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

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

Francis L. Macrina *, Janet Ash Tobian *, Kevin R. Jones *, R. Paul Evans *, and Don B. Clewell **

* Department of Microbiology and Immunology, Virginia Commonwealth University, Richmond, VA 23298, and ** Departments of Oral Biology, and Microbiology and Immunology, Dental Research Institute, University of Michigan, Schools of Medicine and Dentistry, Ann Arbor, MI 48109 (U.S.A.)

(Received March 15th, 1982)
(Revision received June 13th, 1982)
(Accepted July 15th, 1982)

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' Tc') and pVA749 (Em') replicons. This plasmid, designated pVA838, is 9.2 kb in size and expresses Em' in both *E. coli* and *S. sanguis*. Its Cm' marker is expressed only in *E. coli* and may be inactivated by addition of DNA inserts at its internal EcoRI or PvuII sites. The pVA838 molecule also contains unique *SalI*, *SphI*, *BamHI*, *NruI* and *XbaI* 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
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 \textit{S. sanguis} host system. First, we have adapted the “helper plasmid” method originally devised by Gryczan et al. (1980) for use in the \textit{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 \textit{E. coli} and \textit{S. sanguis}. Using this vector, chromosomal fragments first can be cloned in \textit{E. coli} by standard recombinant DNA methodologies. Such \textit{E. coli}-derived plasmid preparations containing oligomers as well as high monomer concentrations of chimeras would transform \textit{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. \textit{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 \textit{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 \( \mu \)g/ml; Cm, 25 \( \mu \)g/ml; Em, 10 \( \mu \)g/ml.

### TABLE I

**Bacterial strains a**

<table>
<thead>
<tr>
<th>Organism</th>
<th>Strain No.</th>
<th>Relevant phenotype</th>
<th>Plasmid</th>
<th>Plasmid size</th>
<th>Comments/reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{Escherichia coli}</td>
<td>V818</td>
<td>Thr(^{-}) Leu(^{-}) Thi(^{-})</td>
<td>pACYC184 (Cm(^{+})Tc(^{+}))</td>
<td>4 kb</td>
<td>Chang and Cohen, 1978</td>
</tr>
<tr>
<td>\textit{Escherichia coli}</td>
<td>V850</td>
<td>Met(^{-}) Thi(^{-}) Gal(^{-})</td>
<td>none</td>
<td>none</td>
<td>DB81 from Julian Davies</td>
</tr>
<tr>
<td>\textit{Streptococcus sanguis}</td>
<td>V288</td>
<td>none</td>
<td>pVA749 (Em(^{+}))</td>
<td>5.2 kb</td>
<td>strain Challis</td>
</tr>
<tr>
<td>\textit{Streptococcus sanguis}</td>
<td>V749</td>
<td>none</td>
<td>pVA749 (Em(^{+}))</td>
<td>5.2 kb</td>
<td>Macrina et al., 1982</td>
</tr>
</tbody>
</table>

\(^{a}\) Abbreviations: Phenotypes: Thr\(^{-}\), requires threonine; Leu\(^{-}\), requires leucine; Thi\(^{-}\), requires thiamine; Met\(^{-}\), requires methionine; Gal\(^{-}\), galactose non-fermenting; NaI\(^{-}\), resistance to nalidixic acid (> 50 \( \mu \)g/ml); Rif\(^{+}\), resistance to rifampicin (>50 \( \mu \)g/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 H₂O; 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 (HindIII 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 CaCl₂-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 replications 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 multi-copy chimeric plasmid, 4 kb in size, that bears Tc' and Cm' determinants. Its restriction cleavage site map is shown in Fig. 1. A unique EcoRI site occurs within its Cm' gene and unique HindIII, BamHI, NruI, SphI and SalI sites occur within its Tc' 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', is also shown in Fig. 1. pVA749 and pACYC184 each were cleaved with
HindIII, mixed in a ratio of 1:1 and ligated in vitro. Ligation products were used to transform E. coli V850 to Cm'. Several Cm' 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' (> 25 μg/ml) pVA838 expressed Em' in E. coli V850. Its efficiency of plating on 10 and 40 μg/ml Em was 100%. The Tc' marker was inactivated by the insertion of pVA749 into the pACYC184 HindIII site. Because the HindIII site of pACYC184 is known to reside in the promoter of the Tc' determinant (Stüber and Bujard, 1981) we attempted to restore the Tc' phenotype by reversing the orientation of the pVA749 insert in pVA838. Accordingly, pVA838 was cleaved with HindIII, self-ligated and used to transform E. coli V850, selecting for Tc'. No Tc' clones were isolated in several experiments. The hypothesis that mutations could be isolated that would restore Tc' promoter function in pVA838 was also tested. In this regard, attempts to select Tc' 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 PvuII sites (see Fig. 1) of pACYC184 were mapped in our laboratory; one lies within and the other just outside the Cm' determinant. pACYC184 derivatives missing the 0.38-kb PvuII fragment, were able to replicate normally in E. coli but, as expected, were unable to confer Cm' (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

<table>
<thead>
<tr>
<th>Plasmid DNA</th>
<th>Origin</th>
<th>Recipient</th>
<th>Transformation frequencies b</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Cm'</td>
</tr>
<tr>
<td>pACYC184</td>
<td>E. coli</td>
<td>E. coli</td>
<td>$8.2 \times 10^{-5}$</td>
</tr>
<tr>
<td>pVA838</td>
<td>E. coli</td>
<td>E. coli</td>
<td>$8.2 \times 10^{-5}$</td>
</tr>
<tr>
<td>pVA838</td>
<td>S. sanguis</td>
<td>E. coli</td>
<td>$6.0 \times 10^{-5}$</td>
</tr>
<tr>
<td>pVA749</td>
<td>S. sanguis</td>
<td>E. coli</td>
<td>-</td>
</tr>
<tr>
<td>pACYC184</td>
<td>E. coli</td>
<td>S. sanguis</td>
<td>$&lt;10^{-8}$</td>
</tr>
<tr>
<td>pVA838</td>
<td>E. coli</td>
<td>S. sanguis</td>
<td>$&lt;10^{-8}$</td>
</tr>
<tr>
<td>pVA838</td>
<td>S. sanguis</td>
<td>S. sanguis</td>
<td>$&lt;10^{-8}$</td>
</tr>
<tr>
<td>pVA749</td>
<td>S. sanguis</td>
<td>S. sanguis</td>
<td>-</td>
</tr>
</tbody>
</table>

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.

A 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' 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.

TABLE IV
Plasmid copy numbers

<table>
<thead>
<tr>
<th>Strain</th>
<th>Plasmid</th>
<th>Penassay or Todd Hewitt broth b</th>
<th>Supplemented M9 broth</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Unamplified</td>
<td>Amplified c</td>
</tr>
<tr>
<td>E. coli V850</td>
<td>pACYC184</td>
<td>16±1.9</td>
<td>51±6.0</td>
</tr>
<tr>
<td>E. coli V854</td>
<td>pVA838</td>
<td>8±0.9</td>
<td>21±2.0</td>
</tr>
<tr>
<td>S. sanguis V749</td>
<td>pVA749</td>
<td>19±1.1</td>
<td>14±1.1</td>
</tr>
<tr>
<td>S. sanguis V839</td>
<td>pVA838</td>
<td>15±1.1</td>
<td>11±1.4</td>
</tr>
</tbody>
</table>

* Chromosome sizes are taken as 2.5X10^9 daltons for E. coli (Cooper and Helmstetter, 1968) and 1.8X10^9 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 spectomycin (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 2.5-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 $M_r \approx 35 \times 10^6$ 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.
[pheromone] synthesized by recipient strains. pPD1 was cleaved into five fragments by EcoRI (21.2 kb, 12.1 kb, 9.2 kb, 6.6 kb and 5.1 kb, see Fig. 2). EcoRI-cleaved pVA838 (approx. 1.5 μg) that had been treated with alkaline phosphatase was mixed with approx. 0.5 μg of EcoRI-cleaved pPD1. Following ligation, this mixture was used to transform *E. coli* V850 to Em'. Em' transformants were recovered at a frequency of approx. $10^{-6}/$survivor. Greater than 80% of all Em' transformants, were Cm'. From these transformants, recombinant plasmids representing pVA838::pPD1 EcoRI-C, pVA838::pPD1 EcoRI-D, and pVA838::pPD1 EcoRI-E chimeras were detected (see Fig. 2, lanes B–D, F–H). In a separate experiment electro- phoretically purified pPD1 EcoRI-B fragment was ligated to EcoRI-cleaved, alkaline phosphatase-treated pVA838. Em' transformants were recovered at a low frequency (approx. $5 \times 10^{-7}$) and several Cm' clones were analyzed and found to contain pVA838::pPD1 EcoRI-B chimeras (Fig. 2, lanes J–L).

The pVA838-derivatives bearing either pPD1 EcoRI-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'. 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 EcoRI-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' 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' 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' expression in *S. sanguis*. The plating efficiency of *S. sanguis* [pVA838] on low Cm concentrations does suggest that the Cm' 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' 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 *EcoRI* and *PvuII* sites that afford insertional inactivation of the Cm' determinant. *PvuII* 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 *BamHI*, *SalI* and *XbaI* 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 *XbaI* 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' gene would also be desirable. We have recently constructed a chimera consisting of pVA749 and pACYC184 joined at their *AvaI* 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 *AvaI* 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' and Cm' genes in *E. coli* thus providing two different insertional inactivation strategies (Fig. 1 and Chang and Cohen, 1978).

ACKNOWLEDGEMENTS

This work was supported by USPHS Grants DE04224 to F.L.M. and DE02731 and AI10318 to D.B.C. and by the Consumer Products Group of the Warner-Lambert Company. F.L.M. is the recipient of Research Career Development Award DE00081 from the National Institute for Dental Research. The helpful suggestions of Dr. D. Behnke are gratefully acknowledged. We are indebted to Jay Shaw who kindly provided his unpublished information on the restriction cleavage analysis of pPD1 and to Dr. Julian Davies for supplying us with *E. coli* DB 11.

REFERENCES


Chang, A.C.Y. and Cohen, S.N.: Construction and characterization of amplifiable multicopy DNA cloning vehicles de-


Communicated by F.E. Young.