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A cloning vector able to replicate in *Escherichia coli* and *Streptococcus sanguis*

(Recombinant DNA; streptococcal shuttle plasmid; pACYC184; pVA749; pVA838)

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SUMMARY

A plasmid that is able to replicate in both *Escherichia coli* and *Streptococcus sanguis* has been constructed by the in vitro joining of the pACYC184 (Cm^r Tc^r) and pVA749 (Em^r) replicons. This plasmid, designated pVA838, is 9.2 kb in size and expresses Em^r in both *E. coli* and *S. sanguis*. Its Cm^r marker is expressed only in *E. coli* and may be inactivated by addition of DNA inserts at its internal *Eco*RI or *Pvu*II sites. The pVA838 molecule also contains unique *Sal*I, *Sph*I, *Bam*HI, *Nru*I and *Xba*I cleavage sites suitable for molecular cloning. pVA838 may be amplified in *E. coli* but not in *S. sanguis*. We have used the pVA838 plasmid as a shuttle vector to clone streptococcal plasmid fragments in *E. coli*. Such chimeras isolated from *E. coli* were readily introduced into *S. sanguis* by transformation.

INTRODUCTION

Considerable effort has been applied recently to the development of molecular cloning systems that use a streptococcal host (Behnke and Gilmore, 1981; Macrina et al., 1980c; 1982; Malke et al., 1981). Such systems have facilitated the study of streptococcal plasmid and chromosomal gene sequences. Using the transformable *S. sanguis* as a host, a number of streptococcal plasmid vectors have been constructed (Behnke and Gilmore, 1981; Macrina et al., 1980c; 1982; Malke et al., 1981). While the cloning of plasmid-derived sequences using such vectors has been straightforward, often chimeric plasmids bearing chromosomal inserts suffer deletions following their entry into the streptococcal cell. This problem appeared to be linked to the transformational requirements imposed on plasmids in the *S. sanguis* and *S. pneumoniae* systems. Namely, monomeric plasmid molecules transform with two-hit kinetics while

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Abbreviations: CCC, covalently closed circular; Cm^r, chloramphenicol resistance; Cm^s, chloramphenicol sensitivity; Em^r, erythromycin resistance; Em^s, erythromycin sensitivity; kb, kilobase pairs; SDS, sodium dodecyl sulfate; Tc^r, tetracycline resistance; Tc^s, tetracycline sensitivity; ::, novel joint; [], indicates plasmid-carrier state.

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oligomeric plasmid molecules transform with one-hit kinetics (Macrina et al., 1981; Saunders and Guild, 1981). In the case of shotgun cloning, the existence of substantial amounts of a single specific chimera or specific multimers generated from a vector-chromosomal fragment mixture would be remote due to the large size of the genome. By contrast, plasmid-plasmid cloning experiments involved relatively few fragment combinations and, thus, appeared to give rise to the needed amounts of specific monomers or oligomeric chimeras.

Two strategies have been employed to improve the efficiency of cloning chromosomal gene fragments in the *S. sanguis* host system. First, we have adapted the "helper plasmid" method originally devised by Gryczan et al. (1980) for use in the *Bacillus subtilis* cloning system (Tobian and Macrina, 1982). This method allows for the recombinational rescue of incoming chimeras by a resident plasmid that is homologous with the vector.

A second strategy we have implemented to circumvent the inherent problems of "shotgun cloning" in streptococci has involved the construction of a shuttle plasmid vector. This plasmid is able to replicate and be used as a cloning vehicle in both *E. coli* and *S. sanguis*. Using this vector, chromosomal fragments first can be cloned in *E. coli* by standard recombinant DNA methodologies. Such *E. coli*-derived plasmid preparations containing oligomers as well as high monomer concentrations of chimeras would transform *S. sanguis* readily, presumably reducing the occurrence of deletion events. In this paper, we report on the construction, characterization and use of such a shuttle plasmid vector.

MATERIALS AND METHODS

(a) Bacterial strains and media

The principal bacterial strains employed in this work are described in Table I. *E. coli* strains were cultured in Penassay Broth (Difco Laboratories, Detroit, MI) or in M9 salts medium supplemented with 1.0% Casamino acids (Difco), 0.3% yeast extract (Difco) and 1% glucose (Davis et al., 1980). LB-broth was used to grow *E. coli* prior to genetic transformation (Davis et al., 1980). Agar was added to a final concentration of 1.5% when solid medium was desired. Streptococci were cultured routinely in Todd Hewitt broth (Difco). Brain Heart Infusion (Difco) containing 1% horse serum was used to grow streptococcal cells to the state of competence. Antibiotic-containing plates always were prepared using Todd Hewitt medium. Antibiotic concentrations employed in media were as follows: Tc, 10 μg/ml; Cm, 25 μg/ml; Em, 10 μg/ml.

TABLE I

Bacterial strains [a]

Organism	Strain No.	Relevant phenotype	Plasmid	Plasmid size	Comments/ reference
Escherichia coli	V818	Thr^- Leu^- Thi^-	pACYC184 (Cm^r Tc^r)	4 kb	Chang and Cohen, 1978
Escherichia coli	V850	Met^- Thi^- Gal^- Nal^r Rif^r $HsdR^-$ $Em^{s,b}$	none		DB11 from Julian Davies
Streptococcus sanguis	V288		none		strain Challis
Streptococcus sanguis	V749		pVA749 (Em^r)	5.2 kb	Macrina et al., 1982

[a] Abbreviations: Phenotypes: Thr^-, requires threonine; Leu^-, requires leucine; Thi^-, requires thiamine; Met^-, requires methionine; Gal^-, galactose non-fermenting; Nal^r, resistance to nalidixic acid (> 50 μg/ml); Rif^r, resistance to rifampicin (>50 μg/ml); $HsdR^-$, host specific restriction deficient.

[b] Carries mutation conferring hypersensitivity to antibiotics, including macrolide lincosamides, fusidic acid and puromycin.

(b) Plasmid DNA isolation and characterization

CCC plasmid DNA was prepared by dye-buoyant density equilibrium centrifugation. The method of Clewell and Helinski (1970), was used for *E. coli* while that described by Macrina et al. (1980c) was used for *S. sanguis*. *E. coli* strains were rapidly screened for plasmid DNA by an adaption of a method obtained from M. Rosenberg (pers. communication). Cells from a 7-ml overnight Penassay broth culture were harvested by centrifugation and suspended in 0.7 ml of a solution containing 15% sucrose, 50 mM EDTA and 1 mg/ml lysozyme. The cell suspension was transferred to a 1.5 ml conical polypropylene centrifuge tube and incubated at 23°C for 10 min. 30 μl of 10% SDS (w/v in water) then was added and the tube inverted gently three times. 50 μl of 5 M potassium acetate was added, mixed by gentle inversion and the tube incubated on ice for 30 min. The tube then was centrifuged at top speed in an eppendorf microfuge (Brinckmann Instruments) for 15 min. About 300 to 500 μl of the clear supernatant was carefully aspirated and transferred to a new 1.5-ml tube. 2 μl of RNase (5 mg/ml, heated for 2 min in a boiling water bath prior to use) was added and the tube incubated for 15 min at 23°C. The RNase-treated supernatant then was extracted once with 0.5 ml of buffer-saturated phenol. The aqueous phase was removed and extracted once with anhydrous ether. 2.5 vols. of ice-cold ethanol then were added to the supernatant, the tube mixed by inversion and centrifuged for 5 min in an eppendorf centrifuge. The supernatant was poured off and the tube was allowed to drain well over a paper towel. 100 μl of water were added to the tube and the DNA pellet was dissolved by agitation on a Vortex mixer for 15 s. 250 μl of ice-cold extranol were added and the tube was incubated at −70°C for at least 30 min. The precipitated DNA was pelleted by centrifugation in an eppendorf centrifuge, the supernatant poured off and the tube allowed to dry under vacuum for 15 min. The DNA pellet was dissolved in 100 μl H_2O; 10 to 30 μl of this preparation were sufficient for visualization of plasmid DNA on agarose gels; such preparations could be cleaved with restriction enzymes, if necessary. Streptococcal cells were screened for plasmid molecules by a previously described method (Macrina et al., 1980b). Plasmid copy number determination was performed according to our previously published methods (Macrina et al., 1980b; c).

Purified plasmid DNA or plasmid restriction digest products were analyzed by vertical agarose gel electrophoresis (Meyers et al., 1976) with appropriate size reference CCC or linear DNA (*Hin*dIII cleaved λ DNA) molecules (Macrina et al., 1978). Restriction endonucleases and T4 DNA ligase were purchased from Bethesda Research Laboratories (Gaithersburg, MD) and enzyme reactions were carried out as previously published (Macrina et al., 1980c). The conditions for DNA ligation and alkaline phosphatase treatment have been described (Macrina et al., 1980c).

(c) Genetic techniques

S. sanguis was genetically transformed according to the procedure of Lawson and Gooder (1970). *E. coli* was transformed by the standard $CaCl_2$-heat shock method (Davis et al., 1980), except that cells were harvested at an absorbance of 0.2 (at 660 nm) rather than 0.5. Plasmid segregation studies were performed according to Macrina et al. (1980a).

RESULTS

(a) Construction of pVA838

Two replicons were chosen to construct our *Escherichia coli-Streptococcus* shuttle plasmid. pACYC184, an *E. coli* plasmid vector was constructed by Chang and Cohen (1978). It is a multicopy chimeric plasmid, 4 kb in size, that bears Tc^r and Cm^r determinants. Its restriction cleavage site map is shown in Fig. 1. A unique *Eco*RI site occurs within its Cm^r gene and unique *Hin*dIII, *Bam*HI, *Nru*I, *Sph*I and *Sal*I sites occur within its Tc^r determinant (see Fig. 1). pVA749 a chimeric streptococcal plasmid 5.2 kb in size, was described by Macrina et al. (1982); its restriction cleavage site map as well as the location of its single selective marker, Em^r, is also shown in Fig. 1. pVA749 and pACYC184 each were cleaved with

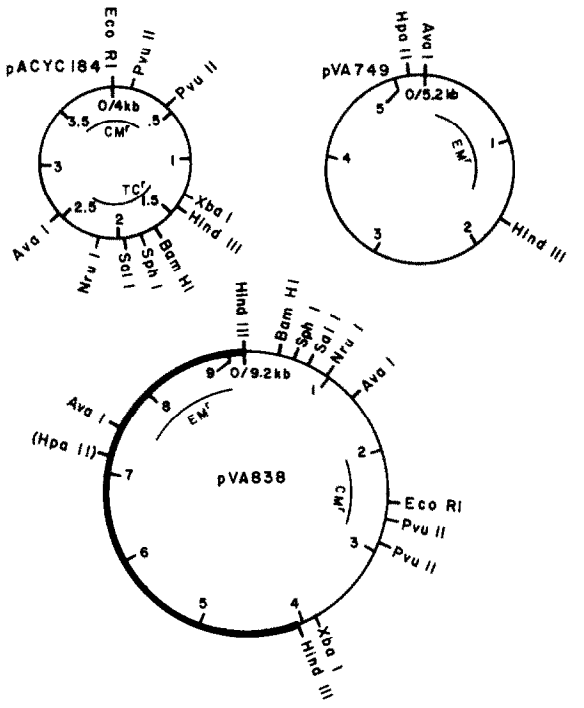

Fig. 1. Plasmid restriction maps. Coordinates (kb) are on the inside of each circular map. The approximate location of the Em^r, Cm^r and Tc^r determinants are indicated. The segments corresponding to pVA749 on the pVA838 chimera are denoted by the thick line. The non-unique *Hpa*II site on pVA838 is shown in parentheses for purposes of physical reference.

TABLE II

Sites for inserting DNA into pVA838

Endonuclease	Recognition site	Comments/reference
*Bam*HI	5'G↓GATCC3'	Chang and Cohen, 1978
*Eco*RI	5'G↓AATTC3'	insertionally inactivates Cm^r (Chang and Cohen, 1978)
*Nru*I	5'TCG↓CGA3'	Schildkraut, I. and Greenough, L.: cited in New England Biolabs catalogue 1982–1983, Beverly, MA, p. 17
*Pvu*II	5'CAG↓CTG3'	insertionally inactivates Cm^r; blunt end replacement of 0.38-kb *Pvu*II fragment
*Sal*I	5'G↓TCGAC3'	Chang and Cohen, 1978
*Sph*I	5'GCATG↓C3'	Fuchs et al., 1980
*Xba*I	5'T↓CTAGA3'	Thomsen and Stinski, 1981

*Hin*dIII, mixed in a ratio of 1:1 and ligated in vitro. Ligation products were used to transform *E. coli* V850 to Cm^r. Several Cm^r transformants were screened for plasmid DNA and found to contain a 9.2-kb plasmid. Restriction enzyme analysis revealed these plasmids to be pVA749::pACYC184 chimeras. One such plasmid, designated pVA838, was characterized by restriction site mapping, and its cleavage map is seen in Fig. 1. Besides Cm^r (>25 μg/ml) pVA838 expressed Em^r in *E. coli* V850. Its efficiency of plating on 10 and 40 μg/ml Em was 100%. The Tc^r marker was inactivated by the insertion of pVA749 into the pACYC184 *Hin*dIII site. Because the *Hin*dIII site of pACYC184 is known to reside in the promoter of the Tc^r determinant (Stüber and Bujard, 1981) we attempted to restore the Tc^r phenotype by reversing the orientation of the pVA749 insert in pVA838. Accordingly, pVA838 was cleaved with *Hin*dIII, self-ligated and used to transform *E. coli* V850, selecting for Tc^r. No Tc^r clones were isolated in several experiments. The hypothesis that mutations could be isolated that would restore Tc^r promoter function in pVA838 was also tested. In this regard, attempts to select Tc^r mutants from *E. coli*[pVA838] were unsuccessful.

Data regarding sites for insertion of passenger DNA into either pACYC184 or pVA749 was compiled from studies done in several laboratories and this information appears in Table II. The *Pvu*II sites (see Fig. 1) of pACYC184 were mapped in our laboratory; one lies within and the other just outside the Cm^r determinant. pACYC184 derivatives missing the 0.38-kb *Pvu*II fragment, were able to replicate normally in *E. coli* but, as expected, were unable to confer Cm^r (unpublished).

(b) Transformation with pVA838

pVA838 isolated from *E. coli* V850 was found to transform readily *S. sanguis*. *S. sanguis* cells carrying pVA838 failed to form colonies on 5 or 10 μg/ml Cm. An inoculum consisting of approx. 5×10^7 cells of *S. sanguis*[pVA838] showed a very

TABLE III

Transformation of *E. coli* and *S. sanguis* [a]

Plasmid DNA	Origin	Recipient	Transformation frequencies [b]	
			Cm^r	Em^r
pACYC184	*E. coli*	*E. coli*	8.2×10^{-5}	–
pVA838	*E. coli*	*E. coli*	8.2×10^{-5}	7.8×10^{-5}
pVA838	*S. sanguis*	*E. coli*	6.0×10^{-5}	9.1×10^{-5}
pVA749	*S. sanguis*	*E. coli*	–	$<10^{-8}$
pACYC184	*E. coli*	*S. sanguis*	$<10^{-8}$	–
pVA838	*E. coli*	*S. sanguis*	$<10^{-8}$	3.2×10^{-5}
pVA838	*S. sanguis*	*S. sanguis*	$<10^{-8}$	4.0×10^{-4}
pVA749	*S. sanguis*	*S. sanguis*	–	1.0×10^{-3}

[a] Competent cells were prepared as described in MATERIALS AND METHODS. In all cases, 0.25 μg of plasmid DNA was added to competent cells. *E. coli* cells were incubated in L-broth for 90 min prior to plating on selective medium. Competent *S. sanguis* cells were exposed to DNA for 30 min, treated with DNase (10 μg/ml) and plated on selective medium.

[b] For *S. sanguis*, frequency was expressed as transformants/recipient at time DNA was added. For *E. coli*, frequency was expressed as transformants/surviving cell following 2 min of 42°C heat shock.

faint lawn of growth following 48 h incubation on 5 μg/ml of Cm. In addition, *S. sanguis*[pVA838] showed about a 10% plating efficiency on 1 μg/ml Cm. *S. sanguis* V288 (devoid of pVA838) failed to display either of these characteristics. The Em^r conferred by pVA838 was fully expressed in *S. sanguis*. *S. sanguis*[pVA838] plated at 100% efficiency on 10 through 200 μg/ml Em. Comparative transformation frequencies using pVA838 isolated from *E. coli* or *S. sanguis* are shown in Table III.

(c) Genetic and physical characterization of pVA838

The genetic stability of pVA838 was examined in *E. coli* V850 and *S. sanguis* V288, following two serial passages in drug-free broth cultures grown at 37°C. In addition, the stabilities of pVA749 and pACYC184 were similarly determined. pVA749 was stable in *S. sanguis* showing a frequency of loss of $<0.1\%$. pACYC184 was lost at a frequency of about 1% from *E. coli* V850. pVA838 was lost at a frequency of about 8–9% from *E. coli* V850 but

TABLE IV

Plasmid copy numbers

Strain	Plasmid	Plasmid copies/chromosomal equivalent [a]			
		Penassay or Todd Hewitt broth [b]		Supplemented M9 broth	
		Unamplified	Amplified [c]	Unamplified	Amplified [c]
E. coli V855 [d]	pACYC184	16±1.9	51±6.0	21±3	330±21
E. coli V854 [d]	pVA838	8±0.9	21±2.0	11±1.0	37± 4.0
S. sanguis V749	pVA749	19±1.1	14±1.1	not done	not done
S. sanguis V839	pVA838	15±1.1	11±1.4	not done	not done

[a] Chromosome sizes are taken as 2.5×10^9 daltons for *E. coli* (Cooper and Helmstetter, 1968) and 1.8×10^9 daltons for *S. sanguis* (Macrina et al., 1977). Numbers represent averages±standard deviation of at least three experiments.

[b] *E. coli* was grown in Penassay broth, *S. sanguis* in Todd Hewitt broth.

[c] Mid log phase cells were treated with either spectinomycin (300 μg/ml for *E. coli*) or chloramphenicol (300 μg/ml for *S. sanguis*) for 8 h prior to preparation of crude cell lysates.

[d] *E. coli* V850 carrying pACYC184 or pVA838.

was stably maintained in *S. sanguis* V288 (frequency of loss $< 0.1\%$).

As can be seen in Table IV, the copy number of pVA838 was depressed relative to pACYC184 in *E. coli*. The pVA838 copy number in *S. sanguis* approached that of pVA749, however. pVA838 could be amplified in *E. coli* by treatment of mid-log grown cells with drugs that blocked protein synthesis. In Penassay broth-grown cells, pACYC184 showed about a 3-fold increase in copy number under standard conditions (8 h of spectinomycin treatment). pVA838 showed about a $2\frac{1}{2}$-fold increase. Neither pVA749 nor pVA838 showed an increase in copy number in *S. sanguis* cells treated with Cm (or spectinomycin, data not shown).

Plasmid amplification properties in *E. coli* could be altered by growth of cells in a supplemented M9-salts broth (Table IV). The unamplified copy numbers of pACYC184 and pVA838 were unremarkable as compared to Penassay broth-grown cells. However, pACYC184 showed an approx. 15-fold increase in copy number in spectinomycin-treated cells. pVA838, on the other hand, showed slightly over a 3-fold increase in copy number in comparably treated *E. coli* cells.

(d) Use of pVA838 as a shuttle cloning vehicle

To directly test the utility of pVA838 we chose to use it to clone fragments of the streptococcal plasmid pPD1. This plasmid is M_r 35×10^6 in size and confers conjugative donor ability and bacteriocin production in *S. faecalis* (Clewell, 1981, and unpublished). In addition, pPD1-containing donor cells respond to the sex pheromone system of *S. faecalis*; i.e., donor cells carrying pPD1 form aggregates when exposed to a small peptide

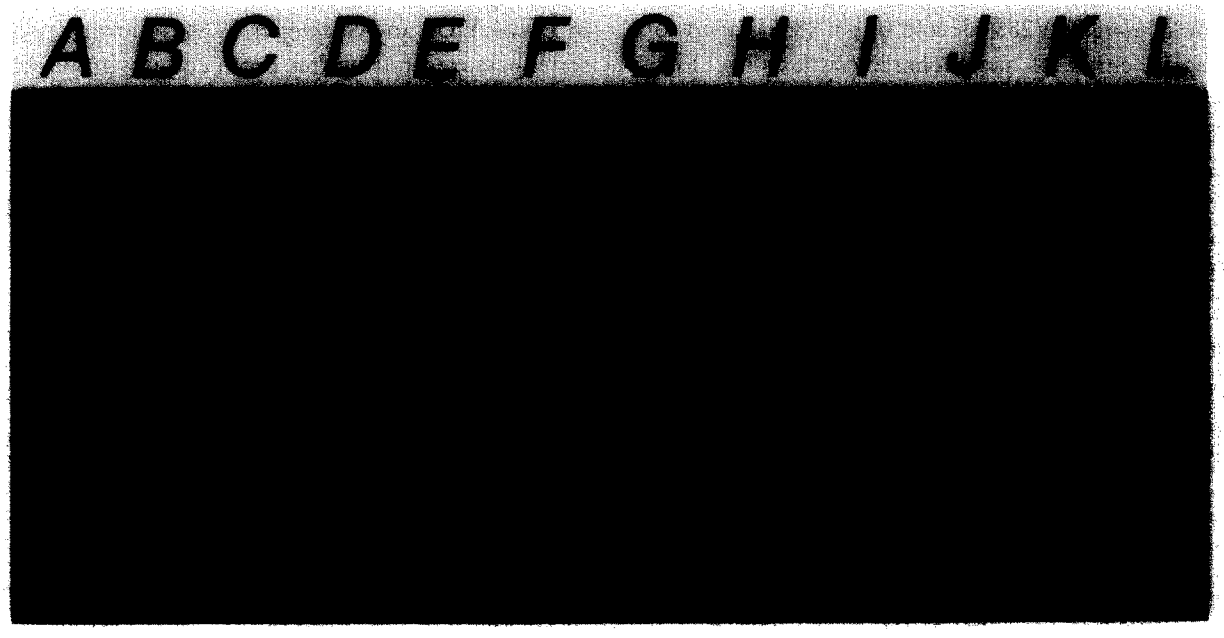

Fig. 2. Analysis of recombinant pPD1:: pVA838 plasmids (see RESULTS, section d). Plasmid DNAs were cleaved with either *Eco*RI (lanes A–E, J–L) or *Ava*I (lanes F–H) and electrophoresed through 0.7% agarose. (A) Linear pVA838 (9.2-kb). (B) *Eco*RI cleavage products of pVA860: 9.2-kb pVA838 and 6.6-kb *Eco*RI-D fragment of pPD1. (C) *Eco*RI cleavage products of pVA861: 9.2-kb pVA838 and 5.1-kb *Eco*RI-E fragment of pPD1 (uppermost band is the partially digested [i.e., linear] pVA861). (D) *Eco*RI cleavage products of pVA862: both fragments [pVA838 and *Eco*RI-C fragment of pPD1] are 9.2–kb. (E) *Eco*RI cleavage products of pPD1. (F–H) *Ava*I cleavage products of pVA860, 861, and 862 respectively. The common fragment corresponds to the *Ava*I-B fragment of pVA838 (kb coordinates 7.5 to 1.3; see Fig. 1). The *Eco*RI-D fragment of pPD1 is known to contain an *Ava*I site (J. Shaw and D. Clewell, unpublished) and this accounts for the three components seen in lane F: (I) Phage λ DNA cleaved with *Hin*dIII; fragment sizes, top to bottom, are 23.7 kb, 9.46 kb, 6.75 kb, 4.26 kb, 2.26 kb and 1.98-kb. (J) pVA838 cleaved with *Eco*RI. (K) pPD1 cleaved with *Eco*RI. (L) pVA876 *Eco*RI cleavage products: 9.2-kb pVA838 and 12.1-kb *Eco*RI-B fragment of pPD1.

[pheromone] synthesized by recipient strains. pPD1 was cleaved into five fragments by *Eco*RI (21.2 kb, 12.1 kb, 9.2 kb, 6.6 kb and 5.1 kb, see Fig. 2). *Eco*RI-cleaved pVA838 (approx. 1.5 μg) that had been treated with alkaline phosphatase was mixed with approx. 0.5 μg of *Eco*RI-cleaved pPD1. Following ligation, this mixture was used to transform *E. coli* V850 to Em^r (Em^r transformants were recovered at a frequency of approx. 10^{-6}/survivor). Greater than 80% of all Em^r transformants, were Cm^s. From these transformants, recombinant plasmids representing pVA838::pPD1 *Eco*RI-C, pVA838::pPD1 *Eco*RI-D, and pVA838::pPD1 *Eco*RI-E chimeras were detected (see Fig. 2, lanes B–D, F–H). In a separate experiment electrophoretically purified pPD1 *Eco*RI-B fragment was ligated to *Eco*RI-cleaved, alkaline phosphatase-treated pVA838. Em^r transformants were recovered at a low frequency (approx. 5×10^{-7}) and several Cm^s clones were analyzed and found to contain pVA838::pPD1 *Eco*RI-B chimeras (Fig. 2, lanes J–L).

The pVA838-derivatives bearing either pPD1 *Eco*RI-B, C, D or E fragments could be readily transformed into *S. sanguis* V288 at frequencies of approx. 10^{-5} per recipient by selecting for Em^r. Such transformants revealed plasmids that were indistinguishable in size from the transforming molecules. *E. coli* and *S. sanguis* clones bearing these chimeric plasmids (i.e., carrying either pPD1 *Eco*RI-B, C, D or E) were tested for ability to elaborate the pPD1 bacteriocin or for their ability to aggregate when exposed to a *S. faecalis* pheromone-producing recipient strain. Neither bacteriocin production nor pheromone-induced aggregation were expressed in *E. coli* or *S. sanguis* host strains containing any of the cloned pPD1 fragments.

DISCUSSION

The *Escherichia-Streptococcus* shuttle plasmid cloning vehicle described in this paper adds an important dimension to the available recombinant DNA systems of the streptococci. Problems associated with the uptake and processing of transforming DNA in the *S. sanguis* host have made the cloning of fragments derived from large DNA molecules (i.e., genomic DNA) difficult. The use of a shuttle plasmid to approach genomic shotgun cloning in the streptococci reduces problems associated with the requirements for the high chimeric monomer concentrations needed to effect two-hit kinetics or for the presence of chimeric oligomers bearing a specific insert. The shuttle plasmid can be used to first construct a streptococcal chromosomal fragment library in *E. coli*. Chimeric plasmids so obtained can then be introduced into *S. sanguis* by transformation. Because any given chimera would represent a homogeneous molecular population, it would transform *S. sanguis* by the normal pathways utilized for a monomeric or oligomeric plasmid species with minimal risk of deletion formation (Saunders and Guild, 1981). Although we have demonstrated the utility of the shuttle plasmid using passenger DNA of streptococcal plasmid origin, we also have successfully employed this system to clone chromosomal fragments from *S. mutans* and *S. faecalis* into *E. coli*. These plasmids were in turn, successfully introduced into *S. sanguis* by genetic transformation (J.A.T. and F.L.M. in preparation).

We have developed our shuttle system around the use of an antibiotic-hyper-sensitive *E. coli* mutant (Table I) so as to be able to use the Em^r marker of pVA749 in this species. Indeed, most commonly used *E. coli* strains can be inhibited by concentrations of 50 to 150 μg/ml Em. In these strains (e.g., *E. coli* C600), the Em^r marker of pVA838 was expressed so as to confer resistance to Em at such drug levels (unpublished). Thus, the pVA838 plasmid may be introduced into and phenotypically identified in most strains of *E. coli*. We have not yet closely examined the problem of pVA838 Cm^r expression in *S. sanguis*. The plating efficiency of *S. sanguis* [pVA838] on low Cm concentrations does suggest that the Cm^r gene is being transcribed and translated. However, this marker cannot be used in any practical way in the *S. sanguis* host at present. Attempts to select moderate or high level Cm^r mutants of *S. sanguis*-[pVA838] have not been fruitful.

pVA838 DNA isolated from either host can be transformed readily into *E. coli* or *S. sanguis* (Table III). Transformation frequencies using *S. sanguis* as a host were consistently higher using *S.*

sanguis-derived vs. *E. coli*-derived pVA838. This may be the result of host-specified restriction; such effects, however, do not significantly impair the functionality of the shuttle system. Studies on plasmid segregation revealed pVA838 to be significantly less stable in *E. coli* (frequency of loss under non-selective conditions, ~ 8-9%) than in *S. sanguis*. The pACYC184 replicon has been reported to be unstable in *E. coli* (Meacock and Cohen, 1980) and the addition of the pVA749 molecule to this replicon appears to have exaggerated this instability for unknown reasons (1% vs. 8-9% loss). In any event, problems with plasmid loss can be effectively and easily dealt with by the constant use of selective pressure (i.e., Em or Cm) on pVA838-harboring cells.

The copy number of pVA838 in *E. coli* V850 is depressed as compared to pACYC184 (Table IV). Both pVA838 and pACYC184 were amplifiable in the absence of protein synthesis (Table IV, columns 2 and 4). These studies (Table IV) revealed the existence of media-specific effects on amplification. Supplemented M9 broth clearly is the medium of choice for amplification of pVA838. The physiologic bases for the superior amplification in supplemented M9 medium remains unclear but somewhat similar observations have been reported for ColE1 (Clewell, 1972) and pBR322 (Norgard et al., 1979) amplification. Consistent with our previous findings for pVA749, the pVA838 shuttle plasmid failed to replicate in the absence of protein synthesis in *S. sanguis*. (Macrina et al., 1982).

Plasmid pVA838 offers a reasonable number of sites for cloning DNA inserts (Table II). Particularly useful are the *Eco*RI and *Pvu*II sites that afford insertional inactivation of the Cm^r determinant. *Pvu*II cleaves to yield blunt-ended molecules thereby allowing one to insert such a molecule formed by any nucleolytic process (blunt-ended-cutting restriction endonucleases, S1, and BAL31 nucleases). Although the *Bam*HI, *Sal*I and *Xba*I cleavage sites of pVA838 do not permit insertional inactivation, molecules cleaved with these enzymes can be treated with alkaline phosphatase to improve recombinant yields. It should be noted that recently Thomsen and Stinski (1981) have cloned fragments as large as 35 kb into the *Xba*I site of pACYC184. Although the largest fragment we have yet to clone into pVA838 was 12.1 kb, this would not seem to be an upper limit.

Plasmid pVA838 is clearly a functional shuttle replicon within the context that it was conceived. However, certain improvements in its design are obvious and desirable. In this connection, we are currently attempting to construct smaller derivatives of pVA838. The restoration of the insertionally inactivatable Tc^r gene would also be desirable. We have recently constructed a chimera consisting of pVA749 and pACYC184 joined at their *Ava*I sites (see Fig. 1). This recombinant plasmid was exceedingly unstable in non-selectively grown *E. coli* cells. We believe this is due to the fact that the pACYC184 *Ava*I site is at or near sequences governing pACYC184 replicative functions (See Meacock and Cohen, 1980). Attempts to obtain stable derivatives of this plasmid are underway. It does, however, express both the Tc^r and Cm^r genes in *E. coli* thus providing two different insertional inactivation strategies (Fig. 1 and Chang and Cohen, 1978).

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