JOURNAL OF BACTERIOLOGY, Oct. 1995, p. 5574–5581 Vol. 177, No. 19
0021-9193/95/$04.00+0

Physical Mapping of the Conjugative Bacteriocin Plasmid pPD1 of *Enterococcus faecalis* and Identification of the Determinant Related to the Pheromone Response

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Received 14 March 1995/Accepted 2 August 1995

The pheromone-responding conjugative bacteriocin plasmid pPD1 (59 kb) of *Enterococcus faecalis* was mapped physically by using a relational clone approach, and transposon analysis with Tn*917* (Em^r) or Tn*916* (Tc^r) facilitated the location of the bacteriocin-related genes in a segment of about 6.7 kb. Tn*917* insertions within a 3-kb region resulted in constitutive clumping. The nucleotide sequence of the region that included the insertions giving rise to constitutive clumping was determined. The region of pPD1 spanned about 8 kb and was found to contain a number of open reading frames, some of which were named on the basis of homologies with two other pheromone-responding plasmids, pAD1 and pCF10. The genes were arranged in the sequence *repB–repA–traC–traB–traA–ipd–traE–traF–orfY–sea-1* with all but *repB* and *traA* oriented in the same (left-to-right) direction. *traC* and *traB* corresponded, respectively, to *traC* and *traB* of pAD1 and to *prgY* and *prgZ* of pCF10.

Certain conjugative plasmids in *Enterococcus faecalis* transfer at a high frequency in broth mating, a phenomenon relating to a response to specific peptide sex pheromones secreted by potential recipients. The sex pheromone induces the synthesis of a surface aggregation substance that facilitates the formation of a mating aggregate (5–7, 14, 15, 18, 48). When a given plasmid is acquired, secretion of the related pheromone is prevented; however, unrelated pheromones continue to be produced. In addition, the plasmid itself determines the production of a peptide that acts as a competitive inhibitor of the corresponding pheromone (8, 10, 11, 27, 32).

Several pheromone-responding plasmids have been reported, and the plasmids in *E. faecalis* have been found to encode such traits as hemolysin-bacteriocin production, UV resistance, and drug resistance (5–7). Of these plasmids, the pheromone-related conjugation systems best studied are pAD1 (60 kb) (6, 7, 9, 12, 23, 33, 35, 36, 40, 41, 44, 45) and pCF10 (54 kb) (4, 30, 37), which confer responses to sex pheromones cAD1 and cCF10, respectively. pAD1 encodes a hemolysin-bacteriocin mediated by the same genetic determinant (2, 3, 5, 26) and belongs to incompatibility group incHly (13, 25). Most (over 90%) of the Hly-Bac plasmids identified in clinical isolates are identical and exhibit extensive homology to pAD1, respond to cAD1, and represent incompatibility group incHly (24, 25, 29). pAD1 is a representative of this group. pCF10 carries a tetracycline resistance determinant located on transposon Tn*925*. Genes involved in regulation of the pheromone response have been identified and are clustered in a 7-kb region on each of the plasmids. In pAD1, these genes are arranged in the order *traE1-iad-traA-traC-traB* (6). The *traE1* product is a key positive regulator for expression of downstream structural genes, including determinant *asa-1* for the aggregation substance and other conjugation-related genes. The *traA* product has been shown to represent a key negative regulator of *traE1* expression. The *traB* product is involved in shutdown of endogenous cAD1 production, whereas the *traC* product is involved in exogenous pheromone binding and/or sensing. *iad* determines iAD1, the competitive inhibitor of endogenous pheromone cAD1. Significant homologies of genes in the regulatory region of pCF10 with equivalent determinants on pAD1 have also been identified (6, 7, 16). The general organizations of the regulatory regions of the two plasmids are similar, and some genes are almost identical (6, 16); other genes that are supposed to be important for regulatory functions (i.e., *traA* and *prgX* of pCF10, *traB* and *prgY* of pCF10, or *traE* of the positive regulator) are not related at the sequence level or differ in gene organization (6, 16).

pPD1 (59 kb) is another well-analyzed plasmid in the pheromone-related conjugation system. pPD1 confers a response to sex pheromone cPD1 and determines bacteriocin production (5, 48). The pheromone-induced synthesis of a proteinaceous microfibrillar substance (aggregation substance) on the cell surface was first resolved in cells containing pPD1 by immunoelectron microscopy (48). Early studies showed that aggregates could be dissociated by a chelating agent such as EDTA (14), and it was subsequently found by studying *E. faecalis* containing pPD1 that phosphate and divalent cations are necessary for aggregation of induced cells (48). Recently, genetic and sequence analyses of the determinant of the pheromone-induced aggregation substance of pPD1 were reported by Galli et al. (21), and they showed that the structural gene has over 90% homology to the aggregation substance gene of pAD1 or pCF10. In their report, they identified two new *Eco*RI fragments, H and I, that were missed in the original report (48) and showed the A-G-C order of *Eco*RI fragments in a clockwise orientation. The genes involved in pheromone response regulation have not been identified in pPD1. In this report, we update further the pPD1 physical map and report the locations of the pheromone response regulatory genes and bacteriocin

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant genotype or phenotype	Plasmid content	Comment(s)	Reference or source
Enterococcus faecalis				
FA2-2	*rif fus*	None	Derivative of JH2	11
JH2SS	*str spc*	None	Derivative of JH2	42
OG1-10	*str*	None	Derivative of OG1	15
DS16	*tet*	pAD1(Hly-Bac); pAD2 (Sm^r Km^r Em^r [Tn*917*])	Clinical isolate	43
DS16C3	*tet*	None	DS16 cured of pAD1 and pAD2	19
OG1X	*str*	None	Proteinase-negative mutant of OG1-10	27
Escherichia coli				
DH1	F^- *recA1 endA1 gyrA96 thi-1 relA1 hsdR17 supE44*	None		38
DH5α	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1* ϕ80*lacZ*ΔM15			Bethesda Research Laboratories
Plasmids				
pPD1	*bac*		59-kb conjugative plasmid from strain 39-5	48
pTV1-ts	*cat erm*; temperature-sensitive replicon		Delivery vector for Tn*917*	49
pAM401	*cat tet*		*E. coli-E. faecalis* shuttle vector	47
pMW119	*bla lacZ*		*E. coli* vector, low copy number	Nippon Gene Co., Ltd., Toyama, Japan

determinant. The results of nucleotide sequence analysis of the regulatory genes are also reported.

MATERIALS AND METHODS

Bacteria, media, and reagents. The strains and plasmids used in this study are listed in Table 1. *E. faecalis* strains were grown in antibiotic medium 3 (Difco, Detroit, Mich.) or Todd-Hewitt broth (Difco). *Escherichia coli* strains were grown in Luria-Bertani medium. Solid media were prepared by addition of 1.5% (wt/vol) agar. Rifampin, spectinomycin, fusidic acid, and streptomycin were kindly provided by Daiichi Pharmaceutical Co., Ltd., Tokyo, Japan; Upjohn Pharmaceutical Ltd.; LEO Pharmaceutical Products, Ballerup, Denmark, and Sankyo Pharmaceutical Co. Ltd.; and Kyowa Hakko Kogyo Co. Ltd., Tokyo, Japan, respectively.

Isolation and manipulation of plasmid DNA. Plasmid DNA was isolated by alkaline lysis methods (17, 38). Plasmid DNA was treated with restriction enzymes and submitted to agarose gel electrophoresis. All restriction enzymes were obtained from Nippon Gene, Toyama, Japan, and used in accordance with the supplier's specifications. Agarose was obtained from Wako Chemicals, Osaka, Japan. A 0.75% agarose concentration was used for estimation of the larger-molecular-size (more than 0.5 kb) molecules, and 2.0% agarose was used for estimation of the smaller fragments (less than 0.5 kb). A "glass milk" method (Gene Clean II kit; Bio 101, Inc., La Jolla, Calif.) was used for elution of the DNA fragments from agarose gels. The eluted fragments were ligated to dephosphorylated, *Eco*RI-digested pAM401 with T4 ligase and introduced into *E. coli* DH1 by electrotransformation (20). Transformants were selected on Luria-Bertani medium agar containing tetracycline (12.5 μg/ml).

Determination of the restriction map of pPD1. Initially, a few *Eco*RI fragments of pPD1 (including A through J) were identified. The molecular sizes of *Eco*RI fragments A through J were 22.5, 13.0, 9.0, 6.9, 5.0, 1.0, 0.6, 0.36, 0.28, and 0.23 kb.

To determine the order of the *Eco*RI fragments, the relational clone set was obtained as shown in Fig. 1. After agarose gel electrophoresis of partially *Eco*RI-digested pPD1 DNA, larger fragments (more than 15 kb) or fragments of around 7.0 to 8.0 kb were eluted and used for cloning. The cloning vector and host strain were pAM401 and *E. coli* DH1, respectively. Thirty-seven clones containing a pPD1 fragment(s) were obtained. Only two of them contained the single fragment D. Others contained two to five fragments. These clones fell into 10 classes of clones that contain *Eco*RI fragments B and F; B, E, and F; B, D, F, and J; B, D, E, F, and J; B, D, E, and F; B, C, and E; B, C, E, F, and G; C and E; C, E, and G; and C and G (Fig. 1). Because only 2 of 37 clones contained a single fragment, the possibility is negligible that these arrays were the results of reconstruction from single fragments. When we used fragments of around 7.0 to 8.0 kb, five classes of clones were obtained that contained fragments D; D and F; D and J; D, J, and H; and D, F, H, and J (Fig. 1). These results indicate that the order of these fragments was GCEBFDJH.

To confirm that *Eco*RI fragment A is located next to *Eco*RI fragment G in counterclockwise order as described previously (21), we cloned *Bgl*II fragment C of pPD1 (Fig. 2) into the *Bam*HI site of pAM401. All 28 clones obtained contained *Eco*RI fragment G plus two other fragments. One of the two fragments was 2.0 kb long, and the other was 5.4 kb long. There was no 0.28-kb small fragment corresponding to *Eco*RI fragment I. These results indicate that *Eco*RI fragment A is located next to *Eco*RI fragment G in the counterclockwise orientation (21).

The above results implied that the remaining fragment, I, was located between *Eco*RI fragments A and H. To confirm this, pPIT7022 (pPD1::Tn*917*), in which Tn*917* is inserted into *Eco*RI fragment A at 21.9 kb from the *Eco*RI-A–G junction, was used to clone the fragment between the *Sal*I site in Tn*917* (39) and the *Bam*HI site in *Eco*RI fragment B of pPD1. The cloning vector and host strain were pMW119 and *E. coli* DH5α, respectively. The resulting clone, designated pMBS221, contained pPD1 *Eco*RI fragments D, F, H, I, and J and portions of A and B (Fig. 1).

DNA sequence analysis. Nucleotide sequence analysis was done by the method described in reference 38. A deletion kit (Nippon Gene) was also utilized. For the pheromone response regulatory region of pPD1, an *Eco*RI-*Sal*I fragment was cloned into vector pMW119. The cloned DNA fragment was digested with *Sal*I and *Sph*I and treated with exonuclease III for different lengths of time, 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 in both orientations with a 373A DNA sequencer (Applied Biosystems).

Generation of pPD1::Tn*917* derivatives. The temperature-sensitive plasmid pTV1-ts (49) was used as a delivery vehicle in the generation of Tn*917* insertions into pPD1. The method used was essentially that described previously (17). An overnight culture of OG1X(pTV1-ts; pPD1) was diluted 1:400 in antibiotic medium 3 broth containing 0.05 μg of erythromycin per ml and 10 μg of chloramphenicol per ml, grown at 30°C for 48 h, and then diluted 1:1,000 in antibiotic medium 3 broth containing 10 μg of erythromycin per ml. The cells were incubated for 18 h at 42°C and mated in broth with recipient FA2-2 cells for 6 h at 37°C as previously described (17). Portions of the mixed culture were then plated on antibiotic medium 3 broth agar plates containing 12.5 μg of erythromycin per ml, 25 μg of fusidic acid per ml, and 25 μg of rifampin per ml for selection of transferable pPD1::Tn*917* derivatives. About ten thousand pPD1::Tn*917* derivatives derived from 3,000 independent matings were examined for this study.

Generation of pPD1::Tn*916* derivatives altered in bacteriocin expression. Derivatives resulting from insertions of Tn*916* into pPD1 were generated as previ-

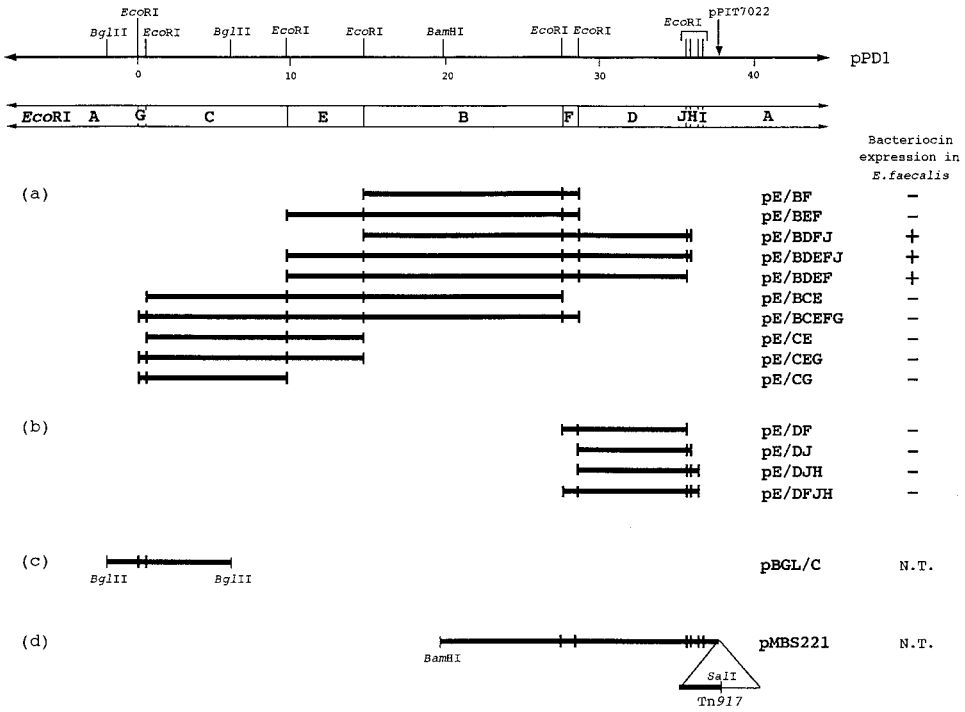

FIG. 1. Relational clones of pPD1. After agarose gel electrophoresis of partially *Eco*RI-digested pPD1 DNA, larger fragments of more than 15 kb (a) or fragments of around 7.0 and 8.0 kb (b) were eluted from the agarose gel and used for cloning. Representatives of the relational clones are shown. (c) A *Bgl*II fragment was cloned into the *Bam*HI site of pAM401. (d) Plasmid pPIT7022 was a derivative of pPD1 with Tn*917* inserted in *Eco*RI fragment A. A fragment between the *Bam*HI site of pPD1 and the *Sal*I site of Tn*917* was cloned. The arrow marked pPIT7022 indicates the position of the Tn*917* insertion in pPIT7022. Clones of pBGL/C and pMBS221 were not tested (N.T.) for bacteriocin expression.

ously described (19, 28). pPD1 was introduced into DS16C3, a plasmid-free derivative of DS16. DS16C3(pPD1) was used as a donor in overnight filter matings with recipient strain FA2-2. Transconjugants were selected on Todd-Hewitt agar plates containing 12.5 μg of tetracycline per ml in addition to antibiotics specific for the recipient (rifampin, 25 μg/ml; fusidic acid, 25 μg/ml). Transconjugants appearing on the selective plates were examined with respect to bacteriocin production by using a soft-agar method (26).

Clumping assay. Detection of aggregation (clumping) with or without a pheromone was done as previously described (14, 15, 23).

Indicator strains for bacteriocin activity. *E. faecalis* OG1-10 or FA2-2, *E. hirae* ATCC 9790, or *S. aureus* FDA209P was routinely used as the indicator strain to detect pPD1 bacteriocin activity. Clinical isolates of *E. faecium, Streptococcus pyogenes* (group A), *S. agalactiae* (group B), *S. sanguis*, *S. pneumoniae*, *Staphylococcus aureus*, or *S. epidermidis* were used to test the bacteriocin spectrum.

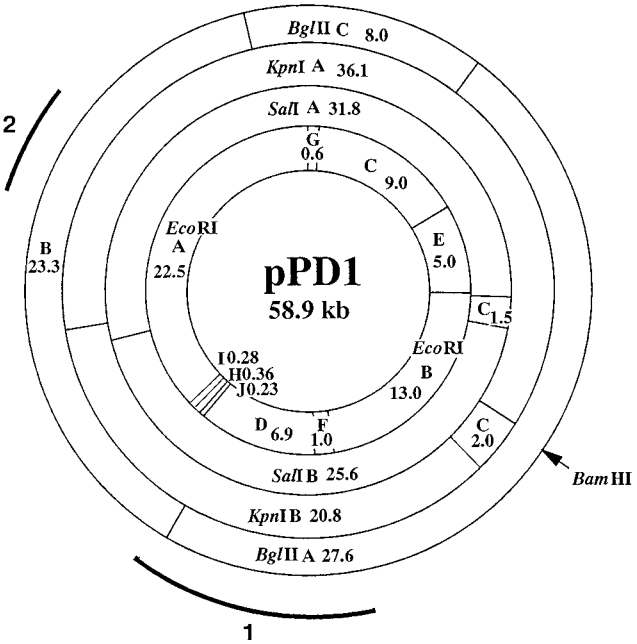

FIG. 2. Physical map of pPD1. Segment 1, shown outside of the map, was a location in which Tn*917* or Tn*916* insertions gave rise to altered bacteriocin expression. Segment 2, also shown outside of the map, was a location in which Tn*917* insertions gave rise to constitutive clumping.

RESULTS AND DISCUSSION

Restriction map of pPD1. As described in Materials and Methods, the relational clone set shown in Fig. 1 contains all of the junctions between the pPD1 *Eco*RI fragments and each clone contains two or more of the fragments. We determined the order of *Eco*RI fragments as AGCEBFDJHI in the clockwise orientation. We digested these clones with *Bgl*II, *Bam*HI, *Sal*I, and *Kpn*I and determined the cleavage sites (Fig. 2).

Constitutively clumping mutants and cloning and sequencing of the pheromone response regulatory region. In plasmid pAD1, insertions at two loci, *traA* and *traB*, resulted in constitutive clumping (22) and plasmid transfer occurred at high frequency with a short (10-min) mating time (22).

Four hundred independent bacteriocinogenic pPD1::Tn*917* derivatives were examined for clumping with or without exposure to the culture supernatant of FA2-2. A total of 25 Tn*917* insertions within the 47- to 50-kb region of the pPD1 map resulted in constitutive clumping (Fig. 3). The region was within the *Eco*RI-A segment. pPD1 DNA was digested with both *Eco*RI and *Sal*I, and an *Eco*RI-*Sal*I fragment (16.7 kb) derived from *Eco*RI fragment A (22.5 kb) was cloned into pMW119. The cloned 16.7-kb *Eco*RI-*Sal*I fragment was digested with *Sal*I and *Sph*I and trimmed with nucleases as de-

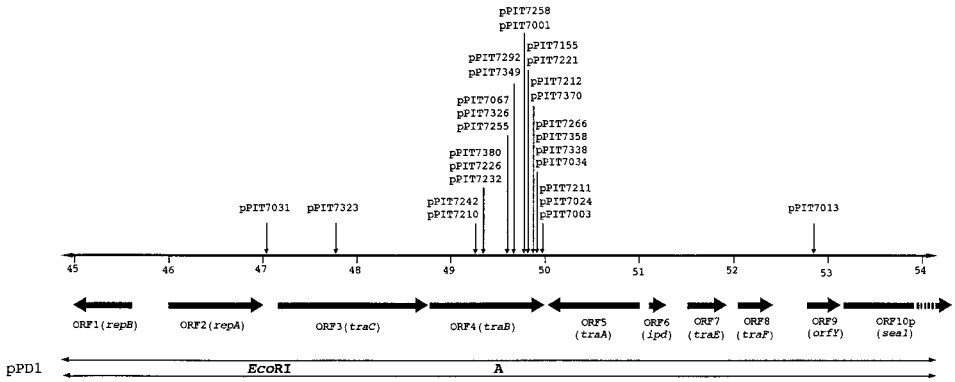

FIG. 3. Map of Tn*917* insertions that resulted in constitutive clumping. Vertical arrows indicate the positions of Tn*917* insertions on the pPD1 physical map. Thick horizontal arrows indicate ORFs on the pPD1 physical map and the directions of transcription of the ORFs. All of the Tn*917* insertions except pPIT7013 gave rise to constitutive clumping mutants.

scribed in Materials and Methods for DNA sequence analysis. The resulting constructions were sequenced in both orientations. In some cases, specific synthetic oligonucleotides served as primers in sequencing reactions. The locations of Tn*917* insertions were determined by using specific synthetic primers that direct to read out from the ends of the transposon (39).

Computer analysis revealed several open reading frames (ORFs). Figure 4 shows the nucleotide sequences of ORFs preceded within 20 bases of the predicted start codon by a good ribosome-binding site and the deduced amino acid sequence.

The precise locations of several Tn*917* inserts in these ORFs were determined by DNA sequence analysis and are marked in Fig. 4. Inserts that resulted in constitutive clumping mapped in various ORFs. The Tn*917* inserts of pPIT7031 and pPIT7323 were mapped in a noncoding region between ORF2 and ORF3 and within ORF3, respectively. Another nine inserts mapped within ORF4 (Fig. 4). The Tn*917* insert of pPIT7003 was the closest to the ORF4 carboxy terminus in the insertions within ORF4. A strain containing pPIT7013 exhibited no altered pheromone response phenotype, and the Tn*917* insert was mapped within ORF9. The positions of the Tn*917* inserts of pPIT7003 and pPIT7013 were separated by a 2.9-kb region in which there were four ORFs, and there was no Tn*917* insert in this region.

Clumping and conjugative transfer of constitutive clumping mutant plasmids. Constitutive clumping mutant plasmids pPIT7031, pPIT7323, and pPIT7003 were used to examine aggregation in cells cultured in broth. pPIT7003 is an ORF4 mutant plasmid. The strain carrying pPIT7022 was used as the wild type with respect to aggregation and transfer of pPD1, and the location of the Tn*917* insertion in pPIT7022 was mapped (see Fig. 7). Virtually all of the cells carrying pPIT7003 clumped, the cells formed large aggregates which sedimented at the bottom of the tube, and the supernatant fluid was clear. Cells carrying pPIT7031 or pPIT7323 formed small aggregates which remained in suspension in the medium, which had a turbid appearance. These findings indicated that clumping of cells carrying the pPIT7031 or pPIT7323 derivative was weaker than that of cells carrying each of the constitutive clumping ORF4 mutant plasmids. Conjugative transfer from *E. faecalis* FA2-2 to *E. faecalis* JH2SS was examined (Fig. 5). pPIT7022 represented normal transfer, which usually required about 60 min before significant transfer occurred, and was shown to give rise to increasing numbers of transconjugants for 2 to 3 h. pPIT7003, pPIT7031, and pPIT7323 exhibited a high degree of transfer early in the mating; they transferred at a relatively high frequency in 10 min, in contrast to the wild type. The transfer frequency of pPIT7003 at 90 min was almost as high as that after 2 to 3 h and about 1 order magnitude higher than that of pPIT7031 or pPIT7323.

DNA sequence analysis of ORFs. A search of the GenBank database revealed that several ORFs have significant homology with genes of the pheromone response regulatory region of pAD1 or pCF10 (Fig. 4 and 6). We performed DNA sequence analysis of ORF3 and ORF4, in which Tn*917* insertions gave rise to constitutive clumping, and the nearby ORFs (Fig. 4 and 6). ORF3, designated *traC*, encoded 545 residues with a molecular mass of 60.7 kDa. The start codon, ATG, is preceded by a good potential ribosome-binding site (GGAGGT) 9 bp upstream. The deduced protein, TraC, has a span of hydrophobic residues typical of a signal sequence at its amino terminus, and there is a potential signal peptidase processing site (40) corresponding to the V-L-A sequence at positions 18 through 20. Comparison of the amino acid sequence of TraC with those of TraC (543 amino acids) (40) and PrgZ (545 amino acids) (37), proteins associated with *E. faecalis* plasmid systems pAD1 and pCF10, respectively, showed very significant similarity, with more than 70 and 87%, respectively, of the aligned amino acid residues being identical. The 20-amino-acid leader peptide of *traC* of pPD1 was completely identical to that of *prgZ* of pCF10. TraC of pAD1 is believed to be located on the cell surface and mediate sex pheromone cAD1 binding (40). Tn*917* insertions in *traC* of pAD1 or *prgZ* do not produce the constitutive clumping phenotype (37, 40, 45).

ORF4, designated *traB*, encoded 384 residues with a molecular mass of 43.5 kDa. The deduced TraB protein has hydrophobic residues in the C-terminal third of its structure. Comparison of the amino acid sequence of TraB with those of TraB (1) and PrgY (37), proteins associated with *E. faecalis* plasmid systems pAD1 and pCF10, respectively, showed significant similarity, with more than 46 and 77%, respectively, of the aligned amino acid residues being identical. The sizes of the proteins were very similar and, as in TraB of pAD1 and PrgY, TraB of pPD1 exhibited strong hydrophobicity in the C-terminal third of its structure. The TraB protein encoded on pAD1 is believed to be involved in shutdown of endogenous pheromone cAD1 in cells carrying plasmid pAD1 (45). Tn*917* insertions in *traB* of pAD1 or *prgY* give rise to constitutive clumping (23, 37, 40, 45).

A segment between the terminator codon of *traC* and the initiator codon of *traB* was 13 bp, and the putative promoter

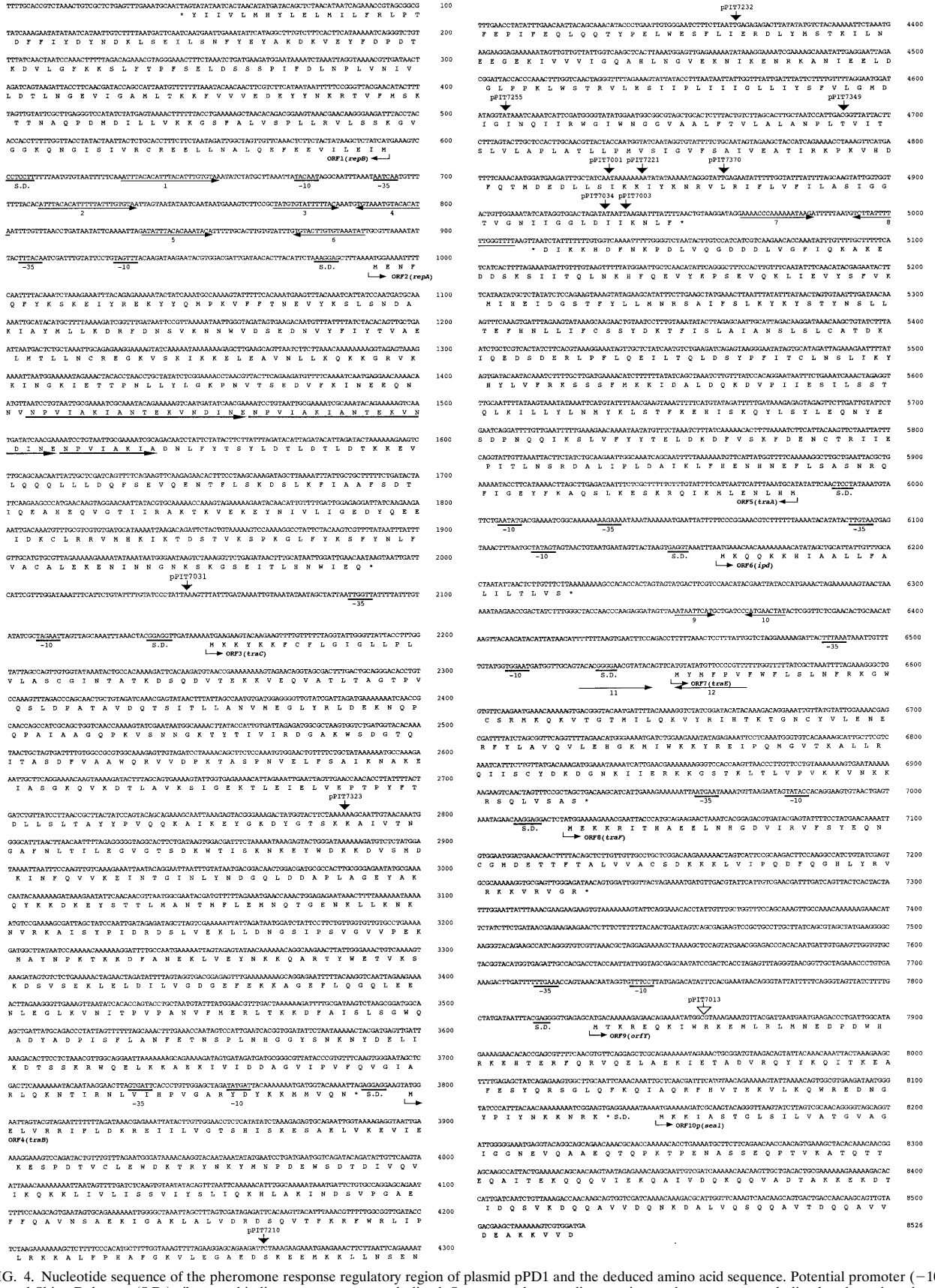

FIG. 4. Nucleotide sequence of the pheromone response regulatory region of plasmid pPD1 and the deduced amino acid sequence. Potential promoter (−10 and −35) and Shine-Dalgarno (S.D.) ribosome-binding sequences are underlined. Sequences that are direct or inverted repeats are underlined and numbered and are described in the text. A direct repeat sequence in ORF2 is underlined and is described in the text. Vertical arrows mark the locations of various Tn*917* insertions that gave rise to constitutive clumping. A vertical open arrow (pPIT7013) marks the location of the Tn*917* insertion that gave rise to normal clumping.

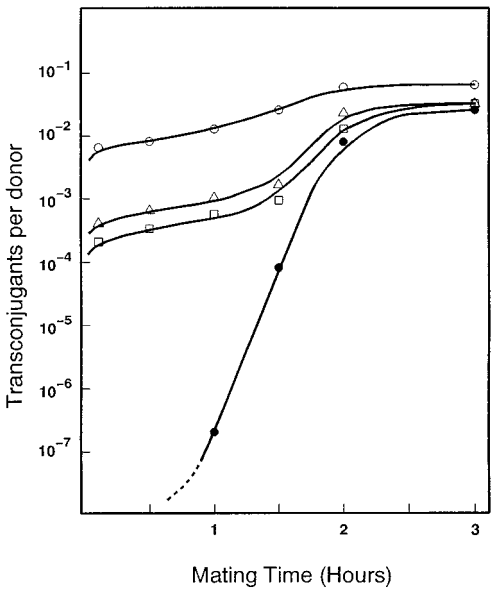

FIG. 5. Kinetics of plasmid transfer of pPD1::Tn*917* derivatives. Mating experiments were performed as previously described (21). The donor strains were FA2-2(pPIT7003), FA2-2(pPIT7031), FA2-2 (pPIT7323), and FA2-2(pPIT7022). The recipient was strain JH2SS. Matings were carried out with initial mixture volumes of 0.05 ml of donors, 0.45 ml of recipients, and 4.5 ml of fresh N2GT broth. The mixtures were incubated at 37°C with gentle shaking. At various times of incubation, 0.1-ml samples were removed, diluted approximately, and plated for donors (erythromycin, 25 μg/ml; rifampin, 25 μg/ml; fusidic acid, 25 μg/ml) and transconjugants (erythromycin, 25 μg/ml; streptomycin, 500 μg/ml; spectinomycin, 250 μg/ml). The number of transconjugants per donor was calculated and plotted as a function of time. Symbols: ○, pPIT7003; △, pPIT7031; □, pPIT7323; ●, pPIT7022.

sequence for *traB* was identified in the C-terminal region of *traC*.

ORF2, designated *repA*, was deduced to encode a protein of 333 amino acids with a molecular mass of 38.6 kDa. The protein encoded by *repA* exhibited extensive homology with the *prgW*-encoded protein of pCF10 (95.5% identical residues) and significant homology with the *repA*-encoded protein of pAD1 (53.7% identical residues) (46). As in the *prgW*-encoded protein, there was a directly repeated 54-bp sequence, followed by an additional repeat of the first 24 bp. RepA and PrgW, encoded on pAD1 and pCF10, respectively, are believed to be proteins required for replication (37, 46).

ORF1, designated *repB*, was oriented in the direction opposite to the other ORFs, except *traA*, in the regulatory region and encodes a predicted protein of 183 amino acids with a molecular mass of 21 kDa. The *repB* gene of pAD1 encodes 282 amino acids. The deduced *repB* product of pPD1 showed 34.1% homology with the first 164 amino residues of the *repB*-encoded amino acid sequence of pAD1 (46).

Strong ribosome-binding sites for *repA* and *repB* were found, and there were two inverted repeats between *repA* and *repB*; one consisted of 16-base inverted repeats separated by 4 bases, and the other consisted of 17-base inverted repeats separated by 20 bases.

There is no obvious transcription termination signal for *repA* or *traC*. A putative transcription termination signal for *traB* consisting of 18-base perfect inverted repeats separated by 9 bases was identified. This structure has an estimated free energy of −15.6 kcal (1 cal = 4.184 J)mol, and this could serve as the transcription termination signal. Conceivably, transcription could initiate from a promoter of *repA*, read through *traC* and *traB*, and terminate at the termination signal downstream of *traB*. The sequence homology suggested that *traB* was involved in shutdown of the endogenous pheromone, resulting in production of cPD1, which then self-induces the system. Like that of pAD1 derivatives with only the *traB* mutation (45), the culture filtrate of cells containing pPD1 with only the *traB* mutation had hardly detectable cPD1 (data not shown). The constitutive clumping of mutants with insertions upstream of *traB* could have resulted from polarity effects. The clumping or the transfer frequency of each of the insertion mutations upstream of *traB* was weaker, or about 1 order of magnitude lower, respectively, than that of the mutation in *traB*. This implied that transcription of *traB* was not completely defective in the insertions upstream of *traB*; some transcription probably also initiates from the *traB* promoter.

ORF5, designated *traA*, encodes 321 residues with a molecular mass of 37.7 kDa. The deduced protein encoded by *traA* exhibited significant homology with TraA (36.6%) of pAD1 (35) and PrgX (21%) of pCF10 (30), which are negative regulatory proteins of pAD1 (45) and pCF10 (16), respectively. All ORFs in the regulatory region were arranged in the same

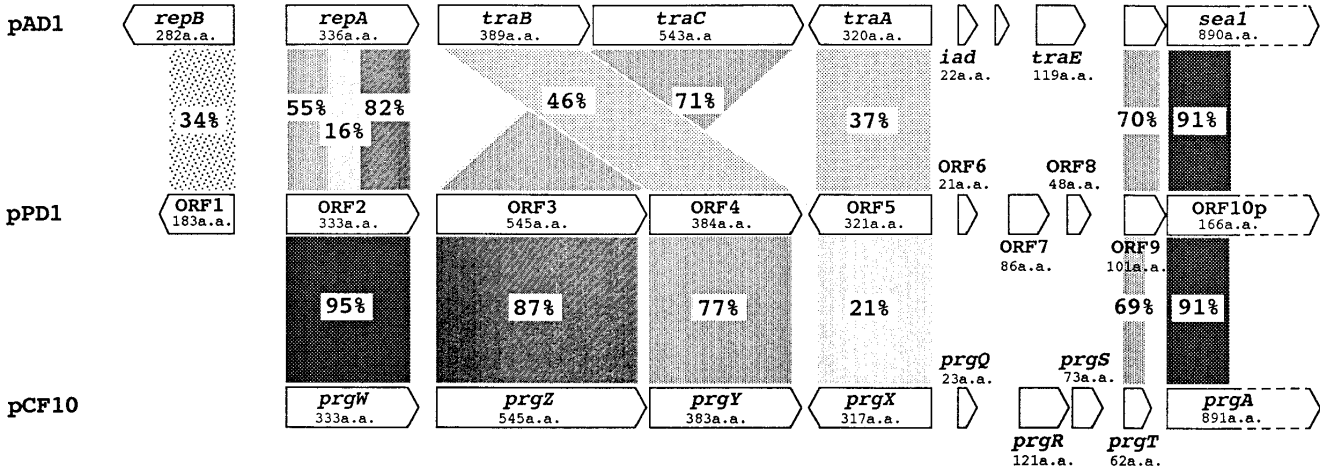

FIG. 6. Graphic comparison of the pheromone response regulatory regions of plasmids pAD1, pPD1, and pCF10 of *E. faecalis*. The values indicating degrees of homology between the gene of pAD1 or pCF10 and the corresponding ORF of pPD1 refer to percent amino acid (a.a.) identity.

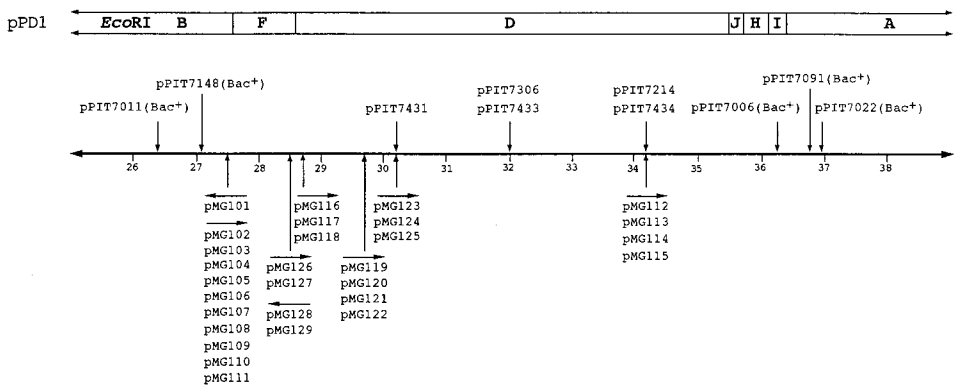

FIG. 7. Map of the bacteriocin-encoding region of pPD1. Vertical arrows above or below the line indicate the positions of Tn*917* and Tn*916* insertions, respectively, on the bacteriocin-encoding region of the pPD1 physical map. pPIT7214, pPIT7306, pPIT7431, pPIT7433, and pPIT7434 are Tn*917* insertions with altered bacteriocin expression. All of the insertions from pMG101 to pMG129 gave rise to altered bacteriocin expression. The orientations of Tn*916* insertions are reflected by arrows which point from the left end toward the right end of the transposon (i.e., the arrowhead corresponds to the right end).

orientation, but like the *traA* and *prgX* genes in pAD1 and pCF10, respectively, *traA* of pPD1 was transcribed in the direction opposite to that of the other ORFs in the regulatory region. A putative transcription termination signal which followed the TAA stop codon for *traA* of pPD1 appeared to be a factor-independent terminator consisting of 18-base perfect inverted repeats separated by 9 bases (Fig. 4).

ORF6 was designated *ipd* as it appears to represent the competitive inhibitor iPD. The *ipd* is located to the right of *traA* and encodes 21 amino acids. The carboxyl-terminal eight residues correspond to those of iPD1 (34).

Other ORFs and the sequence homology to the genes of pAD1 (21, 22, 36) and pCF10 (30) are also shown in Fig. 4 and 6.

Generation and mapping of transposon insertion mutants with altered bacteriocin expression. To map the bacteriocin determinant on pPD1, pPD1::Tn*917* derivatives were examined for bacteriocin expression. The indicator strain for bacteriocin expression was *E. hirae* 9790. Five independent insertion mutants that were altered in bacteriocin expression were isolated from about 10^4 pPD1::Tn*917* derivatives derived from 3,000 independent experiments as described in Materials and Methods. The insertions were designated pPIT7214, pPIT7303, pPIT7431, pPIT7433, and pPIT7434 and were found at three different positions (Fig. 7).

Mutants with altered bacteriocin expression were also isolated by insertion of Tn*916* into pPD1 as described in Materials and Methods. Of 64 mutants isolated from 26 independent mating experiments, 29 were selected for further analysis. These mutants did not express bacteriocin activity. The locations of the Tn*916* insertions were mapped on pPD1 as shown in Fig. 7. The locations of Tn*916* insertions were mapped in *Eco*RI-D, *Eco*RI-F, and a part of *Eco*RI-B. These results were not inconsistent with the results obtained by Tn*917* insertion. From these results, the approximate location of the bacteriocin determinant on pPD1 was observed to include *Eco*RI fragments D and F and a portion of *Eco*RI fragment B (Fig. 7).

Transposon mutagenesis revealed that a minimum of 6.7 kb of pPD1 is required for bacteriocin synthesis. The size was relatively large, suggesting that several genes are required for bacteriocin production.

Bacteriocin activity was examined in the relational clone set. No *E. coli* DH1 derivative containing any member of the relational clone set expressed bacteriocin activity. Each plasmid clone was then introduced into *E. faecalis* OG1X by electrotransformation (20), and the bacteriocin activity was examined. Clones containing *Eco*RI fragments B, D, and F expressed bacteriocin activity (Fig. 1). Clones containing *Eco*RI fragments D and F or F and B did not express bacteriocin activity.

Bacteriocin activities of pPD1, pPD1::Tn*917*, and pPD1::Tn*916* derivatives. The methods and indicator strains used to test bacteriocin activity were those described in Materials and Methods. Bacteriocin of FA2-2(pPD1) was active against *E. faecalis*, *E. hirae* 9790, *E. faecium*, *S. agalactiae*, *S. sanguis*, and *S. aureus*. Bacteriocins are thought to provide producer strains with a selective advantage over other, related strains (3, 5, 31) and usually exhibit a relatively narrow spectrum of susceptible bacteria, whereas, the bacteriocin activity encoded on pPD1 was lethal to a wide variety of gram-positive bacteria. To examine whether pPD1 carried genes for multiple bacteriocins, the pPD1::Tn*917* and pPD1::Tn*916* derivatives with altered bacteriocin expression were examined for bacteriocin activity against various bacteria. All of the derivatives also had altered bacteriocin expression against the various sensitive bacterial species, suggesting that a single bacteriocin exhibited the wide spectrum.

ACKNOWLEDGMENTS

This work was supported by grants 06670283, 06770195, and 07670298 from the Japanese Ministry of Education, Science and Culture.

We thank D. B. Clewell and E. Kamei for helpful advice on the manuscript.

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