JOURNAL OF BACTERIOLOGY, Sept. 1980, p. 1425-1435
0021-9193/80/09-1425/11$02.00/0

Vol. 143, No. 3

Chimeric Streptococcal Plasmids and Their Use as Molecular Cloning Vehicles in *Streptococcus sanguis* (Challis)

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Chimeric plasmids, which were useful as cloning vehicles in a *Streptococcus sanguis* (Challis) host vector system, have been constructed. By using three different strategies of restriction endonuclease digestion and ligation, a deoxyribonucleic acid (DNA) fragment bearing an erythromycin resistance determinant was ligated in vitro to a phenotypically cryptic plasmid from *Streptococcus ferus*. Recombinant plasmids could be recovered after transformation of *S. sanguis* (Challis) with these preparations. Three useful chimeras were constructed. pVA680, 5.5 megadaltons in size, contained a single *Kpn*I site into which passenger DNA may be spliced. pVA736, 5.0 megadaltons in size, contained single *Eco*RI, *Hin*dIII, and *Kpn*I sites into which passenger DNA may be spliced. The *Eco*RI and *Kpn*I sites of pVA736 may be used in combination with one another when ligating DNA into this plasmid. pVA738, 3.7 megadaltons in size, contained single *Hin*dIII and *Ava*I sites into which passenger DNA may be spliced. pVA680, pVA736, and pVA738 were stably maintained as multicopy plasmids in *S. sanguis* (Challis). None of them continued to replicate (amplify) in chloramphenicol-treated cells. By using pVA736 as a vector, we have cloned a chloramphenicol resistance determinant obtained from a large, conjugative streptococcal R plasmid. In addition, chromosomal DNA sequences from *Streptococcus mutans* have been inserted into pVA736 by using the *Kpn*I-*Eco*RI site combination.

Certain species of streptococci long have been recognized as effective colonizers of the human oral cavity (7). Indeed, *Streptococcus mutans* is thought to play a key etiological role in dental caries (tooth decay) in humans (7, 16, 17), and its virulence in animal model systems has been well documented (1, 4, 5, 14, 32). Genetic approaches to the study of *S. mutans* virulence have been made by a number of groups. In this regard, water-insoluble glucan production from sucrose appears to be an important parameter. Mutants defective in ability to synthesize glucan show a dramatic loss of virulence in animal model systems (3, 6, 25, 26, 29, 30). Wild-type revertants of one such mutant recently were reported to regain virulence in an in vivo caries assay (12). Further, mutants of *S. mutans* which hyperproduce insoluble glucans have been reported to show elevated caries production in rats (26).

The study of hypo- and hypercariogenic mutants of *S. mutans* would be greatly aided by a genetic exchange system. We initially surveyed *S. mutans* for the presence of plasmids in hopes of identifying extrachromosomally encoded virulence traits. In contrast to other reports (10, 13), however, we have been unable to ascribe any phenotypic function to the few small plasmids that we have identified in *S. mutans* (19-21). Our most recent efforts in this regard have involved the construction of isogenic strains of *Streptococcus sanguis* (a genetically transformable oral *Streptococcus* sp.) bearing certain cryptic plasmids from *Streptococcus ferus* (formerly *S. mutans* subsp. *ferus* [2, 21]). Although these strains provided a rigorous system in which to test plasmid-encoded phenotypes, no obvious plasmid-specified traits were noted (22). The construction of these strains opened the possibility of developing plasmids that would be useful in a molecular cloning (recombinant DNA) system which employed *S. sanguis* as a host. Such a host vector system would be invaluable in dissecting the chromosomal determinants of *S. mutans* colonization ability and virulence in lieu of a genetic exchange system in this latter host.

Recently, Behnke and Ferretti (Plasmid **3**: 234, 1980) have presented a preliminary report that describes the first successful construction of a chimeric streptococcal plasmid. This plasmid consisted of an erythromycin resistance (Em^r) determinant-bearing fragment from a group A streptococcal R plasmid linked in vitro to a cryptic *S. mutans* plasmid (20).

In this paper, we report on the construction of chimeric plasmids that will be useful as molecular cloning vehicles in *S. sanguis* host systems.

All of these vectors were derived by the in vitro insertion of an Em^r determinant into a 2.8 × 10^6-dalton (2.8-megadalton [Mdal]) phenotypically cryptic plasmid from *S. ferus*. These chimeric plasmid vectors have been characterized with respect to size, copy number, and restriction enzyme site map. The utility of one of these vectors has been demonstrated by using both plasmid and chromosomal fragments as passenger DNA inserts.

MATERIALS AND METHODS

Bacterial strains and media. The strains used in this work are described in Table 1. Strains were grown routinely in Todd-Hewitt broth (Difco Laboratories, Detroit, Mich.). Agar (Difco) was added to a final concentration of 1.5% when solid medium was desired. For genetic transformation studies, cells were grown to competence in brain heart infusion broth (Difco) containing 10% heat-inactivated (56°C for 30 min) horse serum. Erythromycin was added to autoclaved media to a final concentration of 10 μg/ml. Chloramphenicol was used in selective media at a final concentration of 5 μg/ml. All strains were cultivated anaerobically at 37°C.

Chemicals and enzymes. Cesium chloride (technical grade) was obtained from Kawecki Berylco Industries, New York, N.Y. Agarose was obtained from Bethesda Research Laboratories, Rockville, Md. Lysozyme (grade I), protease (grade I), DNase I, RNase T1, ethidium bromide, chloramphenicol, and sodium dodecyl sulfate were purchased from Sigma Chemical Co., St. Louis, Mo. Erythromycin was a gift of The Upjohn Co., Kalamazoo, Mich. Restriction endonucleases, T4 DNA ligase, and bacteriophage lambda DNA were purchased from Bethesda Research Laboratories. Alkaline phosphatase (calf intestine, grade I) was from Boehringer Mannheim Corp., New York, N.Y. *Hin*dIII-digested phage lambda DNA and *Hin*cII-digested φX174 DNA were purchased from New England Biolabs, Beverly, Mass.

Plasmid DNA isolation. Covalently closed circular plasmid DNA was isolated as previously described by Macrina and Scott (20) with minor modification. Specifically, spheroplasts were lysed by the addition of 1% (wt/vol; final concentration) sodium dodecyl sulfate, and this was followed by bulk cellular DNA precipitation in the presence of 1 M NaCl at 4°C as described by Guerry et al. (8). Covalently closed circular DNA was purified by subjecting the high-salt sodium dodecyl sulfate supernatants to cesium chloride-ethidium bromide centrifugation. Isopropanol-extracted, purified plasmid DNA was dialyzed exhaustively against TE buffer (0.01 M Tris, 0.001 M disodium EDTA [pH 8.0]) and stored at 4°C. Purified plasmid DNA or plasmid restriction digest products were analyzed by agarose gel electrophoresis with appropriate size reference covalently closed circular (18, 24) or linear DNA molecules (phage lambda DNA-*Hin*dIII products or φX174 DNA-*Hin*cII products). Transformants were screened for plasmid DNA as previously described (23).

Plasmid copy number was determined by analyzing cells from a 1.5-ml mid-logarithmic-phase culture that had been grown for ~1.5 generations in the presence of 15 μCi of [*methyl*-^{3}H]thymidine per ml (20 Ci/mmol; New England Nuclear Corp., Boston, Mass.). Cells were processed, and lysates were examined by low-voltage electrophoresis as previously described (23). Ethidium bromide-stained chromosomal and plasmid components were cut from the gel, minced, and suspended in 3 ml of Aquasol I (New England Nuclear) scintillation cocktail. These suspensions were incu-

TABLE 1. *Bacterial strains*

Strain	Laboratory no.	Plasmid		Remarks/reference
		Designation	Phenotype	
S. sanguis (Challis)	V288	None		(22, Macrina et al., in press)
S. ferus	V380	pVA380	Cryptic	(21, 22)
		pVA380-1	Cryptic	
S. sanguis (Challis)	V486	pVA1	Em^r[a]	(Macrina et al., in press)
S. sanguis (Challis)	V677	pVA677	Em^r	Spontaneously occurring deletion derivative of pVA1
S. sanguis (Challis)	V685	pVA380-1	Cryptic	(22)
S. sanguis (Challis)	V680	pVA680	Em^r	Recombinant plasmid (this paper)
S. sanguis (Challis)	V736	pVA736	Em^r	Recombinant plasmid (this paper)
S. sanguis (Challis)	V738	pVA738	Em^r	Recombinant plasmid (this paper)
S. sanguis (Challis)	V742	pIP501	Em^{r}Cm^r[b]	(9)

[a] Em^r, Resistance to >400 μg of erythromycin per ml.
[b] Cm^r, Resistance to 5 μg of chloramphenicol per ml.

bated at 37°C with mild reciprocal shaking for 24 h and then counted with a liquid scintillation spectrometer. Recovery of input counts in this system was >90%.

DNA enzymology. Restriction endonuclease digestions were carried out in buffers recommended by Bethesda Research Laboratories with the following exceptions. *Hin*dIII and *Eco*RI (used singly or in combination with one another) were used in a buffer system containing 90 mM Tris-hydrochloride [pH 7.4] and 10 mM $MgCl_2$ (TM buffer [28]). *Hin*dIII-*Ava*I combination digests were performed in Bethesda Research Laboratories *Ava*I buffer (20 mM Tris-hydrochloride [pH 7.4], 30 mM NaCl, 10 mM $MgCl_2$). By using standard Bethesda Research Laboratories buffers, simultaneous double digestions for restriction site mapping were performed as follows: *Hin*dIII-*Ava*I in *Ava*I buffer, *Hin*dIII-*Kpn*I in *Kpn*I buffer, *Hin*dIII-*Hpa*II in *Hpa*II buffer, *Eco*RI-*Ava*I in *Ava*I buffer, *Eco*RI-*Kpn*I in *Kpn*I buffer, *Eco*RI-*Hpa*II in *Hpa*II buffer, *Ava*I-*Hpa*II in *Ava*I buffer, *Ava*I-*Kpn*I in *Kpn*I buffer, and *Hpa*II-*Kpn*I in *Kpn*I buffer. Digestions were performed in 50-μl reaction volumes with 5 to 8 U of enzyme per microgram of DNA (0.5 to 1 μg of total DNA). In all cases, incubation was for 3 h at 37°C. Unless otherwise stated, reactions were stopped by the addition of 0.25 volume of an aqueous solution containing 0.07% bromophenol blue, 33% glycerol, and 7% sodium dodecyl sulfate.

DNA ligase reactions were performed in a final volume of 100 μl. Restriction enzyme digests were first heat inactivated by treatment at 65°C for 5 min. The following components then were added to the 50-μl digest: 22.5 μl of 0.23 M Tris-hydrochloride (pH 7.6)–0.03 M $MgCl_2$–500 μg of bovine serum albumin per ml; 7.5 μl of 1.5 mM ATP; 7.5 μl of 50 mM dithiothreitol; 11.5 μl of water; and 1 μl of T4 DNA ligase (~100 U/ml). Ligase reactions were incubated at 4°C for 18 h and then used in toto to transform competent *S. sanguis* cells.

Alkaline phosphatase treatment was used to remove terminal 5′ phosphates from *Hin*dIII-digested DNA to prevent effective ligation of self-annealed molecules. Two to four micrograms of plasmid DNA first was digested to completion with *Hin*dIII in a reaction volume of 200 to 400 μl. After digestion, the reaction mixture was heat inactivated (65°C for 5 min) to destroy *Hin*dIII activity. The volume of the reaction mixture was brought to 493 μl with TM buffer. A 50-μl amount of 1.0 M glycine (pH 9.5)–10 mM $ZnSO_4$ was then added to the tube. This was followed by the addition of 5 U of alkaline phosphatase. Incubation was for 1 h at 37°C. The reaction was terminated by extraction with an equal volume (~600 μl) of buffer (6 mM Tris-hydrochloride [pH 7.4], 0.1 mM EDTA)-saturated phenol. The phenol extraction step was followed by seven ether extractions (800 μl each) to remove the phenol. The ether-extracted material was dialyzed against TE buffer and stored at −35°C.

Fragments generated by digestion with single restriction endonucleases were designated alphabetically from largest to smallest.

Genetic techniques. The genetic transformation protocol of Lawson and Gooder (15) was used exclusively in this work. Plasmid curing by growth at 42°C was performed as previously described (F. L. Macrina, C. L. Keeler, K. R. Jones, and P. H. Wood, Plasmid, in press).

Containment. The recombinant DNA experiments with streptococcal plasmids fall into exemption category I-E-3 of the current NIH Guidelines for Research Involving Recombinant DNA Molecules. All plasmids or plasmid sequences employed were classed as being indigenous to *S. sanguis* because they may be introduced by naturally occurring genetic transformation and are stably maintained by this host (22; Macrina et al., in press). The introduction of *S. mutans* chromosomal DNA into *S. sanguis* by "shotgun" recombinant techniques falls into exemption category I-E-4 of the NIH guidelines. This ruling was made at the 6–7 March 1980 meeting of the NIH Recombinant DNA Advisory Committee (Fed. Regist. 45:25368, 1980) and was based on the natural transfer (via genetic transformation) of chromosomal sequences from *S. mutans* to *S. sanguis*.

RESULTS

Ligation of an Em^r determinant to a cryptic plasmid from *S. ferus*. We recently reported the introduction of two phenotypically cryptic plasmids from *S. ferus* into *S. sanguis* (22). Because of their small size and multicopy nature, these plasmids seemed ideal candidates for cloning vectors in *S. sanguis*. We sought to determine if the Em^r determinant of pVA1 could be ligated to either or both of these plasmids. pVA1 is a 7.3-Mdal plasmid which confers constitutively expressed erythromycin resistance and is cleaved into four fragments by *Hin*dIII (Fig. 1A, lane c) (Macrina et al., in press). Both cryptic plasmids (pVA380 [2.4 Mdal] and pVA380-1 [2.8 Mdal] [21, 22]) were found to be cleaved once with *Hin*dIII (Fig. 1A, lane b). Approximately 0.5 μg of *Hin*dIII-digested pVA380 and pVA380-1 DNAs (from *S. ferus* V380) was mixed with ~1 μg of *Hin*dIII-digested, alkaline phosphatase-treated pVA1. When reannealed and ligated, this preparation yielded Em^r transformants in *S. sanguis* at a frequency of $\sim 10^{-6}$ transformants per recipient cell. Analysis of six Em^r transformants revealed recombinant plasmids, as evidenced by an increase in molecular size (not shown). One typical recombinant plasmid, designated pVA680, was found to consist of the pVA380-1 (2.8 Mdal) plasmid linked to the *Hin*dIII A fragment (2.7 Mdal) of pVA1. These results allowed the assignment of the Em^r determinant of pVA1 to the *Hin*dIII A fragment. Analysis of parental and recombinant plasmids was extended by using *Hin*fI digestion. Multiple fragments generated by this enzyme allowed the identification of pVA1 and pVA380-1 components in the chimeric pVA680 (Fig. 1B). The insertion of pVA1 sequences into pVA380-1 is assumed to have taken place in the pVA380-1

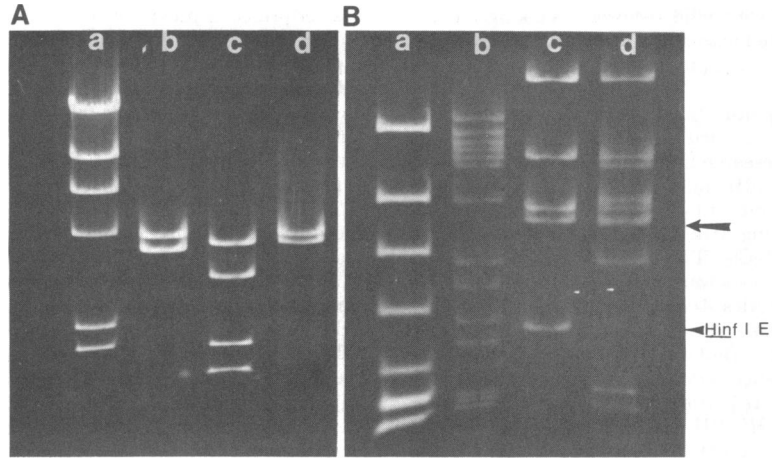

FIG. 1. *Electrophoretic analysis of endonuclease-digested pVA680 and its parental plasmids. (A) 0.7% agarose. Lane a, HindIII-digested phage lambda DNA; sizes (Mdal) from top to bottom are 15, 6.4, 4.3, 2.9, 1.6, and 1.4, respectively. Lane b, HindIII-digested plasmid DNA from S. ferus V380; the upper band corresponds to the linear unit length (2.8 Mdal) of pVA380-1 plasmid DNA, and the lower band corresponds to the linear unit length (2.4 Mdal) of pVA380 plasmid DNA. Lane c, HindIII-digested pVA1; fragment sizes (Mdal) from top to bottom are 2.7, 2.2, 1.3, and 1.1, respectively. Lane d, HindIII-digested pVA680 DNA. (B) 2% agarose. Lane a, HincII digest of ϕX174 DNA providing linear reference fragments from 0.68 (uppermost component) to 0.1 Mdal (lowermost component). Lanes b, c, and d, HinfI digests of pVA1, pVA380-1, and pVA680, respectively. Insertion of the Em^r-bearing fragment (from pVA1) was assumed to occur in the HinfI E fragment of pVA380-1 (indicated by the arrowhead). The arrow indicates a presumed fusion fragment (one member of a doublet in this case) formed as a result of the insertion.*

*Hin*fI E fragment (Fig. 1B, lane c) because this fragment was missing in pVA680 (Fig. 1B, lane d). A *Hin*fI fusion fragment (Fig. 1B, lane d [see arrow]) was noted in pVA680, presumably formed as a result of the insertion event. A restriction site map of pVA680 was constructed by simultaneous double digestion (see Fig. 6). The single *Kpn*I site on pVA680 originated from pVA1 sequences present in the plasmid (Fig. 2 and 3). This *Kpn*I site is part of a region that may be deleted from pVA1 without any effect on its replication or Em^r expression. Restriction site maps of pVA1 and a deletion derivative of pVA1 (pVA677) are shown in Fig. 2. pVA677 is a spontaneously occurring deletion mutant of pVA1 which was found during routine screening of cell lysates of erythromycin-grown V486 cells. From this deletion analysis, we conclude that the *Kpn*I site affords a location at which passenger DNA may be ligated into pVA680.

Construction of pVA736. The construction of pVA680 established the usefulness of the *Hin*dIII site on pVA380-1 for cloning purposes. To further explore the utility of pVA380-1 as a molecular cloning vector, we determined its restriction site map by simultaneous double digestion (Fig. 3; pVA380-1 isolated from V685 [Table 1]). As shown in Fig. 3, the *Hin*dIII site of pVA380-1 was close to a single *Eco*RI site. The positioning of these two enzyme sites prompted us to attempt ligation of the Em^r determinant of pVA1 into pVA380-1 by using *Eco*RI-*Hin*dIII combination digestion (Fig. 2, map of pVA1; note that the *Eco*RI site at ~1.7 kilobases [kb] is in a functionally nonessential region of pVA1). *Eco*RI-*Hin*dIII-digested pVA380-1 DNA (0.5 μg) and identically digested pVA1 DNA (1.0 μg) were mixed, ligated, and used to transform *S. sanguis* V288. Em^r transformants were recovered at a frequency of ~10^{-4} per recipient, and several recombinants that were examined for plasmid content revealed results similar to those shown in Fig. 4. The recombinant plasmid (designated pVA736) shown in Fig. 4A (lane d) consisted of two fragments when simultaneously cleaved with *Eco*RI and *Hin*dIII. The larger component of pVA736 (Fig. 4A, lane d) corresponded to the *Eco*RI-*Hin*dIII-digested pVA380-1 (Fig. 4A, lane c), whereas the smaller component corresponded to the largest *Eco*RI-*Hin*dIII fragment of pVA1. This fragment spans coordinates 9.6 to 1.7 kb on the pVA1 map (Fig. 2) and is known to contain an Em^r determinant (see above). Figure 4B shows parental and recombinant plasmid identities by *Hin*fI digestion, again showing pVA1 sequence insertion into the *Hin*fI E fragment of pVA380-1.

Construction of pVA738. Finally, we decided to attempt the insertion of the Em^r determinant of pVA1 into pVA380-1 by using plas-

mids digested in combination with *Ava*I and *Hin*dIII (the fragments of interest are shown in Fig. 2, pVA1 coordinates ~9.6 to 11 kb and Fig. 3, pVA380-1 coordinates 0 to 3.9 kb). This would provide us with a plasmid vector that would be considerably smaller (3.7 Mdal) than pVA680 (5.5 Mdal) and pVA736 (5.0 Mdal). *Ava*I-*Hin*dIII combination-digested pVA380-1 (0.5 μg) and pVA1 (1.0 μg) were ligated and used to transform V288. Em^r transformants with such preparations were found at a frequency of $\sim 10^{-5}$ per recipient. Analysis of a typical transformant bearing a recombinant plasmid designated pVA738 is shown in Fig. 5. Figure 5A shows

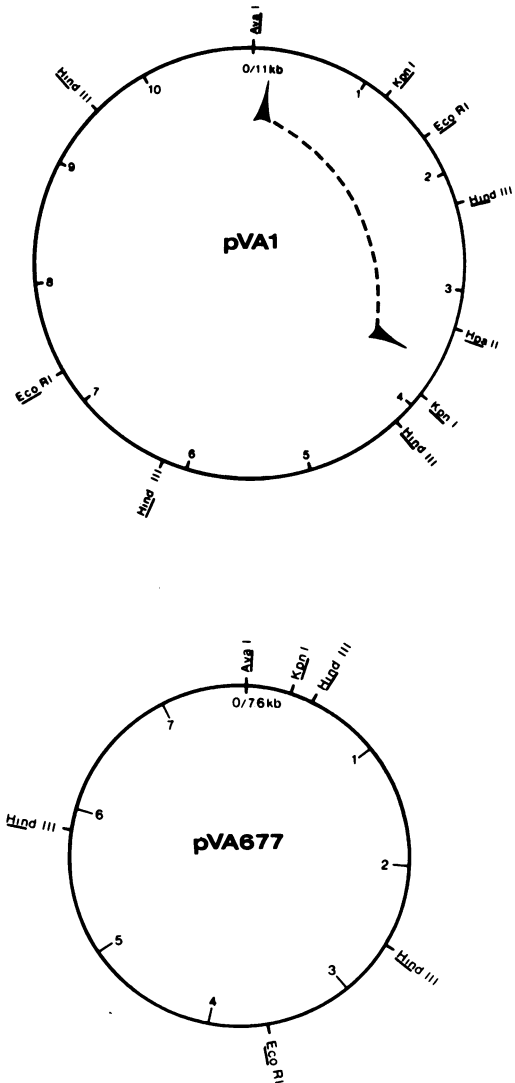

FIG. 2. *Restriction endonuclease site map of pVA1 and pVA677. The dashed line indicates the region deleted from pVA1 in the formation of pVA677.*

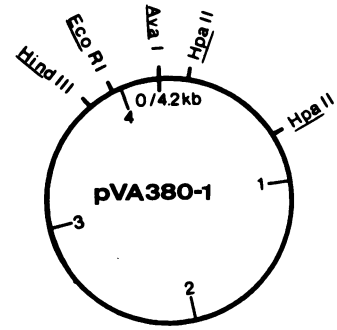

FIG. 3. *Restriction endonuclease site map of pVA380-1.*

*Hin*dIII-*Ava*I combination digests of pVA1 (lane b), pVA380-1 (lane c), and pVA738 (lane d). The large *Hin*dIII-*Ava*I fragment of pVA738 corresponded to the largest *Hin*dIII-*Ava*I fragment of pVA380-1, whereas the small *Hin*dIII-*Ava*I fragment corresponded to the fourth pVA1 fragment. This pVA1 component represented the *Hin*dIII-*Ava*I fragment (Fig. 2, coordinates ~9.6 to 11 kb) known to bear the Em^r determinant. The results shown in Fig. 5B indicate pVA1 sequence insertion into the *Hin*fI E fragment of pVA380-1 (*Hin*dIII site) and the *Hin*fI C fragment (*Ava*I site).

Genetic and physical characterizations of chimeric streptococcal plasmids. The respective molecular sizes of pVA680, pVA736, and pVA738 are shown in Table 2. All three plasmids were found to be present in multiple copies per chromosomal equivalent (Table 2). None of the chimeric plasmids (pVA680, pVA736, and pVA738) continued to replicate in cells treated with inhibitory levels of chloramphenicol (not shown). Specifically, the plasmid copy number remained the same or decreased in cells that were treated with chloramphenicol for 18 to 24 h.

The erythromycin and lincomycin resistance levels conferred by pVA680, pVA736, and pVA738 were unchanged relative to the parental pVA1 plasmid. These were determined to be >400 μg/ml for erythromycin and >100 μg/ml for lincomycin by using a colony unit-forming test (19). Furthermore, the Em^r determinant of pVA1 is known to be expressed constitutively (Macrina et al., in press), and, as expected, constitutive Em^r expression was observed for pVA680, pVA736, and pVA738 (data not shown). As shown in Table 3, pVA1-conferred Em^r was segregated at high frequency from cells grown at 42°C. This is consistent with our previous reports of pVA1 thermosensitive curing (Macrina et al., in press). This was also observed for the pVA1 deletion derivative, pVA677 (Table 3).

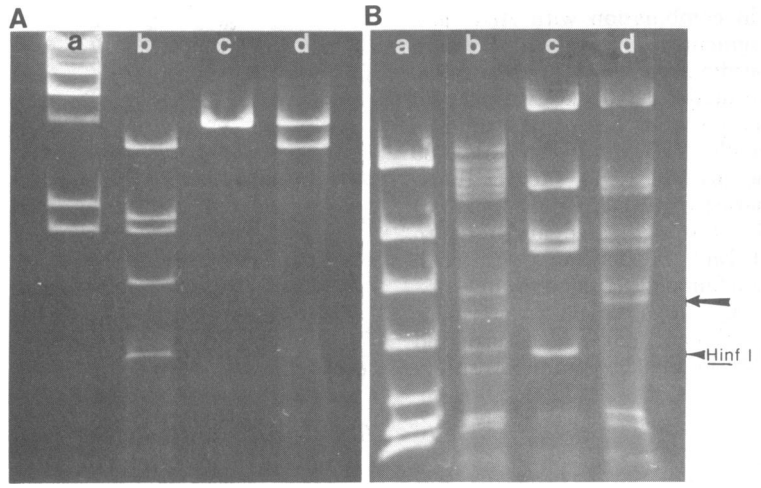

FIG. 4. *Electrophoretic analysis of endonuclease-digested pVA736 and its parental plasmids. (A) 1.0% agarose. Lane a, HindIII-digested phage lambda DNA; sizes (Mdal) from top to bottom are 15, 6.4, 4.3, 2.9, 1.6, 1.4, and 0.3, respectively. Lane b, EcoRI-HindIII-digested pVA1 DNA; fragment sizes (Mdal) from top to bottom are 2.3, 1.5, 1.3, 1.1, 0.7, and 0.4, respectively. Lane c, EcoRI-HindIII-digested pVA380-1 DNA; the fragment corresponds to ~2.7 Mdal, and the 0.1-Mdal EcoRI-HindIII fragment (Fig. 3) was not detected on this concentration of agarose. Lane d, EcoRI-HindIII-digested pVA736 DNA. (B) 2% agarose. Lane a, HincII digest of φX174 DNA providing linear reference fragments from 0.68 (uppermost component) to 0.1 Mdal (lowermost component). Lanes b, c, and d, HinfI digest of pVA1, pVA380-1, and pVA736, respectively. Insertion of the Em^r-bearing fragment (from pVA1) was assumed to occur in the HinfI E fragment of pVA380-1 (indicated by the arrowhead). The arrow indicates a presumed fusion fragment formed as the result of the insertion.*

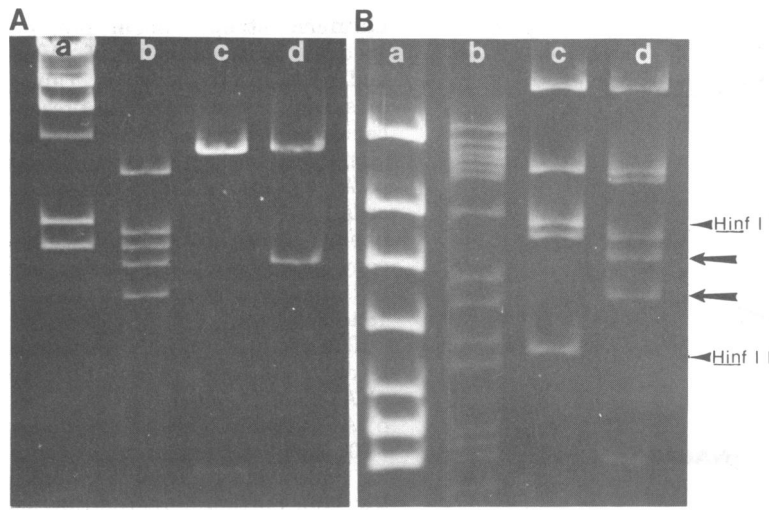

FIG. 5. *Electrophoretic analysis of endonuclease-digested pVA738 and its parental plasmids. (A) 1% agarose. Lane a, HindIII-digested phage lambda DNA; sizes (Mdal) from top to bottom are 15, 6.4, 4.3, 2.9, 1.6, 1.4, and 0.3, respectively. Lane b, HindIII-AvaI-digested pVA1 DNA, fragment sizes (Mdal) from top to bottom are 2.2, 1.5, 1.3, 1.2, and 1.1, respectively. Lane c, HindIII-AvaI-digested pVA380-1 DNA; the larger fragment corresponds to ~2.6 Mdal, and the smaller fragment corresponds to ~0.35 Mdal. Lane d, HindIII-AvaI digest products of pVA738. (B) 2% agarose. Lane a, HincII digest of φX174 DNA providing linear reference fragments from 0.68 (uppermost component) to 0.1 Mdal (lowermost component). Lanes b, c, and d, HinfI digests of pVA1, pVA380-1, and pVA738. Insertion of the Em^r-bearing fragment (from pVA1) was assumed to occur in HinfI E fragment of pVA380-1 (location of the HindIII site) and the HinfI C fragment (location of the AvaI site) (indicated by the arrowheads). The arrows indicate presumed fusion fragments formed as the result of the insertion.*

TABLE 2. *Physical characterization of streptococcal plasmids*

Plasmid	Size (Mdal)		Plasmid DNA of chromosome (%)	No. of plasmid copies[c]
	CCC[a]	*Hin*dIII fragment sum[b]		
pVA1	7.29 ± 0.10[d]	ND	4.98 ± 0.26	~12
pVA677	5.01 ± 0.13	4.97 ± 0.08	5.05 ± 0.25	~18
pVA380-1	2.86 ± 0.09	ND	4.00 ± 0.29	~25
pVA680	5.50 ± 0.23	5.50 ± 0.40	4.30 ± 0.29	~14
pVA736	5.07 ± 0.17	4.96 ± 0.10	5.56 ± 0.48	~20
pVA738	3.70 ± 0.14	ND	2.96 ± 0.20	~14

[a] Determined by electrophoretic migration of covalently closed circular (CCC) molecules through 0.7% agarose as compared with the migration of size reference molecules from *E. coli* V517 (18).

[b] *Hin*dIII fragment sizes estimated by using bacteriophage lambda DNA *Hin*dIII fragments as size reference molecules on 1% agarose electrophoretic gels. ND, Not determined.

[c] Per 1.79 × 10^9-dalton equivalent. Size of the *S. sanguis* chromosome was assumed to be equal to that of *S. mutans* GS-5 as determined by reassociation kinetics (19).

[d] Based on contour length measurements (Macrina et al., in press).

TABLE 3. *Comparison of phenotypes of plasmid-bearing strains*

Strain	Plasmid	Em[r] segregation at 42°C[a]	
		Total no. of colonies scored	Em[r] clones (%)
S. sanguis V486	pVA1	94	96.8
		150	90.0
S. sanguis V677	pVA677	94	84.0
		150	90.0
S. sanguis V680	pVA680	94	0
		150	0
S. sanguis V736	pVA736	94	0
		150	0
S. sanguis V738	pVA738	94	0
		150	0

[a] Loss of erythromycin resistance was less than 5% at 37°C for V486 and V677; all remaining strains showed no segregation of erythromycin resistance at 37°C.

None of the chimeric plasmids appeared to be subject to thermosensitive segregation, and selected Em[r] clones that were recovered from broth cultures grown at 42°C always were found to contain their respective plasmid molecules (data not shown). pVA680, pVA736, and pVA738 (0.5- to 1.0-μg quantities) all transformed *S. sanguis* at frequencies of 10^{-2} per recipient or higher.

Restriction endonuclease site maps of pVA680, pVA736, and pVA738 were constructed by simultaneous double digestion. These maps are shown in Fig. 6.

Utility of pVA736 as a molecular cloning vehicle. The single *Hin*dIII and *Eco*RI sites of pVA736 should reside in functionally nonessential regions because these sites were used to

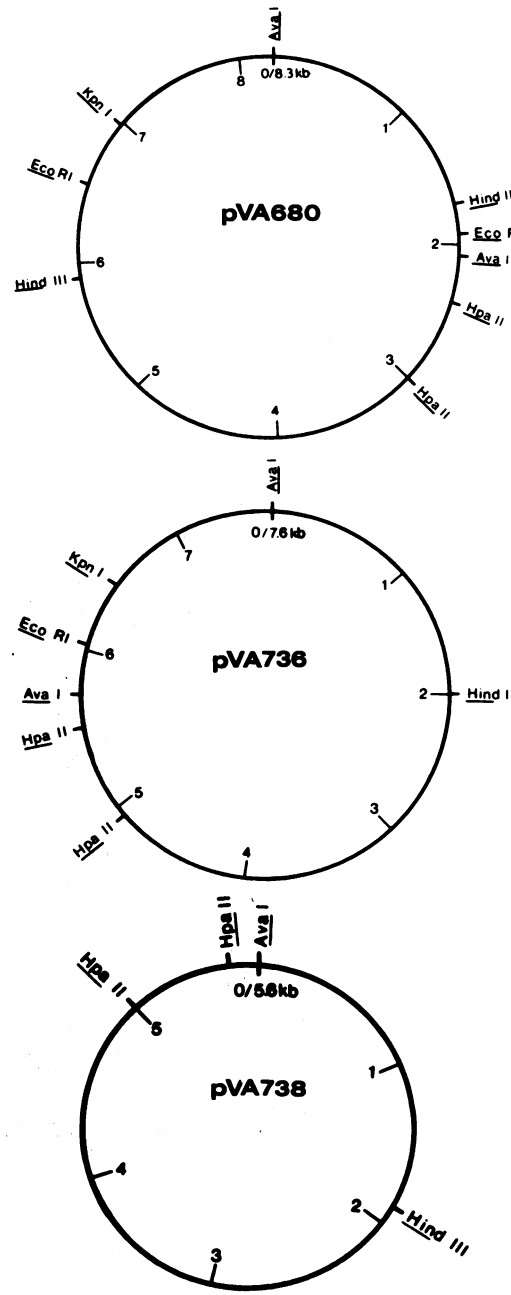

FIG. 6. *Restriction endonuclease site maps of pVA680, pVA736, and pVA738.*

ligate the two component fragments of this chimera. We therefore attempted to use the *Hin*dIII site of pVA736 to insert DNA into this molecule. As a source of passenger DNA for this experiment, streptococcal plasmid pIP501 was used (20 Mdal; confers Em^r and chloramphenicol resistance [Cm^r] [5 μg/ml]) (9, 11). pIP501 may be transferred to *S. sanguis* from *Streptococcus agalactiae* by transformation or conjugation (9; unpublished data). It contains 14 *Hin*dIII sites (9). Alkaline phosphatase-treated, *Hin*dIII-cleaved pVA736 (~0.5 μg) was mixed with ~0.5 μg of *Hin*dIII-digested pIP501. This mixture was reannealed, ligated under standard conditions, and used to transform *S. sanguis*, simultaneously selecting for Em^r (10 μg/ml) and Cm^r (5 μg/ml). Doubly resistant transformants were obtained at a low frequency (~10^{-7} per recipient). Lysates of six doubly resistant clones were screened by agarose gel electrophoresis for plasmid DNA, and all contained recombinant plasmids, based on increased molecular size (data not shown). A plasmid (~10 Mdal) from one such clone was purified and subjected to analysis by *Hin*dIII digestion. These data revealed a recombinant plasmid (designated pVA743) that contained two *Hin*dIII fragments (Fig. 7A, lane d). The larger of these fragments corresponded to the *Hin*dIII-cut pVA736 vector DNA (5.0 Mdal [lane c]), whereas the smaller of the two fragments corresponded to the *Hin*dIII A fragment of pIP501 (4.3 Mdal [lane b]). The sum of the fragments of pVA743 (9.3 Mdal) compared well with the value of covalently closed circular pVA743 DNA sized by migration through 0.7% agarose (9.1 ± 0.3 Mdal). pVA743 DNA could be used to transform *S. sanguis* (Challis) at high frequency by using either erythromycin (10 μg/ml) or chloramphenicol (5 μg/ml) in selective media. Certain of the recombinants from the pIP501 cloning experiments (3/6) were found to contain a second *Hin*dIII fragment from pIP501 in addition to the Cm^r-bearing *Hin*dIII A fragment. These were not further characterized. Expression of the Cm^r determinant of pVA743 and the parental pIP501 plasmids was examined comparatively. As expected, pIP501 expressed Cm^r in an inducible fashion (induction dose =

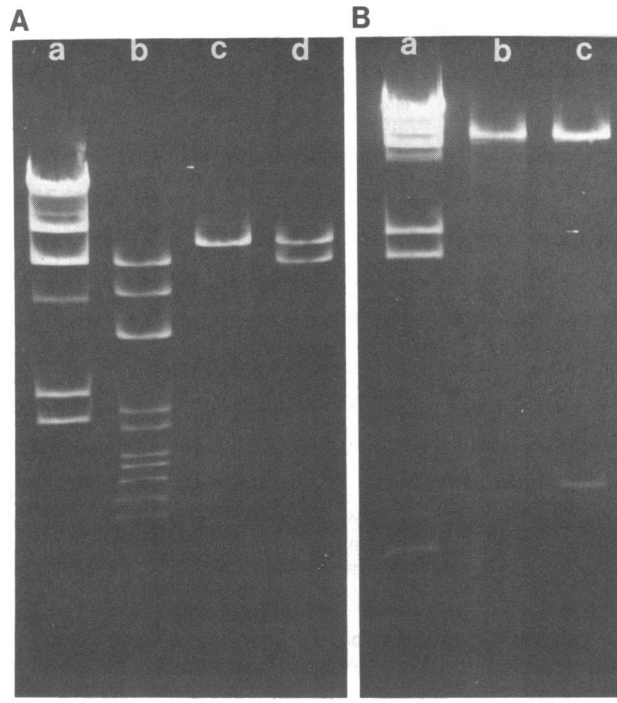

FIG. 7. *Recombinant plasmids derived from pVA736. (A) 1% agarose. Lane a, HindIII digest products of phage lambda DNA; sizes (Mdal) from top to bottom are 15, 6.4, 4.3, 2.9, 1.6, and 1.4, respectively. Lane b, HindIII digest of pIP501. Lane c, HindIII digest of pVA736. Lane d, HindIII digest of pVA743. (B) 1.5% agarose. Lane a, HindIII digest of phage lambda DNA; sizes (Mdal) from top to bottom are 15, 6.4, 4.3, 2.9, 1.6, 1.4, and 0.3, respectively. Lane b, EcoRI-KpnI digest of pVA736; larger fragment is ~4.75 Mdal, and the smaller fragment is 0.25 Mdal. Lane c, EcoRI-KpnI digest of pVA745; inserted (small) fragment corresponds to 0.5 Mdal.*

0.5 μg of chloramphenicol per ml; challenge dose = 10 μg of chloramphenicol per ml) (9). pVA743-mediated Cm^r was similarly expressed under such conditions (data not shown).

Finally, we attempted a cloning experiment designed to test the use of the *Kpn*I and *Eco*RI sites of pVA736 (Fig. 6). pVA736 was digested with *Kpn*I and *Eco*RI (Fig. 7B, lane b). Chromosomal DNA from *S. mutans* 6715-13 (prepared as previously described [19]) was similarly digested with these two enzymes. A 0.5-μg amount of digested pVA736 DNA was mixed with ~6 μg of *Kpn*I-*Eco*RI-cut chromosomal DNA. After reannealing and ligation, this mixture was used to transform *S. sanguis* employing selection for the Em^r marker of pVA736. Transformants were detected at a frequency of ~4 × 10^{-6} per recipient. Half of the 12 clones screened in this experiment were found to contain plasmids that were equal to or smaller than pVA736. The remainder displayed an increased molecular size consistent with the addition of a chromosomal insert between the *Kpn*I and *Eco*RI sites of pVA736. One isolate (designated V745) was examined and found to contain a plasmid which yielded fragments equal to the *Kpn*I-*Eco*RI-digested pVA736 DNA (~4.7 Mdal) and a chromosomal insert of ~0.5 Mdal (Fig. 7B, lane c).

DISCUSSION

The introduction of small multicopy plasmids, like pVA380-1, into *S. sanguis* (22) was of key importance to the development of the molecular cloning system described in this report. Specifically, *S. sanguis* (unpublished data) and other oral streptococci (19, 21) have been found to largely lack indigenous plasmids. In fact, only one naturally occurring plasmid has been reported in *S. sanguis* (31), and its low copy number and genetic instability made it somewhat undesirable as a cloning vehicle. Thus, our direct or indirect introduction of plasmids into *S. sanguis* has been aimed specifically at the eventual utilization of such replicons as cloning vehicles in this host (21, 22; Macrina et al., in press).

Standard recombinant DNA methods have allowed us to successfully link the Em^r determinant from pVA1 with the phenotypically cryptic streptococcal plasmid pVA380-1 by using three different ligation strategies. Ligation at *Hin*dIII sites allowed the construction of pVA680 (Fig. 1 and 2) and the localization of the Em^r determinant to the *Hin*dIII A fragment (2.7 Mdal) of pVA1. The deletion of the right-hand side of this pVA1 *Hin*dIII A fragment (0 to 3.7 kb [Fig. 2]) was known to have no effect on the expression of Em^r. Hence, the *Kpn*I site of pVA680 was assumed to reside in a functionally nonessential region of this molecule. The assignment of the Em^r determinant of pVA1 to the *Hin*dIII A fragment and the information relating to deletions in this fragment (Fig. 2) led to the design of the pVA736 plasmid. The ligation of *Eco*RI-*Hin*dIII-generated fragments of pVA380-1 and pVA1 resulted in the formation of the predicted chimera (pVA736). The construction of pVA738 with the *Hin*dIII-*Ava*I fragments from pVA1 and pVA380-1 (Fig. 5 and 6) was also accomplished. Taken together, these data allow the specific assignment of the Em^r determinant on the pVA1 map (Fig. 2). The Em^r gene must reside between the *Hin*dIII site at 9.6 kb and the *Ava*I site at 11 kb. Neither of these sites was in the Em^r determinant nor did they effect Em^r expression (Table 3). The usefulness of pVA380-1 (Fig. 3) as a cloning vehicle for DNA sequences bearing a directly selectable marker is apparent from our studies. Namely, the single *Eco*RI, *Hin*dIII, and *Ava*I sites all evidently reside in a nonessential region with respect to the replicative functions of pVA380-1. Thus, any of these sites may be used alone or in paired combinations to insert DNA (e.g., drug resistance determinants) into this plasmid. This system should facilitate the dissection of multiple resistance-conferring streptococcal plasmids.

pVA680, pVA736, and pVA738 are relatively small in size and were present to the extent of multiple copies per chromosomal equivalent (Table 2), making them desirable as molecular cloning vehicles. None of these vectors, including pVA1 and pVA677, appears to be transferred by conjugation among the streptococci (data not shown; D. LeBlanc, personal communication). pVA680, pVA736, and pVA738 do not appear to undergo amplication (selective replication in the absence of protein synthesis), and because they contain only one selectable marker, they do not afford the opportunity to utilize insertional inactivation (27). The replication functions of all three of these chimeras appeared to be governed by the pVA380-1 portion of the recombinant molecules. Their thermostability (Table 3) in *S. sanguis* was consistent with pVA380-1 replication (22). pVA1 inheritance, on the other hand, is known to be temperature sensitive in cells grown at 42°C (Table 3; Macrina et al., in press). The use of the *Kpn*I and *Eco*RI sites for inserting DNA into pVA736 was directly demonstrated (Fig. 7B). In addition, this confirms the utility of the *Kpn*I site for inserting passenger DNA into pVA680 as well. The *Hin*dIII site used to insert DNA into pVA736 was formed identically to the single *Hin*dIII site of pVA738 (Fig. 5 and see above). Hence, the successful use of the pVA736 site (Fig. 7A) confirms that the

*Hin*dIII site of pVA738 may be used to insert DNA into this latter plasmid. The use of the pVA738 *Ava* I site was not directly tested, but because this site was used to ligate the component parts of pVA738, it is unlikely that it occurs in some newly formed essential plasmid sequence.

The *Kpn*I site of pVA736 (and pVA680) offers a unique advantage for cloning with this plasmid. *Kpn*I digestion yields a protruding 3'OH end which is an excellent primer for terminal deoxynucleotidyl transferase (27). The *Kpn*I recognition sequence is 5'GGTAC↓C3'. Consequently, the addition of a "tail" of deoxycytidine to the *Kpn*I-digested vector plasmid results in the restoration of the *Kpn*I site. When such deoxycytidine-containing molecules are allowed to anneal with passenger DNA which has been "tailed" with deoxyguanosine, the inserted DNA will be flanked by *Kpn*I sites. Passenger DNA so inserted into pVA736 thus could be removed from the chimeric plasmid by *Kpn*I digestion. This system will afford a highly efficient recovery of recombinant molecules while, at the same time, obviating the problem of using several different restriction enzymes when performing "shotgun" experiments. An additional advantage of the pVA736 vector is the use of the *Kpn*I and *Eco*RI sites in combination. Simultaneous digestion with these enzymes renders a pVA736 molecule that is unable to self-anneal. When present with an excess of *Kpn*I-*Eco*RI-digested passenger DNA, the formation of recombinant molecules is greatly favored. A final point worth noting is that pVA1 and pVA677 (Tables 1 to 3) may be used as molecular cloning vehicles themselves. Deletion mapping (Fig. 1) indicates that the single *Hpa*II site of pVA1 (~3.3 kb) lies in a functionally nonessential region and, hence, could be used as a site into which passenger DNA might be inserted. Secondly, the successful construction of pVA738 (see above) indicates that the single *Ava*I sites on pVA1 and pVA677 reside in a nonessential plasmid sequence, thus allowing their use in cloning experiments. These plasmids are readily cured by growth of their host strain at 42°C, and this property would be most useful in confirming traits borne by cloned sequences. Of equal importance is that these temperature-sensitive plasmids should allow the construction of chimeras (e.g., bearing specific drug resistance genes) that will afford a ready assay for transposable gene sequences in the streptococci.

We tested the utility of pVA736 by cloning a Cm^r determinant into its *Hin*dIII site (Fig. 7). These experiments permitted us to assign the Cm^r determinant to the largest *Hin*dIII fragment (fragment A) of pIP501 (Fig. 7). The inducible expression of chloramphenicol resistance by the chimeric pVA743 suggests that regulatory machinery for the Cm^r determinant as well as the structural gene reside on the cloned *Hin*dIII A fragment of pIP501. We plan to exploit this system further to study the regulation of both the Cm^r and Em^r genes carried by pIP501. The utility of the *Kpn*I and *Eco*RI sites of pVA736 was clearly demonstrated (Fig. 7B) by the insertion of *S. mutans* DNA into this vector using a shotgun approach. We plan to use the *Kpn*I and *Eco*RI sites of pVA736 as well as the tailing method (see above) to construct an *S. mutans* gene library in *S. sanguis*. In this fashion, we anticipate being able to identify important *S. mutans* gene sequences. One potential problem uncovered in these experiments was the detection of deletion-bearing plasmids after transformation with newly formed recombinant molecules (Fig. 7B and see above). Site-specific deletion of streptococcal R plasmids has been suggested, and pVA1 is, in fact, thought to have been formed by such an event (Macrina et al., in press). Further work with the vectors developed here is needed to more fully appreciate problems stemming from deletion formation in this system. If significant, such problems would be likely to be vector specific (i.e., associated with plasmid-borne sequences) and could be dealt with by using a number of strategies (e.g., in vitro or in vivo vector shortening or use of vectors which are not prone to deletion formation). Alternatively, the deletion formation could be due to recombinational events between plasmid vectors carrying specific *S. mutans* chromosomal sequences that share high homology with *S. sanguis*. If so, this would underscore the need for a recombination-deficient mutant of *S. sanguis*. Such mutants currently are being sought in an attempt to further refine the *S. sanguis* host system.

In sum, the cloning systems described here, although usable, are still at an early stage of development. The construction of plasmids with multiple selectable markers would, of course, be desirable from the standpoint of an insertional inactivation system. In this regard, pVA743 affords this possibility, and we plan to determine its restriction site map and to isolate in vitro shortened derivatives of this molecule. At present, however, the cloning systems outlined here open a variety of new approaches to geneticists studying streptococci. They will allow the dissection of large streptococcal R plasmids so as to study the regulatory and structural gene sequences of resistance determinants. Similarly, the genetic basis for plasmid-mediated conjugal transfer in the streptococci can be systematically explored. Most importantly, in our view, studies

aimed at the genetic basis of chromosomal determinants of virulence and colonization in *S. mutans* are now within reach. Indeed, *S. sanguis* provides an ideal genetic and biochemical background for the study of the organization and expression of such *S. mutans* genes.

ACKNOWLEDGMENTS

P. L. Shipley's critical review of this manuscript is acknowledged and appreciated. We thank Carroll Carter and Olivia Washington for assistance in manuscript preparation.

F.L.M. is the recipient of Research Career Development Award DE00081 from the National Institute of Dental Research. This work was supported by Public Health Service grant DE 04224 from the National Institute of Dental Research.

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