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Identification, Characterization, and Nucleotide Sequence of a Region of *Enterococcus faecalis* Pheromone-Responsive Plasmid pAD1 Capable of Autonomous Replication

KEITH E. WEAVER,[1]* DON B. CLEWELL,[2] AND FLORENCE AN[2]

Department of Microbiology, School of Medicine, University of South Dakota, Vermillion, South Dakota 57069,[1] and Department of Biologic and Materials Sciences, School of Dentistry, The University of Michigan, Ann Arbor, Michigan 48109-0402[2]

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A 5-kbp region of pAD1, previously shown to be capable of supporting replication, copy control, and stable inheritance of the plasmid, was cloned into a replicon probe vector and subjected to transposon insertional mutagenesis. Transposon inserts identifying essential replication, copy control, and stability functions were isolated. Deletion of stability functions not essential for replication resulted in delimitation of a basic replicon. The complete DNA sequence of this ≈3-kbp region and the precise positions of several transposon inserts were determined, and the phenotypic effects of the transposon inserts were correlated with the physical locations of individual determinants. The following three genes, apparently involved in plasmid maintenance, were identified; *repA*, which encodes a protein required for replication; *repB*, which encodes a protein involved in copy control; and *repC*, which may be involved in stable inheritance. In addition, two clusters of repeats composed of a consensus sequence, TAGTARRR, were identified, one located between the divergently transcribed *repA* and *repB* genes and another located downstream of *repC*. The region between *repA* and *repB* contained 25 repeats divided into two subregions of 13 and 12 repeats separated by 78 bp. The region located downstream of *repC* contained only three repeats but may be essential for plasmid replication, since deletion of this determinant resulted in loss of ability to replicate in *Enterococcus faecalis*. We hypothesize that the repeat units represent protein-binding sites required for assembly of the replisome and control of plasmid copy number. Another region of unrelated repeat units that may also be involved in replication is located within the *repA* gene. Possible mechanisms of action of these determinants are discussed.

Enterococcus faecalis plasmid pAD1 is an ≈60-kbp conjugative, low-copy (one to four copies per chromosomal equivalent) plasmid which carries determinants that encode a cytolytic exotoxin (30) and resistance to UV light (4). The cytolytic exotoxin has been implicated as a virulence factor (16–18), and several studies on its mechanism of action have been published (11, 24). In addition, another enterococcal plasmid closely related to pAD1, pBEM10, has been reported to encode a β-lactamase-type determinant (20). The pheromone-responsive conjugation system of pAD1 and related plasmids is probably one of the best-understood transfer systems encoded by plasmids of gram-positive bacteria and has been recently reviewed (5, 9). Briefly, the conjugative functions encoded on such plasmids remain repressed until the host cell is exposed to a specific peptide sex pheromone secreted by plasmid-free cells. Pheromone exposure induces production of a surface protein, called an aggregation substance, which facilitates aggregation of plasmid-containing and plasmid-free cells, initiating the intimate contact necessary for plasmid transfer. Determinants required for DNA processing and transfer are also induced. Upon acquisition of the plasmid, the pheromone specific for that plasmid is shut down by an unknown mechanism and a related plasmid-encoded peptide which functions as a competitive inhibitor of pheromone function is produced. The DNA sequences of genes that encode the aggregation substance from three pheromone-responsive plasmids (pAD1, pCF10, and pPD1) have been determined and found to be highly homologous (10). All three aggregation substances contain RGD motifs that may be involved in the observed aggregation substance adhesion to eukaryotic cells (19), implicating the aggregation substance as an important adhesin for *Enterococcus faecalis*. Finally, some of the pheromones themselves have been implicated as possible chemotactic factors for lymphocytes and may act to facilitate an effective host immune response to invading enterococci (24).

Although the conjugative mechanisms of pAD1 have been well studied, little is known about the determinants which control plasmid maintenance functions required for replication and stable inheritance. Indeed, except for the family of small, multicopy, broad-host-range plasmids known to replicate via single-stranded intermediates (reviewed in reference 12) and related theta replicating, broad-host-range plasmids pAMβ1 and pIP501 (1, 2), little is known about the maintenance functions of plasmids native to gram-positive bacteria in general. Previous work on pAD1 had identified an ≈5-kb region of DNA sufficient to support replication, copy control, and stable inheritance (32, 35). This maintenance region was found to be immediately adjacent to the pheromone response regulatory region, an ≈7-kb region that encodes all determinants required for pheromone shutdown, inhibitor production, and transduction of the pheromone signal. Some circumstantial evidence indicated that expression of plasmid conjugation and maintenance functions are linked. For example, a mutation that effects stable inheritance of the plasmid was found to alter expression of a pheromone-inducible promoter (33).

To understand fully the biology of the pheromone-responsive plasmids, it is necessary to determine their mechanisms

* Corresponding author.

of replication and stable inheritance. In this report, we describe the identification and characterization of a pAD1-encoded basic replicon capable of supporting replication in *E. faecalis* and of maintaining the plasmid at the appropriate copy number. Correlation of DNA sequence data and phenotypic analysis of several transpositional insertion mutants within the basic replicon have identified determinants involved in replication, copy control, and stability of the plasmid. Possible mechanisms of replication and copy control are discussed.

MATERIALS AND METHODS

Bacterial strains and plasmids. Plasmid constructions and other DNA manipulations were performed with *Escherichia coli* JM101 (IBI, New Haven, Conn.), HB101 (GIBCO/BRL, Gaithersburg, Md.), or DH5α (Promega, Madison, Wis.) (23). Determination of the maintenance characteristics of the pAD1 replicon and its derivatives was performed primarily with *E. faecalis* OG1X, a streptomycin-resistant, extracellular protease-deficient derivative of OG1S (15). Incompatibility experiments were performed with *E. faecalis* UV202, a recombination-deficient, rifampin- and fusidic acid-resistant derivative of JH2-2 (37).

Most of the plasmids used in and constructed for this study are depicted graphically in Fig. 1 and 2 and described in the text. Hybrid plasmid pDAK102 (Fig. 1), the primary focus of this study, is a 14.3-kb plasmid consisting of ≈5.4 kb of pAD1 DNA previously identified as sufficient for directing plasmid replication in *E. faecalis* (33, 35). The source of pAD1 DNA, pDAK2300E, was previously described (35). The remainder of pDAK102 is composed of replicon probe vector pAM201, which was constructed from shuttle vector pWM401 (36) as shown in Fig. 1. Replication of pDAK102 is supported in *E. coli* by the pACYC184 replicon present on pAM201 and in *E. faecalis* by the cloned pAD1 replicon. The two vector *cat* genes allow selection for chloramphenicol resistance (Cm^r) in both *E. coli* and *E. faecalis*. Transposon inserts and subclones of pDAK102, as well as other pAD1 replicon derivatives, are shown in Fig. 2, and their characteristics are described in Results. Other plasmids used as reference or control plasmids in particular experiments will be described where appropriate.

Media, enzymes, and reagents. *E. coli* was grown in Luria broth (23). *E. faecalis* OG1X was grown in nutrient broth no. 2 (Oxoid Ltd., London, England) supplemented with 0.1 M Tris buffer (pH 7.5) and 0.2% glucose (N2GT medium). Strain UV202 was grown in Todd-Hewitt broth (Difco Laboratories, Detroit, Mich.). Agar (1.8%; Difco) was added for preparation of solid media. Antibiotics were generally used in the following concentrations: ampicillin, 100 μg/ml; streptomycin, 1 mg/ml; rifampin, 25 μg/ml; fusidic acid, 25 μg/ml; erythromycin, 10 μg/ml. Chloramphenicol was used at either 10 or 25 μg/ml, depending on the experiment. 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal; Sigma Chemical Co., St. Louis, Mo.) was used at a concentration of 40 μg/ml.

DNA purification, manipulation, and transformation. Large-scale plasmid purifications were performed by CsCl-ethidium bromide equilibrium density gradient ultracentrifugation as previously described (6). Small-scale plasmid preparations from *E. faecalis* were performed by the modified alkaline lysis procedure previously described (32), and those from *E. coli* were performed by the boiling method of Holmes and Quigley (13). Plasmid constructs were analyzed by digestion with various restriction enzymes and separation

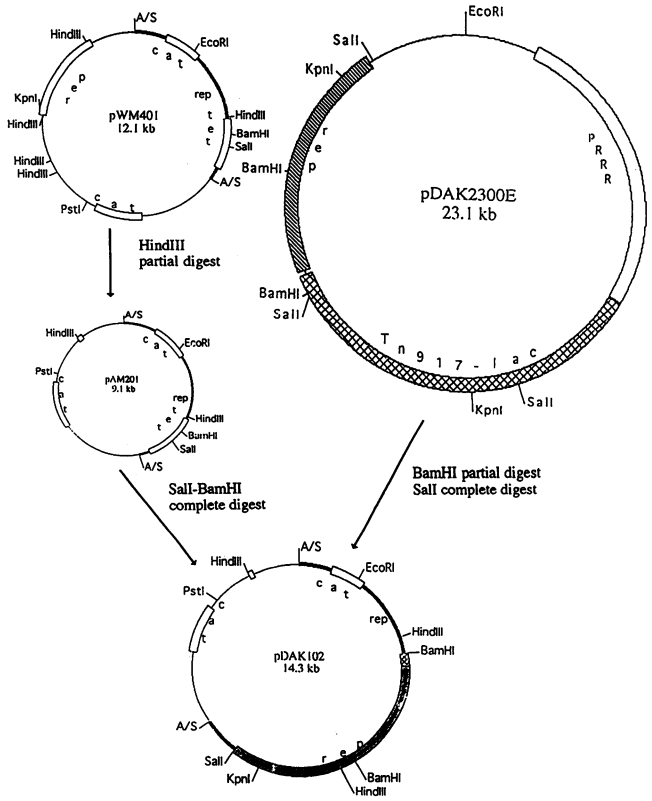

FIG. 1. Construction of pDAK102, a hybrid plasmid containing pACYC184 and pAD1 replicons. Replicon probe vector pAM201 was constructed from shuttle vector pWM401 (36) by partial digestion with *Hin*dIII, which resulted in deletion of the pIP501 replicon. Therefore, pAM201 is capable of replication in *E. coli*, supported by the pACYC184 replicon, but not in *E. faecalis*. pAD1 miniplasmid pDAK2300E (35) was used as the source of the pAD1 replicon. pDAK2300E is a miniplasmid consisting of the pAD1 *Eco*RI B fragment and the B30 Tn*917-lac* insert located in *traB*. pDAK2300E and pAM201 were digested with *Bam*HI and *Sal*I; pDAK2300E was partially digested with *Bam*HI to obtain some fragments which were not cut at the *Bam*HI site located near the center of the pAD1 maintenance region. Digested samples were mixed, ligated, and introduced into OG1X. The resultant plasmid, pDAK102, contains two *cat* genes, one expressing Cm^r in *E. coli* and the other expressing Cm^r in *E. faecalis*. In each plasmid map, the thicker black line represents the pACYC184 sequence, the cross-hatched box represents the pAD1 replicon, and the double cross-hatched box represents Tn*917-lac*. PRRR on pDAK2300E stands for the pheromone response regulatory region. Note that pDAK102 contains ≈300 bp of Tn*917-lac* on the counterclockwise side of the pAD1 replicon.

of restriction fragments on 0.8% agarose gels. Restriction enzymes were purchased from New England BioLabs (Beverly, Mass.) and used under the conditions recommended by the manufacturer. Tn*5* transposon mutagenesis of pDAK102 was performed with *E. coli* by the method of de Bruijn and Lupski (8), modified as previously described (38). Tn*917* and Tn*917-lac* mutagenesis was carried out as previously described (14, 31). The effects of Tn*917* insert 710, present on pAD1 derivative pAM710, on plasmid copy number were previously described by Ike and Clewell (14). Tn*917-lac* insert 2016 and Tn*917* insert 107 are described here for the first time.

Plasmid constructs were introduced by electroporation

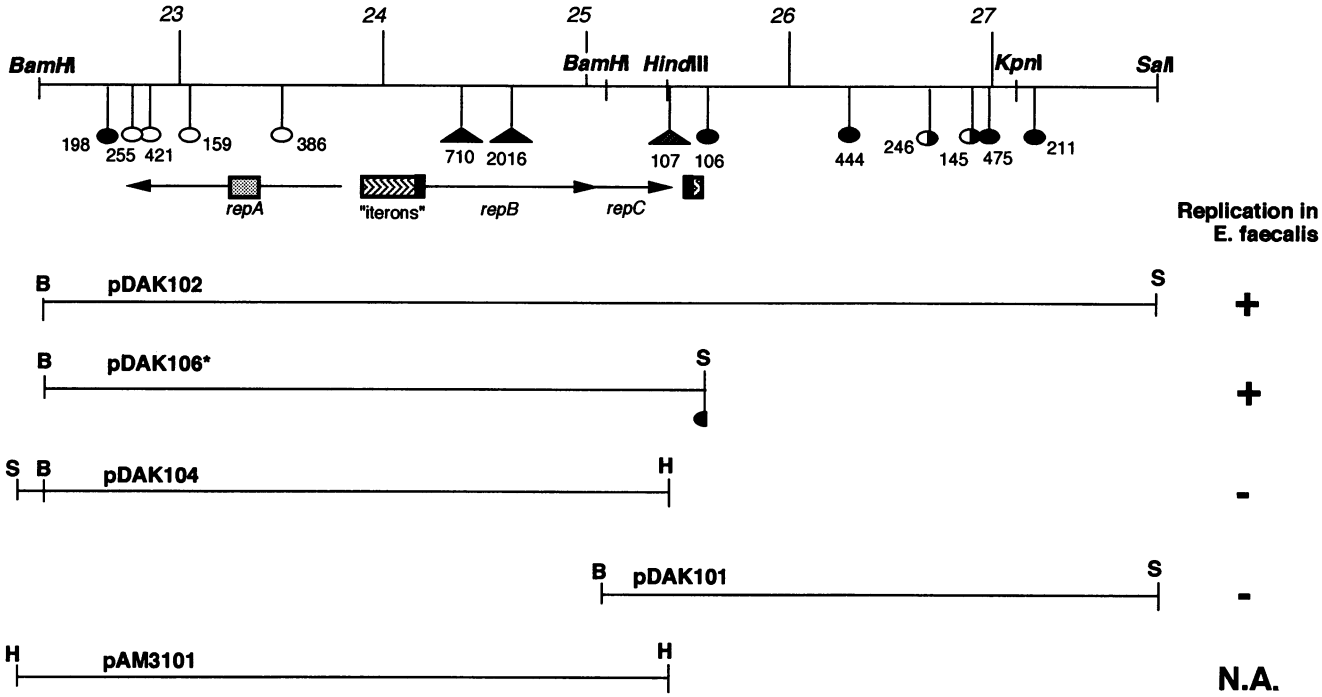

FIG. 2. Insertional mutagenesis and subcloning of the pAD1 maintenance region. The top line represents the portion of pAD1 DNA cloned in pDAK102. The *Bam*HI site marking the left end of this fragment is derived from Tn*917-lac* and is not a native pAD1 restriction site. Markers with circular heads represent Tn*5* transposon inserts constructed in *E. coli* on pDAK102. Markers with triangular heads represent Tn*917* (710 and 107) or Tn*917-lac* (2016) inserts isolated on other pAD1 derivatives in *E. faecalis*; these inserts are not present on pDAK102. Marker fill patterns represent the following maintenance phenotypes: filled heads, no detectable effect; open heads, no replication in *E. faecalis*; half-filled heads, loss of plasmid stability; stippled heads (710 and 2016), increased copy number and loss of stability; dark stripes (107), loss of stability. Insert 2016 forms a productive *lacZ* fusion read left to right. Below the insert map are represented the maintenance determinants identified on the basis of DNA sequence analysis. Depicted are the putative coding sequences for the three structural genes encoded within the basic replicon (labelled arrows indicate directions of transcription), direct and inverted repeats that may be involved in origin function and transcriptional regulation. The hatched boxes represent the TAGTARRR iterons, and the stippled box represents the unrelated *repA* internal direct repeats. The black boxes represent two unrelated inverted repeats. The left inverted repeat contains a TAGTARRR repeat between the two arms. The right inverted repeat may represent a transcriptional terminator. Lines shown below the insert map represent the extent of pAD1 DNA present in several maintenance region subclones. Both pDAK106* and pDAK101 are pAM201 clones, like pDAK102. pDAK106* was constructed by deletion of a *Sal*I fragment bordered by an internal Tn*5* site on insert 106 and the *Sal*I site marking the right end of the maintenance region. pDAK101 was isolated fortuitously from the cloning experiment depicted in Fig. 1. pDAK104 consists of the maintenance region DNA indicated cloned into replicon probe vector pMTL21C (26; kindly provided by N. Minton). The source for the pAD1 DNA present in pDAK104 was pDAK2300E (Fig. 1). pMTL21C was used for this construction because a unique *Hin*dIII site is not available in pAM201. Both the *Bam*HI and *Sal*I sites marking the left end of the fragment are derived from Tn*917*. pAM3101, which was used to determine most of the nucleotide sequence of the maintenance region, contained the pAD1 DNA to the left of the *Hin*dIII site and extends to a *Hin*dIII site at approximately 22.2 kb on the pAD1 map inserted in pBluescript. It does not contain the Tn*917-lac* remnant which contributes the *Bam*HI site present on the other constructs depicted here. Since pBluescript does not contain a suitable antibiotic resistance marker, the replication competence of this construct was not tested in *E. faecalis* (N.A.).

into *E. coli* JM101 by using a BRL Cell-Porator Electroporation System I and Voltage Booster. Cells were prepared and electroporated as prescribed by the manufacturer. On a few occasions, constructs were introduced into HB101 competent cells purchased from GIBCO/BRL in accordance with the procedure described by the manufacturer. Plasmid constructs were introduced into *E. faecalis* by electroporation of glycine-treated cells as described by Cruz-Rodz and Gilmore (7). In most cases, when plasmid DNA was to be transferred from an *E. coli* strain to an *E. faecalis* strain, as was often the case with pDAK102 and its derivatives, DNA was purified by the rapid-boiling method rather than by large-scale preparations. Precipitated DNA was resuspended in 5 μl of sterile water and used directly to electroporate prepared *E. faecalis* cells. Electroporation with small-scale plasmid preparations was generally very efficient, resulting in total transformants of 100 to >1,000 cells. In other cases, plasmid DNA purified from large-scale preparations from either *E. coli* or *E. faecalis* were used.

Characterization of pAD1 replicon functions. pDAK102 and its derivatives constructed in *E. coli* were examined first for the ability to replicate in *E. faecalis*. Plasmids determined to be incapable of replication and/or establishment in *E. faecalis* were identified by the failure to transform either OG1X or UV202 by electroporation. DNA purified by both large- and small-scale preparations was tested. Controls with equal or smaller amounts of either pDAK102 or a derivative previously shown to be able to replicate in *E. faecalis* were run with each experiment to ensure that the electroporation procedure worked properly.

Once replication was established, plasmid stability, copy number, and incompatibility were determined. Stability was determined as previously described (33). Briefly, cultures were grown overnight in medium containing the appropriate

antibiotic to ensure that nearly all cells contained plasmid DNA at the beginning of the experiment. Cells were then diluted $\approx 10^{-4}$ in antibiotic-free medium and maintained in the log phase from that point by periodic dilution. At 24-h intervals, aliquots were plated on antibiotic-free solid medium, grown overnight, and then transferred to antibiotic-containing plates by replica plating or by transferring individual colonies with toothpicks. Maintenance of the plasmid was then expressed as percent maintenance of the plasmid-encoded antibiotic resistance marker.

Plasmid copy number was determined by fractionating extracts of [^{3}H]thymidine-labelled cultures by CsCl-ethidium bromide centrifugation (6) or by comparison of test and internal-standard plasmid yields from small-scale alkaline lysate preparations (29). In general, the latter procedure was used as an initial screen for copy number defects and the former was used for more accurate copy number determinations on plasmids showing abnormal copy numbers. The internal standard used was pAM378, a pAM373 derivative marked with Tn*918* (tetracycline resistance [Tc^r]). pAM378 was introduced into strains containing the plasmid of interest by filter matings as previously described (3). Plasmid DNA was then purified by alkaline lysis and cut with *Sal*I. Plasmid bands were then separated on 0.8% agarose gels, stained with ethidium bromide, and photographed with Polaroid positive-negative instant pack film (Fotodyne, New Berlin, Wis.). Densitometric tracings of the photographic negative were made on a Zeineh Soft Laser Scanning Densitometer (Biomed Instruments Inc., Chicago, Ill.). Areas under peaks were determined by integration and adjusted for differences in the sizes of the fragment measured. The ratio of the yield of the test plasmid to that of the standard plasmid (pAM378) was determined and compared to a control ratio, determined on the same gel, of pDAK102 to pAM378. Results provided a test plasmid copy number relative to that of pDAK102. To test this procedure, copy numbers of pDAK102 and pDAK2016E, a miniplasmid derivative containing the 2016 insert (Fig. 2) on the pAD1 *Eco*RI B fragment, were determined by [^{3}H]thymidine labelling. pAM378 was then introduced into cells containing each plasmid and the ratio of test plasmid yield to standard-plasmid yield was determined. It was determined by the [^{3}H]thymidine labelling procedure that the pDAK102 copy number was equivalent to that of wild-type pAD1. Both procedures indicated that the copy number of pDAK2016E was two- to threefold higher than that of pDAK102, confirming the value of the method utilizing an internal standard for screening mutants for possible copy number defects.

Incompatibility was determined by introducing test plasmids, generally, Cm^r pDAK102 derivatives, by electroporation into an *E. faecalis* UV202 strain containing pAM2005. pAM2005 (34) is a nonconjugative pAD1::Tn*917-lac* derivative. An intact pAD1 derivative was used as a resident to rule out the possibility that an auxiliary replicon, unrelated to that cloned on pDAK102, was capable of supporting replication in the presence of an incompatible plasmid. A nonconjugative derivative was used to eliminate complications in the incompatibility data caused by aggregation or transfer of the resident plasmid back into segregants. After electroporation, Cm^r transformants were selected and several were streaked for isolation of single colonies on fresh chloramphenicol-containing plates. Single-colony isolation was necessary because of background growth of chloramphenicol-sensitive colonies which occurred on selective plates. Twenty-five individual colonies from each streak plate were then screened for maintenance of the unselected resident plasmid by transferring with toothpicks to erythromycin-containing plates. Loss of the resident plasmid was indicative of incompatibility. pWM401 (36; Fig. 1), a Cm^r shuttle vector which utilizes the unrelated pIP501 replicon for maintenance in *E. faecalis*, was used as a compatible test plasmid control.

DNA sequencing, sequence analysis, and identification of protein products of postulated open reading frames (ORFs). The DNA sequence of both strands of the identified pAD1 basic replicon was determined by compiling sequence information from several different plasmid constructs. Dideoxy sequencing was performed by using Sequenase (United States Biochemical, Cleveland Ohio) in accordance with the instructions of the manufacturer. Most of the replicon sequence was determined by using pAM3101, a pBluescript construct (Stratagene, La Jolla, Calif.) containing a pAD1 *Hin*dIII fragment extending from 22.2 to 25.3 kb on the pAD1 map (manuscript in preparation). This fragment contains the left half of the maintenance region to the *Hin*dIII site (Fig. 2) and a portion of the neighboring *traB* gene. It does not contain the Tn*917-lac* remnant which supplied the *Bam*HI site used to construct pDAK102. Sequencing was performed on nested deletions of the insert, constructed with an ExoIII/Mung Bean Deletion Kit (Stratagene). A few difficult-to-sequence gaps were filled in by using synthetic primers complementary to the adjacent sequence. The remainder of the sequence of the replicon, from the *Hin*dIII site to the 106 insert, was determined from two constructs. One strand was sequenced directly from pDAK106* by using a primer, 5′AAAGGTTCCGTTCAGGA3′, complementary to a sequence within the inverted repeat of Tn*5*. Since one of the inverted repeats was deleted in the construction of pDAK106*, this primer allowed sequencing toward and across the *Hin*dIII site. The other strand was sequenced from a pTZ18R (Pharmacia [Piscataway, N.J.] phagemid cloning vector, a gift from R. Lindahl) construct containing the DNA between the *Hin*dIII site and insert 145 (Fig. 2). The positions of transposon inserts were determined either by sequencing out from the transposon by using a primer complementary to one transposon end or by sequencing toward the transposon by using a synthetic primer complementary to the sequence believed to be adjacent to the transposon on the basis of restriction mapping data. DNA sequence analysis was performed by using DNA Inspector Ile (Textco, Inc., West Lebanon, N.H.) or MacVector (IBI). Comparison to GenBank data was accomplished by using the Protein Identification Resource, National Biomedical Research Foundation, Washington, D.C. Identification of protein products of putative ORFs was performed by in vitro transcription-translation by using a DNA-directed translation kit (Amersham Co., Arlington Heights, Ill.).

Nucleotide sequence accession number. The GenBank accession number of the sequence reported here is L01794.

RESULTS

Characterization of the pAD1 maintenance region and identification of the pAD1-encoded basic replicon. Previous characterization of pAD1 miniplasmid constructs (33, 35) identified an ≈5-kb region of pAD1 DNA, located between 23 and 28 kb on the pAD1 map, sufficient to support plasmid replication in *E. faecalis*. To characterize the pAD1 replicon further, this region of DNA was cloned in replicon probe vector pAM201 by the strategy shown in Fig. 1. The resulting plasmid, pDAK102, replicates in *E. coli* by utilizing the vector-encoded pACYC184 replicon and in *E. faecalis* by

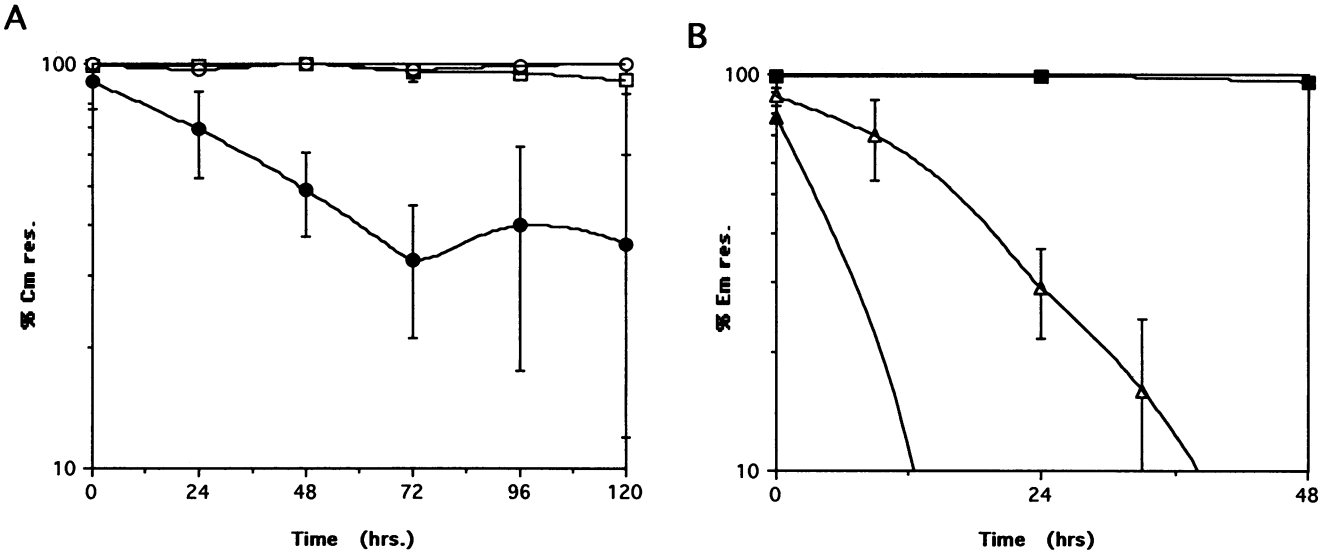

FIG. 3. Stability of pAD1 maintenance region mutants. Plasmid stability was monitored by periodic sampling of cultures maintained in the log phase in antibiotic-free medium as previously described (33). Experiments were done with an OG1X host grown in N2GT medium. Since growth rates remained relatively steady throughout the experiment and since no significant differences in growth rate were observed for the various plasmid-containing strains, for simplicity percent maintenance is graphed against time rather than generations. (A) Stability of minimal replicon pDAK106*. Controls used were pDAK102 and pDAK106 (pDAK102 containing the 106 insert, the parent plasmid of pDAK106*). ○, pDAK106; ●, pDAK106*; □, pDAK102. Cm res., Chloramphenicol resistance (B) Stability of pDAK2016E and pDAK107E. These two plasmids are miniplasmid derivatives of pAD1 consisting of the *Eco*RI B fragment and the 2016 or 107 insert, respectively, constructed as previously described (33). Miniplasmids were used because in contrast to the parental plasmids, they are nonconjugative. Therefore, stability can be measured without concern for conjugative transfer back to plasmid-free segregants. pDAK2300E, a similarly constructed miniplasmid with a Tn*917-lac* insert located within *traB*, outside of the maintenance region, was used as a control. ■, pDAK2300E; △, pDAK2016E, ▲, pDAK107E. All lines shown, except that for pDAK107E, are an average of three experiments, and error bars show standard deviations. Stability of pDAK107E was determined several times, and the line shown represents the greatest stability observed. A 15-h time point where 3% Em^r was observed is off the scale. Em res., erythromycin resistance.

utilizing the cloned pAD1 replicon and expresses Cm^r in both hosts. In *E. faecalis*, pDAK102 is maintained at a copy number of two to four per chromosomal equivalent, identical to that observed with wild-type pAD1, expresses incompatibility against pAD1 derivatives, and is stably maintained, showing <5% loss over 120 generations (Fig. 3A), indicating that sufficient information is present on the cloned fragment to support plasmid maintenance in a manner indistinguishable from that of wild-type pAD1.

Plasmid pDAK102 was then subjected to Tn*5* transposon mutagenesis in *E. coli*, and the maintenance characteristics of various inserts within the cloned replicon were determined following reintroduction into *E. faecalis*. The positions of relevant inserts, confirmed by sequence analysis, and their phenotypes are shown in Fig. 2. Three general classes on Tn*5* insertion mutants were identified. Plasmids containing inserts 198, 106, 444, 475, and 211, marked with filled circles in Fig. 2, had maintenance characteristics in *E. faecalis* indistinguishable from those of pDAK102. Plasmids containing inserts 255, 421, 159, and 386, marked with open circles, could not be reintroduced into *E. faecalis*, indicating that these inserts disrupt a genetic determinant required for pAD1 maintenance or establishment. Plasmids containing inserts 246 and 145, marked with half-filled circles, were maintained in ≤20% of cells after overnight growth in the absence of selection, indicating that they disrupt a pAD1-encoded determinant required for stable inheritance.

Also shown in Fig. 2 are the positions of two Tn*917* (710 and 107) inserts and one Tn*917-lac* (2016) insert previously isolated during transposon mutagenesis of the intact pAD1 plasmid. The 710 insert present on plasmid pAM710 is a pAD1 copy number mutant previously described by Ike and Clewell (14). pAM710 is maintained at a copy number about fivefold higher than that of wild-type pAD1. The 2016 insert caused a two- to threefold increase in plasmid copy number. In addition, the *lacZ* gene of the 2016 insert forms a productive fusion that is expressed constitutively at a relatively high level (an average of ≈60 Miller units) compared with other Tn*917-lac* fusions previously constructed on pAD1, e.g., uninduced *tra* genes (32). The 107 insert showed no apparent change in plasmid copy number. The segregational stability of two pAD1 miniplasmid derivatives containing the 2016 and 107 inserts, pDAK2016E and pDAK107E, respectively, was determined. As shown in Fig. 3B, inserts 107 and 2016 resulted in decreased plasmid stability.

To identify the basic replicon, three maintenance region subclones were constructed (Fig. 2). pDAK101 was isolated fortuitously during pDAK102 construction and consists of the indicated pAD1 DNA in pAM201. Not surprisingly, this construct could not be introduced into *E. faecalis*, presumably because it lacks the determinant identified by transposon mutagenesis as essential for replication. pDAK106* is a deletion derivative of pDAK102 containing only the pAD1 maintenance region DNA to the left of the 106 insert. pDAK104 contains the pAD1 replicon DNA to the left of the *Hin*dIII site cloned into replicon probe vector pMTL21C (26) (kindly provided by Nigel Minton). Thus, pDAK104 lacks 211 bp of pAD1 DNA present in pDAK106* (see sequence data below). Despite this small difference in genetic information, pDAK106* could be introduced into *E. faecalis*

while pDAK104 could not. Although we cannot formally rule out the possibility that pMTL21C adversely affects the function of the cloned pAD1 replicon, the simplest explanation for this finding is that a determinant essential for plasmid maintenance is present on the DNA missing from pDAK104. Therefore, pDAK106* closely defines the border of an element of the basic replicon.

The maintenance characteristics of pDAK106* were determined as described in Materials and Methods. The copy number of pDAK106* was indistinguishable from that of pDAK102. pDAK106* also expressed incompatibility against a nonconjugative derivative of pAD1, pAM2005. Plasmids pWM401, pDAK102, pDAK106 (pDAK106 is a pDAK102::Tn*5* derivative containing the 106 insert; it is the parent plasmid of pDAK106* [Fig. 2]), and pDAK106* (plasmid structures are depicted graphically in Fig. 2) were introduced by electroporation into an *E. faecalis* strain containing pAM2005, an intact pAD1 derivative containing a Tn*917-lac* insert (encoding Em^r) within *traE*, disrupting the conjugative system. Cm^r electroporants were obtained and streaked for isolation, and then 25 colonies were screened for maintenance of the unselected Em^r marker. In an average of seven or eight isolates from three separate experiments, the respective plasmids produced 100, 41.7, 0, and 58.5% maintenance of Em^r. It should be noted that although pDAK102 and pDAK106* transformants showed average pAM2005 maintenance percentages of 42 and 58%, respectively, a large degree of variability was observed between individual isolates. Thus, in two isolates that received pDAK106*, loss of pAM2005 was complete (0% maintenance). In the remaining five isolates, maintenance was >50% but always <100%. A similar pattern was observed with pDAK102. Retention was not due to recombination between the two plasmids, since no cointegrates were observed in plasmid preparations of heteroplasmid-containing cells. We have no explanation for the nearly all-or-nothing nature of incompatibility expression, although it is possible that bacteriocin production by pAM2005 helps sustain its presence if it does not segregate out after the first few generations following introduction of the incompatible plasmid. In contrast to pDAK102 and pDAK106*, introduction of pDAK106 (pDAK102::Tn*5* 106 insert) always resulted in complete loss of pAM2005. Therefore, it appears that the 106 insert results in a higher level of incompatibility than is observed with pDAK102. This effect is lost when the DNA to the right of the 106 insert is deleted, as in pDAK106*. The reason for this phenomenon is unknown. As expected, no loss of pAM2005 was observed when compatible control plasmid pWM401 was introduced into pAM2005-containing strains.

As shown in Fig. 3A, pDAK106* is less stable than either pDAK106 or pDAK102, indicating that deletion of the DNA to the right of the 106 insert, but not the 106 insert itself, results in loss of a stability determinant. This result is consistent with transposon mutagenesis studies that indicate the presence of a stability determinant identified by inserts 145 and 246 (Fig. 2). In some experiments, a partial and temporary increase in the percentage of pDAK106*-containing cells remaining after 72 h was observed, resulting in the shoulder seen in Fig. 3A. This has been observed with several unstable pAD1 derivatives, and in longer-term experiments, plasmid levels always began to fall again and eventually dropped below 10% (data not shown). Whether this is a significant characteristic of the pAD1 maintenance system or merely an artifact is unknown.

DNA sequence and analysis of the basic replicon. The sequence of the pAD1 DNA contained in plasmid pDAK106* is shown in Fig. 4. Analysis of the replicon revealed three potential ORFs that encode proteins longer than 50 amino acids and are preceded by reasonable Shine-Dalgarno sequences within 6 bp of the initiation codon. The amino acid sequences of these putative protein products, along with potential −10 and −35 RNA polymerase binding sites, are shown in Fig. 4. The genes that encode these proteins will be referred to as *repA*, *repB*, and *repC*. *repA* is transcribed in a leftward direction relative to the map in Fig. 2 and encodes a protein of ≈39.0 kDa. *repB* and *repC* are both transcribed in a rightward direction and encode proteins of 33.0 and 14.4 kDa, respectively. The 3′ end of *repB* overlaps the *repC* coding sequence by five nucleotides. It is possible that *repB* and *repC* are cotranscribed, although potential −10 and −35 boxes were identified upstream of both genes. A 22-bp palindrome that has a single mismatch and ends with a series of T's was identified downstream of *repC* and may function as a transcription terminator. As shown in Fig. 5, an in vitro transcription-translation reaction programmed with pDAK106* produced three novel proteins not produced by pDAK255*. pDAK255* is a pDAK102 derivative that lacks all of the pAD1 DNA to the right of the 255 Tn*5* insert (Fig. 1) and has the same Tn*5* remnant as pDAK106*. Therefore, pDAK255* makes an appropriate control since it should produce all relevant vector- and Tn*5*-encoded proteins but no pAD1-encoded proteins. The unique pDAK106*-encoded proteins had approximate molecular masses of 43, 35, and 17.5 kDa, in reasonable agreement with the products of *repA*, *repB*, and *repC*, respectively. Reactions programmed with pDAK102 produced a fourth novel protein that may be encoded by DNA deleted in pDAK106*.

In addition to the three *rep* genes, the sequence reveals the presence of several structural elements that may represent sites of DNA-protein interaction. The most striking of these is a series of 25 8-bp direct repeats with a consensus sequence of TAGTARRR located between the divergently transcribed *repA* and *repB* genes. These 25 8-bp repeats are separated into two groups of 13 and 12 end-to-end repeats with an intervening nonrepetitive sequence of 78 bp. Another single repeat was located 18 bp from the last repeat in the group of 12 iterons. This repeat was situated between the two arms of an imperfect (one mismatch) 14-bp palindrome. The left arm of this palindrome overlaps the −10 site of *repB*. Therefore, if this palindrome were to form a stem-loop structure, a TAGTARRR site would be located within the loop and the −10 sequence of *repB* would be located within the left side of the stem. Another three repeats were located ≈70 bp downstream of the *repC* stop codon and immediately adjacent to the presumptive transcription terminator mentioned above. The TAGTARRR sequence was found nowhere else within the replicon. These repeats are reminiscent of iterons which perform origin and copy control functions in several plasmids native to gram-negative bacteria and possibly in *Clostridium perfringens* plasmid pIP404 (22), suggesting that these sequences function as the pAD1 origin of replication. Interestingly, nonreplicative subclone pDAK104 lacks the three repeats 3′ to the *repC* gene, as well as the C-terminal 10 amino acids of the *repC* product and the putative transcription terminator. It is tempting to propose that the failure of pDAK104 to replicate in *E. faecalis* results from loss of these repeats. Given the distribution of this element, it seems very likely that the TAGTARRR sequence is an important protein-binding site, possibly involved in the formation of a nucleoprotein complex required for pAD1

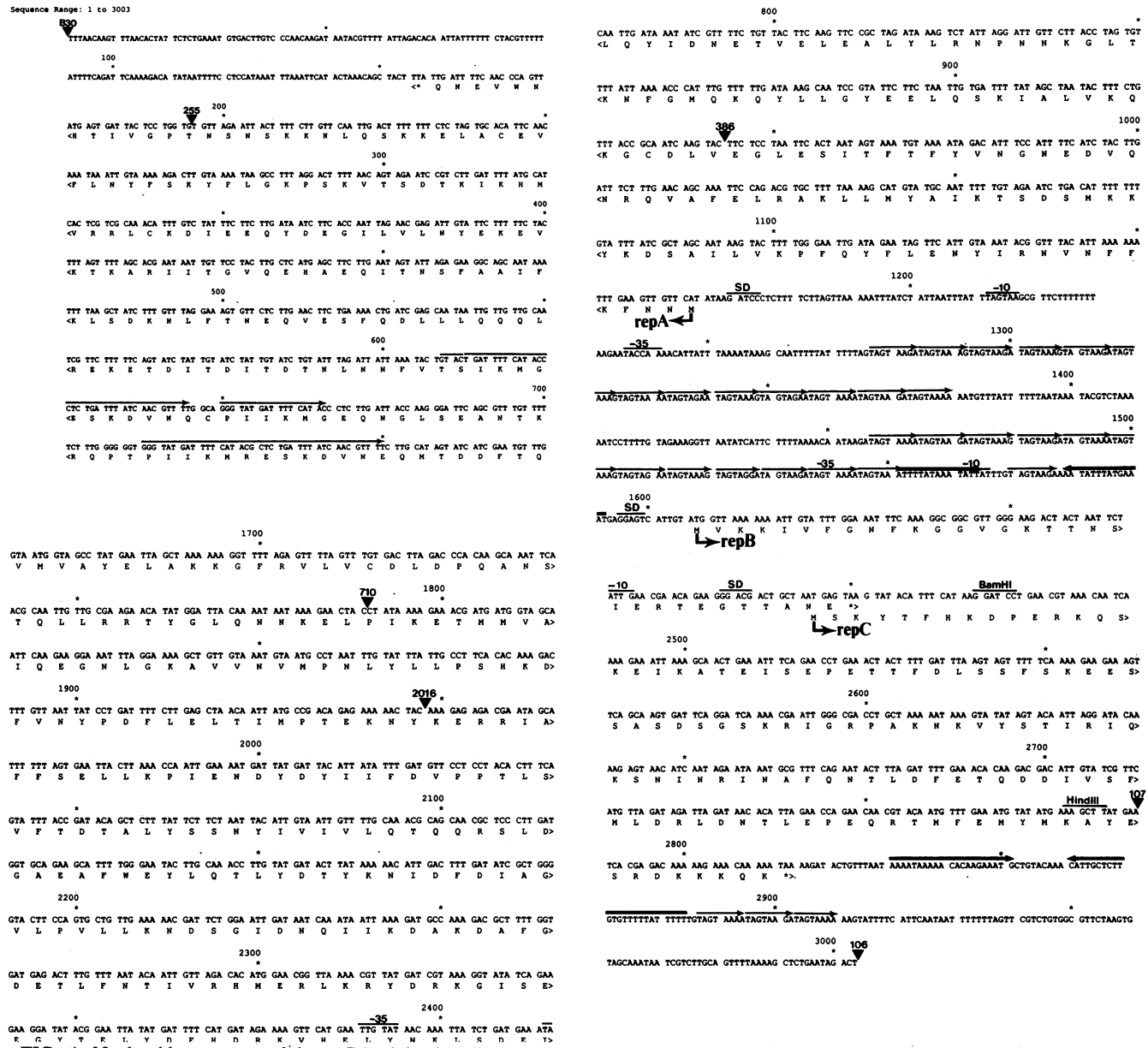

FIG. 4. Nucleotide sequence of the pAD1 minimal replicon. The nucleotide sequence shown begins at the site of the Tn*917-lac* insert of pDAK2300E which was used to construct pDAK102 and extends to the site of Tn*5* insert 106. These inserts mark the left and right borders, respectively, of the pAD1 minimal replicon. The predicted amino acid sequences of plasmid-encoded proteins RepA, RepB, and RepC are shown beneath the nucleotide sequence, and their direction of transcription is indicated by arrows. Putative −10 and −35 regions and Shine-Dalgarno sites (SD) are labeled. Direct-repeat sequences within *repA* and the TAGTARRR repeats are underlined with solid-line arrows. Inverted repeats are indicated with converging thick arrows. Insert sites of selected transposon mutants are marked with inverted triangles, and the insert numbers are indicated above them. *Bam*HI and *Hin*dIII sites are labelled as points of reference.

replication and in the transcriptional or posttranscriptional regulation of both *repA* and *repB*.

Another set of two 33-bp direct repeats and another related 17-bp repeat between, unrelated to the TAGTARRR repeats, are located within the *repA* coding region. Whether these repeats act as a DNA site for interaction with some other determinant or the resulting amino acid repeats perform some role in the function of the *repA* product has yet to be determined.

The amino acid sequences of all three *rep* gene products and the putative iteron DNA sequences were compared with the GenBank data base to identify any potential relationships to other known plasmids. No significant homology to either RepC or the iteron sequences was identified with the FASTA program. The protein sequence of RepA showed significant homology (initn score, 295) to the product of a single ORF present on *Lactobacillus helveticus* plasmid pLJ1 (28). The most significant homology was in the amino terminus of the protein, where 35.8% identity and 69% similarity were observed over a 134-amino-acid overlap. Two smaller homologous regions were found near the carboxyl terminus. No function has been assigned to the pLJ1 protein, but the

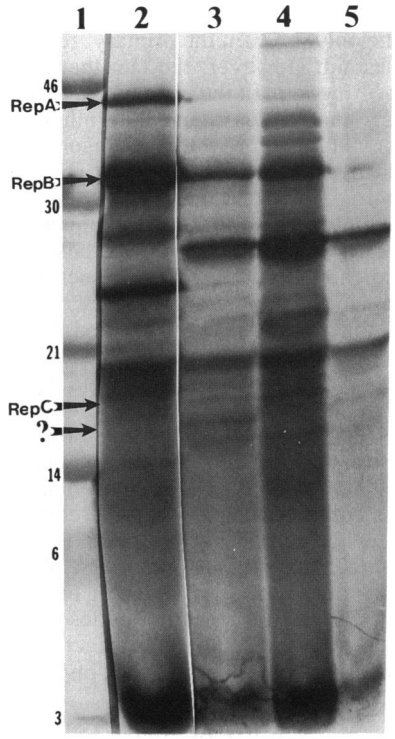

FIG. 5. Identification of putative protein products of *repA*, *repB*, and *repC*. Plasmids pAM3101 pDAK102, pDAK106*, and pDAK255* were used to program an in vitro transcription-translation reaction with an Amersham DNA-directed translation kit in accordance with the manufacturer's instructions. Plasmid pDAK255* is a deletion derivative of pDAK102 lacking all cloned pAD1 DNA to the right of the 255 insert (Fig. 2). The same side of Tn*5* remains in pDAK255* as in pDAK106*. Therefore, all vector-related sequences present in pDAK106* are also present in pDAK255* but all putative pAD1 replicon-encoded genes are deleted. The extent of the pAD1 DNA present in pAM3101, pDAK102, and pDAK106* is shown in Fig. 2. pAM3101 contains the indicated DNA in pBluescript, while the rest are in pAM201, which most likely explains the number of proteins produced from pAM3101 that are not present in the others. The positions of three protein products present in pDAK102 and pDAK106* but lacking in pDAK255* are marked and labelled. RepA and RepB proteins are also visible in pAM3101 along with a protein migrating slightly faster than RepC which may represent a truncated derivative of this protein. A fourth protein, encoded by pDAK102 but not by pDAK106*, is also shown and labelled with a question mark. Lanes: 1, molecular size markers labelled alongside in kilodaltons; 2, pAM3101; 3, pDAK102; 4, pDAK106*; 5, pDAK255*. The expected molecular masses of RepA, RepB, and RepC are 39, 33, and 14.4 kDa, respectively. Lane 2 was obtained from a shorter exposure of the same gel and spliced into the appropriate position.

fact that it is the only plasmid-encoded ORF suggests that it encodes the essential initiator protein. Interestingly, homology with RepA was also detected in mouse interferon response-binding factor 1 (39, initn score, 208). The most significant homology also appeared to occur in the amino-terminal end. The significance of this homology for protein function is unknown.

Several proteins potentially involved in plasmid or chromosomal replication control were found to have significant homology to RepB (initn score, >100). The strongest homology was found between RepB and two proteins of unknown function recently identified near the chromosomal origins of replication of *Pseudomonas putida* and *Bacillus subtilis* (sequences entered in GenBank but not published), showing almost 30% identity over 250 of the 281 amino acids. We also found significant homology to three plasmid-encoded proteins, the RepA proteins of *Agrobacterium tumefaciens* plasmids pRiA4b and pTiB6S3 (21, 27) and ORF7 of *Chlamydia psittaci* plasmid pCpA1 (unpublished data). These three proteins showed 23 to 29% identity to pAD1 RepB in overlaps of at least 180 amino acids. The precise function of RepA in the *A. tumefaciens* plasmids is unknown, but it is not essential for plasmid maintenance in either case. In all cases, homology was strongest in the amino-terminal end of RepB.

Correlation of transposon insert location and mutant phenotype. The precise locations of several transposon inserts were determined by DNA sequence analysis. Sites are marked on the sequence shown in Fig. 4. Tn*5* inserts 255 and 386 were located within the coding sequence for RepA. Since inserts 421 and 159 mapped between 255 and 386 by restriction mapping, it is likely that they are also located in RepA. The fact that all four inserts also result in loss of the ability of pDAK102 to replicate in *E. faecalis*, along with the observed RepA homology to the single ORF of *L. helveticus* plasmid pLJ1, indicates that RepA is required for plasmid replication and may be the replication initiator. Tn*917* and Tn*917-lac* inserts 710 and 2016 are both located within *repB*. Both of these inserts resulted in an increase in plasmid copy number, implicating the *repB* product in copy control. In addition, the 2016 Tn*917-lac* insert is in the appropriate orientation for formation of a *repB-lacZ* transcriptional fusion, which it has been observed to do. Tn*917* insert 107 was located within the 3′ end of *repC*, adjacent to the *Hin*dIII site. This insert resulted in loss of plasmid stability, perhaps indicating that the *repC* product is involved in some mechanism required for stable inheritance and interacts with the determinant identified by the 246 and 145 Tn*5* inserts, although more work is required to confirm this. The position of the 107 insert causes loss of nearly the same number of amino acids from the *repC* product as does deletion of the *Hin*dIII-*Sal*I fragment formed in the construction of pDAK104. This indicates that the failure of pDAK104 to replicate in *E. faecalis* cannot be due to the loss of RepC function alone, since derivatives containing the 107 insert can replicate. Whether the failure of pDAK104 to replicate is due to an additive effect of the loss of RepC and other functions or loss of a single function between the *Hin*dIII site and the 106 insert (e.g., the 26-bp repeat) has yet to be determined.

DISCUSSION

Analysis of several previously constructed pAD1 miniplasmids had identified a functional replicon located on an ≈5-kb DNA fragment immediately adjacent to the pheromone response-regulatory region (33, 35). In this study, we further delimited the basic replicon to a fragment of pAD1 DNA of ≈3 kb which supports the replication of pDAK106*. The basic replicon supported replication in *E. faecalis* at a copy number similar or identical to that of wild-type pAD1. A decrease in stability was observed relative to plasmids containing the entire 5-kb maintenance region. This loss of stability may be accounted for by the deletion of a determinant, identified by Tn*5* inserts 246 and 145, that has been implicated as essential for stable plasmid inheritance.

Three ORFs, whose genes we have named *repA*, *repB*, and *repC*, are encoded within the basic replicon. Potential

protein products for all three ORFs have been identified by in vitro transcription-translation. *repA* is transcribed in a leftward direction and encodes a protein of 336 amino acids with a molecular mass of ≈39 kDa. Transpositional insertion mutations within *repA* resulted in inability of the plasmid to become established in *E. faecalis*, suggesting that the *repA* product is essential for replication and perhaps functions as the replication initiator. Supporting this conclusion is the finding that the *repA* gene product shows homology with the product of the single ORF of *L. helveticus* plasmid pLJ1. However, no homology was detected between pLJ1 and the pAD1 replicon at the DNA level, even within the internal *repA* repeats and the iterons, indicating that if RepA is the replication initiator it may recognize an origin with a sequence different from that of the pLJ1 origin. *repB* and *repC* are transcribed in a rightward direction and encode proteins of 281 amino acids (≈32 kDa) and 123 amino acids (≈14.4 kDa), respectively. Transpositional *repB* insertion mutations resulted in an increase in plasmid copy number, implicating the *repB* gene product as a negative regulator of plasmid copy number. The *repB* gene product showed homology to several proteins of unknown function which are encoded close to bacterial chromosome and plasmid replication origins. We identified a single transposon insert in the carboxyl-terminal end of the *repC* product which caused a dramatic loss of plasmid stability. These results indicate that the *repC* product is involved in regulating stable inheritance, perhaps by interacting with the stability determinant located in the nonessential right half of the maintenance region. However, because only one insert was identified and because it resulted only in the deletion of eight amino acids, more proof is required to assign a role to the RepC protein conclusively.

In addition to the identified protein-coding sequences, several interesting structural features were identified within the basic replicon. The most striking is a series of 25 8-bp direct repeats with the consensus sequence TAGTARRR located between the divergently transcribed *repA* and *repB* genes. These repeats are reminiscent of the iterons involved in replication and copy control in plasmids native to gram-negative bacteria. Another three TAGTARRR repeats were identified ≈1,300 bp to the right of the iterons, just downstream of *repC*. Finally, an individual TAGTARRR repeat was located 18 bp from the last iteron repeat, between two arms of a 14-bp palindrome, the 5′ arm of which overlaps the putative *repB* −10 sequence. Considering the arrangement or these iterons with respect to *repA* and *repB* and the demonstrated involvement of iterons in the replication and copy control of other plasmids, it is tempting to propose that the TAGTARRR sequence represents a binding site for a component of the replication apparatus, perhaps the *repA* or *repB* product. Binding to the multiple repeats in the iteron sequences may be required for the formation of a nucleoprotein complex which initiates replication. Interaction between the iterons and the three repeats 3′ to *repC* by looping may be required for replicon function. This would explain the failure of pDAK104 to replicate. Binding to the TAGTARRR sequences could also both obscure the *repB* −35 sequence and disrupt or in some other way alter the structure of the region of dyad symmetry overlapping the *repB* −10 sequence, thereby controlling *repB* transcription. Although the iterons do not overlap the *repA* controlling elements, it is still possible that a protein bound to them could interact in some way with bound RNA polymerase and regulate transcription either positively or negatively.

Although the isolated replicon is capable of supporting plasmid replication in *E. faecalis* at a copy number similar to that of pAD1, we have not rigorously proven that this replicon is the only, or even the primary, replicon present on the intact plasmid. However, the fact that the cloned replicon expresses incompatibility against intact pAD1 derivatives suggests that at least one determinant essential for replication or stability (e.g., multimer resolution or partition) of pAD1 is located on the cloned replicon. Also, transposon insertions within the *repB* gene result in an increase in plasmid copy number of both pAD1 miniplasmid derivatives and intact pAD1 plasmids containing these inserts. At the least, this indicates that the replicon described can be used by pAD1 and may become the primary replicon when its copy control functions are short-circuited. It is also possible that the cloned replicon represents a parasite rolling-circle replicon (12) that is not normally used by the plasmid. However, this seems unlikely since none of the identified determinants displayed homology to known determinants required for replication of rolling-circle plasmids, and no determinants homologous to known rolling-circle plus or minus origins could be identified on the cloned replicon. In addition, no evidence of structural instability or accumulation of high-molecular-weight multimers, phenomena common in rolling-circle plasmids containing foreign DNA, were observed in any of the constructs described here. The precise role of the cloned replicon in intact pAD1 replication and stable inheritance requires further investigation.

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