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Characterization of the *Streptococcus mutans* Plasmid pVA318 Cloned into *Escherichia coli*

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We further characterized the cryptic plasmid pVA318 of *Streptococcus mutans*. It had a contour length of 5.64 ± 0.26 kilobases and a guanine-plus-cytosine content of 32 to 34 mol %. Upon cloning the pVA318 plasmid into the vector pBR322 in *Escherichia coli*, we made the following observations. The expression of tetracycline resistance by *Hin*dIII-cloned chimeras, where the insert was in the tetracycline resistance promoter, depended on the orientation of the pVA318 insert. Both *Hin*dIII-cloned chimeras segregated from *polA*(Ts) cells at a nonpermissive temperature. Chimeric molecules cloned with *Pst*I initially showed much instability; the reason for this is unknown, although stable variants were obtained. Both *Hin*dIII-cloned variants and a *Pst*I-cloned chimera produced a pVA318-specific protein of approximately 20,000 molecular weight in *E. coli* minicells. The biological function of this protein is not known; it had no bacteriocin activity against *S. mutans* or group A *Streptococcus* indicator strains, and it did not appear in the *E. coli* periplasm. We constructed a map of pVA318 for restriction endonucleases *Hin*dIII, *Hpa*I, *Pst*I, and *Hae*III. A previously reported *Bam*HI site in pVA318 did not appear in the pVA318 portion of any of our chimeric clones.

A small plasmid, first demonstrated by Dunny et al. (9), is infrequently present in *Streptococcus mutans* serotype *c* and *e* strains, as demonstrated by its presence in 4 of 86 *S. mutans* strains surveyed by Macrina et al. (23, 24). The 3.6-megadalton plasmids in those four independent isolates were identical in size and in restriction digest patterns for five restriction endonucleases; thus, those four isolates were considered to contain an identical, disseminated plasmid (24). The mode of its dissemination or any selective advantage that it might confer to strains harboring it is unknown. No phenotype associated with this plasmid has been identified (23).

There have been subsequent reports of other plasmids besides this cryptic prototype; a plasmid-containing strain of *S. mutans* (no serotype indicated) was reported by LeBlanc and Lee (19), and plasmids were found in *S. mutans* subsp. *ferus* isolates (25). But generally the cryptic prototype plasmid appears to be the predominant plasmid in *S. mutans*, especially from human isolates. In screening *S. mutans* from pedodontic patients with rampant caries, we have found the cryptic plasmid twice (J. Hansen, Y. Abiko, and P. Caufield, unpublished data).

Work in this laboratory to examine the biochemical and genetic bases for *S. mutans* odontopathogenesis has involved cloning of *S. mutans* genes into *Escherichia coli* (J. P. Robeson and R. Curtiss III, Abstr. Annu. Meet. Am. Soc. Microbiol. 1980, D53, p. 46; J. C. Hsu, M. Smorawinska, Y. Abiko, and R. Curtiss III, Abstr. Annu. Meet. Am. Soc. Microbiol. 1980, D54, p. 47; Y. Abiko and M. Smorawinska, Abstr. Annu. Meet. Am. Soc. Microbiol. 1980, D55, p. 47; Y. Abiko, M. Smorawinska, J. C. Hsu, J. P. Robeson, J. B. Hansen, R. G. Holt, and R. Curtiss III, J. Dental Res. **59**(special issue B):949, 1980).

We used this approach to further the description of a prototype, pVA318, of the small cryptic *S. mutans* plasmid (24) in terms of both physical characterization and gene products in *E. coli*. A preliminary report of part of this work has appeared (J. B. Hansen and Y. Abiko, Abstr. Annu. Meet. Am. Soc. Microbiol. 1980, H47, p. 116).

MATERIALS AND METHODS

Bacterial strains and growth media. Bacterial strains used are listed in Table 1. *S. mutans* was grown in brain heart infusion (BHI; Difco Laboratories) at 37°C or on BHI agar (Difco) plates anaerobically under 4 to 10% CO_2 (GasPak, BBL Microbiology Systems) at 37°C.

Liquid minimal media (ML [6]) for growth of *E. coli* strains contained 0.5% glucose and the following supplements (per milliliter) as needed: 20 μg of L-isoleucine, 40 μg of DL-threonine, 40 μg of thymine, 40

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TABLE 1. *Bacterial strains*

Strain	Description	Source or reference
E. coli		
χ1849	F⁻ *tonA53 dapD8 minA1 purE41 supE42* Δ(*gal-uvrB*)*47* λ⁻ *minB2 his-53 nalA25 metC65 oms-1* T3ʳ Δ(*bioH-asd*)*29 ilv-277 cycB2 cycA1 hsdR2*	This laboratory
χ1891	F⁻ *thr-16 tsx-63 purE41 supE42* λ⁻ Δ*trpE63 his-53 nalA23 srl-2* Δ*thyA57* T3ʳ *mtlA9 polA12*(Ts) *cycB2 cycA1*	This laboratory
χ1953	F⁻ *tonA53 dapD8 lacY1 supE44* Δ(*gal-uvrB*) *47* λ⁻ *nalA29* Δ*thyA57 hsdS3*	This laboratory
CSR603	F⁻ *thr-1 leuB6 proA2 phr-1 recA1 argE3 thi-1 uvrA6 ara-14 lacY1 galK2 xyl-5 mtl-1 rpsL31 tsx-33* λ⁻ *supE44*	*E. coli* Genetic Stock Center[a]
S. mutans		
V318	Biotype c, contains plasmid pVA318	F. Macrina[b]; 25
HS6	Serotype *a*	T. Shiota[c]
BHT	Serotype *b*	T. Shiota
JC-1	Serotype *c*	T. Shiota
PS-14	Serotype *c*	T. Shiota
Ingbritt	Serotype *c*	T. Shiota
OMZ176	Serotype *d*	T. Shiota
LM7	Serotype *e*	T. Shiota; 9
OMZ175	Serotype *f*	T. Shiota
6715	Serotype *g*	T. Shiota
AHT	Serotype *g*	T. Shiota
S. sanguis, Challis (pDB201)	Contains plasmid pDB201	D. Behnke[d]; 2
Group A *Streptococcus*, UAB405	Clinical isolate	Department of Pediatrics[c]

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μg of adenine, 50 ng of biotin, 2 μg of thiamine, and 50 μg of DL-*meso*-diaminopimelic acid (DAP). To grow strains for minicell harvests, 0.5% Casamino Acids (Difco) replaced individual amino acids except DAP.

Complex medium for *E. coli* growth was LB (20) supplemented with DAP. For plates, media were solidified by adding 1.2% agar (Difco). Solid media for antibiotic selection and screening contained either 25 μg of ampicillin (Lederle Laboratories) or 20 μg of tetracycline (Lederle) per ml. To test tetracycline minimal inhibitory concentrations (MICs), cells were streaked with an inoculating loop on LB agar supplemented with various concentrations of tetracycline and monitored for independent colony formation after incubation at 37°C.

Isolation of plasmid DNA. Plasmid pVA318 deoxyribonucleic acid (DNA) was isolated from *S. mutans* strain V318. The cells from either 500-ml or 1-liter cultures were lysed by various methods (16, 19, 23). Plasmid DNA was purified by CsCl-ethidium bromide density equilibrium centrifugation as previously described (16), and DNA was extracted with neutral phenol. When necessary, we concentrated DNA by precipitation with 2 volumes of ethanol and 0.1 volume of 2 M sodium acetate buffer (pH 5.3) at −20°C for 30 min.

Various procedures were used to isolate plasmid DNA from *E. coli* strains: some procedures were to simply screen the size of covalently closed circular (CCC) DNA in clones, some were to isolate small amounts of DNA pure enough for restriction endonuclease digestion, and some were to isolate large amounts of purified plasmid DNA.

To obtain DNA for screening plasmid sizes, we used the colony lysis method of Eckhardt (10), the rapid alkaline extraction procedure of Birnboim and Doly (3), or a scaled-down protocol by J. P. Robeson (personal communication) of a Triton X-100 cleared lysis technique.

Small amounts of DNA purified enough for endonuclease digestion or transformation were obtained by the acid phenol method of Zasloff et al. (36) scaled to small volumes, the rapid alkaline extraction procedure of Birnboim and Doly (3), or the Robeson minilysate procedure (above) combined with neutral phenol extraction. The Birnboim and Doly method is the most satisfactory one, being simple and giving better quality of results.

For larger-scale isolation, 1 liter of late-logarithmic-phase cells in ML was amplified by continued incubation (12 to 17 h) in the presence of 170 μg of chloramphenicol per ml (Sigma Chemical Co.). Cells

were lysed by the method of Guerry et al. (15), with the addition of a freeze-thaw step after lysozyme digestion to facilitate lysis. Plasmid DNA was purified by CsCl-ethidium bromide equilibrium density centrifugation, as above.

Plasmid DNA was stored in 1 mM ethylenediaminetetraacetic acid (EDTA)–10 mM tris(hydroxymethyl)-aminomethane (Tris)-hydrochloride buffer (pH 8.0) at 4°C.

Enzymes. The restriction endonucleases *Hin*dIII, *Hae*III, and *Eco*RI were the kind gifts of G. Cardineau, of P. Chan, and of M. Smorwinska and E. Hendrickson, respectively. *Pst*I was isolated by the method of Sato et al. (32). T4 polynucleotide ligase was the kind gift of C. K. K. Nair. We also used commercial preparations of *E. coli* DNA polymerase I (Boehringer Mannheim Corp.), of alkaline phosphatase (Worthington Biochemicals Corp.), and of T4 polynucleotide kinase and the restriction endonucleases *Bam*HI, *Pst*I, *Hpa*I, and *Sal*I (Bethesda Research Laboratories).

Buffers for endonuclease digestion were as described in the Bethesda Research Laboratories catalog. Digestion was for 30 min at 30°C for *Pst*I and at 37°C for the other endonucleases. The reactions of all endonucleases were stopped by heating at 65°C for 5 min.

We ligated endonuclease-digested plasmid DNAs by incubating 4 μg of DNA at 12°C for 16 h in a 50-μl reaction mixture that contained 10 mM Tris-hydrochloride (pH 7.5), 10 mM $MgCl_2$, 10 mM dithiothreitol, 1.0 mM adenosine triphosphate (ATP), and 1 U of T4 polynucleotide ligase.

Agarose gel electrophoresis of DNA. DNA was electrophoresed in 0.7% agarose (Sigma) slab gels. The running buffer was 40 mM Tris-hydrochloride–5 mM sodium acetate–1 mM EDTA, adjusted to pH 7.6 with acetic acid (1). DNA was loaded after mixing with a one-fifth volume of dye (33% glycerol–7% sodium dodecyl sulfate [SDS]–0.17% bromophenol blue [Sigma]). After electrophoresis, gels were stained in a solution of 2 μg of ethidium bromide per ml and photographed with ultraviolet light, Polaroid T57 film, and a red filter.

DNA size standards for agarose gels included the plasmid pBR322 (33), the size reference plasmids from a multiple-plasmid-containing *E. coli* strain (22), and λ *c*I857 DNA, which was the kind gift of M. Smorawinska.

Transformation and selection of clones that contained chimeric plasmids. Transformation of *E. coli* cells with plasmid pBR322 DNA or with chimeric plasmids consisting of plasmid pVA318 cloned into pBR322 followed the $MgCl_2$ method of G. S. Gill (personal communication). An overnight standing culture was diluted 1:100 into 20 ml of LB and grown with shaking at 37°C to an optical density of about 0.07 at 600 nm. The culture was chilled in an ice-water bath, and cold $MgCl_2$ was added to a final concentration of 100 mM. After 20 min on ice and then centrifugation at 4°C, the cells were suspended in 0.4 ml of transformation buffer (50 mM $MgCl_2$–75 mM KCl–100 mM $CaCl_2$–10 mM Tris-hydrochloride, pH 8.0). Plasmid DNA (10 to 500 ng in 1 to 10 μl) and 0.2 ml of cells were mixed, held at room temperature for 25 min, and then heat shocked for 5 min in a 30°C water bath. To allow phenotypic expression, an equal volume of LB was added, and cells were incubated at 37°C for 30 min. Dilutions were plated on antibiotic selective agar.

To screen for insertional inactivation of one of the cloning vector pBR322 antibiotic resistance genes, transformant colonies selected for either ampicillin (Ap^r) or tetracycline (Tc^r) resistance were patched to LB agar supplemented with antibiotic corresponding to the unselected antibiotic resistance gene.

Alternatively, *E. coli* transformant colonies were screened for the presence of pVA318 DNA by a method based on the colony hybridization technique of Grunstein and Hogness (14). Replicate transformant colonies were grown on nitrocellulose filters (HAWP; Millipore Corp.; 0.45-μm pore size; boiled for 5 min in three changes of water and sterilized by autoclaving) laid on LB agar plates supplemented with antibiotic. The filters were then placed serially on double layers of Whatman no. 1 paper saturated with the following solution: first, 15 min on 0.5 M NaOH–1.5 M NaCl; second, 10 min on 1 M Tris-hydrochloride (pH 7.6); and third, 10 min on 0.2 M Tris-hydrochloride (pH 7.6) in 2× SSC (SSC is 0.15 M NaCl plus 0.015 M sodium citrate, pH 7.0). The filters were then dried at room temperature, baked for 3 h at 80°C, incubated for 3 h in Denhardt solution (8) at 65°C, and dried. The filters were hybridized with pVA318 DNA labeled by nick translation with [α-^{32}P]ATP (New England Nuclear Corp.) as described by Rigby et al. (30). About 10^6 cpm of DNA in 5× SSC and 50% formamide was incubated with each filter for 18 to 24 h at 37°C. Filters were then serially washed: first, 30 min in 5× SSC–50% formamide at 42°C; second, 10 min in 6× SSC at room temperature; and third, 10 min in 2× SSC at room temperature. After drying, filters were autoradiographed at −70°C for 12 h.

Restriction endonuclease mapping. Since we were interested in a method generally applicable to mapping DNA inserted into the pBR322 *Hin*dIII site, we used the following approach; this approach is, however, dependent on the fact that the inserted DNA of interest, pVA318, lacked sites for *Bam*HI and *Sal*I endonucleases.

The chimeric plasmid DNA was digested with *Bam*HI, and the ends were dephosphorylated with alkaline phosphatase and end labeled with [γ-^{32}P]ATP (New England Nuclear) by the method of Maxam and Gilbert (26). Then, one labeled end was cut off by *Sal*I digestion and the DNA was partially digested with restriction endonucleases of interest. The partial digests were electrophoresed in agarose slab gels and visualized by autoradiography. The small labeled end cut off with *Sal*I electrophoresed ahead of the partial digest fragments. Adjunct mapping data also came from single and double digests of unlabeled DNA electrophoresed in agarose gels to determine size.

Contour length measurement of plasmid DNA. Plasmids pVA318 and pBR322 relaxed by thermal hydrolysis (13) were mixed together and mounted for electron microscopy by the basic protein film technique (7). Experimental details were as described elsewhere (16).

Plasmid base pair content. pVA318 DNA was centrifuged to equilibrium in a Beckman model E analytical ultracentrifuge equipped with mirror optics

and electronic speed control; the solvent was CsCl in 10 mM Tris-hydrochloride–1 mM EDTA (pH 8.0) (34). The *Micrococcus lysodeikticus* standard DNA had a buoyant density of 1.7271 g/cm^3 determined relative to simian virus 40 DNA (R. Woodward and J. Lebowitz, personal communication). The fraction of guanine-plus-cytosine (G + C) base pairs in the pVA318 molecule was calculated from the buoyant density by the revised equation of Woodward and Lebowitz (35).

Minicell analysis of plasmid gene products. Plasmid-containing minicells of *E. coli* strain χ1849 derivatives were isolated by standard methods (for review, see reference 12). Minicells from a late-logarithmic-phase culture in ML were purified by one differential centrifugation and two discontinuous sucrose density gradient sedimentations. Purified minicells from a 60-ml culture were incubated in ML without DAP for 20 min at 37°C and then labeled by the addition of 1 μCi of a uniformly ^{14}C-labeled amino acid mixture (New England Nuclear; 0.1 mCi/ml). Cells were washed and then lysed by 5 min of boiling in 50 μl of sample buffer (2.3% SDS–5% β-mercaptoethanol–10% glycerol–62.5 mM Tris-hydrochloride, pH 6.8). SDS-polyacrylamide gel electrophoresis (6% stacking gel and 10% separating gel) of 15-μl samples followed the method of Laemmli and Favre (18). Molecular mass standards from Bio-Rad Laboratories included phosphorylase B (94,000 daltons), bovine serum albumin (68,000 daltons), ovalbumin (43,000 daltons), carbonic anhydrase (30,000 daltons), soybean trypsin inhibitor (21,000 daltons), and lysozyme (14,300 daltons). The gel was impregnated with a scintillator (Enhance; New England Nuclear), dried, and fluorographed.

Cold osmotic shock to release periplasmic proteins. To determine whether a plasmid-specified protein was translocated to the periplasm, plasmid-containing minicells radioactively labeled as above were treated by a small-scale cold osmotic shock (17). Samples of the shock fluid and of minicells before and after osmotic shock were washed, lysed, and solubilized as above. Electrophoresis and fluorography of solubilized minicells and shock fluid were as described above.

Bacteriocin assays. Strains tested for bacteriocin production were grown (as spots made with a loop) on BHI agar medium overnight, treated with $CHCl_3$ vapor for 10 min, and overlaid with 3 ml of complex soft agar (0.7% agar) medium seeded with several drops of a logarithmic-phase indicator strain. Plates were examined after 24 h of incubation for a clear zone around the strains tested.

Alternatively, sonic fluids of 12-ml samples of mid-logarithmic-phase *E. coli* derivatives carrying chimeric plasmids were made by 1 min of sonication with a microtip at maximum power (Branson Sonifier, model 185) and intermittent cooling. After removal of debris by 15 min of centrifugation at 17,000 × *g* at 4°C, 20-μl samples of the sonic fluid were spotted on top of indicator cells in soft agar overlays.

RESULTS

Contour length and base pair content of pVA318. We measured the contour length of pVA318 by electron microscopy, using pBR322 as an absolute (i.e., having a known sequence) size standard on the same grid. One hundred and fifty-four of the 4,362 base pair (33) pBR322 molecules and 49 of the pVA318 molecules were measured. pVA318 was calculated to have a size of 5.64 ± 0.26 kilobases (kb). This is about 5% less than the previous size determination of 3.6 megadaltons (23), which did not use an absolute standard.

The base pair content of pVA318 was calculated to be 32 to 34 mol % G + C by analytical CsCl density equilibrium centrifugation.

Restriction endonuclease sites in pVA318. It will be easier to describe the construction of pVA318::pBR322 chimeras by first examining their restriction endonuclease maps shown in Fig. 1, even though this order of presentation is the inverse of the actual experimental chronology. The mapping of the restriction endonuclease sites in pVA318 and the finding of single sites for the endonucleases *Pst*I and *Hae*III are all data we compiled as the result of analysis of chimeric molecules.

Figure 1 shows a linear representation of two *Hin*dIII-cloned pVA318::pBR322 chimeras and one *Pst*I-cloned pVA318::pBR322 chimera that also appeared to have IS*1* inserted into it.

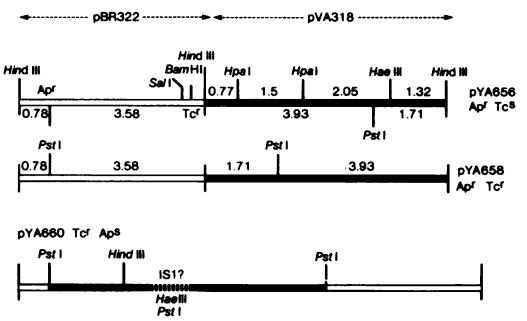

FIG. 1. *Physical maps of three pVA318::pBR322 plasmids: pYA656, pYA658, and pYA660. In these linear representations of the three plasmids, with ends at a HindIII site, the pBR322 portion is indicated by the light line and the pVA318 portion is indicated by the dark. The plasmid phenotype for ampicillin (Ap) and tetracycline (Tc) resistance or sensitivity is indicated next to the plasmid designation. In addition, for pYA656, the regions of pBR322 coding for Ap and Tc resistance gene products are identified: the PstI site is in the Ap^r structural gene, the BamHI site is in the Tc^r gene, and the HindIII site is in the promoter for the Tc^r gene (33). Distances between endonuclease sites are given in kilobases. For ease of representation of pYA658 and pYA660, only the endonuclease sites for HindIII and PstI are shown. pYA660 may contain IS1 (see Results). The multiple HaeIII sites for pBR322 are not shown in the maps.*

Note several properties of the 4.36-kb (33) vector plasmid pBR322. It has a single *Hin*dIII site in the tetracycline resistance promoter: foreign DNA cloned at that site could result in tetracycline sensitivity. Insertion of DNA at the *Pst*I site of pBR322 will inactivate a structural gene for β-lactamase and cause ampicillin sensitivity.

Chimeric plasmids pYA656 and pYA658, which differed in the orientation of the inserted DNA, were found to have only one *Bam*HI site, in their pBR322 portion. This absence of a *Bam*HI site in pVA318 was also seen for pYA660, a *Pst*I-cloned pVA318::pBR322 chimera in *E. coli*. All of these data contradict an earlier report of a single *Bam*HI site in pVA318 (24).

Figure 1 shows endonuclease sites in pVA318 for pYA656. Using the single *Hin*dIII site as zero, *Hpa*I sites mapped at 0.77 and 2.27 kb, a *Pst*I site mapped at 3.93 kb, and a *Hae*III site mapped at 4.32 kb.

It is evident from the maps that *Pst*I endonuclease allowed determination of the orientation of pVA318 cloned into the *Hin*dIII site of pBR322: digestion of pYA656 with *Pst*I gave two fragments of 2.5 and 7.5 kb after gel electrophoresis, whereas pYA658 had fragments of 4.7 and 5.3 kb.

Construction of tetracycline-sensitive pVA318::pBR322 chimeras. *Hin*dIII-digested pVA318 and pBR322 plasmid DNAs were annealed, ligated, and transformed into *E. coli* strain χ1849 with selection for Ap^r. Twenty-four of 550 Ap^r clones tested were Tc^s; the rest were Tc^r.

The plasmid DNA of 12 of these 24 Tc^s Ap^r clones was isolated and examined for size and restriction endonuclease digest patterns by agarose gel electrophoresis. One clone had DNA the size of a pBR322 dimer, 8.7 kb, and was not investigated further. Ten clones were pVA318::pBR322 chimeras in size, 10 kb, and showed bands of 2.5 and 7.5 kb after *Pst*I digestion. Thus, the 10 all apparently had the same orientation as pYA656 (Fig. 1). Five of these 10 were also digested with other endonucleases; all showed a single cut with *Eco*RI and *Bam*HI and gave two fragments identical in size to pBR322 and pVA318 with *Hin*dIII. The 12th clone examined was a pVA318::pBR322 chimera of the same orientation as above, but with a small deletion which included the pVA318 *Hae*III site.

Construction of tetracycline-resistant pVA318::pBR322 chimeras. The finding that all Tc^s pVA318::pBR322 chimeras examined were of the same orientation (e.g., pYA656; Fig. 1) naturally suggested that clones of the other orientation would be phenotypically Tc^r. To test this, we digested pYA656 DNA with *Hin*dIII endonuclease and religated the fragments to randomize the orientation of the pVA318 insert. We transformed χ1849 with such randomized DNA, selected for Ap^r, and then tested 250 colonies for hybridization with radioactively labeled probe pVA318 DNA. Fifty colonies hybridized with the probe; of these 50, 33 had the phenotype Ap^r Tc^s, and 17 had the phenotype Ap^r Tc^r. The plasmid DNA isolated from several clones was examined for size as CCC molecules and as linear molecules (after *Eco*RI digestion), for maintenance of the fragments identical in size to pVA318 and pBR322 after *Hin*dIII digestion, and for orientation of the pVA318 insert in pBR322 as tested by *Pst*I digestion.

Plasmid DNA from all four Ap^r Tc^s clones examined was identical in these tests to the pYA656 configuration; plasmid DNA from all three Ap^r Tc^r clones examined was identical to that of pYA658. Thus, the hypothesis of the effect of orientation on Tc^r expression was supported. The plasmids designated pYA656 and pYA658 (see Fig. 1) are representative clones from each orientation of *Hin*dIII-cloned pVA318::pBR322 chimeras. pYA656 and pYA658 have each been serially isolated as DNA and retransformed in *E. coli* without alterations in size or specified phenotype.

Since the expression of Tc^r by clones of the pYA658 type might be due to initiation of transcription at a promoter sequence on pVA318 upstream of the pBR322 Tc^r genetic region, we compared the tetracycline MIC of χ1849 derivatives which contained either pBR322 or two independent isolates each of the pYA656 or pYA658 type. The χ1849, plasmid-free control had an MIC of about 1 μg/ml. The clones that contained chimeras of the pYA656 type showed MICs of about 10 μg/ml. Both pBR322 and chimeras of the pYA658 type had similar MICs of about 140 μg/ml. Thus, a different level of promoter activity of pBR322 and pYA658 was not evident with regard to tetracycline antibiotic resistance expression.

***Pst*I-cloned pVA318::pBR322 chimeras.** Cloning pVA318 into the *Pst*I site of pBR322 was problematic. At first we identified two such clones by colony hybridization, but successive DNA isolations from each clone revealed the chimeric plasmid to be decreasing in size with time. When we identified, by the phenotype Ap^s Tc^r, 11 clones of χ1849 with pVA318 cloned in the *Pst*I site of pBR322 and immediately examined their plasmid DNAs for size, three had deletions and one had an insertion. Four of the clones that contained plasmids of the expected size, 10 kb, were immediately grown for large-scale plasmid isolation, but the plasmid DNA

recovered from all four showed extensive deletion. By transforming *E. coli* strains CSR603 and χ1953 with a small-scale lysate made from one of the above *Pst*I-derived clones immediately after construction, and which contained both 10-kb plasmids and smaller plasmids as shown by agarose gel electrophoresis, two stable variants of *Pst*I-cloned pVA318::pBR322 were isolated. One, pYA660, appeared to have IS*1* inserted into the pVA318 portion of the chimeric molecule at the site shown in Fig. 1. The evidence for this was a 0.8-kb size increase of the *Hin*dIII-digested pVA318 portion and new sites for *Pst*I and *Hae*III (29) that mapped together in the pVA318 segment. The other variant, pYA661, was 10 kb, equal in size to pYA656 and pYA658, and maintained the *Pst*I and *Hin*dIII sites expected for a *Pst*I-cloned pVA318::pBR322 chimera that had not undergone any molecular rearrangement.

Figure 2 shows a comparison on agarose gel electrophoresis of the *Pst*I-cloned pYA660 and pYA661 plasmids with the *Hin*dIII-cloned pYA656. Lanes 1 through 3 show, in order, intact pYA656, pYA661, and pYA660. The lower band of each was CCC plasmid; pYA660 appeared larger than the 10-kb pYA656 and pYA661. Lanes 4 through 6 show pYA656, pYA661, and pYA660, respectively, after digestion with *Hin*dIII. The *Hin*dIII-digested pYA656 gave two bands: the upper was linear pVA318 (5.6 kb), and the lower was linear pBR322 (4.4 kb). The sizes of the *Hin*dIII digestion fragments of pYA661 (7.5 and 2.5 kb) and pYA660 indicate the orientation of the pVA318 insert in the pBR322 vector (see also Fig. 1). Furthermore, the large *Hin*dIII fragment of pYA660 had increased size compared with the large, 7.5-kb *Hin*dIII fragment of pYA661. Lanes 7 through 9 show pYA656, pYA661, and pYA660, respectively, after digestion with *Pst*I. pYA661 shows the parental pVA318 and pBR322 fragments (compare with *Hin*dIII-digested pYA656, lane 4), and the sizes of the *Pst*I fragments of pYA656 in lane 7 indicate the orientation of the inserted pVA318 in the pBR322 vector for this recombinant plasmid (compare with *Hin*dIII-digested pYA661, lane 5; see Fig. 1). *Pst*I digestion of pYA660 (lane 9) gave three fragments sized approximately 4.1, 3.1, and 2.8 kb (the fourth, uppermost band is an incomplete digestion product of about 7 kb).

Lane 10 contains λ *c*I857 digested with *Eco*RI (fragment sizes of 3.38, 4.80, ~5.74 [the average of two fragments close in size], 7.55, and 21.8 kb; the top band is an incomplete digestion product).

When each of these *Pst*I-cloned chimeras, pYA660 and pYA661, was transformed into χ1849, they remained stable in size. This suggested that the 10-kb pYA661 might have some as yet undetected alteration, since prior attempts to purify a stable variant from the same lysate by repeated, serial transformation in χ1849 were not successful.

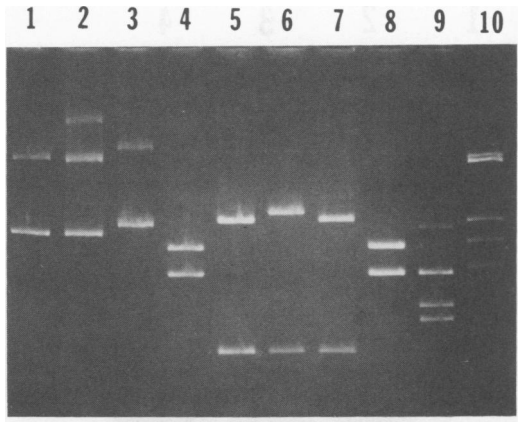

FIG. 2. *Slab agarose gel electrophoresis of pVA318::pBR322 plasmids. For samples in which DNAs were digested with restriction endonucleases, the enzyme is identified after the DNA designation. DNAs are as follows: lane 1, pYA656; lane 2, pYA661; lane 3, pYA660; lane 4, pYA656/HindIII; lane 5, pYA661/HindIII; lane 6, pYA660/HindIII; lane 7, pYA656/PstI; lane 8, pYA661/PstI; lane 9, pYA660/PstI; lane 10, λ cI857/EcoRI. See Materials and Methods for details.*

Phenotypic expression of pVA318::pBR322 chimeras. Replication of pBR322 was dependent on the chromosomal *polA* gene. Maintenance of a pVA318::pBR322 chimera in an *E. coli* strain without a functional *polA* gene product would require pVA318 replication. However, when pYA656 and pYA658 were transformed into the *polA*(Ts) strain χ1891, the Ap^r phenotype was not maintained at nonpermissive temperatures. Thus, the pVA318 replicator appeared either nonfunctional or *polA* dependent in *E. coli*.

SDS-polyacrylamide gel electrophoresis of lysates of minicells containing pYA656, pYA658, or pYA661 all revealed a major, pVA318-specific gene product with a molecular weight of approximately 20,000. Figure 3 (lane 1) shows the χ1849 plasmid-free control, with proteins, translated from *E. coli* very stable messenger ribonucleic acid (mRNA) (21), of apparent sizes 39,000, 37,000, and 12,000 daltons. The other three faint bands are proteins specified by chromosomal genes, resulting from residual contamination of the minicell preparation with whole cells. Lane 2 shows χ1849(pBR322). The extra protein of approximately 40,000 daltons corresponds to the largest Tc^r gene product of pBR322 (33), and

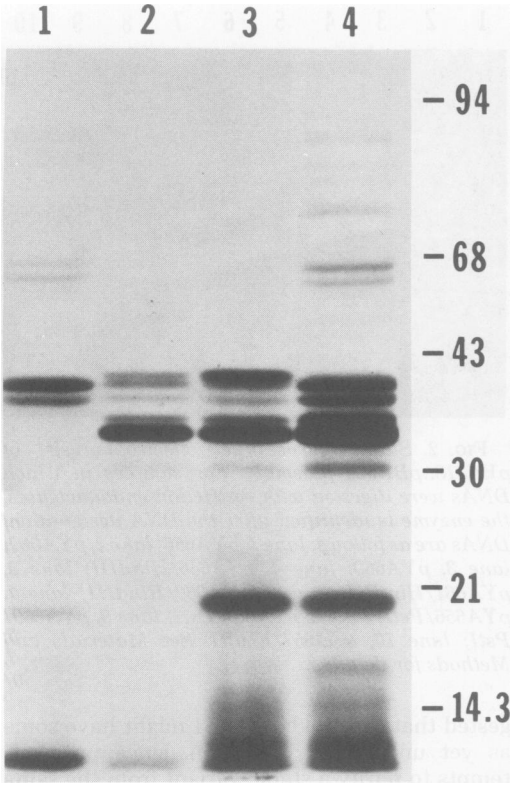

FIG. 3. *Fluorograph, after SDS-polyacrylamide gel electrophoresis, of radioactively labeled proteins from E. coli χ1849 minicells containing pVA318::pBR322 plasmids. Lane 1, plasmid-free χ1849; lane 2, χ1849(pBR322); lane 3, χ1849(pYA658); lane 4, χ1849(pYA656). Position and size (in kilodaltons) of molecular mass standards is indicated to the right. See Materials and Methods for details.*

proteins exhibiting sizes of 34,000, 33,000, and 30,000 daltons may be the Ap^r gene products (11, 33). The 40,000-dalton Tc^r gene product was present for the Tc^r chimera pYA658 (lane 3) but missing for the Tc^s plasmid pYA656 (lane 4). Both pYA656 and pYA658 showed a major protein of 20,000 daltons. As expected for insertional inactivation at the pBR322 *PstI* site, minicells containing pYA661 did not exhibit the 33,000- and 30,000-dalton proteins for ampicillin resistance; however, a faint 34,000-dalton band was still produced (data not shown).

Evidence that the 20,000-dalton pVA318-specific gene product was not translocated to the periplasm of *E. coli* is seen in Fig. 4. The enzyme β-lactamase acted as the control: it was released from the periplasm as the result of osmotic shock. If the 20,000-dalton protein is periplasmic, it should similarly be released. Lane 1 is the fluorograph of the labeled minicell protein pattern after electrophoresis through an SDS-polyacrylamide gel for χ1849(pYA656) (corresponds to lane 3 of Fig. 3 except that fewer 39,000- and 37,000-dalton stable mRNA translation products are seen). Lane 3 is the pattern produced by washed minicells after osmotic shock; some of the 33,000-dalton Ap^r gene product has been removed by osmotic shock, as compared with lane 1. Lane 2 shows the labeled 33,000-dalton protein band, evidently the periplasmic β-lactamase, present in the shock fluid. All three lanes contained samples from an equivalent amount of input cells; the mobility of the shock fluid band was slightly retarded by the large sample volume applied (160 μl). The 20,000-dalton, pVA318-specific gene product did not appear in the shock fluid at all, and therefore was probably not periplasmic in location.

We used a clinical group A *Streptococcus* strain, UAB405, as an indicator to test for bacteriocin production by 10 different *S. mutans* strains of various serotypes (Table 1). UAB405 was not sensitive to *S. mutans* strain AHT, HS6, BHT, PS-14, OMZ176, or 6715; it was sensitive to strains Ingbitt, OMZ175, LM7, and JC-1. The latter two strains contained pVA318-like plasmids (9, 23; Hansen et al., unpublished data).

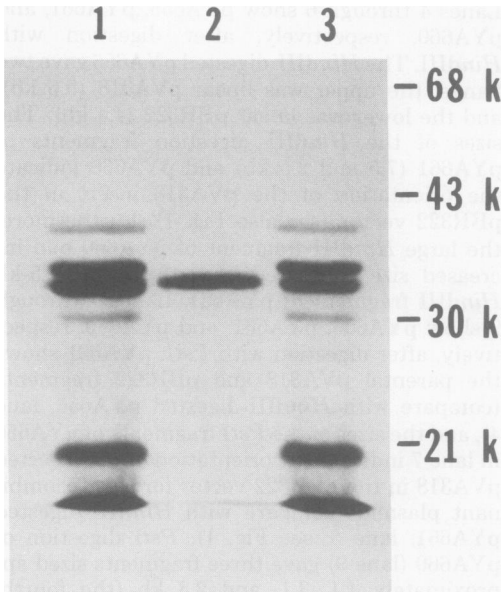

FIG. 4. *Effect of cold osmotic shock on radioactively labeled proteins from χ1849(pYA656) minicells. Fluorograph after SDS-polyacrylamide gel electrophoresis shows radioactively labeled proteins from different preparations of χ1849(pYA656) minicells: lane 1, minicells lysed without osmotic shock; lane 2, shock fluid of labeled minicells; lane 3, minicells lysed after osmotic shock. Position and size (in kilodaltons) of molecular mass standards is indicated to the right. See Materials and Methods for details.*

However, UAB405 did not show sensitivity to spots of grown, chloroform-treated cells or sonicated lysates of χ1849 derivatives that contained pYA656, pYA658, or pYA661 (data not shown). Also, an *S. sanguis* Challis strain containing a derivative of pVA318 (pDB201 [2]) did not show bacteriocin production when tested with the same indicator.

In bacteriocin screening with the 10 abovementioned *S. mutans* strains, we observed that the two strains which contained pVA318-like plasmids LM7 and JC-1 were uniquely sensitive to a bacteriocin produced by *S. mutans* strain BHT. The possibility that presence of the pVA318 plasmid might confer sensitivity to this bacteriocin was examined; neither *E. coli* nor *S. sanguis* strains with pVA318 derivatives in them showed any such sensitivity (data not shown).

DISCUSSION

Besides measuring a contour length of 5.64 ± 0.26 kb for pVA318, as compared with a standard of known base pair size, we found a base pair content for the plasmid of 32 to 34 mol % G + C. There are four different genospecies of *S. mutans*, and the four have been proposed as different subspecies (4, 5). The genospecies *S. mutans* subsp. *mutans* includes *S. mutans* strains with serotypes *c*, *e*, and *f*; thus, the strains from which pVA318-like plasmids have been isolated are all in that subspecies (24, 28). In addition, of the four genospecies, *S. mutans* subsp. *mutans* is closest in moles percent G + C to pVA318, being 36 to 38 (4, 5).

In our map of pVA318 there is no *Bam*HI site since pVA318 was not cut by this endonuclease in any of our chimeras. The initial report of a *Bam*HI site in pVA318 specified conditions of threefold enzyme excess and 16 h of digestion (24). We have not been able to repeat those results with pVA318 DNA.

Our examination of pVA318 cloned into pBR322 in *E. coli* covered several aspects. First was the observation that *Pst*I-cloned pVA318::pBR322 showed much instability initially. We do not know the reason for this instability or, conversely, the reason for present stability of the variants pYA660 and pYA661.

With *Hin*dIII-cloned chimeras of either orientation and a *Pst*I-cloned chimera, we examined aspects of the pVA318 phenotype in *E. coli*. The plasmid does not appear able to mediate *polA*-independent replication. However, a 20,000-molecular-weight, pVA318-specific protein was produced by pYA656, pYA658, and pYA661. The fact that the protein is the same size for all three plasmids suggests that the promoter and transcription regions of the gene are distinct from both the *Hin*dIII and *Pst*I sites of pVA318 and were not interrupted in constructing the chimeras.

Finding evidence of the protein prompted us to test our *E. coli* derivatives that contained pVA318::pBR322 plasmids for bacteriocin activity, even though no apparent phenotype (including bacteriocin production) was conferred by the presence of pVA318-like plasmids in *S. mutans* strains (23). We found no evidence of bacteriocin production or sensitivity conferred by pVA318-like plasmids in *E. coli* or *S. sanguis*.

The 20,000-dalton protein did not appear to have a periplasmic location in *E. coli*; this contrasts with findings that several *S. mutans* extracellular proteins are translocated to the periplasm of *E. coli* hosts that contain the cloned *S. mutans* genes (Y. Abiko and J. Robeson, personal communication). Thus, contrapositively, we might expect that *S. mutans* strains carrying pVA318 will produce a 20,000-dalton protein that is not extracellular, but instead will be found in the bacterial cytoplasm or the cell membrane.

Several observations concerning the three reported proteins of the pBR322 Ap^r region (11, 33) were unexpected. Of the three proteins sized 34,000, 33,000, and 30,000 daltons (our estimates), it appeared to be the 33,000-dalton protein that was located in the *E. coli* periplasm rather than the smaller 30,000-dalton protein (Fig. 4). Perhaps the minor, 30,000-dalton protein is the product of premature termination or improper processing. Could the 34,000-dalton protein be an intracellular precursor form before processing? The anomalous observation of a residual, faint 34,000-dalton band for the Ap^s pYA661 plasmid (data not shown) conflicts with this, but may be misleading if this faint band of pYA661 is not identical to the intense 34,000-dalton produced by pYA656 and pYA658, but merely one of similar size.

We should note that our estimated molecular sizes for the Ap^r, Tc^r, and stable mRNA gene products were reproducibly slightly larger than (about 10%), but still consistent with, several literature values (e.g., see references 11, 21, and 33). The literature values themselves vary among investigators. In three separate SDS-polyacrylamide gels we ran, the range of the calculated molecular weights for the pBR322 Ap^r proteins or for the higher-molecular-weight stable mRNA proteins varied no more than 3% from the values we have reported (see Results). Our calculated molecular size for the low-molecular-weight stable mRNA protein varied slightly more: it averaged 12,500 daltons, with a range of 11,800 to 13,500 daltons.

The production by pVA318::pBR322 derivatives of one, relatively small pVA318-specific protein with molecular size distinct from that of pBR322 gene products contrasts with the four proteins readily seen by minicell analysis that are made by pBR322, especially in light of the fact that pVA318 is 25% larger than pBR322. However, this one protein is produced in relatively large amounts in *E. coli*, as judged by comparison with pBR322 gene products (Fig. 3 and 4). We do not know whether the pattern of gene expression of pVA318 chimeras in *E. coli* is similar to that of intact pVA318 in *S. mutans*.

The ability to obtain large amounts of pVA318 DNA when it is cloned into an amplifiable vector in *E. coli* should aid those doing finer physical mapping of the plasmid. Besides our mapping work, Behnke and Ferretti (2) have also reported a similar map of *Hin*dIII and *Hpa*I sites for pVA318. Either *Hin*dIII with its single site reported by Macrina and Scott (24) or the two endonucleases we identified and mapped that cleave pVA318 once, *Hae*III and *Pst*I, may be used for cloning with this plasmid. After the extension of 3′ end of duplex DNA with single-stranded homopolymer by treatment with terminal transferase, both *Hae*III and *Pst*I sites can be reconstructed by joining with polydeoxyguanosine-polydeoxycytidine homopolymers the plasmid and an insert of randomly sheared source DNA; this will result in two sites on either side of the inserted DNA (27). Recombinant DNA cloning in streptococci has already been initiated (2, 21a).

What role might pVA318-like plasmids have in *S. mutans* biology? A general belief is that cryptic plasmids must have some still unknown contribution in the host cell's biological fitness, at least when the plasmid has more coding capacity than that needed for its own maintenance. If the 20,000-dalton protein is produced by pVA318 when it is in *S. mutans*, it might be expected to provide some as yet unknown selective advantage to its host. However, progress in understanding that depends on fortuitous discovery of phenotypic traits conferred by cryptic plasmids.

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LITERATURE CITED

1. **Aaij, C., and P. Borst.** 1972. The gel electrophoresis of DNA. Biochim. Biophys. Acta **269**:192–200.
2. **Behnke, D., and J. J. Ferretti.** 1980. Molecular cloning of an erythromycin resistance determinant in streptococci. J. Bacteriol. **144**:806–813.
3. **Birnboim, H. C., and J. Doly.** 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. **7**:1513–1523.
4. **Coykendall, A. L.** 1976. On the evolution of *Streptococcus mutans* and dental caries, p. 703–712. *In* H. M. Stiles, W. J. Loesche, and T. C. O'Brien (ed.), Microbial Aspects of Dental Caries, (a special supplement to Microbiological Abstracts), vol. III. Information Retrieval Inc., Washington, D.C.
5. **Coykendall, A. L.** 1977. Proposal to elevate the subspecies of *Streptococcus mutans* to species status, based on the molecular composition. Int. J. Syst. Bacteriol. **27**: 26–30.
6. **Curtiss, R., III.** 1965. Chromosomal aberrations associated with mutations to bacteriophage resistance in *Escherichia coli*. J. Bacteriol. **89**:28–40.
7. **Davis, R. W., M. Simon, and N. Davidson.** 1971. Electronmicroscope heteroduplex methods for mapping regions of base sequence homology in nucleic acids. Methods Enzymol. **21D**:413–428.
8. **Denhardt, D.** 1966. A membrane-filter technique for the detection of complementary DNA. Biochem. Biophys. Res. Commun. **23**:641–646.
9. **Dunny, G. M., N. Birch, G. Hascall, and D. B. Clewell.** 1973. Isolation and characterization of plasmid deoxyribonucleic acid from *Streptococcus mutans*. J. Bacteriol. **114**:1362–1364.
10. **Eckhardt, T.** 1978. A rapid method for the identification of plasmid desoxyribonucleic acid in bacteria. Plasmid **1**:584–588.
11. **Fling, M. E., and L. P. Elwell.** 1980. Protein expression in *Escherichia coli* minicells containing recombinant plasmids specifying trimethoprim-resistant dihydrofolate reductases. J. Bacteriol. **141**:779–785.
12. **Frazer, A. C., and R. Curtiss III.** 1975. Production, properties and utility of bacterial minicells. Curr. Top. Microbiol. Immunol. **69**:1–84.
13. **Freifelder, D., and R. Dewitt.** 1977. Thermal hydrolysis as a means of opening supercoiled circles of DNA. Gene **1**:385–387.
14. **Grunstein, M., and D. S. Hogness.** 1975. Colony hybridization: a method for the isolation of cloned DNAs that contain a specific gene. Proc. Natl. Acad. Sci. U.S.A. **72**:3961–3965.
15. **Guerry, P., D. J. LeBlanc, and S. Falkow.** 1973. General method for the isolation of plasmid deoxyribonucleic acid. J. Bacteriol. **116**:1064–1066.
16. **Hansen, J. B., and R. H. Olsen.** 1978. Isolation of large bacterial plasmids and characterization of the P2 incompatibility group plasmids pMG1 and pMG5. J. Bacteriol. **135**:227–238.
17. **Hazelbauer, G. L., and S. Harayama.** 1979. Mutants in transmission of chemotactic signals from two independent receptors of *E. coli*. Cell **16**:617–625.
18. **Laemmli, U. K., and M. Favre.** 1973. Maturation of the head of bacteriophage T4. J. Mol. Biol. **80**:575–599.
19. **LeBlanc, D. J., and L. N. Lee.** 1979. Rapid screening procedure for detection of plasmids in streptococci. J. Bacteriol. **140**:1112–1115.
20. **Lennox, E. S.** 1955. Transduction of linked genetic characters of the host by bacteriophage P1. Virology **1**:190–206.
21. **Levy, S. B.** 1975. Very stable prokaryotic messenger RNA

in chromosomeless *Escherichia coli* minicells. Proc. Natl. Acad. Sci. U.S.A. **72**:2900–2904.

21a. **Macrina, F. L., K. R. Jones, and P. H. Wood.** 1980. Chimeric streptococcal plasmids and their use as molecular cloning vehicles in *Streptococcus sanguis* (Challis) J. Bacteriol. **143**:1425–1435.

22. **Macrina, F. L., D. J. Kopecko, K. R. Jones, D. J. Ayers, and S. M. McCowen.** 1978. A multiple plasmid-containing *Escherichia coli* strain: convenient source of size reference plasmid molecules. Plasmid **1**:417–420.

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

24. **Macrina, F. L., and C. L. Scott.** 1978. Evidence for a disseminated plasmid in *Streptococcus mutans*. Infect. Immun. **20**:296–302.

25. **Macrina, F. L., S. S. Virgili, and C. L. Scott.** 1977. Extrachromosomal gene system in *Streptococcus mutans*. Adv. Exp. Med. **107**:859–868.

26. **Maxam, A. M., and W. Gilbert.** 1977. A new method for sequencing DNA. Proc. Natl. Acad. Sci. U.S.A. **74**:560–564.

27. **Nelson, T., and D. Brutlag.** 1979. Addition of homopolymers to the 3'-ends of duplex DNA with terminal transferase. Methods Enzymol. **68**:41–50.

28. **Newbrun, E.** 1978. Cariology. The Williams & Wilkins Co., Baltimore, Md.

29. **Ohtsubo, H., and E. Ohtsubo.** 1978. Nucleotide sequence of an insertion element, IS1. Proc. Natl. Acad. Sci. U.S.A. **75**:615–619.

30. **Rigby, P. W. J., M. Dieckmann, C. Rhodes, and P. Berg.** 1977. Labelling deoxyribonucleic acid to high specific activity *in vitro* by nick translation with DNA polymerase I. J. Mol. Biol. **113**:237–251.

31. **Rodriguez, R. L., R. Tait, J. Shine, F. Bolivar, H. Heyneker, M. Betlach, and H. W. Boyer.** 1977. Characterization of tetracycline and ampicillin resistant plasmid cloning vehicles, p. 73–84. *In* W. A. Scott and R. Werner (ed.), Molecular cloning of recombinant DNA, vol. 13, Miami Winter Symposia. Academic Press, Inc., New York.

32. **Sato, D. I., F. R. Blattner, and J. Davies.** 1976. The isolation and partial purification of a new restriction endonuclease from *Providencia stuartii*. Nucleic Acids Res. **3**:343–353.

33. **Sutcliffe, J. G.** 1979. The complete nucleotide sequence of the *Escherichia coli* plasmid pBR322. Cold Spring Harbor Symp. Quant. Biol. **43**:77–90.

34. **Vinograd, J., and J. E. Hearst.** 1962. Equilibrium sedimentation of macromolecules and viruses in a density gradient, p. 372–422. *In* L. Zechmeister (ed.), Progress in the chemistry of organic natural products, vol. 20. Springer-Verlag, Vienna.

35. **Woodward, R. S., and J. Lebowitz.** 1980. A revised equation relating DNA buoyant density to guanine plus cytosine content. J. Biochem. Biophys. Methods **2**:307–309.

36. **Zasloff, M., G. D. Ginder, and G. Felsenfeld.** 1978. A new method for the purification and identification of covalently closed circular DNA molecules. Nucleic Acids Res. **5**:1139–1151.