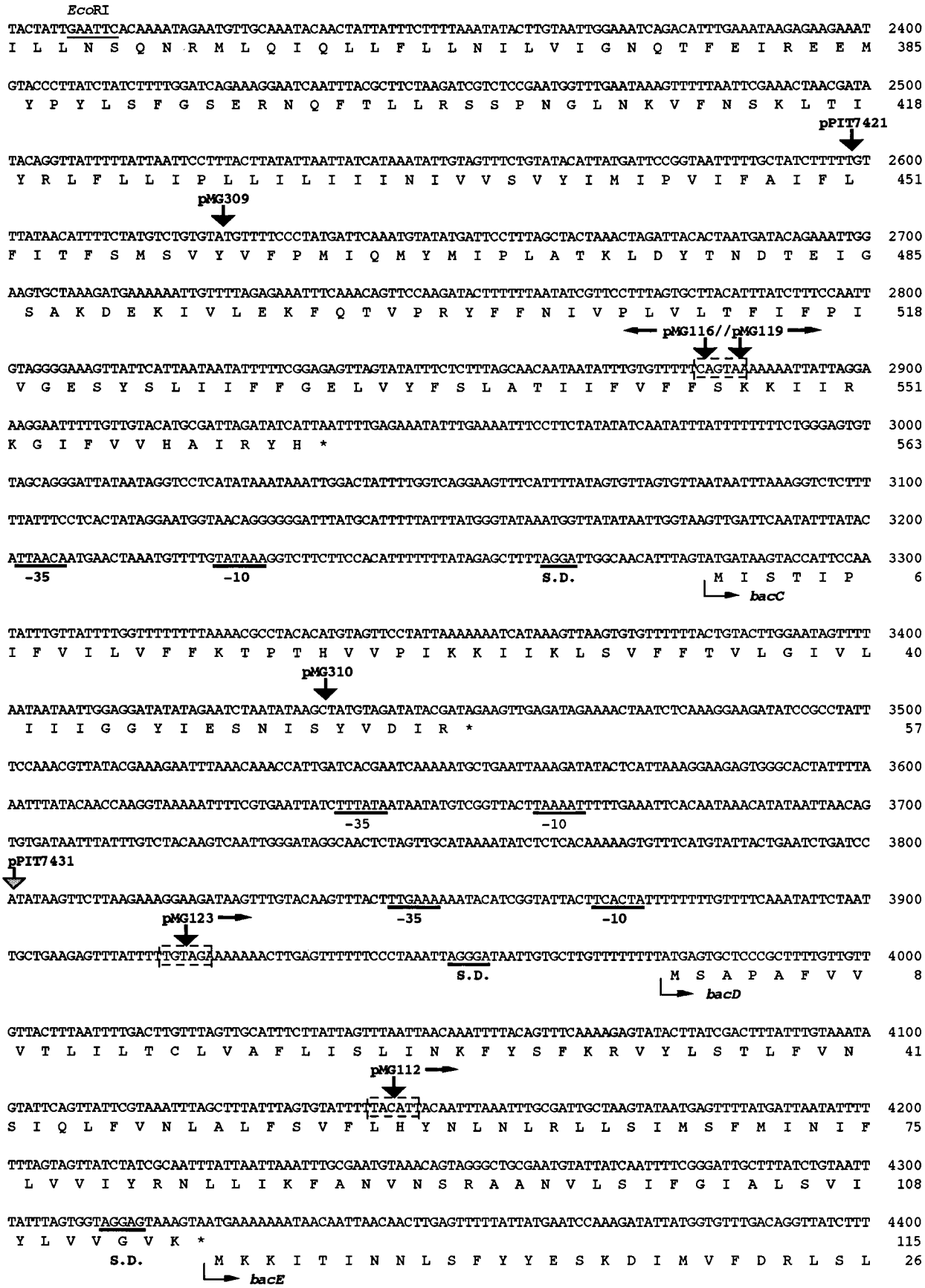


FIG. 2—*Continued.*

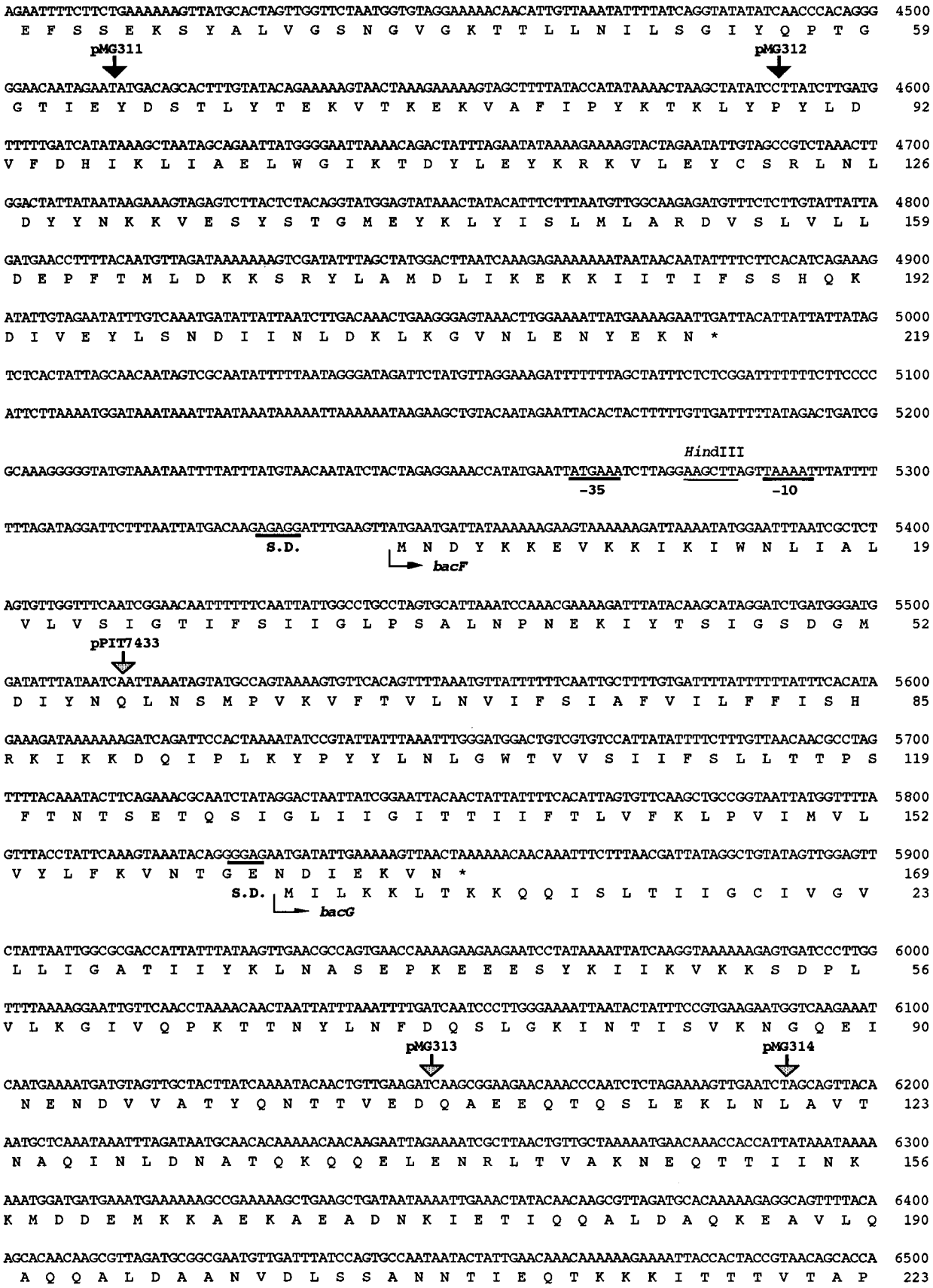


FIG. 2—*Continued.*

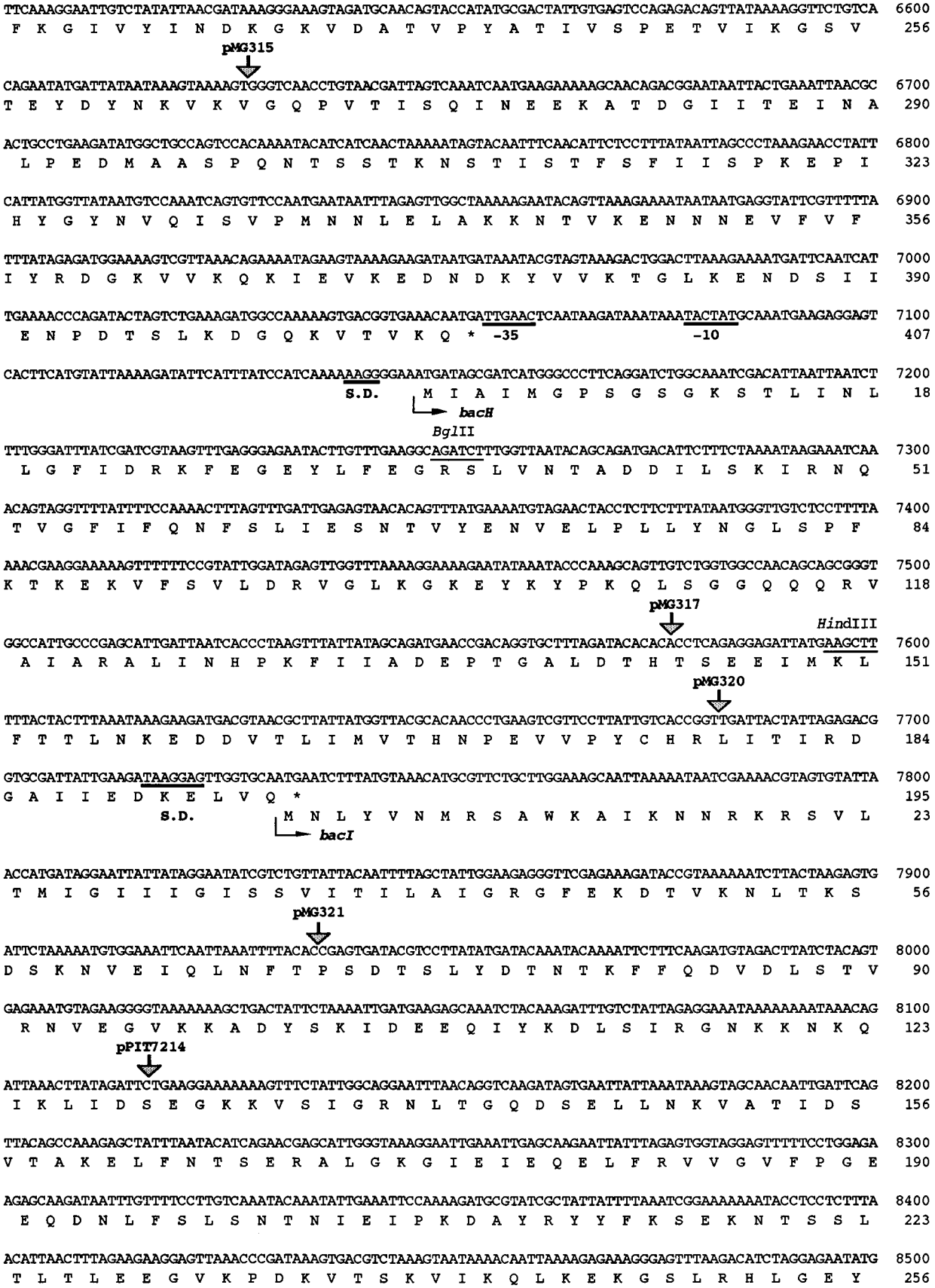


FIG. 2—*Continued.*

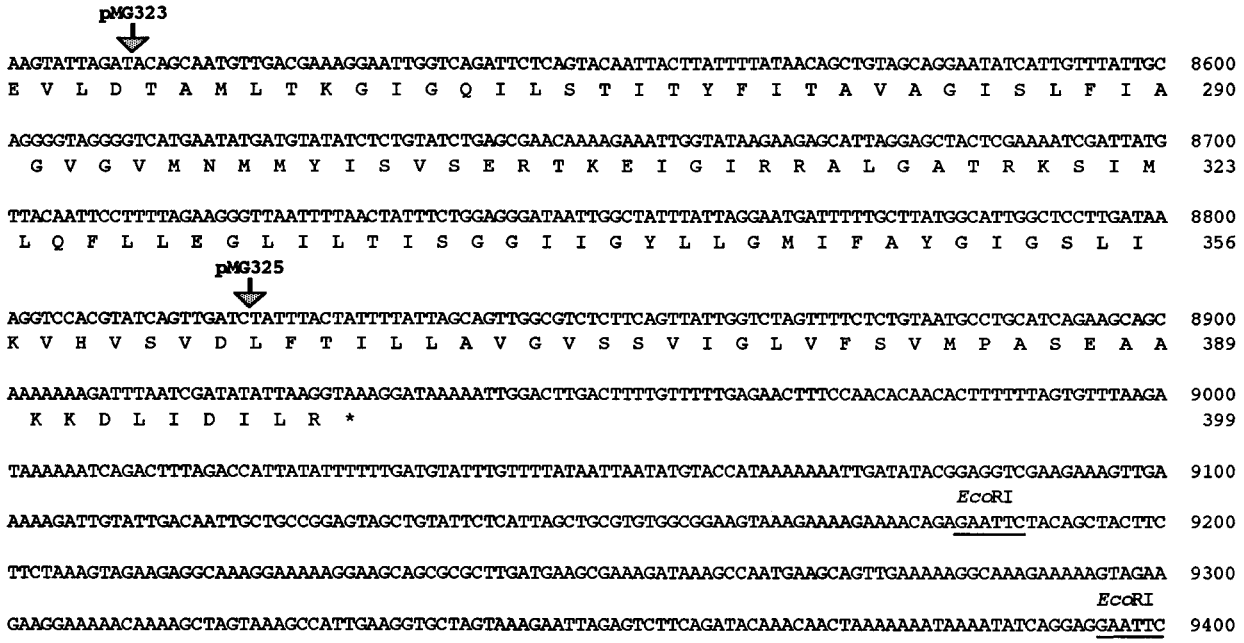


FIG. 2. Nucleotide sequence of the bacteriocin 21 determinant of plasmid pPD1 and the deduced amino acid sequence. The potential promoter (−10 and −35) and Shine-Dalgarno (S.D.) (44) ribosome-binding sequence are underlined. Inverted-repeat sequences are indicated by two horizontal arrows. The vertical arrows on the nucleotide sequence indicate the location of transposon insertions. The open arrowheads indicate complete or normal bacteriocin expression. The stippled arrowheads indicate leaky bacteriocin expression. The solid arrowheads indicate no bacteriocin expression. The six nucleotides included in the dashed boxes indicate the target sequences of the Tn*916* insertions (8). The horizontal arrows beside the identification number for the Tn*916* insertions indicate the orientation of Tn*916* insertion (the arrowhead corresponds to the right end of Tn*916*).

ferred to UV202(pMG333) by broth mating, and the transconjugants were examined for bacteriocin expression. [The proximal insertion mutants pMG116(pPD1 *bacB*::Tn*916*) and pMG112(pPD1 *bacD*::Tn*916*) were available for the complementation studies with pMG333; therefore, pMG116 was used in this study.] To determine whether the two plasmids were compatible in each transconjugant, the plasmid DNAs of the transconjugant were examined by agarose gel electrophoresis. It was confirmed that the plasmids exist compatibly in the transconjugant (data not shown). Representative results showing bacteriocin expression are shown in Fig. 5. UV202 (pMG333, pMG116) produced a bacteriolytic zone that was larger than that of the wild-type strain but similar in size to that produced by UV202 carrying the cloned bacteriocin determinant of plasmid pHT1. On the other hand, the transconjugant UV202(pMG333, pPIT7214), which contained plasmids containing the intact proximal half of the bacteriocin determinant, produced bacteriolytic zones similar to that produced by UV202 carrying pMG333, which contains the cloned proximal half of the determinant of pPD1. This indicated that the bacteriocin expression of pMG333 was not affected by an increased gene dosage of the proximal-half determinants of pPIT7214. The results of the complementation studies indicate that BacB of pMG333 could act in *trans* and that the distal determinants of pMG116 also could act in *trans* to complement the deleted distal half of the determinant in pMG333; they also indicate that complementation causes a level of bacteriocin expression similar to that of the strain containing pHT1.

To examine whether the products of the proximal half of the determinant and the distal half of the determinant complement extracellularly for complete bacteriocin expression, extracellular complementation analysis was performed with strains containing pMG333 and pMG116. If the products of the proximal half of the determinant and the distal half of the determinant complement extracellularly on the soft agar plate containing the indicator strain, a bacteriolytic zone larger than that of the strain containing pMG333 would be observed at the streak junction (28). Cross-streaks of UV202 containing pMG333 or pMG116 were made on bacteriocin assay plates as previously described (28). Complementation of bacteriocin activity was not observed at the streak junction on the bacteriocin assay plate (data not shown).

DISCUSSION

The *E. faecalis* bacteriocin 21 (Bac21) is active against a wide variety of bacteria including *E. faecalis* itself and *Staphylococcus aureus* (19). Transposon analysis with Tn*917* (Em^r) and Tn*916* (Tc^r) has shown that the bacteriocin-related genes are located in a segment of about 6.9 kb, which lies between kb 27.6 and 34.5 of the pPD1 map (19), and that these genes determine a single bacteriocin (19). Based on this data, we have now cloned a 9.4-kb fragment originating from the kb 26.4-to-35.8 region of the pPD1 map. The cloned fragment conferred complete bacteriocin expression and contained nine ORFs designated *bacA* to *bacI*, which are carried on an 8.5-kb segment from kb 27.1 to 35.6 of the pPD1 map.

In previous studies, only six independent Tn*917* insertion mutants with insertions into the bacteriocin 21 determinant had been isolated from about 10^4 pPD1::Tn*917* derivatives in 3,000 independent experiments (19). For the Tn*916* insertion mutants, 29 independent insertion mutations were mapped to the upper region of the determinant (19). In this study, we have determined the precise locations of the Tn*916* and Tn*917* insertion mutations and have isolated Tn*5* insertion mutants with insertions into the cloned bacteriocin determinant. Five of six Tn*917* insertion mutations were mapped to *bacB*, *bacF* and *bacI*. In the 29 Tn*916* insertion mutants, the Tn*916* insertions

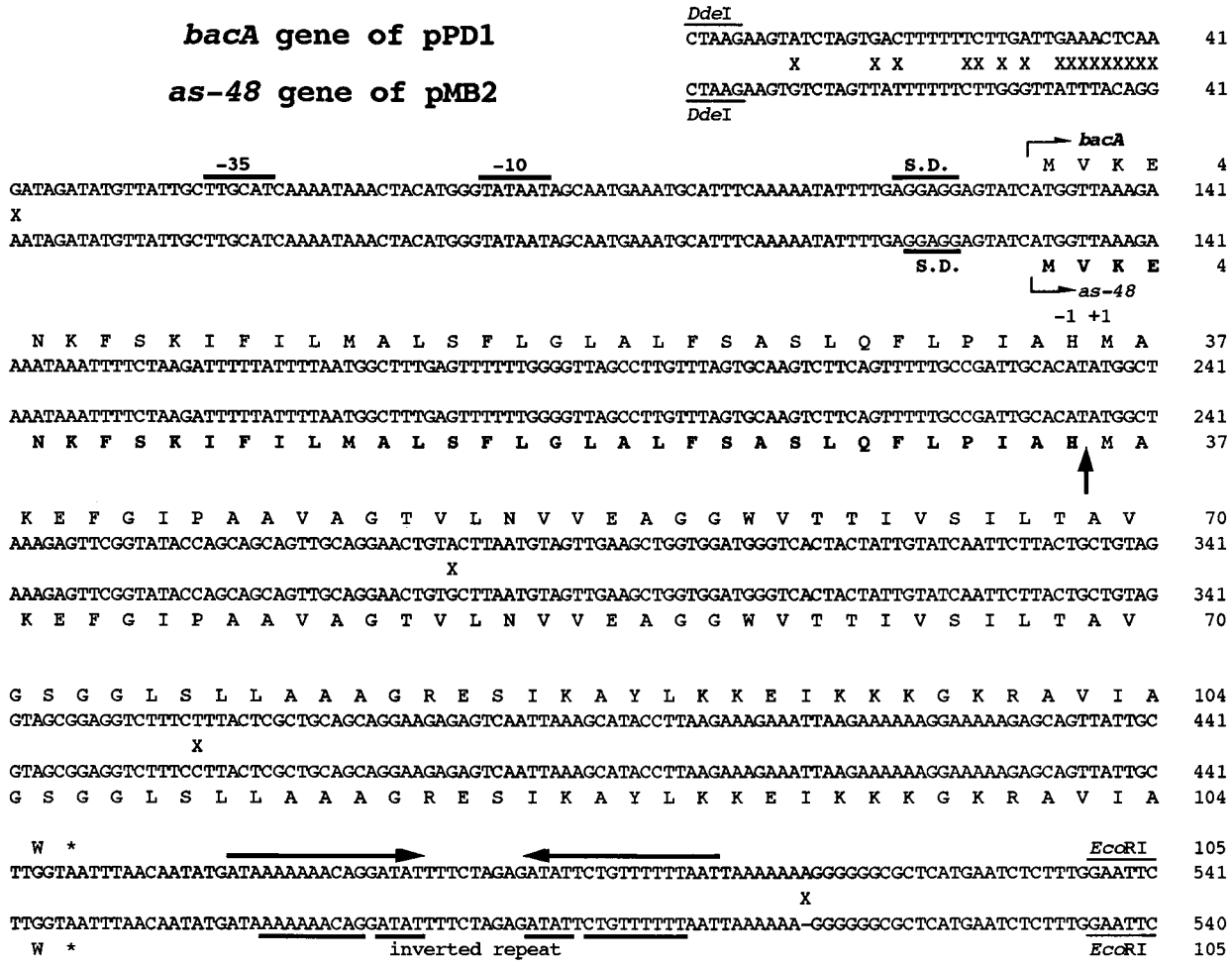


FIG. 3. Comparison of the amino acid sequence of the predicted BacA protein of bacteriocin 21 with the amino acid sequence of the predicted precursor of the peptide antibiotic AS-48. Symbol X indicates nonidentical nucleotides. The vertical arrow shows the processing site of the precursor peptide. Each horizontal line or arrow indicates the deduced promoter sequence, ribosome-binding site, and inverted repeat sequence in order from upstream to downstream.

into the ORFs were found to be located in *bacB* and *bacD*. On the other hand, Tn*5* inserted into seven different ORFs. Together with the results of transposon mutagenesis, it was shown that two regions of the bacteriocin 21 determinant were involved in bacteriocin expression. One region included the *bacA* to *bacE* genes in the proximal half of the determinant. Mutation of each of these genes completely disrupted bacteriocin expression. The other region involved the *bacF* to *bacI* genes, which are located in the distal half of the determinant. Insertions into each of these genes resulted in a reduction in bacteriocin expression. Presently, we cannot exclude any potential polar effects on the adjacent gene(s) by transposon insertions.

Deletion mutant analysis showed that deletions of the gene(s) in the distal half of the determinant resulted in reduced bacteriocin expression. The smallest fragment necessary for a degree of bacteriocin expression contained the proximal half of the determinant. These results indicate that the distal half of the determinant was necessary for complete bacteriocin expression. The complementation studies with the pPD1 *bacB* insertion mutant and the cloned proximal half of the determinant indicate that the distal half of the determinant could complement intracellularly, resulting in complete bacteriocin expression in the presence of the proximal region, and that an insertion in *bacB* did not result in any polar effects on the expression of the distal half of the determinant.

The complete homology of the deduced amino acid sequence encoded on *bacA* with that of the AS-48 protein (37) indicated that *bacA* was the structural gene for the bacteriocin 21 determinant (*bac21*) and encoded the precursor of Bac21. AS-48 is a peptide antibiotic and is active against a wide variety of gram-positive and gram-negative bacteria (37). This determinant has not yet been cloned, but biochemical analysis of the AS-48 mature protein and sequence analysis of the structural gene for the AS-48 peptide have been reported (21, 37). The *as-48* gene encodes a 105-amino-acid protein. The deduced precursor protein has a 35-residue signal sequence at its amino terminus and is processed to the mature 70-amino-acid AS-48 protein during secretion (21, 22, 37). The mature protein is modified to produce an active cyclic molecule by the linkage of the methionine residue (M^{+1}) to the C-terminal tryptophan residue (W^{+70}) (a peptide head-to-tail linkage) (21, 37). It is believed that several specific proteins are necessary for the posttranslational modification of the AS-48 precursor protein during secretion (37). These proteins involve a signal peptidase and a protein to produce the head-to-tail linkage of the mature 70-residue AS-48 protein (37). Nine ORFs involved in an

```
      1                                                  50                                                 100
BacH: MIAIMGPSGSGKSTLINLLGFIDRK--FEGEYLFEGRSLVNTADDILSKIRNQTVGFIFQNFSLIESNTVY-----ENVELPLLYNGLSPFKTKEKVFSVLDRVGLK
       * * * ********  *           *  *  *  *       *                 *  *         *                    *    * *
HlyB: VIGIVGRSGSGKSTLTKLIQRFYIP--ENGQVLIDGHDLALADPNWLRRQVGVVLQDNVLLNRSIIDNISL-----ANPGMSVEKVIYAAKLAGAHDFISELREGYN
LcnC: KLTIVGMSGSGKSTLVKLLVNFFQP--TSGTITLGGIDLQQFDKHQLRRLINYLPQQPYIFTGSILDNLLL----GANENASQEEILKAVELAEIRADIEQMQLGYQ
PedD: KITIVGMSGSGKTTLAKLLVGFFEPQEQHGEIQINHHNISDISRTILRQYINYVPQEPFIFSGSVLENLLL----GSRPGVTQQMIDQACSFAEIKTSIENLPQGYH
CylB: KVAIVGRSGSGKSTLLKLLAGLLQP--SNGEILYEGYPLSNNSNNRRNIFYVNQNAHIFNETIEKNISLEFK---PNSSINEKKRLKGSMSKSKMDEVLLGIPQYEK
SpaB: RVAIVGPNGSGKSTFIKLLTGLYEV--QQGDILINGINIKELDMDSYMNQIAALFQDFMKYEMTLKENIGFGQIDKLHQTNKMHEVLDIVRADFLKSHSSYQFDTQL
NisT: LTAIVGKNGSGKSTLVKIISGLYQP--TMGIIQYDKMRSSLMPEEEYQKNISVLFQDFVKYELTIRENIGLSDLSSQWEDEKIIKVLDNLGLDFLKTNNQYVLDTQL

                                                  150
BacH: GKEYKYPKQLSGGQQQRVAIARALINHPKFIIADEPTGALDTHTSEEIMKLFTTLNKEDDVTLIMVTHNPEVVPYCHRLITIRDGAIIEDKELVQ   1-195
             ***** ** ****** * **  * ** * ***      **            * *   *    *    * *    * * *
HlyB: TIVGEQGAGLSGGQRQRIAIARALVNNPKILIFDEATSALDYESEHVIMRNMHKIC--KGRTVIIIAHRLSTVKNADRIIVMEKGKIVEQGKHKE 498-689 (33.3%)
LcnC: TELSSDASSLSGGQKQRIALARALLSPAKILILDEATSNLDMITEKKILKNLLPL----DKTIIFIAHRLSVAEMSHRIIVVDQGKVIESGSHVD 501-702 (30.1%)
PedD: TRLSESGFNLSGGQKQRLSIARALLSPAQCFIFDESTSNLDTITEHKIVSKLLFMK---DKTIIFVAHRLNIASQTDKVVVLDHGKIVEQGSHRQ 505-708 (29.0%)
CylB: TIVSENGSNFSGGQRQKIALARAFYSNVNTLLLDEPTSAMDNISEFEVFSNLLDE----KRTVITVAHRISTVKNFDKIILMDNGEIVCIGKHED 491-692 (28.0%)
SpaB: GLWFDEGRQLSGGQWQKIALARAYFREASLYILDEPSSALDPIAEKETFDTFFSLS--KDKIGIFISHRLVAAKLADRIIVMDKGEIVGIGTHEE 382-579 (26.7%)
NisT: GNWFQEGHQLSGGQWQKIALARTFFKKASIYILDEPSAALDPVAEKEIFDYFVALS--ENNISIFISHSLNAARKANKIVVMKDGQVEDVGSHDV 371-577 (26.4%)
```

FIG. 4. Comparison of the amino acid sequence of the predicted BacH protein of bacteriocin 21 with the amino acid sequence of the C-terminal region of ATP-binding export proteins. The thin black bars show the two conserved A and B sites located at residues 6 to 14 and 31 to 35, respectively. The numbers in parentheses show the percent homology between the BacH amino acid sequence and each ATP-binding export protein. HlyB, exporter for *E. coli* alpha-hemolysin (16); LcnC, exporter for the *Lactococcus lactis* bacteriocin lactococcin A; PedD, exporter for the *Pediococcus acidilactici* pediocin PA-1 (38); CylB, exporter for the *E. faecalis* hemolysin/bacteriocin (Hly/Bac) (23); SpaB, exporter for the *Bacillus subtilis* subtilin (5, 34); NisT, exporter for the *Lactococcus lactis* nisin (14).

8.5-kb fragment of pPD1 were required for the bacteriocin 21 expression. The genes *bacB* to *bacI* could be involved in processing to form the mature bacteriocin protein, modification to the active bacteriocin, secretion of the bacteriocin, and host immunity to the bacteriocin activity.

The BacH protein is highly conserved in the C-terminal region, which is characteristic of the ATP-binding export proteins (11, 15, 16, 23, 25, 52). The deduced amino acid sequence of the BacH protein consists of 195 residues, whereas the majority of the bacterial ATP-binding export proteins are relatively large and consist of approximately 600 to 750 residues. Most of the bacterial proteins are composed of a membrane-spanning domain (MSD) located in the N-terminal region and an ATP-binding cassette (ABC) domain located in the C-terminal region. The ABC domain consists of two conserved A and B sites that form an ATP-binding pocket. There are conserved sequences located around the A and B sites, which in turn are located between residues 10 and 34 and residues 115 and 150, respectively, of the ABC domain. The A site is the ATP-binding site and contains the consensus sequence GXGKST. The B site is a hydrophobic strand of parallel β-pleated sheet terminated by an aspartate residue. The BacH protein has a highly conserved sequence located between residues 4 and 18, which includes the consensus GXGKST sequence for the putative ATP-binding domain. The aspartate-containing conserved sequence is located between residues 110 and 141 of the sequence. This suggests that the BacH protein acts as an ABC. The BacH protein lacks an amino acid sequence corresponding to the MSD of the ABC export protein. Comparison of the amino acid sequences of the neighboring *bacF*, *bacG*, and *bacI* genes did not show any significant homology to any other MSD reported for the ABC transport proteins. As described above, the proximal half of the determinants encoded production of the bacteriocin, but complete expression of the bacteriocin was not obtained without the distal half of the determinant. Although the precise function of the distal half of the determinant has not yet been determined, these genes may be involved in export of the bacteriocin; however, there is also the possibility that these sequences encode a positive regulator for bacteriocin expression.

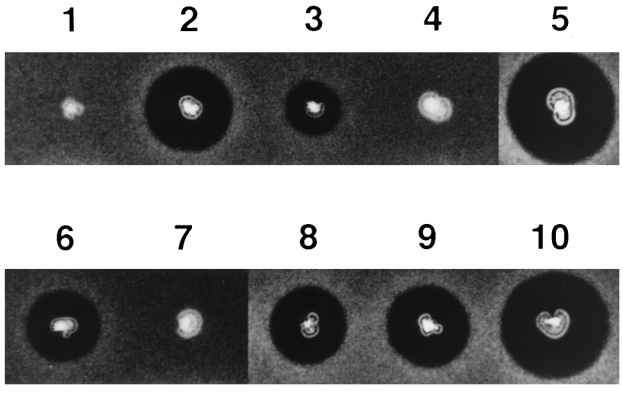


FIG. 5. Bacteriocin expression assay by the soft agar method with *E. faecalis* OG1X carrying representative pPD1 bacteriocin derivatives. The indicator strain was *S. aureus* FDA209P. 1, plasmid-free UV202; 2, UV202 [pPIT7022 (pPD1::Tn*917*)], wild type; 3, UV202[pPIT7214 (pPD1 *bacI*::Tn*917*)], representative of reduced bacteriocin expression; 4, UV202[pMG116 (pPD1 *bacB*::Tn*917*)], a representative nonbacteriocinogenic mutant; 5, FA2-2(pHT1), a cloned bacteriocin 21 determinant; 6, UV202(pMG317)(pHT1 *bacH*::Tn*5*), a representative reduced-bacteriocin mutant; 7, UV202(pMG301)(pHT1 *bacB*::Tn*5*), a nonbacteriocinogenic mutant; 8, UV202(pMG333), a pHT1 deletion mutant containing *bacA* through *bacE*; 9, UV202(pPIT7214, pMG333); 10, UV202(pMG116, pMG333).

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REFERENCES

1. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moor, J. G. Seidman, J. A. Smith, and K. Struhl.** 1987. Current protocols in molecular biology. John Wiley & Sons, Inc., New York, N.Y.
2. **Booth, M. C., C. P. Bogie, H.-G. Sahl, R. J. Siezen, K. L. Hatter, and M. S. Gilmore.** 1996. Structural analysis and proteolytic activation of *Enterococcus faecalis* cytolysin, a novel lantibiotic. Mol. Microbiol. **21:**1175–1184.
3. **Brock, T. D., B. Peacher, and D. Pierson.** 1963. Survey of the bacteriocins of enterococci. J. Bacteriol. **86:**702–707.

4. **Chow, J. W., L. A. Thal, M. B. Perri, J. A. Vazquez, S. M. Donabedian, D. B. Clewell, and M. J. Zervos.** 1993. Plasmid-associated hemolysin and aggregation substance production contribute to virulence in experimental enterococcal endocarditis. Antimicrob. Agents Chemother. **37:**2474–2477.
5. **Chung, Y. J., M. T. Steen, and J. N. Hansen.** 1992. The subtilin gene of *Bacillus subtilis* ATCC 6633 is encoded in an operon that contains a homolog of the hemolysin B transport protein. J. Bacteriol. **174:**1417–1422.
6. **Clewell, D. B.** 1981. Plasmids, drug resistance, and gene transfer in the genus *Streptococcus*. Microbiol. Rev. **45:**409–436.
7. **Clewell, D. B.** 1993. Bacterial sex pheromone-induced plasmid transfer. Cell **73:**9–12.
8. **Clewell, D. B., S. E. Flannagan, Y. Ike, J. M. Jones, and C. Gawron-Burke.** 1988. Sequence analysis of termini of conjugative transposon Tn*916*. J. Bacteriol. **170:**3046–3052.
9. **Clewell, D. B., P. K. Tomich, M. C. Gawron-Burke, A. E. Franke, Y. Yagi, and F. Y. An.** 1982. Mapping of *Streptococcus faecalis* plasmids pAD1 and pAD2 and studies relating to transposition of Tn*917*. J. Bacteriol. **152:**1220–1230.
10. **Cogue, T. M., J. E. Patterson, J. M. Steckelberg, and B. E. Murray.** 1995. Incidence of hemolysin, gelatinase, and aggregation substance among enterococci isolated from patients with endocarditis and other infections and from feces of hospitalized and community-based persons. J. Infect. Dis. **171:**1223–1229.
11. **Doolittle, R. F., M. S. Johnson, I. Husain, B. V. Houten, D. C. Thomas, and A. Sancar.** 1986. Domain evolution of a prokaryotic DNA repair protein and its relationship to active-transport proteins. Nature (London) **323:**451–453.
12. **Dunny, G. M., B. L. Brown, and D. B. Clewell.** 1978. Induced cell aggregation and mating in *Streptococcus faecalis*: evidence for a bacterial sex pheromone. Proc. Natl. Acad. Sci. USA **75:**3479–3483.
13. **Ehrenfeld, E. E., and D. B. Clewell.** 1987. Transfer functions of the *Streptococcus faecalis* plasmid pAD1: organization of plasmid DNA encoding response to sex pheromone. J. Bacteriol. **169:**3473–3481.
14. **Engelke, G., Z. Gutowski-Eckel, M. Hammelmann, and K.-D. Entian.** 1992. Biosynthesis of lantibiotic nisin: genomic organization and membrane localization of the NisB protein. Appl. Environ. Microbiol. **58:**3730–3743.
15. **Fath, M. J., and R. Kolter.** 1993. ABC transporters: bacterial exporters. Microbiol. Rev. **57:**995–1017.
16. **Felmlee, T., S. Pellett, and R. A. Welch.** 1985. Nucleotide sequence of an *Escherichia coli* chromosomal hemolysin. J. Bacteriol. **163:**94–105.
17. **Franke, A., and D. B. Clewell.** 1981. Evidence for a chromosome-borne resistance transposon in *Streptococcus faecalis* capable of "conjugal" transfer in the absence of a conjugative plasmid. J. Bacteriol. **145:**494–502.
18. **Fujimoto, S., H. Hashimoto, and Y. Ike.** 1991. Low cost device for electrotransformation and its application to the highly efficient transformation of *Escherichia coli* and *Enterococcus faecalis*. Plasmid **26:**131–135.
19. **Fujimoto, S., H. Tomita, E. Wakamatsu, K. Tanimoto, and Y. Ike.** 1995. Physical mapping of the conjugative bacteriocin plasmid pPD1 of *Enterococcus faecalis* and identification of the determinant related to the pheromone response. J. Bacteriol. **177:**5574–5581.
20. **Galli, D., A. Friesenegger, and R. Wirth.** 1992. Transcriptional control of sex pheromone-inducible genes on plasmid pAD1 of *Enterococcus faecalis* and sequence analysis of a third structural gene for (pPD1-encoded) aggregation substance. Mol. Microbiol. **6:**1297–1308.
21. **Galvez, A., G. Gimenez-Gallego, M. Maqueda, and E. Valdivia.** 1989. Purification and amino acid composition of peptide antibiotic AS-48 produced by *Streptococcus* (*Enterococcus*) *faecalis* subsp. *liquefaciens* S-48. Antimicrob. Agents Chemother. **33:**437–441.
22. **Galvez, A., M. Maqueda, M. Martinez-Bueno, and E. Valdivia.** 1991. Permeation of bacterial cells, permeation of cytoplasmic and artificial membrane vesicles, and channel formation on lipid bilayers by peptide antibiotic AS-48. J. Bacteriol. **173:**886–892.
23. **Gilmore, M. S., R. A. Segarra, and M. C. Booth.** 1990. An HlyB-type function is required for expression of the *Enterococcus faecalis* hemolysin/bacteriocin. Infect. Immun. **58:**3914–3923.
24. **Gilmore, M. S., R. A. Segarra, M. C. Booth, C. P. Bogie, L. R. Hall, and D. B. Clewell.** 1994. Genetic structure of the *Enterococcus faecalis* plasmid pAD1-encoded cytolytic toxin system and its relationship to lantibiotic determinants. J. Bacteriol. **176:**7335–7344.
25. **Goebel, W., and J. Hedgpeth.** 1982. Cloning and functional characterization of the plasmid-encoded hemolysin determinant of *Escherichia coli*. J. Bacteriol. **151:**1290–1298.
26. **Ike, Y., and D. B. Clewell.** 1984. Genetic analysis of the pAD1 pheromone response in *Streptococcus faecalis*, using transposon Tn*917* as an insertional mutagen. J. Bacteriol. **158:**777–783.
27. **Ike, Y., and D. B. Clewell.** 1992. Evidence that the hemolysin/bacteriocin phenotype of *Enterococcus faecalis* subsp. *zymogenes* can be determined by plasmids in different incompatibility groups as well as by the chromosome. J. Bacteriol. **174:**8172–8177.
28. **Ike, Y., D. B. Clewell, R. A. Segarra, and M. S. Gilmore.** 1990. Genetic analysis of the pAD1 hemolysin/bacteriocin determinant in *Enterococcus faecalis*: Tn*917* insertional mutagenesis and cloning. J. Bacteriol. **172:**155–163.
29. **Ike, Y., R. A. Craig, B. A. White, Y. Yagi, and D. B. Clewell.** 1983. Modification of *Streptococcus faecalis* sex pheromones after acquisition of plasmid DNA. Proc. Natl. Acad. Sci. USA **80:**5369–5373.
30. **Ike, Y., S. E. Flannagan, and D. B. Clewell.** 1992. Hyperhemolytic phenomena associated with insertions of Tn*916* into the hemolysin determinant of the *Enterococcus faecalis* plasmid pAD1. J. Bacteriol. **174:**1801–1809.
31. **Ike, Y., H. Hashimoto, and D. B. Clewell.** 1984. Hemolysin of *Streptococcus faecalis* subspecies *zymogenes* contributes to virulence in mice. Infect. Immun. **45:**528–530.
32. **Ike, Y., H. Hashimoto, and D. B. Clewell.** 1987. High incidence of hemolysin production by *Enterococcus* (*Streptococcus*) *faecalis* strains associated with human parenteral infection. J. Clin. Microbiol. **25:**1524–1528.
33. **Jett, B. D., H. G. Jensen, R. E. Nordquist, and M. S. Gilmore.** 1992. Contribution of the pAD1-encoded cytolysin to the severity of experimental *Enterococcus faecalis* endophthalmitis. Infect. Immun. **60:**2445–2452.
34. **Klein, C., C. Kaletta, N. Schnell, and K.-D. Entian.** 1992. Analysis of genes involved in biosynthesis of the lantibiotic subtilin. Appl. Environ. Microbiol. **58:**132–142.
35. **LeBlanc, D. J., L. N. Lee, D. B. Clewell, and D. Behnke.** 1983. Broad geographical distribution of a cytotoxin gene mediating beta-hemolysis and bacteriocin activity among *Streptococcus faecalis* strains. Infect. Immun. **40:**1015–1022.
36. **Martinez-Bueno, M., A. Galvez, E. Valdivia, and M. Maqueda.** 1990. A transferable plasmid associated with AS-48 production in *Enterococcus faecalis*. J. Bacteriol. **172:**2817–2818.
37. **Martinez-Bueno, M., M. Maqueda, A. Galvez, B. Samyn, J. V. Beeumen, J. Coyette, and E. Valdivia.** 1994. Determination of the gene sequence and the molecular structure of the enterococcal peptide antibiotic AS-48. J. Bacteriol. **176:**6334–6339.
38. **Marugg, J. D., C. F. Gonzalez, B. S. Kunka, A. M. Ledeboer, M. J. Pucci, M. Y. Toonen, S. A. Walker, L. C. M. Zoetmulder, and P. A. Vandenbergh.** 1992. Cloning, expression, and nucleotide sequence of genes involved in production of pediocin PA-1, a bacteriocin from *Pediococcus acidilactici* PAC1.0. Appl. Environ. Microbiol. **58:**2360–2367.
39. **Nakayama, J., K. Yoshida, H. Kobayashi, A. Isogai, D. B. Clewell, and A. Suzuki.** 1995. Cloning and characterization of a region of *Enterococcus faecalis* plasmid pPD1 encoding pheromone inhibitor (*ipd*), pheromone sensitivity (*traC*), and pheromone shutdown (*traB*) genes. J. Bacteriol. **177:**5567–5573.
40. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
41. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
42. **Segarra, R. A., M. C. Booth, D. A. Morales, M. M. Huycke, and M. S. Gilmore.** 1991. Molecular characterization of the *Enterococcus faecalis* cytolysin activator. Infect. Immun. **59:**1239–1246.
43. **Shaw, J. H., and D. B. Clewell.** 1985. Complete nucleotide sequence of macrolide-lincomide-streptogramin B resistance transposon Tn*917* in *Streptococcus faecalis*. J. Bacteriol. **164:**782–796.
44. **Shine, J., and L. Dalgarno.** 1974. The 3′ terminal sequence of *E. coli* 16S ribosomal RNA: complementarity to nonsense triplets and ribosome binding sites. Proc. Natl. Acad. Sci. USA **71:**1342–1346.
45. **Tanimoto, K., F. Y. An, and D. B. Clewell.** 1993. Characterization of the *traC* determinant of the *Enterococcus* hemolysin-bacteriocin plasmid pAD1: binding of sex pheromone. J. Bacteriol. **175:**5260–5264.
46. **Tanimoto, K., and D. B. Clewell.** 1993. Regulation of the pAD1-encoded sex pheromone response in *Enterococcus faecalis*: expression of the positive regulator TraE1. J. Bacteriol. **175:**1008–1018.
47. **Tanimoto, K., and T. Iino.** 1985. Additional genes essential for replication of the mini-F plasmid from origin I. Mol. Gen. Genet. **198:**358–359.
48. **Tanimoto, K., H. Tomita, and Y. Ike.** 1996. The *traA* gene of the *Enterococcus faecalis* conjugative plasmid pPD1 encodes a negative regulator for the pheromone response. Plasmid **36:**55–61.
49. **Tomich, P. K., F. Y. An, and D. B. Clewell.** 1980. Properties of erythromycin-inducible transposon Tn*917* in *Streptococcus faecalis*. J. Bacteriol. **141:**1366–1374.
50. **Tomich, P. K., F. Y. An, S. P. Dample, and D. B. Clewell.** 1979. Plasmid-related transmissibility and multiple-drug resistance in *Streptococcus faecalis* subsp. *zymogenes* DS16. Antimicrob. Agents Chemother. **15:**828–830.
51. **Tomita, H., S. Fujimoto, K. Tanimoto, and Y. Ike.** 1996. Cloning and genetic organization of the bacteriocin 31 determinant encoded on the *Enterococcus faecalis* pheromone-responsive conjugative plasmid pYI17. J. Bacteriol. **178:**3585–3593.
52. **Wagner, W., M. Vogel, and W. Goebel.** 1983. Transport of hemolysin across the outer membrane of *Escherichia coli* requires two functions. J. Bacteriol. **154:**200–210.
53. **Wirth, R., F. Y. An, and D. B. Clewell.** 1986. Highly efficient protoplast transformation system for *Streptococcus faecalis* and a new *Escherichia coli-S. faecalis* shuttle vector. J. Bacteriol. **165:**831–836.
54. **Yagi, Y., and D. B. Clewell.** 1980. Recombination-deficient mutant of *Streptococcus faecalis*. J. Bacteriol. **143:**966–970.
55. **Yagi, Y., R. E. Kessler, J. H. Show, D. E. Lopatin, F. Y. An, and D. B. Clewell.** 1983. Plasmid content of *Streptococcus faecalis* strain 39-5 and identification of a pheromone (cPD1)-induced surface antigen. J. Gen. Microbiol. **129:**1207–1215.