Gene. 25 (1983) 145–150
Elsevier

GENE 863

Short Communications

Novel shuttle plasmid vehicles for *Escherichia-Streptococcus* transgeneric cloning

(Recombinant DNA; *E. coli* and *S. sanguis* vectors; pACYC184; pVA856; pVA891)

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

*Department of Microbiology and Immunology, Virginia Commonwealth University, Richmond, VA 23298 (U.S.A.) Tel. (804) 786-9745 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.) Tel. (313) 763–0117.*

(Received April 28th, 1983)
(Revision received June 1st, 1983)
(Accepted June 15th, 1983)

SUMMARY

A novel plasmid vector that is able to replicate both in *Escherichia coli* and in *Streptococcus sanguis* is described. This 9.2-kb plasmid, designated pVA856, carries Cm^r, Tc^r and Em^r determinants that are expressed in *E. coli*. Only the Em^r determinant is expressed in *S. sanguis*. Both the Cm^r and the Tc^r of pVA856 may be insertionally inactivated. This plasmid affords several different cleavage-ligation strategies for cloning in *E. coli* followed by subsequent introduction of chimeras into *S. sanguis*. In addition, we have modified a previously described *E. coli-S. sanguis* shuttle plasmid [pVA838; Macrina et al., Gene 19 (1982) 345–353], so that it is unable to replicate in *S. sanguis*. The utility of such a plasmid for cloning and selecting sequences enabling autonomous replication in *S. sanguis* is demonstrated.

INTRODUCTION

The principal strategies for implementing molecular cloning in naturally transformable streptococcal hosts have been recently reviewed (Macrina et al., 1982a,b). Using suitable vectors (Macrina, 1980a), plasmid-derived sequences may be readily cloned by standard methods. However, the cloning of host chromosomal fragments has been problematic owing to the requirements placed on plasmids entering genetically competent, transformable streptococci. Because plasmid transformation requires two-hit kinetics with monomers, or the entrance of oligomeric forms, the probability of generating specific transformants from a ligation mixture of chromosomal fragments and vector is extremely low (Macrina et al., 1981; 1982a,b; Saunders and Guild, 1981). The net effect of such cloning has been to yield

* To whom correspondence and reprint requests should be sent at Box 678, MCV Station, Richmond, VA 23298 (U.S.A.).

Abbreviations: Cm^r, chloramphenicol resistance; Cm^S, chloramphenicol sensitivity; Em^r, erythromycin resistance; Em^s, erythromycin sensitivity; kb, kilobase pairs; Tc^r, tetracycline resistance; Tc^s, tetracycline sensitivity; ::, novel joint, [ ], indicates plasmid-carrier state.

recombinant molecules of limited value due to the presence of extensive deletions.

One strategy that has been employed successfully to circumvent this problem is to use a marker-rescue system called "helper-plasmid cloning" by Gryczan et al. (1980). We have employed this method to clone a chromosomal tetracycline-resistance determinant from *S. mutans* in *S. sanguis* (Tobian and Macrina, 1982). Behnke (1982) has also described a similar streptococcal marker rescue system. As an alternative to "helper-plasmid cloning", the construction and use of an *Escherichia-Streptococcus* shuttle plasmid vector recently has been reported (Macrina et al., 1982c).

In this communication, we describe the construction of a new, useful shuttle vector that affords a variety of cloning strategies, including two modes of insertional inactivation. In addition, a novel vector that allows the cloning of streptococcal replication origins has been derived from the previously reported shuttle vector, pVA838 (Macrina et al., 1982c).

EXPERIMENTAL

Two bacterial strains were used in this work. *E. coli* DB11, kindly supplied by Dr. Julian Davies, has been described previously (Macrina et al., 1982c). Its principal features are hypersensitivity to antibiotics (including macrolides, fusidic acid and puromycin) and restriction deficiency. *S. sanguis* V288 is our laboratory designation for the Challis strain, which is naturally transformable. Media, drug concentrations, plasmid isolation, restriction endonuclease site mapping, copy number determination, plasmid segregation studies, cloning methods and transformation protocols were as previously published (Macrina et al., 1982c). Streptococcal transformation frequency was expressed as transformants per μg DNA per viable recipient at the time of DNA addition.

The previously reported *Escherichia-Streptococcus* shuttle vehicle pVA838, was constructed by the in vitro ligation of *Hin*dIII-cleaved pACYC184 (Chang and Cohen, 1978) and *Hin*dIII-cleaved pVA749 (Macrina et al., 1982a). pVA838 is 9.2 kb in size and confers Em^r and Cm^r in *E. coli*, while only the Em^r determinant is expressed in *S. sanguis*. As a cloning vehicle in *E. coli*, the Cm^r determinant can be inactivated by the insertion of fragments into its *Eco*RI or *Pvu*II sites. Five additional enzyme sites (*Bam*HI, *Sph*I, *Sal*I, *Nru*I, *Xba*I) are also available for cloning into pVA838 but these do not inactivate either drug resistance determinant.

We since have constructed a novel shuttle replicon, designated pVA856, consisting of pACYC184 and pVA749 linked together at their

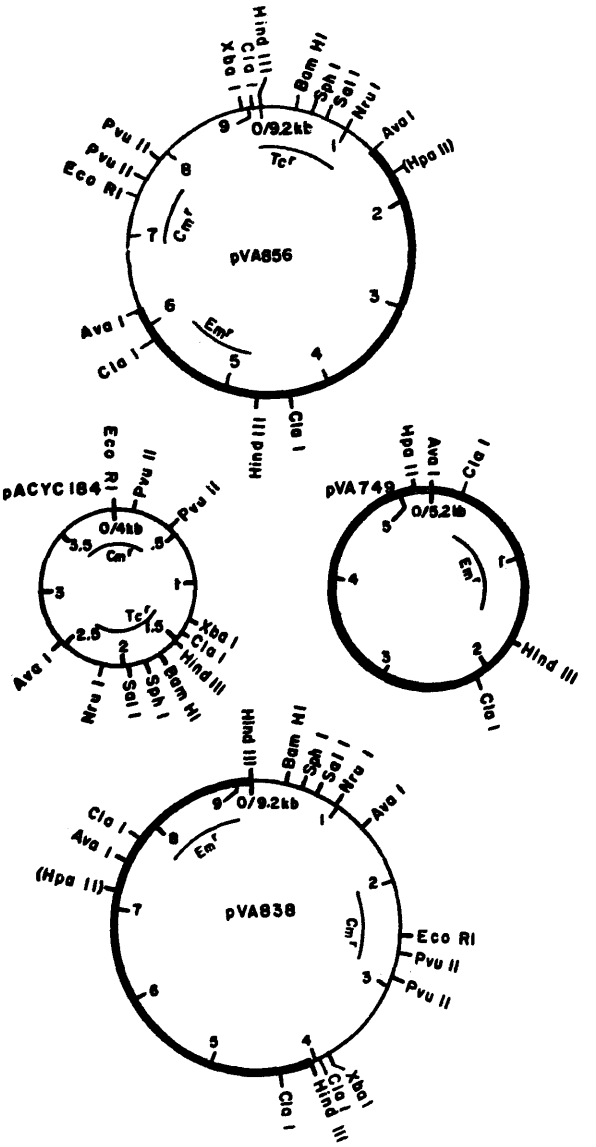

Fig. 1. Plasmid restriction maps. Coordinates (kb) are on the inside of each circular map. The approximate location of the Em^r, Cm^r and Tc^r determinants are indicated. The segments of pVA838 and pVA856 corresponding to pVA749 and pACYC184 are indicated by thick and thin lines, respectively. Plasmids pVA856 and pVA838 may be propagated in either *E. coli* or *S. sanguis*. Plasmid pACYC184 replicates in *E. coli* but not in *S. sanguis*. Plasmid pVA749 replicates in *S. sanguis* but not in *E. coli*.

unique *Ava*I sites. A restriction endonuclease site map of pVA856 and each of its component replicons is shown in Fig. 1. For comparative purposes a map of pVA838 is also illustrated. In contrast to pVA838, all of the resistance determinants of the parental replicons are preserved in pVA856. Hence, passenger DNA may be cloned into pVA856 at the *Bam*HI, *Sph*I, *Sal*I or *Nru*I sites (see Macrina et al., 1982c) and inserts monitored via the inactivation of Tc^r. In addition, inserts into pVA856, employing the *Eco*RI or *Pvu*II (fragment replacement) sites, can be detected by Cm^r inactivation.

Although the pVA856 shuttle vector provides more versatility in terms of insertion possibilities, it does present a disadvantage as compared to pVA838. Continued passage of *E. coli*[pVA856] under nonselective conditions resulted in the extensive loss of all plasmid markers indicating plasmid segregation (Table I). This was verified by the examination of numerous cell lysates prepared from drug-sensitive segregants (not shown). Some instability of pVA856 also was noted in *S. sanguis* grown nonselectively (Table I). In contrast, pVA838 was relatively stably inherited in nonselectively grown cells.

The instability of pVA856 in *E. coli* may be due to the fact that some pACYC184 replication machinery is near the *Ava*I site (see Meacock and Cohen, 1980). Its instability in *S. sanguis* remains unexplained because we have demonstrated that the *Ava*I site on pVA749 lies in a dispensible area (Macrina et al., 1982a). In any event, growth of pVA856-containing cells in the presence of one or more of the appropriate antibiotics eliminates the problems associated with plasmid segregation. pVA856, like pVA838, may be amplified, albeit modestly in *E. coli* but not in *S. sanguis* (Table I; see Macrina et al., 1982c).

Plasmid pVA856 is being successfully used by us and others to clone both plasmid and chromosomal sequences of streptococcal origin in *E. coli*. Once constructed, these molecules may be transformationally introduced into *S. sanguis* as purified monomeric and/or oligomeric chimeras, greatly reducing the risk of deletion formation of cloned sequences (see Macrina et al., 1982b).

The restriction endonuclease cleavage map of pVA838 (see Fig. 1) enabled us to predict that a derivative of this plasmid missing the *Cla*I-B (approx. 4.3 to 7.8 kb) and *Cla*I-C (approx. 4.0 to

TABLE I

Copy number and plasmid stability

Plasmid	Host	Plasmid copies/ Chromosome equivalent [a]		Frequency of plasmid marker loss [c]	
		Unamplified	Amplified [b]	at 37°C (%)	at 42°C (%)
pVA856	*E. coli*	13	33	98	N.D.
pVA856	*S. sanguis*	14	N.D. [d]	22	N.D.
pVA838	*E. coli*	12	35	9	N.D.
pVA838	*S. sanguis*	13	N.D.	< 1	N.D.
pVA891	*E. coli*	15	36	19	N.D.
pVA1	*S. sanguis*	12	N.D.	6	85
pVA961	*S. sanguis*	12	N.D.	18	74

[a] Chromosome sizes were taken as 2.5×10^9 Dal for *E. coli* (Cooper and Helmstetter, 1968) and 1.8×10^9 Dal for *S. sanguis* (Macrina et al., 1977). Results are presented as the average of at least three experiments. Plasmid copy number was determined by measuring and comparing radiolabelled plasmid and chromosomal DNA recovered from agarose gels, exactly as described in Macrina et al. (1980b).

[b] Mid-log phase cells were treated with 300 μg/ml spectinomycin for 6 h prior to preparation of crude cell lysates. The relatively modest amplification (2–3-fold) of the shuttle vectors compared to pACYC184 (15-fold; Macrina et al., 1982c) remains unexplained.

[c] Plasmid marker segregation was performed as described by Macrina et al. (1980a). Several colonies, picked from solid selective medium, were grown overnight at 37°C in drug-free broth. A sample of this culture was diluted 1:10000 in fresh, drug-free broth and grown to stationary phase at 37°C. Cells from this culture then were grown as single colonies on solid, drug-free medium and scored for resistance phenotypes by replica plating. Results are presented as the average of several experiments.

[d] Not done.

4.3 kb) fragments would replicate in *E. coli* and still retain Cm^r and Em^r. Accordingly, about 0.3 μg of pVA838 was cleaved with *Cla*I, ligated and transformed into *E. coli* DB11 selecting for Cm^r and Em^r. Transformants were recovered and the majority of those examined contained a 5.4-kb plasmid. The Cm^r and Em^r levels in these transformants were indistinguishable from *E. coli*[pVA838] (growth on 25 μg/ml and 10 μg/ml of drug, respectively).

One such transformant was selected for further study. Its plasmid, designated pVA891, was purified and a restriction endonuclease cleavage map was determined (Fig. 2). Comparison with Fig. 1 confirms that pVA891 represents a self-ligated *Cla*I-A fragment of pVA838. Copy number determinations and amplification properties of pVA891 were similar to pVA838, however, the former plasmid was somewhat less stable in *E. coli* as compared to its parental replicon (Table I).

The 1.5-kb streptococcal sequence left on pVA891 (shown as the thick line in Fig. 2) contains the Em^r determinant. None of the streptococcal plasmid replication machinery remains (see Macrina et al., 1982a). Consistent with this observation, pVA891 failed to yield Em^r transformants when it was added to genetically competent *S. sanguis* cells. None of the unique cleavage sites on pVA891 (Fig. 2) occur within the Em^r gene; hence DNA fragments that sustain autonomous replication in *S. sanguis* can be cloned into this plasmid using any of the ten restriction sites shown in Fig. 2.

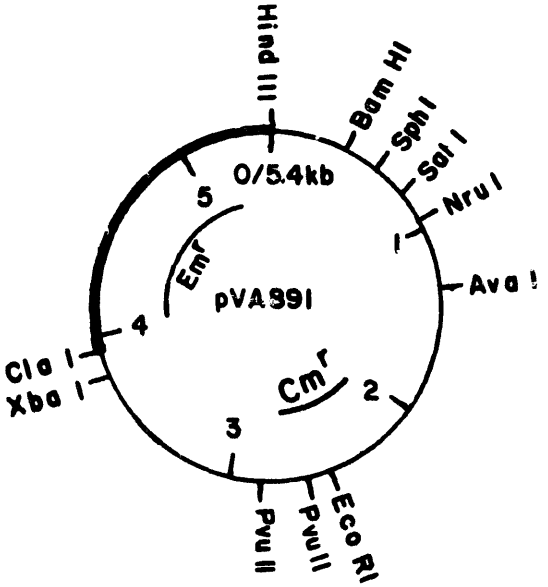

Fig. 2. Restriction map of pVA891. This plasmid was derived by the in vitro removal of the *Cla*I-B and *Cla*I-C fragments of pVA838 (see Fig. 1).

To test the utility of pVA891 we mixed (1:1) and ligated *Eco*RI-cleaved pVA1 with *Eco*RI-cleaved pVA891 (approx. 0.3 μg of each plasmid). pVA1 (a deletion derivative of the conjugative pAMβ1 plasmid) is a 10.4-kb streptococcal plasmid bearing an Em^r gene (Macrina et al., 1980b). Its replication in *S. sanguis* is thermosensitive. It is cleaved by *Eco*RI at two sites yielding 5.3- and 5.1-kb fragments (A and B, respectively). The ligation mixture was used to transform *S. sanguis* with selection for Em^r. Transformants were recovered at a frequency of about 9×10^{-5} and about one-third of the transformants contained a plasmid that was slightly larger in size than pVA1. One such representative plasmid (designated pVA961) was examined by a number of

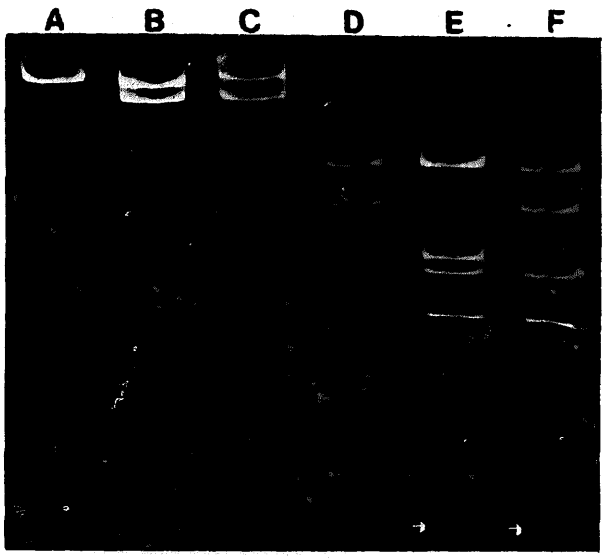

Fig. 3. Restriction analysis of vector and recombinant plasmids. Fragment sizes were determined by comparison with the migration of *Hin*dIII-cleaved phage λ DNA. Lanes: A, pVA891 (see Fig. 1) cleaved with *Eco*RI; B, pVA1 (Macrina et al., 1980b) cleaved with *Eco*RI showing fragments of 5.3 (A fragment) and 5.1 kb (B fragment); C, pVA961 cleaved with *Eco*RI showing fragments corresponding to the 5.4-kb pVA891 component (upper component) and the 5.1-kb *Eco*RI-B pVA1 component; D, pVA891 cleaved with *Eco*RI and *Hin*dIII; E, pVA1 cleaved with *Eco*RI and *Hin*dIII. The smallest *Hin*dIII-*Eco*RI fragment is barely visible and its position has been indicated with an arrow; F, pVA961 cleaved with *Eco*RI and *Hin*dIII. The upper two components correspond to the *Hin*dIII-*Eco*RI fragments of pVA891 (see lane D) while the lower four components correspond to four of the fragments liberated by *Hin*dIII and *Eco*RI digestion of pVA1 (see lane E). These four fragments are known to comprise the *Eco*RI-B fragment of pVA1 (see Macrina et al., 1980b).

criteria and found to be a pVA1-*Eco*RI-B::pVA891 recombinant. Restriction endonuclease analysis of pVA961 revealed two *Eco*RI fragments (Fig. 3, lane C). The larger fragment corresponded to *Eco*RI-linearized pVA891 (Fig. 3, lane A) and the smaller one to pVA1-*Eco*RI-B (Fig. 3, lane B). *Eco*RI + *Hin*dIII double digestion of pVA961 yielded six fragments. (Fig. 3, lane F). The four smallest fragments corresponded to the *Eco*RI-*Hin*dIII and *Hin*dIII fragments of pVA1 (Fig. 3, lane E) that are known to comprise the *Eco*RI-B fragment of pVA1 (see pVA1 map in Macrina et al., 1980b). The two largest *Eco*RI-*Hin*dIII fragments of pVA961 corresponded to the pVA891 components (Fig. 3, lane D). The largest *Eco*RI-*Hin*dIII fragment of pVA961 co-migrated with the largest *Eco*RI-*Hin*dIII of pVA1 (Fig. 3, lane E). However, the pVA961 fragment contained unique *Pvu*II and *Cla*I sites that establish its origin as pVA891 (not shown). Two lines of genetic evidence support our conclusions regarding the structure of pVA961. First, this plasmid displayed thermosensitive replication in *S. sanguis* (Table I, lines 6 and 7, columns 5 and 6) consistent with the pVA1-phenotype. Second, pVA961 could be transformed successfully into *E. coli* V850, where it replicated stably and expressed Em^r and Cm^s. These results were consistent with pVA961 being an insertionally inactivated pVA891 derivative. We conclude that the *Eco*RI-B fragment of pVA1 contains an origin of replication. This observation also has been made by D. LeBlanc and L. Lee (personal communication).

DISCUSSION

In addition to affording a tool to isolate and study the nature of streptococcal plasmid replicators, pVA891 should be employable as a vehicle for mapping cloned *S. sanguis* genes, using the methodology developed by Haldenwang et al. (1980) in *Bacillus subtilis*. Specifically, streptococcal genes cloned into pVA891 in *E. coli*, which might be identified phenotypically or immunologically, could be introduced into *S. sanguis*, where the recombinant plasmid would be expected to integrate into the chromosome via a homologous pairing and a Campbell-like recombination event. The net result would be the genomic incorporation of the pVA891 replicon in toto, which would be monitored by stable Em^r. This directly selectable gene would now be linked to the gene of interest, which would have been duplicated following the integration event (see Haldenwang et al., 1980). This would afford several genetic options, including linkage analysis and gene inactivation (if the cloned sequence was defective).

ACKNOWLEDGEMENTS

This work was supported by USPHS Grants DE04224 to F.L.M. and DE02731 and AI10328 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. We are indebted to Dr. Julian Davies for supplying us with *E. coli* DB11.

REFERENCES

Behnke, D.: Marker rescue allows direct selection for recombinant plasmids in streptococci. Mol. Gen. Genet. 188 (1982) 161–163.

Chang, A.C.Y. and Cohen, S.N.: Construction and characterization of amplifiable multicopy DNA cloning vehicles derived from the P15A cryptic miniplasmid. J. Bacteriol. 134 (1978) 1141–1156.

Cooper, S. and Helmstetter, C.: Chromosome replication and the division cycle of *E. coli* B/r. J. Mol. Biol. 31 (1968) 519–540.

Gryczan, T., Contente, S. and Dubnau, D.: Molecular cloning of heterologous chromosomal DNA by recombination between a plasmid vector and a homologous resident plasmid in *Bacillus subtilis*. Mol. Gen. Genet. 177 (1980) 459–467.

Haldenwang, W.G., Banner, C.D., Ollington, J.F., Losick, R., Hoch, J.H., O'Connor, M.B. and Sonenshein, A.L.: Mapping a cloned gene under sporulation control by insertion of a drug resistance marker into the *Bacillus subtilis* chromosome. J. Bacteriol. 142 (1980) 90–98.

Macrina, F.L., Reider, J.L., Virgili, S.S. and Kopecko, D.J.: Survey of the extrachromosomal gene pool of *Streptococcus mutans*. Infect. Immun. 17 (1977) 215–226.

Macrina, F.L., Keeler, C.L., Jones, K.R. and Wood, P.H.: Molecular characterization of unique deletion mutants of the streptococcal plasmid pAMβ1. Plasmid 4 (1980a) 8–16.

Macrina, F.L., Jones, K.R. and Wood, P.H.: Chimeric streptococcal plasmids and their use as molecular cloning vehicles in *Streptococcus sanguis* (Challis). J. Bacteriol. 143 (1980b) 1425–1435.

Macrina, F.L., Jones, K.R. and Welch, R.A.: Transformation of *Streptococcus sanguis* with monomeric pVA736 plasmid deoxyribonucleic acid. J. Bacteriol. 146 (1981) 826–830.

Macrina, F.L., Tobian, J.A., Jones, K.R. and Evans, R.P.: Molecular cloning in the streptococci, in Hollaender, A., DeMoss, R., Kaplan, S., Konisky, J., Savage, D. and Wolfe, R. (Eds.), Genetic Engineering of Micro-organisms for Chemicals, Plenum, New York, 1982a, pp. 195–210.

Macrina, F.L., Tobian, J.A., Evans, R.P. and Jones, K.R.: Molecular cloning strategies for the *S. sanguis* host-vector system, in D. Schlessinger (Ed.), Microbiology–1982. American Society for Microbiology, Washington, DC, 1982b, pp. 234–238.

Macrina, F.L., Tobian, J.A., Jones, K.R., Evans, R.P. and Clewell, D.B.: A cloning vector able to replicate in *Escherichia coli* and *Streptococcus sanguis*. Gene 19 (1982c) 345–353.

Meacock, P.A. and Cohen, S.N.: Partitioning of bacterial plasmids during cell division: a cis-acting locus that accomplishes stable plasmid inheritance. Cell 20 (1980) 529–542.

Saunders, C.W. and Guild, W.: Pathway of plasmid transformation in Pneumococcus: open circular and linear molecules are active. J. Bacteriol. 146 (1981) 517–526.

Tobian, J.A. and Macrina, F.L.: Helper plasmid cloning in *Streptococcus sanguis*: cloning of a tetracycline resistance determinant from the *Streptococcus mutans* chromosome. J. Bacteriol. 152 (1982) 215–222.

Communicated by S.R. Kushner.