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Physical and Genetic Analyses of Streptococcal Plasmid pAMβ1 and Cloning of Its Replication Region

DONALD J. LEBLANC* AND LINDA N. LEE

Laboratory of Molecular Microbiology, National Institute of Allergy and Infectious Diseases, Fort Detrick, Frederick, Maryland 21701

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Plasmid pAMβ1, originally isolated from *Streptococcus faecalis* DS5, mediates resistance to the MLS (macrolide, lincosamide, and streptogramin Bα) group of antibiotics. A restriction endonuclease map of the 26.5-kilobase (kb) pAMβ1 molecule was constructed by using the enzymes *Ava*I, *Hpa*II, *Eco*RI, *Pvu*II, *Kpn*I, *Bst*EII, *Hpa*I, *Hha*I, and *Hin*dIII. A comparison of this map to those of four independently isolated deletion derivatives of pAMβ1 located the MLS resistance determinant within a 2-kb DNA segment and at least one conjugative function within an 8-kb region. The 5.0-kb *Eco*RI-B fragment from pAMβ1 was ligated onto the 4.0-kb *Escherichia coli* plasmid vector, pACKC1, and used to transform *E. coli* HB101. The 9.0-kb chimeric plasmid was then used to transform *Streptococcus sanguis* Challis with concurrent expression of the *E. coli* kanamycin resistance determinant. The 5.0-kb *Eco*RI-B fragment from pAMβ1 was subsequently used as a vector to clone a streptomycin resistance determinant from a strain of *Streptococcus mutans* containing no detectable plasmid DNA. Subcloning experiments, using a *Hin*dIII partial digest of pAMβ1 DNA, narrowed the replication region of this plasmid to a 2.95-kb fragment.

Plasmid pAMβ1, originally identified in a clinical isolate of *Streptococcus faecalis* DS5 (8), mediates resistance to erythromycin, which is representative of the MLS phenotype (resistance to macrolides, lincosamides, and streptogramin Bα; 40). Plasmid pAMβ1 was transformed into the Challis strain of *S. sanguis* (21) and into a competent group F *Streptococcus* strain (20). The self-transmissibility of pAMβ1 was demonstrated when the group F transformant was used as a conjugative donor for three different species of viridans group streptococci (22). Subsequently, pAMβ1 was shown to be transmissible among members of virtually every species of *Streptococcus* (7), and to certain strains of *Lactobacillus casei* (14), *Staphylococcus aureus* (13, 35), and *Bacillus subtilis* (19).

Several MLS resistance plasmids, similar in size (26 to 34 kilobases [kb]) to pAMβ1, have been isolated from strains representing various species of *Streptococcus* (7). A number of these have been transferred, by conjugation, to different species of *Streptococcus* and to *S. aureus* (7). DNA-DNA homology studies (12, 15, 41) and restriction endonuclease analyses (17) have suggested that these plasmids are closely related. Two independently isolated MLS plasmids, pAC1 from *Streptococcus pyogenes* and pAMβ1, were virtually identical (41). It would appear, then, that the MLS plasmids have been widely disseminated in nature.

Whereas the pAMβ1-like plasmids are readily transferred to a number of gram-positive bacterial species, most plasmids of streptococcal origin are not. The majority of streptococcal plasmids that we have examined, including a few that mediate an MLS resistance phenotype, cannot be used to transform competent streptococci, nor can they be transferred by conjugation to species of a Lancefield group other than that from which they originated. We have been interested in studying those genetic determinants that contribute to plasmid host-range. Since pAMβ1 is representative of the broad host-range streptococcal plasmids, we have begun an extensive physical and functional analysis of this molecule. We present in this communication (i) a detailed restriction endonuclease cleavage map of pAMβ1; (ii) the locations of the MLS resistance determinant, at least one *tra* function, and the replication region, on this map; and (iii) the results of cloning experiments that delineate further the replication gene(s) of pAMβ1.

* Corresponding author.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. The bacterial strains used in this study are described in Table 1. The *Escherichia coli* plasmid vector, pACKC1, was constructed by the religation of combined *Hae*II digestion fragments from pACYC177 (6) and pACYC184 (6) by V. Burdett. It is a 3.97-kb hybrid plasmid consisting of the kanamycin (Km) resistance determinant from pACYC177 and the replicon and chloramphenicol (Cm) resistance determinant from pACYC184. When DNA is inserted into the single *Eco*RI site of pACKC1, the Cm resistance determinant is inactivated.

L broth (reference 11, p. 201) was used for the cultivation of all *E. coli* strains. Streptococci were grown in brain heart infusion (BHI) (Difco Laboratories) broth, or in LCM (33) broth supplemented with 10 mM D-glucose. Solid medium contained 1.5% agar (Difco). Antibiotics employed for the selection of resistant transformant colonies were: Em, 10 μg/ml; streptomycin (Sm), 1 to 2 mg/ml; and Km, 50 μg/ml for *E. coli* and 500 μg/ml for *Streptococcus sanguis*. The insertional inactivation of Cm resistance among *E. coli* transformants containing pACKC1 was determined in the presence of 5 μg of Cm per ml. Incubations were at 37°C in all cases.

Transformation and conjugation. Transformation of *E. coli* was by the $CaCl_2$ procedure of Mandel and Higa (30), as described in Maniatis et al. (31, p. 250–251). The *S. sanguis* Challis strain was prepared for transformation as follows: six passages in LCM broth containing 10 mM D-glucose were followed by serial dilutions ranging from 10^{-1} to 10^{-9}, in

TABLE 1. Bacterial strains

Strain	Plasmid	Phenotype[a]		Reference, source, or derivation
		Plasmid	Chromosomal	
S. sanguis Challis				
DL1			Em^s Km^s Sm^s	21; Transformable strain of *S. sanguis*
DL125	pAMβ1	Em^r Con^+	Em^s Km^s Sm^s	Transformation of DL1 with purified pAMβ1 DNA from DL812; this study
DL206	pDL206	Km^r Cm^s	Em^s Km^s Sm^s	Transformation of DL1 with a mixture of chimeric plasmids including pDL206; this study
DL206-C			Em^s Km^s Sm^s	Spontaneous Km^s isolate from DL206; this study
DL212	pDL212	Sm^r Em^s	Em^s Km^s Sm^s	Transformation of DL1 with religated mixture of *Eco*RI-B fragment of pAMβ1 and *Eco*RI-digested DL5 DNA; this study
DL214	pDL214	Em^r	Em^s Km^s Sm^s	Transformation of DL1 with religated *Hin*dIII partial digest of pAMβ1; this study
DL216	pDL216	Em^r	Em^s Km^s Sm^s	Transformation of DL1 with religated *Hin*dIII partial digest of pAMβ1; this study
V480	pVA2	Em^r Con^-	Em^s Km^s Sm^s	Contains deletion derivative of pAMβ1; from F. Macrina (Medical College of Virginia, Richmond)
V486	pVA1	Em^r Con^-	Em^s Km^s Sm^s	27; Contains deletion derivative of pAMβ1; from F. Macrina
S. anginosus-constellatus				
DL8			Em^s	20; Transformable strain of *S. anginosus-constellatus*
DL812	pAMβ1	Em^r Con^+	Em^s	20; pAMβ1 transformant of strain DL8
DL177	pDL177	Em^r Con^-	Em^s	Single-colony isolate of DL812 containing a deletion derivative of pAMβ1; this study
S. faecalis				
DL9	pAMβ1	Em^r	Em^s Gm^r	17; JH201 containing pAMβ1; from V. Burdett (Duke University, Durham, N.C.)
DL186	pMVΔβ1 pMV158	Em^s Con^+ Tc^r Con^-	Em^s Gm^r	17; JH201 containing pMV158 (4) and a deletion derivative of pAMβ1; from V. Burdett
S. mutans				
DL5			Sm^r	25; Porcine isolate of *S. mutans* resistant to 20 mg of Sm per ml
E. coli				
HB101			Km^s Cm^s Em^r	3; From M. Martin (NIH, Bethesda, Md.)
DL199	pACKC1	Km^r Cm^r	Km^s Cm^s Em^r	Transformation of HB101 with purified pACKC1 obtained from V. Burdett; this study
DL205	pDL206	Km^r Cm^s	Km^s Cm^s Em^r	Transformation of HB101 with religated mixture of *Eco*RI-digested pACKC1 and pAMβ1 DNA; this study
DL205-C			Km^s Cm^s Em^r	Spontaneous Km^s isolate from DL205; this study
V850			Km^s Cm^s Em^s	28; Em^s isolate of *E. coli* obtained from F. Macrina

[a] Abbreviations: Em, erythromycin; Km, kanamycin; Sm, streptomycin; Gm, gentamycin; Tc, tetracycline; Cm, chloramphenicol; Con, conjugative; r, resistant; s, sensitive; +, positive; −, negative.

BHI broth supplemented with 10 mM D-glucose and 1% heat-inactivated horse serum. Incubation was continued for 18 to 20 h. The dilution tube (usually 10^{-8} or 10^{-9}) with an optical density at 660 nm of 0.02 to 0.05 contained the most highly competent cells. For each transformation, 450 μl of a competent cell culture was mixed with 0.5 to 1.0 μg of transforming DNA in 50 μl of sterile 0.1 × SSC (24; 1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and incubated for 4 h under anaerobic conditions (GasPak; BBL Microbiology Systems). Samples of diluted and undiluted transformation cultures were then spread on the appropriate selective media. Conjugation experiments, to assess the transmissibility of pAMβ1 and its deletion derivatives, were conducted as described previously (24).

Plasmid isolation. *E. coli* strains were screened for the presence of plasmid DNA according to Birnboim and Doly (2). Plasmids were detected in streptococcal isolates by the method of LeBlanc and Lee (23). Large-scale preparations of plasmid DNA were obtained from *E. coli* cultures as described in Maniatis et al. (reference 31, p. 90–91), and from streptococcus strains as described previously (24), with the following alteration. Lysozyme treatment of streptococci, other than *S. faecalis*, was in the presence of 50 mM Tris-hydrochloride (pH 8.0) instead of 50 mM Tris–10 mM EDTA.

Enzyme reactions and DNA manipulations. All restriction endonucleases and T4 DNA ligase were purchased from Bethesda Research Laboratories. Calf intestine alkaline phosphatase was purchased from Boehringer-Mannheim Biochemicals.

Restriction endonuclease digests were carried out according to supplier-recommended assay conditions with the addition of bovine serum albumin at a final concentration of 500 μg/ml. Double digests were performed simultaneously when compatible reaction conditions existed and sequentially when vastly different conditions were required. Alkaline phosphatase treatment of digested DNA and ligation reactions were performed according to Davis et al. (reference 11, p. 138). The transfer of DNA fragments from agarose gels to nitrocellulose filters (37), the purification of DNA from agarose (39), the preparation of ^{32}P-labeled probes by nick translation (32), and conditions for DNA-DNA hybridization (38) were as described with minor modifications as indicated previously (24).

Plasmid curing frequencies. The stability of a plasmid, or hybrid molecule, under nonselective conditions was assessed by the following procedure. A single colony was picked from an agar plate containing the appropriate selective antibiotic and transferred to 10 ml of broth medium without antibiotic. After incubation for 18 h, 1 ml was

TABLE 2. Restriction endonuclease fragments of pAMβ1

Fragment	Fragment size[a] (kb) after digestion with:								
	*Hin*dIII	*Hha*I	*Bst*EII	*Hpa*I	*Eco*RI	*Pvu*II	*Kpn*I	AvaI	*Hpa*II
A	4.1	6.4	18.7	21.7	21.5	18.0	19.3	26.5	26.5
B	3.45	3.2	5.7	3.0	5.0	8.5	7.2		
C	3.2	2.6	2.1	1.8					
D	3.0	2.1							
E	2.1	1.9							
F	1.95	1.7							
G	1.85	1.5							
H	1.45	1.4							
I	1.2	1.3							
J	1.15	1.1							
K	1.05	1.05(2)							
L	0.75	0.7							
M	0.7	0.4							
N	0.5								

[a] Fragment sizes were determined from their migration rates in agarose gels relative to fragments of known sizes derived from *Eco*RI or *Hin*dIII digestion of phage λ DNA and *Hae*III digestion of φX174 RF DNA.

transferred to 9 ml of fresh medium and the culture was incubated until it reached the mid-exponential phase of growth. The culture was then serially diluted, and samples were spread on agar medium containing no antibiotic. After incubation for 48 h, 250 colonies were transferred with sterile toothpicks to agar plates with and without added antibiotic. Both sets of plates were scored for growth after incubation for 48 h.

Plasmid copy number determinations. The number of plasmid copies per chromosome equivalent for each strain tested was determined from the total cell DNA isolated from 20 ml of an exponential-phase culture grown in the presence of a selective antibiotic. The shearing and purification of total cellular DNA were as described previously (24). The amount of plasmid and chromosomal DNA derived from each culture was estimated from the incorporated counts ([^{3}H]thymidine) sedimenting in the respective bands of a dye-buoyant density gradient. Copy numbers were determined from the known sizes of the plasmids and an average chromosome size of 2.8×10^3 kb (27).

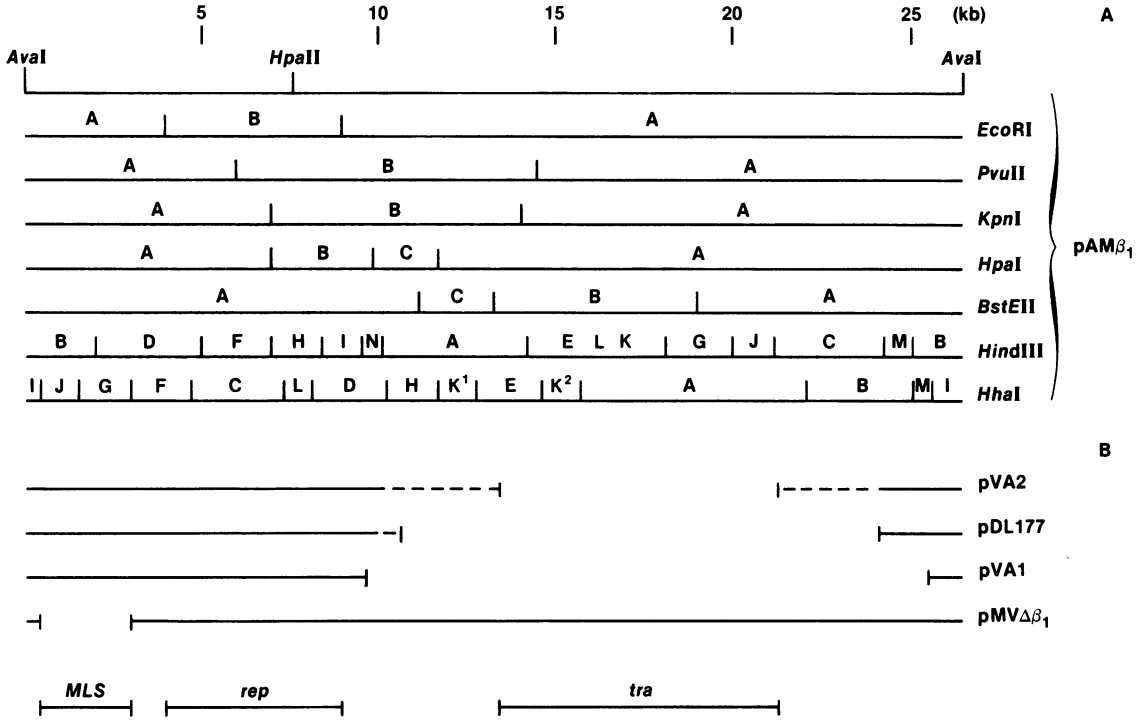

FIG. 1. Restriction endonuclease map of pAMβ1 and four pAMβ1 deletion derivatives. (A) Locations of restriction endonuclease cleavage sites on pAMβ1, relative to a single *Ava*I site. (B) Maps of deletion derivatives of pAMβ1 and proposed locations of the pAMβ1 MLS (resistance), *rep* (replicon), and *tra* (transfer) functions, based on the presence or absence of these functions in host strains harboring the derivatives. Radiolabeled pVA2 DNA did not hybridize to the *Hin*dIII-E, -G, -J, -K, and -L fragments of pAMβ1. Portions of the *Hin*dIII-A and -C fragments of pAMβ1 were present in pVA2 as a single fusion fragment. The extent of the deletion into each of these two fragments could not be determined accurately, which is reflected in the broken lines on the linear map of pVA2.

RESULTS

Construction of a restriction endonuclease map of pAMβ1. Plasmid DNA was purified from two streptococcal strains, *S. sanguis* Challis DL125 and *S. anginosus-constellatus* DL812. No obvious differences existed between plasmid preparations obtained from either strain, as determined from restriction endonuclease digests with nine different enzymes. Table 2 shows the numbers and sizes of DNA fragments obtained by digestion of the 26.5-kb pAMβ1 molecule with these enzymes.

The following approach was employed in the construction of the restriction endonuclease map of pAMβ1 illustrated in Fig. 1A. A preliminary map was obtained from double and triple digests, using various combinations of the nine enzymes listed in Table 2. The arrangement of the fourteen *Hin*dIII fragments was confirmed by the following experiments. *Hin*dIII-digested pAMβ1 DNA was electrophoretically separated and transferred to nitrocellulose filters by the method of Southern (37). The filter blots were incubated with one of the five purified, ^{32}P-labeled fragments obtained from the sequential digestion of pAMβ1 with *Eco*RI and *Bst*EII. By comparing autoradiograms showing fragments that hybridized to the ^{32}P-labeled probes, we were able to localize individual *Hin*dIII fragments within the *Eco*RI plus *Bst*EIII-derived fragments. This same approach was employed to localize the *Hha*I fragments of pAMβ1. Further confirmation of the physical map was provided by similar analyses of four deletion derivatives of pAMβ1.

The identification, mapping, and phenotypic characterization of pAMβ1 deletions permitted the preliminary assignment of plasmid-mediated functions to specific regions of the map (Fig. 1B). One of these deletion derivatives, pMVΔβ1, no longer mediated resistance to MLS antibiotics and had lost approximately 2.0 kb of DNA. The transfer functions of pMVΔβ1 were not affected by the deletion, as evidenced by its ability to mobilize pMV158, a nonconjugative tetracycline resistance plasmid (4) also present in the *S. faecalis* host strain (data not shown; V. Burdett, personal communication). Three of the deletion derivatives of pAMβ1—pVA1, pVA2 and pDL177—were transferred to competent cultures of *S. sanguis* Challis (DL1) and the group F strain (DL8) by transformation. When representative Em-resistant transformants were used as donors in conjugation experiments with the plasmid-free recipient strain, *S. faecalis* JH2-2 (24), no Em-resistant transconjugants were obtained (frequencies $<10^{-9}$ per donor CFU). *S. sanguis* Challis (strain DL125) and the group F strain (DL812), containing pAMβ1, served as Em resistance donors at frequencies of 2.4×10^{-6} and 4.3×10^{-4}, respectively. Similar results were obtained with a *S. mutans* recipient. All three of the nonconjugative deletion derivatives shared the loss of a common segment, between the 13.4 and 21 kb points on the map, of the pAMβ1 molecule. Since all four deletion derivatives retained the smaller *Eco*RI-B fragment, we postulated that the replication function(s) of pAMβ1 might be contained within this 5-kb fragment.

Construction of an *E. coli*/*S. sanguis* shuttle vector. The *E. coli* plasmid pACKC1 was used for the cloning of *Eco*RI-digested pAMβ1 DNA. *Eco*RI-digested pACKC1 was treated with alkaline phosphatase, ligated to *Eco*RI-digested pAMβ1 DNA, and used to transform *E. coli* HB101. Of 106 colonies that were obtained on medium containing Km, 79 were sensitive to Cm. Twenty of these Km^r Cm^s isolates were examined for the presence of plasmid DNA. Each of these clones contained one or more plasmids ranging in size from 4.0 to 26.0 kb, based on their migration rates in agarose gels. Two DNA pools, each containing the combined plasmids from 10 of the 20 isolates, were used to transform a competent culture of *S. sanguis* DL1. One of the pools yielded four Km-resistant colonies. Plasmid DNA was isolated from each of the four Km-resistant transformants. They all contained a plasmid that migrated in an agarose gel at the same rate as a plasmid from one of the 10 *E. coli* isolates that contributed to the transforming DNA pool. This *E. coli* strain, DL205, and one of the Km-resistant *S. sanguis* isolates, DL206, were chosen for further studies.

Restriction endonuclease digests of the plasmids isolated from strains DL205 and DL206 were compared with digests of pAMβ1 and pACKC1. *Eco*RI digests of these four plasmids (Fig. 2A) suggested that both the plasmid from strain DL205 (lane 1) and that from strain DL206 (lane 2) were composed of pACKC1 (lane 4) and the *Eco*RI-B fragment of pAMβ1 (lane 3). From the pAMβ1 map (Fig. 1A), we predicted that the *Eco*RI-B fragment would contain *Hin*dIII fragments F, H, D″ (the smaller of the two fragments obtained by cleavage of the *Hin*dIII-D fragment by *Eco*RI), and I′ (the larger of the two fragments derived from the cleavage of the *Hin*dIII-I fragment with *Eco*RI). *Eco*RI-

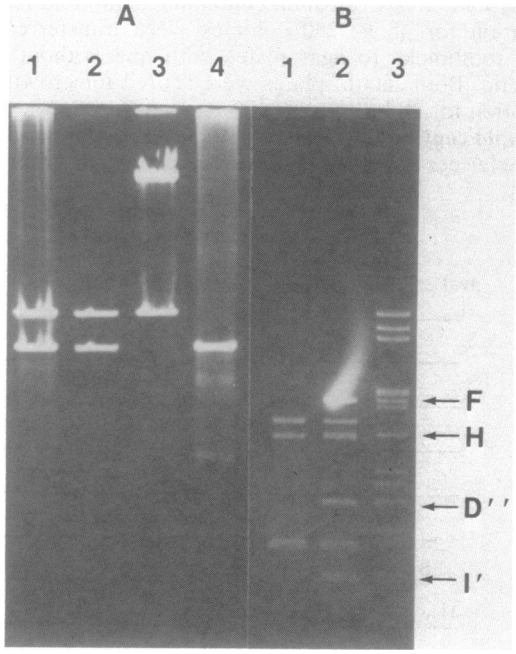

FIG. 2. Restriction endonuclease analysis of pAMβ1, pACKC1, and the chimeric plasmid, pDL206. (A) *Eco*RI digests of pDL206 from *E. coli* DL205 (lane 1), pDL206 from *S. sanguis* DL206 (lane 2), pAMβ1 from *S. sanguis* DL125 (lane 3) and pACKC1 from *E. coli* DL199 (lane 4). The DNA bands in lanes 1 and 4 were from digests of plasmid preparations obtained by the method of Birnboim and Doly (2). Only the two bands of high intensity in lane 1, and the single high-intensity band in lane 4, were associated with the respective plasmids after purification by dye-buoyant density gradient centrifugation. The plasmids in lanes 2 and 3 were purified from dye-buoyant density gradients. (B) *Eco*RI-*Hin*dIII double digests of pACKC1 (lane 1), pDL206 from strain DL206 (lane 2), and pAMβ1 (lane 3), all purified from dye-buoyant density gradients. The arrows point to the intact *Hin*dIII-F and -H fragments and to the -D″ (the smaller of the two fragments obtained by cleavage of the *Hin*dIII-D fragment by *Eco*RI) and -I′ (the larger of the two fragments derived from the cleavage of the *Hin*dIII-I fragment with *Eco*RI) of pAMβ1 (lane 3).

TABLE 3. Transformation of *E. coli* and *S. sanguis* with pACKC1, pAMβ1, and pACKC1 plus pAMβ1 chimeras

Plasmid	Source	No. of transformants of the following strain per μg of DNA:				
		E. coli HB101	*E. coli* V850		*S. sanguis* DL1	
		Km	Km	Em	Km	Em
pACKC1	DL199 (*E. coli*)	8.0×10^4	2.6×10^4	0	0	0
pAMβ1	DL125 (*S. sanguis*)	ND[a]	0	0	0	2.8×10^5
pDL206	DL205 (*E. coli*)	2.2×10^4	6.3×10^2	0	3	0
pDL206	DL206 (*S. sanguis*)	7.5×10^3	8.8×10^4	0	3.2×10^6	0

[a] ND, Not done; strain HB101 is resistant to Em.

*Hin*dIII double digests (Fig. 2B) showed that the plasmids from DL206 (lane 2) and from DL205 (data not shown) contained all of the fragments of pACKC1 (lane 1) and the *Hin*dIII-F, -H, -D″, and -I′ fragments from pAMβ1 (lane 3).

Phenotypic studies. *E. coli* HB101 is sensitive to Km but resistant to Em. *E. coli* V850 is sensitive to both antibiotics and is able to express the MLS resistance determinant of pAMβ1 when it is ligated to an *E. coli* vector plasmid (28). The transformability of these two *E. coli* strains and *S. sanguis* DL1 by pACKC1, pAMβ1, and the chimeric plasmid pDL206 (isolated from *E. coli* DL205 and from *S. sanguis* DL206) was examined (Table 3). The results clearly showed that the *E. coli* strains could be transformed by pACKC1, but not by pAMβ1, whereas *S. sanguis* transformants were obtained with pAMβ1 but not with pACKC1. In addition, no Em resistance was associated with pACKC1, and no Km resistance was associated with pAMβ1. The pACKC1 plus pAMβ1 (*Eco*RI-B fragment) chimera, pDL206, isolated from either *E. coli* DL205 or *S. sanguis* DL206 transformed all three strains to Km resistance. In three separate transformation experiments, using pDL206 DNA purified from *E. coli* DL205, only three to six *S. sanguis* transformants were obtained per μg of DNA. However, when pDL206 was isolated from any of the *S. sanguis* transformants and used to transform *S. sanguis* DL1, the number of transformants produced was always between 10^5 and 10^6 per μg of DNA. These results support previous data (20, 22) that suggested the presence of a restriction-modification system in the Challis strain of *S. sanguis*.

Since mutants of *S. sanguis* resistant to concentrations of Km as high as 1 mg/ml have been isolated, it was necessary to demonstrate that the Km resistance exhibited by DL206 was due to the presence of the chimeric plasmid, pDL206 (Table 4). *S. sanguis* DL1 was able to grow in the presence of 64 μg of Km per ml. When this strain contained pDL206, it was able to grow in the presence of 500 μg of the antibiotic per ml, but reverted to its original level of resistance upon loss of the plasmid. The presence of either pACKC1 or the hybrid plasmid (pDL206) in *E. coli* HB101 resulted in a 1,000-fold increase in resistance to Km.

Cloning of an *S. mutans* streptomycin resistance determinant. The results presented above suggested that the replication gene(s) of pAMβ1 were entirely contained in the *Eco*RI-B fragment of this plasmid. However, it was also possible that replicative functions of pACKC1 were contributing to the maintenance of the chimeric plasmid in *S. sanguis*. To eliminate this possibility, we used the *Eco*RI-B fragment of pAMβ1 as a vector to clone a streptococcal resistance determinant. The cloned DNA was obtained from *S. mutans* DL5, which is able to grow in the presence of 20 mg of streptomycin (Sm) per ml and contains no detectable plasmid DNA. The *Eco*RI-B fragment of pAMβ1, separated

TABLE 4. Resistance of *E. coli* and *S. sanguis* host cells to kanamycin

Strain	Plasmid	Resistance to Km (μg/ml)
S. sanguis Challis		
DL1	None	64
DL206	pDL206	500
DL206-C	None	64
E. coli		
HB101	None	1
DL199	pACKC1	1,000
DL205	pDL206	1,000
DL205-C	None	2

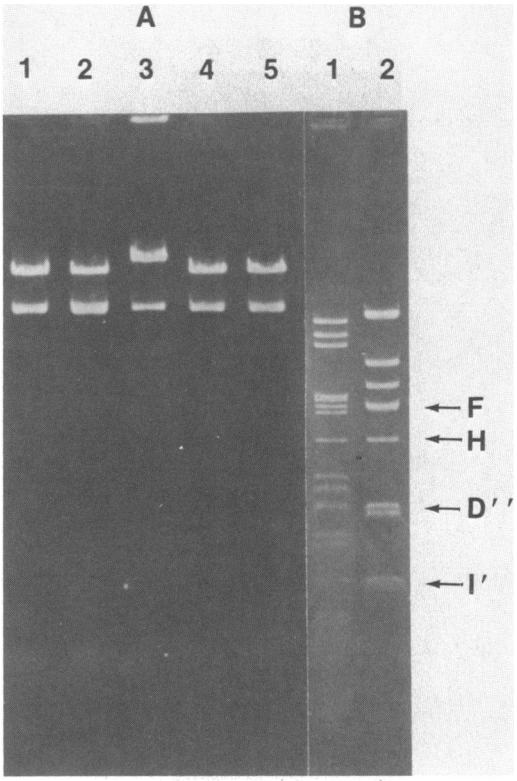

FIG. 3. Restriction endonuclease analysis of pAMβ1 plus *S. mutans* hybrid plasmids. (A) *Eco*RI digests of plasmid DNA isolated from four steptomycin-resistant clones (lanes 1, 2, 4, 5) and pAMβ1 (lane 3). (B) *Eco*RI-*Hin*dIII double digest of pAMβ1 (lane 1) and pDL212 (lane 2). All plasmids were purified from dye-buoyant density gradients. The arrows point to the *Hin*dIII-F, -H, -D″, and -I′ fragments of pAMβ1 (lane 1B), as described in the legend to Fig. 2.

from the larger A fragment on a neutral sucrose gradient, was ligated to *Eco*RI-digested DNA from *S. mutans* DL5. The ligation mixture was used to transform *S. sanguis* DL1. Four of six Sm-resistant isolates obtained from this experiment contained detectable plasmid DNA. *Eco*RI digests of purified plasmids from these four strains were compared with an *Eco*RI digest of pAMβ1 (Fig. 3A). The 5-kb *Eco*RI-B fragment of pAMβ1 (lane 3) appeared to be common to all four plasmids. The presence of the pAMβ1 *Eco*RI-B fragment in plasmid DNA from one of these clones (strain DL212) was confirmed by a *Hin*dIII-*Eco*RI double digest (Fig. 3B). As expected, the hybrid plasmid (lane 2) contained the *Hin*dIII-F, -H, -D″, and -I′ fragments of pAMβ1 (lane 1). The four additional fragments in lane 2 were derived from the *S. mutans* DNA. The size of the cloned fragment, calculated from the sizes of these four *Hin*dIII fragments, was 10.2 kb. *S. sanguis* DL212, like *S. mutans* DL5, was resistant to greater than 20 mg of Sm per ml. Spontaneous mutants of *S. sanguis* DL1, resistant to 1 mg of Sm per ml, have been obtained. However, only strain DL212 and transformants of *S. sanguis* DL1 obtained with plasmid DNA from strain DL212 were able to grow in the presence of 20 mg of Sm per ml. Strain DL1 as well as plasmid-free derivatives of strain DL212 were sensitive to less than 50 μg of Sm per ml.

Subcloning of the replication region of pAMβ1. In an attempt to define the limits of the replication region of pAMβ1, a *Hin*dIII partial digest of pAMβ1 DNA was

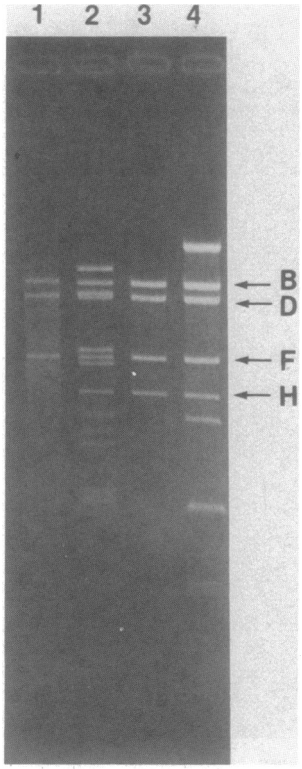

FIG. 4. *Hin*dIII restriction analyses of pAMβ1 and derivatives. Fragment patterns of plasmid DNA isolated from pAMβ1-*Hin*dIII-digested and self-ligated subclones DL216 (lane 1) and DL214 (lane 3) were compared with those of pAMβ1 (lane 2) and the pAMβ1 deletion derivative, pVA2 (lane 4). All plasmids were purified from dye-buoyant density gradients. The arrows point to the *Hin*dIII-B, -D, -F, and -H fragments of pAMβ1 (lane 2).

TABLE 5. Spontaneous plasmid curing frequencies under nonselective conditions[a]

Strain	Plasmid present	Loss of resistance to[b]:	% Spontaneously cured	
			Expt 1	Expt 2
DL9	pAMβ1	Em	<0.4	<0.4
DL125	pAMβ1	Em	8.0	1.2
DL31	pVA2	Em	<0.4	<0.4
DL206	pDL206	Km	64.0	61.0
DL212	pDL212	Sm	2.4	6.0
DL214	pDL214	Em	<0.4	0.8
DL216	pDL216	Em	4.0	1.6

[a] Single-colony isolates of strains on medium containing the appropriate selective antibiotic were allowed to grow in the absence of antibiotics for 15 to 20 generations before being scored for loss of the resistance trait.

[b] Abbreviations: Em, erythromycin; Km, kanamycin; Sm, streptomycin.

religated and used to transform strain DL1. Sixty-five Em-resistant colonies were isolated and screened for plasmid DNA. Two isolates, representing clones containing the smallest plasmids observed in agarose gels, were chosen for further analyses. Complete *Hin*dIII digests of plasmid DNA purified from these two strains, DL214 and DL216, were compared with *Hin*dIII digests of pAMβ1 and pVA2 (Fig. 4). The smallest plasmid obtained from the subcloning experiment, pDL216 (lane 1), contained the *Hin*dIII-B, -D, and -F fragments of pAMβ1 (lane 2) but was missing the H fragment present in pDL214 (lane 3). The *Hin*dIII-B fragment, which contains the MLS resistance determinant, is not required for replication since it was not present in either pDL206 or pDL212. A segment of DNA that included the 1-kb *Eco*RI plus *Hin*dIII-D junction fragment and the 1.95-kb *Hin*dIII-F fragment was common to all of the pAMβ1 derivatives.

Plasmid stability and copy number determinations. We examined the stability of pAMβ1, and several of the plasmid derivatives described above, by determining rates of spontaneous curing under nonselective conditions (Table 5). Among those strains harboring whole or partial pAMβ1 molecules, or the pAMβ1 plus *S. mutans* hybrid plasmid, greater than 90% of the population had retained the resident plasmid after 15 to 20 generations in the absence of selective pressure. However, strain DL206, containing the pAMβ1 plus pACKC1 chimeric plasmid, was significantly less stable under nonselective conditions.

Experiments were performed to determine whether the various alterations of pAMβ1 had any effects on plasmid copy number (Table 6). Plasmid pAMβ1 was maintained at a copy number that was relatively independent of the streptococcal host species. The copy number obtained for pVA2 (deleted of a segment of DNA far removed from the replication functions but tentatively associated with conjugative transfer) was nearly sixfold higher than that observed with *S. sanguis* harboring intact pAMβ1. The remaining *S. sanguis* strains studied, harboring the pAMβ1 plus *E. coli* chimera (pDL206), the pAMβ1 plus *S. mutans* hybrid plasmid (pDL212), or the pAMβ1 *Hin*dIII subclones (pDL214 and pDL216), yielded plasmid copy numbers that were approximately 9- to 10-fold higher than *S. sanguis* containing whole pAMβ1.

DISCUSSION

A detailed restriction endonuclease map of the broad host-range streptococcal plasmid, pAMβ1, was constructed by

TABLE 6. Plasmid copy number determinations for pAMβ1 and pAMβ1 derivatives

Strain	Plasmid	Copy no.	
		Expt 1	Expt 2
DL9	pAMβ1	7	7
DL812	pAMβ1		7
DL125	pAMβ1	1	9
DL31	pVA2	27	31
DL206	pDL206	55	35
DL212	pDL212	45	38
DL214	pDL214	39	52
DL216	pDL216	61	39

standard mapping techniques. Forty of forty-two cleavage sites obtained by the digestion of pAMβ1 with nine different restriction enzymes were located on the map. The precise arrangement of the *Hin*dIII-E, -K, and -L fragments (Fig. 1A) could not be determined. The same mapping approach was used to determine the locations and extent of deletions on four independently isolated spontaneous derivatives of pAMβ1 (Fig. 1B). The ability of three of the deletion derivatives (pVA1, pVA2, and pDL177) to replicate and express the MLS resistance phenotype in *S. sanguis* and a group F *Streptococcus* was confirmed by transformation experiments. When these transformants were used as donors in conjugation experiments, no MLS-resistant transconjugants were obtained under conditions that resulted in high frequencies of pAMβ1 transfer. All three of these deletion derivatives were missing a common segment of the pAMβ1 molecule, suggesting that at least one conjugation function was located in this region. Preliminary attempts to clone restriction endonuclease fragments from this region of pAMβ1 have been unsuccessful.

A fourth deletion derivative of pAMβ1, pMVΔβ1, was missing a 2-kb segment of the original molecule. The *S. faecalis* strain (DL186) containing this plasmid was sensitive to the MLS group of antibiotics. The transfer functions of this derivative remained intact since it was able to mobilize pMV158, a nonconjugative tetracycline resistance plasmid (4) present in the same host strain. Transconjugants containing pMVΔβ1 and pMV158 were also MLS sensitive. The 2-kb deletion in pMVΔβ1 provided a rough localization of the MLS resistance determinant on the map of pAMβ1 (Fig. 1B). Macrina and associates (26, 28) cloned this determinant by using an *Ava*I plus *Hin*dIII-derived fragment from pAMβ1. The cloned fragment corresponds to the *Ava*I, *Hin*dIII-B and -D fragment junction site in Fig. 1A. Recent experiments in our laboratory have shown that the MLS resistance determinant is completely contained within the 1.1-kb *Hha*I-J fragment of pAMβ1 (Fig. 1 and 5).

Figure 5, which illustrates an 11-kb segment of the 26.5-kb pAMβ1 molecule, summarizes the results of experiments designed to delineate the replication region of this plasmid. The *E. coli* plasmid, pACKC1, which does not replicate in *S. sanguis*, was used to clone the 5.0-kb *Eco*RI-B fragment of pAMβ1 in *E. coli* HB101. The resultant chimeric plasmid, pDL206, was used to transform a competent culture of *S. sanguis*. Plasmid pDL206 replicated and expressed the *E. coli* kanamycin resistance determinant in *S. sanguis* transformants. Further evidence that the *Eco*RI-B fragment contained the replication functions was obtained when this fragment was used as a vector to clone a chromosomal streptomycin resistance determinant from *S. mutans*. Subcloning experiments using religated *Hin*dIII partial digests of pAMβ1 provided two additional plasmids, pDL214 and pDL216, which helped to delineate further the replication region. The combined results of the cloning and subcloning experiments located the replicon function(s) within a 2.95-kb region of the pAMβ1 map.

Several reports have described the expression of genetic information derived from gram-positive bacteria in *E. coli* (5, 9, 10, 18, 28). However, the ability of gram-positive bacteria to phenotypically express *E. coli* chromosomal, or plasmid, genes appears to be rare. Goldfarb et al. (16) were able to demonstrate the expression of Tn*9*-associated chloramphenicol (Cm) resistance in *B. subtilis* only after the Tn*9* regulatory elements were supplanted by *B. subtilis* DNA. The *E. coli* gene coding for thymidylate synthetase apparently cannot be expressed by *B. subtilis* unless the DNA is integrated into the chromosome (34). Schottel et al. (36) demonstrated

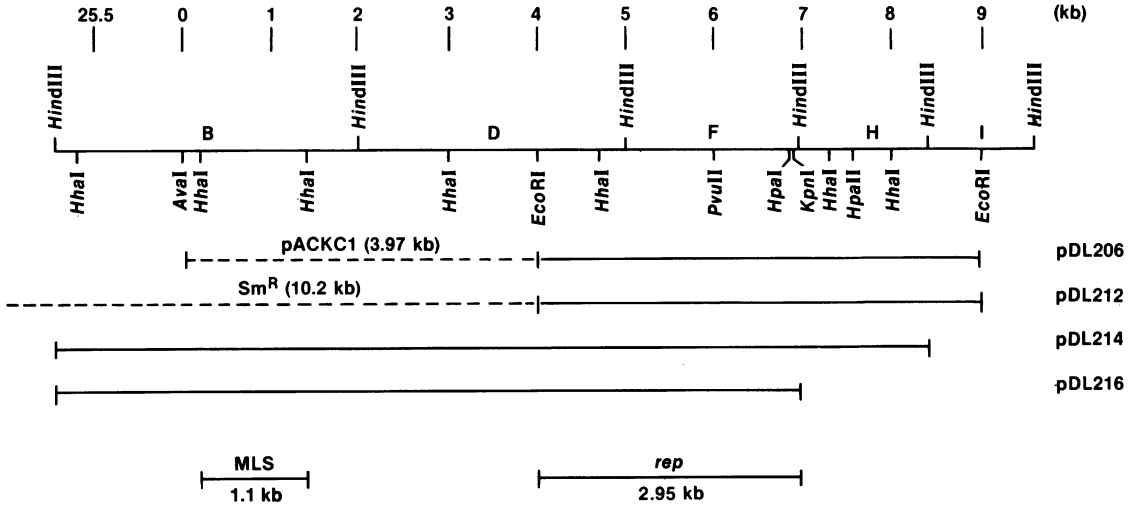

FIG. 5. Locations of pAMβ1 replication and MLS resistance functions. An 11.05-kb region of the pAMβ1 restriction endonuclease map is represented. Solid lines represent pAMβ1 DNA present in plasmids pDL206, pDL212, pDL214, and pDL216. Broken lines indicate pACKC1 (pDL206) and *S. mutans* DNA (pDL212) mediating resistance to Km and Sm, respectively. The 2.95-kb *rep* (replication) region was common to all four plasmid molecules.

that hybrid plasmids constructed from the *Streptomyces lividans* plasmid, pSLPIII, plus either pACYC184 (Cm resistance) or pACYC177 (Km resistance) could replicate and express their respective antibiotic resistance phenotypes in either *E. coli* or *S. lividans*. The Cm resistance gene of pACYC184 was not transcribed in *S. lividans* from the normal *E. coli* promotor (36). Whether this is also true for the expression of the Km resistance gene of pACYC177 by *S. lividans*, or by the *S. sanguis* strain DL206 described in this communication, has not been determined.

Intact pAMβ1 was present in each of the three streptococcal species examined at five to seven copies per genome equivalent. All of the partial pAMβ1 plasmids, including the 18.5-kb deletion derivative, pVA2, were present in *S. sanguis* at 30 to 50 copies. A gene product associated with the transmissibility of pAMβ1, possibly involved in transfer replication, may influence the copy number of the intact plasmid. A number of MLS plasmids contain, within their respective replication regions, a set of closely spaced *Hpa*I, *Kpn*I, *Hin*dIII restriction sites (1, 29). This area of the replicon, located between the 6.75 and 7.0 kb points on the map of pAMβ1 (Fig. 1 or 5), is associated with the regulation of copy number in at least one MLS plasmid (1). Experiments are currently in progress to determine whether this is also true for the pAMβ1 replicon.

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