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Survey of the Extrachromosomal Gene Pool of *Streptococcus mutans*

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Fifty strains of *Streptococcus mutans* independently isolated from human dental plaque were examined for the presence of covalently closed circular plasmid deoxyribonucleic acid (DNA). Cesium chloride-ethidium bromide centrifugation of [^{3}H]thymidine-labeled, Sarkosyl-lysed cells revealed that 2 of the 50 strains contained plasmid DNA. The plasmid DNA from these strains was characterized by velocity and equilibrium centrifugation and by electron microscopy. The plasmids in these strains were virtually identical in size, with molecular weights of 3.6×10^6 and 3.7×10^6. Both were present to the extent of approximately 20 molecules per genome equivalent. Interlocked catenated dimeric molecules of each plasmid were readily detected by velocity sedimentation and electron microscopy. These plasmid-containing strains were compared with representative plasmid-free *S. mutans* strains by using such criteria as bacteriocin production, antibiotic susceptibility, and hemolysis of mammalian erythrocytes. Although no correlation of phenotype to plasmid content could be made, production of bacteriocin-like activity differed significantly between the two plasmid-containing *S. mutans* isolates. Thus, although the plasmids in these two isolates appeared identical by the criteria of molecular weight, presence of dimers, and copy number, they appeared to be harbored by two distinct *S. mutans* strains.

The occurrence of extrachromosomal deoxyribonucleic acid (DNA) in bacteria has been shown to have wide-reaching implications relative to a variety of important phenotypes. The genetic information carried by bacterial extrachromosomal elements (plasmids) may confer such properties as multiple antibiotic and metal ion resistance; productions of toxins, antigens, and bacteriocins (antibiotic proteins); conjugal donor ability; and a diversity of metabolic and catabolic capabilities. Plasmids in the streptococci may confer nearly all of the above-mentioned phenotypes. Plasmids conferring drug resistance (R plasmids) have been demonstrated in Lancefield group A (4), B (21), and D (5, 7) streptococci. A plasmid that is able to mediate the conjugal transfer of itself as well as a separate tetracycline resistance plasmid has been described for group D streptococci (22). This same conjugative plasmid (designated pAMγ1 [12]) has been shown to encode the production of beta-hemolysin and two bacteriocin-like substances (12). In addition, at least one streptococcal plasmid (pAMα1, found in group D *Streptococcus faecalis*) contains a tetracycline resistance gene bordered by directly repeated DNA sequences, which enable selective amplification of this gene (5, 36). Evidence supporting the plasmid linkage of lactose fermentation and proteolytic activity in group N streptococci (*S. lactis*) has recently been forwarded (13).

The evolutionary and clinical significance of plasmid DNA has stimulated an interest in the occurrence and function of these genetic elements in the streptococci of the oral cavity. This group of organisms appears to have a significant role in human oral ecology. These streptococci are known to colonize both the hard and soft tissues of the mouth, and this colonization is a critical component of bacterial plaque formation and growth (16, 17). *S. mutans* has been shown to promote the formation of dental caries in animals (16, 17), and this species is thought to be cariogenic in humans as well. Dunny et al. (11) first reported the presence of a small plasmid (molecular weight, 3×10^6) in *S. mutans* LM-7. No phenotypic function has yet been ascribed to this plasmid, however. Reports from Clewell's laboratory (6) and our laboratory (32)

have since reported the absence of extrachromosomal elements in a total of 22 well-characterized laboratory strains of this species. In this paper we report the results of a survey of 50 isolates of *S. mutans* obtained from human dental plaque samples. Only 2 of the 50 strains were found to contain plasmid DNA. The plasmid DNA was characterized by velocity centrifugation and electron microscopy; however, we were unable to identify any phenotypic traits unique to the plasmid-containing strains.

MATERIALS AND METHODS

Media. Todd-Hewitt (TH) broth (Difco Laboratories, Detroit, Mich.) and brain heart infusion (BHI; Difco) were used as complex liquid media. Agar (Difco) was added to give a final concentration of 1.5% when a solid medium was desired. Mitis salivarius agar (Difco) was used to evaluate colonial morphology of the *S. mutans* strains. Carbohydrate fermentation was carried out with phenol red broth base or phenol red agar base (Difco). Carbohydrates were filter sterilized and added to these media to give a final concentration of 0.5%. All culturing was performed routinely under an atmosphere of 90% N_2 and 10% CO_2, using the methodologies previously described (20).

Bacterial strains. Isolates of *S. mutans* from human dental plaque were kindly provided by the following individuals: I. L. Shklair, Naval Dental Research Institute, Great Lakes, Ill.; J. Tanzer, University of Connecticut, Farmington; J. Bulkacz, Medical College of Georgia, Augusta; W. Loesche, University of Michigan, Ann Arbor. Upon receipt, strains were checked for mannitol and sorbitol fermentation and for typical-appearing raised, "frosted-glass" colonial morphology on mitis salivarius agar. All strains fermented these two carbohydrates and displayed this colonial morphology consistent with *S. mutans*. Since in vitro culturing of *S. mutans* sometimes results in colonial morphology alteration and/or loss of virulence in animals, we were interested in minimizing the number of serial transfers these strains experienced. The following procedure was thus developed for the storage and routine use of *S. mutans* in our laboratory. After confirmation of the above-mentioned phenotypes, several BHI agar plates were inoculated (from the originally received culture, if possible) so as to give confluent growth. After incubation at 37°C for 24 h, cells (which had not been subjected to more than three serial passages) from these plates were scraped into 30 ml of BHI broth containing 30% glycerol. This suspension (usually $\sim 10^{10}$ cells per ml) was stored at −35°C in a 100-ml screw-cap bottle. Use of the strains stored in this fashion was as follows. Approximately 0.25 ml was removed from this bottle and allowed to grow in TH or BHI broth. The cells in the broth culture then were checked by Gram staining, mannitol and sorbitol fermentation tests, and colonial morphology on mitis salivarius agar. Three or four BHI or TH agar plates were then inoculated with this culture and incubated at 37°C for 24 to 48 h. These plates (stored at 4°C) were used as the inoculum source for experiments during a time period that usually lasted about 4 weeks. This entire process was repeated at that time if additional use of the strain was desired. By this method, a freezer bottle will last several years (before restocking is necessary) even in the case of extensively used strains. We conclude that this procedure ensures an acceptable standard of control in our strain maintenance and provides reasonable assurance of genetic stability.

Biochemicals and drugs. Lysozyme (grade I, crystallized three times), protease (grade I, from *Streptomyces griseus*), sucrose (grade I, for sucrose gradients), ethidium bromide (EB), sodium dodecyl sulfate, and bacitracin were purchased from the Sigma Chemical Co. (St. Louis, Mo.). Technical-grade cesium chloride was purchased from Kawecki Berylco Industries, Inc., New York, N.Y. Sarkosyl NL97 (sodium dodecyl sarcosinate) was the gift of the Ciba-Geigy Corp., Greensboro, N.C.

[*Methyl*-^{3}H]thymidine (20 Ci/mmol) was purchased from Amersham/Searle, Arlington Heights, Ill. [2-^{14}C]thymidine was purchased from New England Nuclear, Boston, Mass.

Antibiotic-containing paper disks for screening antibiotic susceptibility patterns were obtained from Difco. Streptomycin sulfate was purchased from Sigma. Kanamycin sulfate was a gift from K. Price, Bristol Laboratories, Syracuse, N.Y., and gentamicin was a gift from J. T. Baldini, Schering Corporation, Kenilworth, N.J.

Biotyping of *S. mutans*. Biotyping of the clinical isolates of *S. mutans* was performed by the method of Shklair and Keene (34). Carbohydrate fermentations were generally determined on solid medium by stabbing the test strain into a plate containing phenol red agar base plus 0.5% carbohydrate. Up to six strains could be tested per plate. All carbohydrate fermentations were carried out anaerobically.

Serological testing. The presence of the Lancefield group antigen (27) was assayed by the standard protocol recommended by the Center for Disease Control (*Recommended Method for the Use of Streptococcal Antisera*, Center for Disease Control, Atlanta, Ga., April 1970). HCl extracts prepared from TH-grown cells were tested against Lancefield group E antisera obtained from Wellcome Reagents Division, Research Triangle Park, N.C. Positive and negative control strains always were included.

Antibiotic susceptibility testing. Strains were screened for susceptibility to 11 different antibiotics as follows. A TH agar plate was inoculated by streaking with a cotton swab that had been immersed in a mid-log-phase TH broth culture of the strain to be tested. Antibiotic-containing disks were aseptically transferred to these plates (six disks per plate), and the plates were incubated for 24 h under N_2-CO_2. An antibiotic disk that did not produce a zone of inhibition of 2 cm or more in diameter was noted, and the minimal inhibitory concentration of the antibiotic in question was determined by the sensitive colony-forming-unit test as previously described (29).

Bacteriocin testing. Assessment of bacteriocin-

like activity was performed by an agar overlay technique. Up to six tester strains were inoculated onto a TH agar plate by stabbing with a sterile toothpick. After 48 h of incubation under N_2-CO_2 at 37°C, the plates were overlaid with 3.5 ml of molten (tempered to 45°C) TH-soft agar (0.75% agar in TH broth) containing 0.3 ml of an overnight TH broth culture of the indicator strain. Zones of inhibition surrounding the tester colony were noted after 24 h of incubation at 37°C under N_2-CO_2.

Radioactive labeling and gentle lysis of *S. mutans*. The procedure routinely used in the examination of strains for plasmid DNA was as follows. An overnight TH broth culture of the strain to be examined was used to inoculate prewarmed TH broth contained in a 50-ml side-arm Erlenmeyer flask. The inoculum was added to give a final optical density at 660 nm (OD_{660}) of ~0.1 as measured on a Bausch & Lomb Spectronic 20 spectrophotometer. The volume of the culture was adjusted to 20 ml. Growth at 37°C under N_2-CO_2 was monitored spectrophotometrically, and, at an OD_{660} of ~0.2, [^{3}H]thymidine was added to give a final concentration of 20 μCi/ml. The culture was allowed to grow in the presence of isotope until the OD_{660} reached 0.38. Then 1 g of solid glycine was added to the culture, and incubation continued for 45 min. During this 45-min time period there was little increase in OD_{660}, but ^{3}H incorporation into trichloroacetic acid-insoluble, alkali-resistant material continued at a constant rate. This incubation in glycine has been shown to render *S. mutans* very susceptible to detergent lysis after treatment with lysozyme and protease. A complete description of this system may be found in a separate publication (32).

After glycine treatment of the culture, cells were harvested by centrifugation (12,000 × *g* at 4°C) and washed twice with sterile ice-cold water. Sedimented cells (from the 20-ml culture) were suspended in 1 ml of 25% (wt/vol) glucose prepared in 0.005 M ethylenediaminetetraacetate-0.01 M tris(hydroxymethyl)aminomethane (ET buffer; final pH, 8.5). Lysozyme (0.25 ml of a 6-mg/ml stock solution in ET buffer) was added to this suspension, and the cells were incubated for 2 h at 37°C. Protease (0.25 ml of a 6-mg/ml stock solution in ET buffer) that had been self-digested for 1 h at 37°C was then added. Incubation was continued for 30 min. A 1.5-ml volume of a 2% solution of Sarkosyl NL97 (in ET buffer) was added to this suspension, and the mixture was gently swirled. Cells and detergent then were allowed to stand for 20 min at 23°C. Lysis that occurred during this time was accompanied by a marked clearing in the cell suspension and a dramatic increase in viscosity. Lysis as measured by release of trichloroacetic acid-insoluble material from centrifuged cell lysates (see reference 32) was always greater than 90%.

Dye-buoyant density centrifugation. Cell lysates prepared in the above fashion were cleared by centrifugation in a Sorvall SS34 rotor at 4°C for 30 min at 20,000 rpm (~48,000 × *g*), using a Sorvall RC-5 Superspeed refrigerated centrifuge. Approximately 2 to 2.5 ml of supernatant fluid could be decanted after this clearing step, which usually resulted in the sedimenting of about 60 to 70% of the ^{3}H radioactivity. A 2-ml volume of this cleared lysate was mixed with 1.9 ml of sterile water, 1.6 ml of a 2-mg/ml EB solution (in phosphate buffer, pH 7), and 5.1 g of CsCl. This mixture then was placed in a polyallomer centrifuge tube, overlaid with light mineral oil, and centrifuged in a Beckman 50 Ti rotor, using a Beckman L5-50 preparative ultracentrifuge. Centrifugation was for 60 h at 40,000 rpm and 20°C. Fractionation of gradients and assay of radioactivity were as previously described (35), except that Amersham/Searle organic counting scintillant liquid scintillation cocktail was employed in lieu of 2,5-bis[2-(5-*tert*-butylbenzoxazolyl)]-thiophene (BBOT)-toluene.

The analysis of crude lysates was essentially the same as that described above, except that, after lysis by Sarkosyl, the lysate was subjected to shearing by being drawn in and out of a 1-ml pipette 20 times. Usually 0.8 ml of the sheared lysate (plus 3.1 ml of water; EB and CsCl, same as above) was analyzed by dye-buoyant density equilibrium centrifugation. Fractions representing covalently closed circular (CCC) plasmid DNA obtained from the analysis of either cleared or crude lysates were pooled and extracted two times with isoamyl alcohol to remove EB. This material then was dialyzed against TES buffer [0.05 M NaCl, 0.05 M tris(hydroxymethyl)-aminomethane, and 0.005 M ethylenediaminetetraacetate, pH 8.5]. Such preparations of purified plasmid DNA were stored at −35°C.

Sedimentation velocity centrifugation. The conditions and methods for both neutral and alkaline sucrose gradient centrifugation and fractionation have been described previously in detail (35). Samples to be analyzed by using alkaline sucrose gradients were first adjusted to 0.25 N NaOH and allowed to stand for 10 min at room temperature before being layered onto the gradient. Recovery of input radioactivity from sucrose gradients was always greater than 85%. Preparation of ^{14}C-labeled R6K plasmid DNA for use as a sedimentation reference was accomplished as previously described (35).

Isolation of bulk cellular DNA. Strains used in studies on the kinetics of DNA reassociation were as follows: *E. coli* B (χ8 from the collection of R. Curtiss III); *S. mutans* GS-5 and SL1 (from A. Coykendall). DNA was extracted from 2-liter cultures grown to late log phase in BHI broth. The procedure (appropriately scaled-up) was exactly as described by Bøvre and Szybalski (1). The following modifications were employed in treating the *S. mutans* strains. Lysozyme was added to a final concentration of 1.2 mg/ml and incubated for 2 h at 37°C followed by protease treatment (final concentration, 1.2 mg/ml) for 30 min at 37°C prior to freezing. The addition of sodium dodecyl sulfate to effect lysis was followed by a 30-min incubation at 60°C. Two or three freeze-thaw steps (ethanol-dry ice, 60°C water bath) were usually implemented to complete lysis. All other steps in the DNA purification were identical to those described by Bøvre and Szybalski (1). The DNA finally was dialyzed against 1× SSC buffer (0.15 M NaCl, 0.015 M sodium citrate) and stored at −35°C. DNA prepared by this method had an absorbance ratio (A_{260}/A_{280}) equal to or greater than 2.0

Free-solution kinetics of DNA reassociation. Measurement of DNA renaturation was performed by the method of Bradley (2). The DNA preparation was adjusted so as to effect a concentration of 8× SSC and then suitably diluted to give an A_{260} of 0.8. The DNA was sheared by sonic oscillation in a Biosonik III (Bronwill Scientific, Rochester, N.Y.) at a rheostat setting of 90. A total of 3 min of sonic oscillation was used (10 s of oscillation followed by 20 s of rest). The vessel containing the DNA was immersed in an alcohol-ice bath during sonic treatment. The sheared DNA sample was subjected to low-speed centrifugation (10,000 × *g*, 4°C) to remove metal originating from the titanium sonic probe. Formamide was added to the sheared DNA sample to a final concentration of 25% (vol/vol). Addition of formamide was such that the SSC concentration was reduced to 6×. The DNA (concentration, ca. 40 μg/ml) was heat-denatured in a recording spectrophotometer with an attached temperature programmer (Gilford Instruments Lab, Oberlin, Ohio). The denaturing temperature was maintained for 20 min after attainment of maximal hyperchromicity at 270 nm (270 nm was used to minimize formamide absorbance). The temperature was then adjusted to 20 to 25°C below the T_m (midpoint of hyperchromic shift in 6× SSC). Reassociation at this temperature was monitored until the DNA was approximately 75% reassociated. The slopes of the reassociation plots were determined from points obtained in the 2- to 3-h range. Genome sizes were computed by comparisons of slopes between a biological standard and the unknown. No correction was made to compensate for purported reassociation rate differences due to differences in base composition of DNA. *E. coli* B, having a genome size of 2.88×10^9 (18), was used as the biological standard in determining the molecular weight of the *S. mutans* SL1 genome. The reassociation temperature for these determinations (*E. coli* B [T_m = 84°C in 6× SSC] and *S. mutans* SL1 [T_m = 80°C in 6× SSC]) was 65°C. SL1 DNA then was used as the biological standard in determining the *S. mutans* GS-5 genome size. The reassociation temperature for these determinations (*S. mutans* SL1 [T_m = 80°C in 6× SSC] and *S. mutans* GS-5 [T_m = 76°C in 6× SSC]) was 55°C.

Electron microscopy of DNA. Purified plasmid DNA was converted to the open circular (OC) form by exposure to either 95°C for 12 to 15 min or timed gamma irradiation in a ^{60}Co irradiator (Gammacell 220). For contour length determinations, OC DNA, spread onto a 0.25 M ammonium acetate hypophase, was picked up on Parlodion-coated copper grids and rotary-shadowed with platinum-palladium (23, 25). Grids were examined and photographed in a Joel 100C electron microscope (Joel, Inc., Tokyo, Japan). Molecular measurements were obtained from electron-image plate projections (Besseler 45 MCRX enlarger) by use of a Numonics Corp. electronic graphics calculator. Either the pSC101 (6.02×10^6 [9.1 kilobase pairs]) or pSC185 (9.2×10^6 [13.9 kilobase pairs]) plasmid molecule served as an internal length standard (25).

RESULTS

Examination of clinical *S. mutans* isolates for plasmid DNA. Prior to examination for plasmid DNA, strains were verified as *S. mutans* by the criteria of colonial morphology on mitis salivarius agar and ability to ferment mannitol and sorbitol. Strains were radioactively labeled and lysed as described above. Lysis by the glycine treatment regimen was always greater than 90%. Cleared Sarkosyl lysates of 48 of the strains examined by CsCl-EB centrifugation displayed only a single component at a position in the gradient consistent with contaminating chromosomal DNA. In some instances, a "shoulder" approximating the position occupied by CCC plasmid DNA was observed. Although such "shoulders" could be repeatedly seen in independent experiments, we were never able to demonstrate CCC DNA in these strains.

Two (V310 and V318) of the 50 strains displayed two bands after analysis by CsCl-EB centrifugation; the lower band was consistent with CCC plasmid DNA. Figure 1 depicts representative radioactivity profiles of CsCl-EB gradients for strains V310 and V318. In both instances, lower (plasmid DNA) band components are seen at fraction 15, while contaminating chromosomal DNA is seen as a peak occurring at about fraction 22.

Velocity sedimentation analysis of CCC DNA from *S. mutans* V310 and V318. Plasmid DNA recovered from CsCl-EB gradients was purified by dialysis and subjected to velocity sedimentation centrifugation. Figure 2 presents data obtained for both plasmid preparations after centrifugation through 5 to 20% neutral and alkaline sucrose gradients. Despite the independent origin of V310 and V318 and their differing physiological properties (see below), their plasmid contents appear very similar, if not identical. Neutral sucrose gradient centrifugation reveals a major component sedimenting at fraction 8 in these gradients. Based on cosedimentation studies with ^{14}C-labeled R6K plasmid DNA (sedimentation coefficient, 51S), this component corresponds to an *s* value of 22S. Of interest in both these neutral sucrose gradient profiles is the minor component seen at about fraction 13. This peak generally falls in the 30S to 36S range and usually comprises about 20% of the total radioactivity of the gradient. The leading region of this component (fractions 14 through 19 in both panels A and C) always appears as a gradual slope in contrast to the well-defined nature of the 22S component.

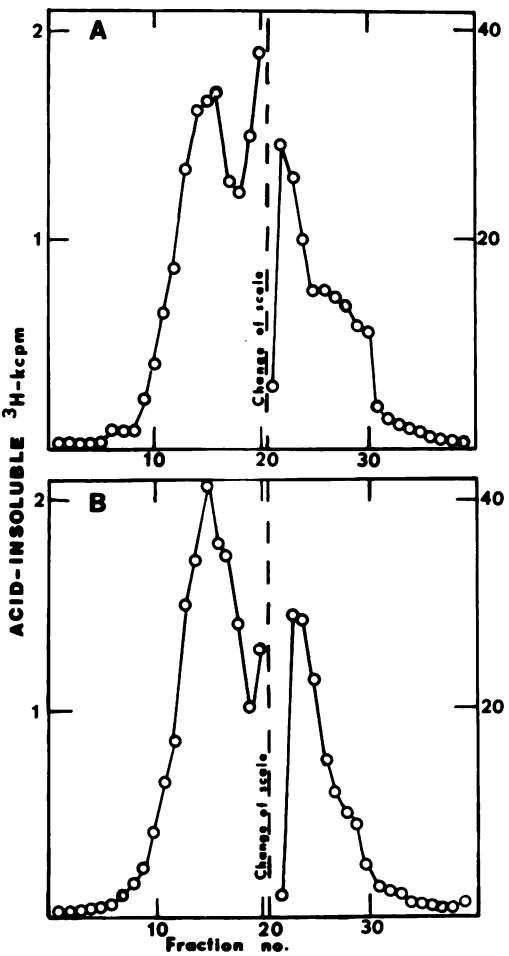

FIG. 1. *Dye-buoyant density gradient analysis of S. mutans V310 and V318. Strains V310 and V318 were radiolabeled, lysed, and processed as described in the text. Samples of cleared lysates were centrifuged to equilibrium in CsCl-EB gradients. Gradients were fractionated into tubes, and 10-μl samples of each fraction were spotted onto Whatman 3MM filter paper disks. The disks were processed as described and counted by liquid scintillation spectrometry to yield the ³H-radioactivity profiles. Increasing density is from right to left. (A) S. mutans V310; (B) S. mutans V318.*

This characteristic shape suggested that this minor component consisted of high-molecular-weight forms of the 22S plasmid, e.g., interlocked and/or oligomeric catenated dimers. Indirect evidence supporting the presence of interlocked dimer plasmid molecules is presented in Fig. 3. CCC DNA obtained from CsCl-EB gradients of cleared lystes was centrifuged to equilibrium in a second CsCl-EB gradient. In such gradients the lower band corresponding to CCC DNA is seen at about fraction 20, and the component representing open circular and/or linear DNA is seen at fraction 28. In both cases, however, the lower band is skewed toward the less-dense side. This intermediate region (between the CCC and OC components) represents the position where interlocked plasmid dimers consisting of one open and one closed circular monomer would occur (23, 25). Definitive evidence in support of the presence of interlocked dimeric molecules is given below.

Alkaline sucrose gradient analyses of plasmid DNA preparations from strains V310 and

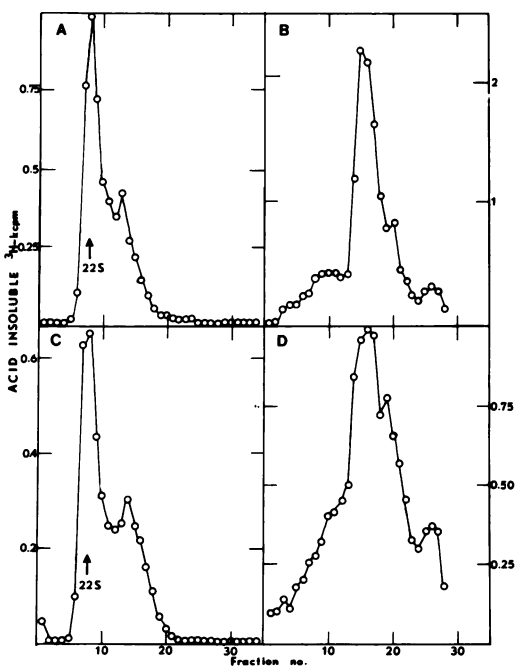

FIG. 2. *Velocity sedimentation analysis of CCC DNA from strains V310 and V318. Neutral and alkaline sucrose gradient analyses were performed on purified plasmid preparations obtained from CsCl-EB gradients. Conditions for neutral gradients were: Beckman SW41 Ti rotor; 40,000 rpm; 15°C for 170 min. Conditions for alkaline sucrose gradients were: Beckman SW50.1 rotor; 45,000 rpm; 23°C for 100 min. Sedimentation is from left to right. (A) Neutral gradient ³H-radioactivity profile of CCC DNA from strain V310. (B) Alkaline gradient ³H-radioactivity profile of CCC DNA from strain V310. (C) Neutral gradient ³H-radioactivity profile of CCC DNA from strain V318. (D) Alkaline gradient ³H-radioactivity profile of CCC DNA from strain V318. Sedimentation coefficient of the major component in both neutral sucrose gradients was previously determined by co-sedimentation with differentially ¹⁴C-labeled R6K plasmid DNA (35).*

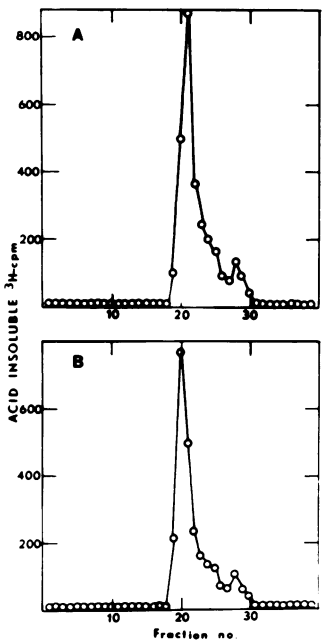

FIG. 3. *Dye-buoyant density centrifugation of purified plasmid DNA. Plasmid DNA, purified from CsCl-EB gradients prepared from cleared Sarkosyl lysates, was recentrifuged in CsCl-EB. Increasing density is from right to left. Conditions of centrifugation were: Beckman SW41 Ti rotor; 35,000 rpm; 20°C for 60 h. (A) pVA310. (B) pVA318.*

V318 are presented in Fig. 2. The major, fast-moving DNA species is seen in both gradients at about fraction 15. This corresponds to the 22S component seen on neutral sucrose gradients. This rapid sedimentation in alkali verifies the CCC nature of this DNA. Components sedimenting at about fractions 19 and 20 are also seen, consistent with the 30S- to 36S-sedimenting species seen in neutral sucrose gradients. The trailing component seen in both gradients at about fraction 10 is thought to represent the alkali denaturation product of a CCC-OC interlocked dimer. This species would move more slowly than the CCC monomer in alkali (26).

Electron microscopy of plasmid DNA. Purified plasmid DNA obtained from CsCl-EB gradients of Sarkosyl-cleared lysates (Fig. 1) was examined in an electron microscope. Fractions representing lower bands from such gradients were pooled and dialyzed before examination. No attempt was made to separate the major (22S) DNA species from the minor fast-moving DNA species. Streptococcal plasmid molecules always were mixed with plasmids of known molecular weight before spreading onto grids. Contour lengths of the streptococcal and reference plasmids were measured by using photographs taken from the same grid square. These studies revealed the predominating plasmid species in strains V310 and V318 to be a single molecular species in the 3.6×10^6-molecular-weight range. The molecular weight data are given in Table 1. We have assigned these plasmids the designations pVA310 (from strain V310) and pVA318 (from strain V318) in accordance with the plasmid nomenclature suggested by Novick et al. (30). From the data in Table 1 we conclude that pVA310 and pVA318 are statistically indistinguishable from one another with respect to molecular weight. Further examination of plasmid preparations obtained from strains V310 and V318 did yield high-molecular-weight forms consisting of interlocked circular catenates. The occurrence of these molecules was consistent with the velocity and equilibrium sedimentation data previously discussed (Fig. 2 and 3 and related discussion). A composite of representative electron micrographs of pVA310 and pVA318 plasmid preparations is presented in Fig. 4. Based on contour length measurements, we conclude that the interlocked molecules are dimers of pVA310 and pVA318. When large numbers of molecules (>500) were scanned in the electron microscope, the catenated forms represented about 3% of the total molecules seen.

Estimation of number of plasmid copies per chromosome. Quantitation of plasmid DNA may be readily accomplished by comparison with the amount of chromosomal DNA observed after CsCl-EB centrifugation of crude Sarkosyl lysates. Using such information, determination of the number of plasmid copies per genome equivalent can be determined if the molecular weights of both the plasmid and host genome are known. Since no data on the genome size of *S. mutans* were available, we obtained molecular-weight estimates from two well-characterized *S. mutans* strains, SL1 and GS-5. Free-solution reassociation kinetics of denatured DNA were measured and compared

TABLE 1. *Contour length measurements and molecular size of S. mutans plasmid DNA*

Plasmid	N[a]	Kilobase equivalent	Megadaltons[b]
pVA310	27	5.55 ± 0.09[c]	3.68 ± 0.06
pVA318	21	5.47 ± 0.12[d]	3.62 ± 0.08

[a] Number of molecules measured.
[b] Molecular size ± standard error.
[c] Measurements made by using pSC185 (13.9 kilobases) as a reference molecule (25).
[d] Measurements made by using pSC101 (9.1 kilobases) as a reference molecule (25).

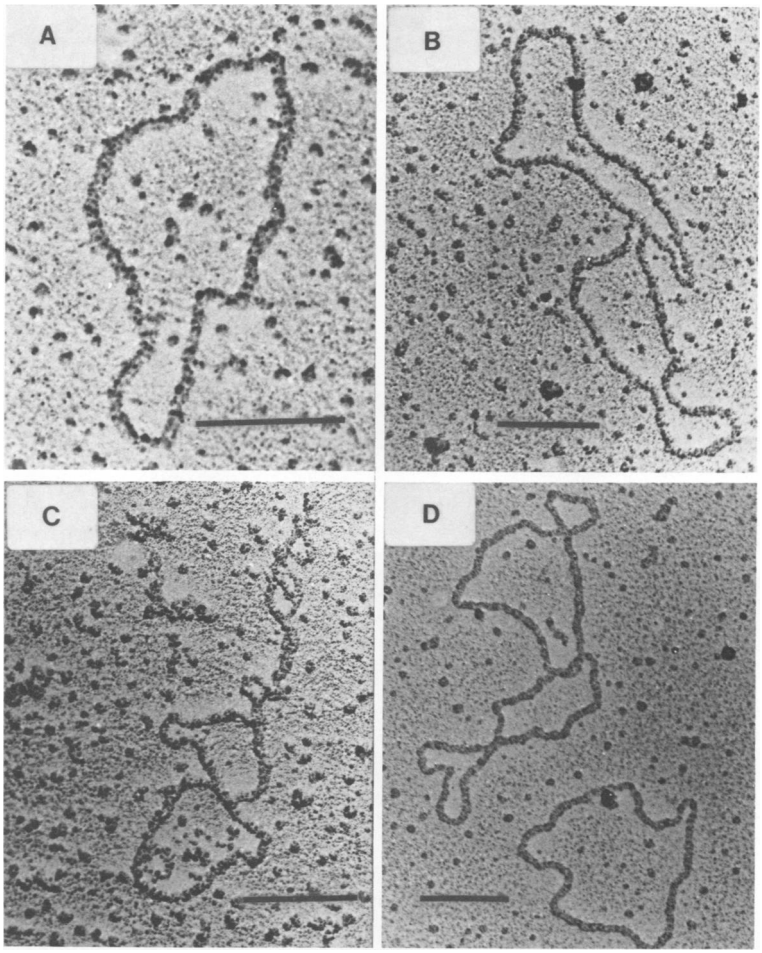

FIG. 4. *Electron micrographs of purified plasmid DNA. (A) OC of pVA310. (B) Catenated dimer of pVA310, consisting of interlocked OC molecules. (C) Catenated dimer of pVA310, consisting of a CCC monomer interlocked with an OC monomer. (D) Catenated dimer of pVA318, consisting of two interlocked OC monomers; an OC monomeric molecule of pVA318 is also seen in this panel. The scale in each photograph represents 0.25 μm.*

with the rate of reassociation of DNA from a genome of known molecular size. The molecular-weight values from these two *S. mutans* strains may be seen in Table 2. First the molecular weight of strain SL1 was calculated with *E. coli* B DNA as a reference. Then the molecular weight of the GS-5 genome was determined from its reassociation kinetics, using strain SL1 as the biological standard. Strain GS-5 belongs to the same biotype (I; see references 8 and 34 and Table 4) as strains V310 and V318. We will assume a molecular-weight value of 1.79×10^9 for all strains belonging to this biotype.

Crude lysates of strains V310 and V318 were analyzed on CsCl-EB gradients, and the amounts of plasmid DNA were compared with that of chromosomal DNA by summation of radioactivity under the respective peaks. The data (Table 3) were quite similar for both strains. The plasmid represented approximately 4% of the chromosomal DNA in each strain, reflecting some 20 plasmid molecules per genome equivalent. This estimate includes the monomeric molecular components of the catenated dimers known to be present in the lower band of such gradients.

Physiological studies of selected strains. In attempting to identify plasmid-specified phenotypes, we comparatively studied the plasmid-containing *S. mutans* strains V310, V318, and V303 (3×10^6 multicopy plasmid-containing

TABLE 2. *Genome size estimates for S. mutans*

Strain	Antigenic group[a]	Genetic group[b]	Guanine plus cytosine (mol%)[c]	Genome size (daltons, $\times 10^9$)
SL1	*d*	III	45.1	1.60 ± 0.08[d]
GS-5	*c*	I	37.7	1.79 ± 0.15[e]

[a] Unique antigens as determined by Bratthall (3).
[b] Based on DNA sequence homology by Coykendall, using molecular hybridization studies (8).
[c] See reference 8.
[d] Molecular-size estimate ± standard deviation, using *E. coli* B DNA as a standard. Molecular size of the *E. coli* B genome equal to 2.88×10^9 daltons (18). *S. mutans* genome size estimate based on an average of three determinations.
[e] Molecular-size estimate ± standard deviation, using *S. mutans* SL1 DNA as a standard. Based on an average of three determinations.

TABLE 3. *Plasmid copy numbers*

Plasmid	Plasmid molecular size (daltons, $\times 10^6$)	Plasmid DNA as % of chromosome[a]	Plasmid copies per 1.79×10^9-dalton chromosomal equivalent
pVA310	3.7	4.1	~20
pVA318	3.6	4.0	~20

[a] Based on an average of at least two independent determinations.

strain from Clewell's laboratory [11]). Five nonplasmid-containing strains (two well-characterized laboratory strains [V153 and V286, see reference 8] and three recent plaque isolates [V316, V327, and V333]) were included in these studies as well. Table 4 lists these strains and relevant phenotypic data. All of these strains were subjected to biotyping by the scheme of Shklair and Keene (34). This scheme is based on fermentation of key carbohydrates, the production of acid from mannitol in the presence of bacitracin, and the production of ammonia from arginine. The scheme correlated consistently with both serological typing (based on the Bratthall [3] *S. mutans*-specific antigens and the Lancefield E antigen) and the genetic groupings (based on DNA-DNA homology) of Coykendall (8). The strains tested all belonged to biotype I or V. The previously reported plasmid-containing strain (11), V303, belonged to biotype V in contrast to V310 and V318, which represented biotype I. Specifically, V303 possessed the Lancefield E antigen and failed to ferment melibiose, whereas V310 and V318 failed to react with Lancefield E antiserum but did ferment melibiose.

The production of bacteriocins (antibiotic proteins) is known to be plasmid specified in both gram-negative and gram-positive bacte-

TABLE 4. *Selected characteristics of principal S. mutans strains used in this study*

Strain no. (this laboratory)	Carbohydrate fermentation[a]					Ammonia from L-arginine[b]	Serotype (if known)	Shklair and Keene biotype[c]	Source and/or alternate designation
	Mannitol	Sorbitol	Raffinose	Melibiose	Acid from mannitol in presence of 2 U of bacitracin/ml				
V153	+	+	+	+	+	−	Bratthall *c*	I	GS-5; from A. Coykendall (8)
V286	+	+	+	+	+	−	Bratthall *c*	I	NCTC 10449; from A. Coykendall (8)
V303	+	+	+	−	+	−	Lancefield E[d]	V	LM-7; from D. Clewell (11)
V310	+	+	+	+	+	−	No E antigen[d] present	I	Human plaque isolate; from I. Shklair
V316	+	+	+	+	+	−	ND[e]	I	Human plaque isolate; from I. Shklair
V318	+	+	+	+	+	−	No E antigen[d] present	I	Human plaque isolate; from I. Shklair
V327	+	+	+[f]	−	+	−	ND	V	Human plaque isolate; from I. Shklair
V333	+	+	+[f]	−	+	−	ND	V	Human plaque isolate; from I. Shklair

[a] +, Acid produced; −, no acid produced.
[b] +, NH_3 produced; −, NH_3 not produced.
[c] See reference 34; based on characteristics presented in columns 1 through 6 of this table.
[d] Confirmed by protocol described in Materials and Methods.
[e] Not done.
[f] Acid production weak at 48 h.

ria. Since bacteriocin production is prevalent in the *S. mutans* group (10, 19, 33), we tested the three plasmid-containing strains for their ability to produce bacteriocins (Table 5). All three strains were active against a clinically isolated group A *Streptococcus*; however, the range of activity against the *S. mutans* strains varied markedly. V310 showed the widest activity; V318 showed an intermediate activity, and V303 was not active against any of the *S. mutans* strains used in these studies. Strain V310 killed both of the other plasmid-containing strains (V303 and V318) but itself was not susceptible to any bacteriocin activity. These data do not establish any correlation between plasmid presence and bacteriocin production.

The plasmid-containing and plasmidless *S. mutans* strains were also examined for resistance to antibiotics while we were testing the hypothesis that one or more of these extrachromosomal elements were R plasmids. Strains were first screened by a disk diffusion assay.

TABLE 5. *Bacteriocin-like activity patterns of plasmid-containing S. mutans strains*

Indicator strain[a]	Activity of strain[b]:		
	V303	V310	V318
V153	−	+	−
V286	−	+	+
V303	−	+	−
V310	−	−	−
V316	−	+	−
V318	−	+	−
V327	−	+	−
V333	−	+	+
Streptococcus[c] group A (V373)	+	+	+

[a] See Table 4 for strain description.

[b] +, Bacteriocin production as defined by a 1-cm (diameter) or greater zone of inhibition surrounding the producing colony; −, no production.

[c] Kindly provided by Esther Figuly, Bureau of Microbiological Sciences, Consolidated Laboratory Services, Commonwealth of Virginia, Richmond.

The strains listed in Table 4 proved to be susceptible to penicillin, ampicillin, chloramphenicol, erythromycin, novobiocin, tetracycline, clindamycin, and cephalothin. Resistance of a varying degree was noted for the aminoglycoside antibiotics, and relative resistance levels thus were determined for each of the strains (Table 6). Clinically significant resistance to streptomycin and kanamycin was noted in all strains except V333; however, no correlation can be made as regards this resistance and the presence of any of the plasmids known to exist in strain V303, V310, or V318. Gentamicin resistance levels were not clinically significant in any of the strains tested.

Finally, all of the strains listed in Table 4 were tested for their hemolytic properties on blood agar (5% blood in BBL blood agar base). Human, sheep, horse, and rabbit blood agar plates were used in these studies. With two exceptions, no hemolysis was observed with any of these strains. However, strains V310 (plasmid containing) and V333 (plasmidless) displayed a beta-type hemolysis on rabbit blood agar plates. Thus, there appeared to be no correlation between plasmid content and rabbit blood hemolysis.

DISCUSSION

Based on the examination of 50 fresh human plaque isolates of *S. mutans* in this study, the frequency of occurrence of extrachromosomal DNA is 4%. This value is somewhat low when compared with the occurrence of plasmids in other gram-positive and gram-negative bacteria (15) but is consistent with earlier screening studies using well-characterized laboratory reference strains of this species (6, 32). Namely, 1 strain (LM-7) of the 20 screened contained a

TABLE 6. *Susceptibility of selected S. mutans strains to antibiotics*

Strain	Susceptibility[a] to antibiotic:											
	Gentamicin			Kanamycin					Streptomycin			
	1[b]	5	10	1	10	20	30	50	1	10	20	30
V153[c]	+	+	−	+	+	+	+	−	+	+	+	−
V286	+	−	−	+	+	+	+	−	+	+	+	−
V303	+	+	−	+	+	+	+	−	+	+	+	−
V310	+	+	−	+	+	+	−	−	+	+	+	−
V316	+	+	−	+	+	+	−	−	+	+	+	−
V318	+	+	−	+	+	+	+	−	+	+	+	−
V327	+	+	−	+	+	+	+	−	+	+	+	−
V333	+	+	−	+	+	−	−	−	−	−	−	−

[a] +, Growth; −, no growth.

[b] Concentration of antibiotic in micrograms per milliliter.

[c] See Table 4 for strain description.

small plasmid (molecular weight, 3×10^6) present in multiple copies per chromosomal equivalent. Our rationale in implementing the current studies with fresh plaque isolates was to exclude the possibility that the laboratory strains had lost any plasmid DNA due to excessive subculture in vitro. The results presented in this paper, taken together with those of earlier work (6, 32), clearly establish the infrequent occurrence (<10%) of extrachromosomal elements in *S. mutans*. In this vein, it should be pointed out that CCC plasmid DNA easily converted to the OC form might go undetected by the CsCl-EB centrifugation technique. At present, however, we have no evidence to indicate this to be a problem in our screening studies.

Physical characterization of the plasmids reported in this work indicated that they are identical in molecular weight (3.6×10^6) and are both present to the extent of about 20 copies per chromosomal equivalent (Tables 1 and 3). This latter parameter was estimated after the determination of the genome molecular weight of a plasmid-free *S. mutans* strain (GS-5) belonging to the same biotype (Table 4) as the two plasmid-containing strains (V310 and V318). Genome weight determination of a second *S. mutans* (SL1) strain was necessitated so that this strain could serve as a biological standard for comparison with the GS-5 strain (the moles percent guanine-plus-cytosine content of SL1 is intermediate [8] relative to that of *E. coli* B [18] and GS-5 [8]). The genome size estimations (Table 2) of both of the *S. mutans* strains examined here are not dissimilar to those obtained for other streptococcal species by DNA reassociation kinetics (e.g., 1.47×10^9 daltons for *S. faecalis* and 1.27×10^9 daltons for *S. pyogenes* [28]).

Further evidence supporting the similarity between pVA310 and pVA318 lies in the presence of the high-molecular-weight forms (interlocked catenated dimers) in both of the strains containing these plasmids. Work in other plasmid systems (*E. coli* [26] and *Staphylococcus aureus* [31]) has established that such interlocked molecules are replicative intermediates in the plasmid life cycle and that these dimers are ultimately processed to yield two monomeric CCC molecules. It seems reasonable to speculate that the dimeric catenanes of pVA310 and pVA318 are replicative intermediates. When examined by velocity sedimentation (Fig. 2), the amount of catenated molecules of pVA310 and pVA318 usually represented between 15 and 25% of the total plasmid DNA recovered from the lower band of preparative CsCl-EB gradients. Interlocked circular dimers of the R6K plasmid of *E. coli* (26×10^6-dalton plasmid conferring streptomycin and ampicillin resistance) may be readily isolated by CsCl-EB centrifugation (24, 26, 29). Such dimers may be present to the extent of 20 to 30% of the total plasmid DNA (24; unpublished data). This value is similar to the amount of catenated molecules seen in preparations of pVA310 and pVA318. The low percentage (3 to 5%) of dimers seen in the electron microscope is a minimum estimate and is undoubtedly due to the inability to view large enough populations of molecules by this technique.

The 3×10^6-dalton plasmid of *S. mutans* LM-7 (designated pAM7 by Clewell; personal communication) shares a striking size resemblance to pVA310 and pVA318. In fact, we have been unable to show a statistically significant difference in the contour lengths of pVA310, pVA318, and pAM7 (unpublished data). However, the amount of pAM7 plasmid DNA relative to chromsomal seen on CsCl-EB gradients of crude lysates averages about 1.7% (11, 32). This represents a plasmid copy number of 10 molecules per 1.79×10^9 genomic equivalents. This is in sharp contrast to the copy number of 20 for pVA310 and pVA318.

Our physiological comparison of the plasmid-containing and non-plasmid-containing *S. mutans* strains (Tables 4 through 6) fails to provide any clues as to possible plasmid function in this species. The bacteriocin data (Table 5), however, are useful in establishing that the three plasmid-containing strains represent unique bacteriocin-producing types. Thus, although pVA310 and pVA318 seem to be identical extrachromosomal DNA systems, they appear to be present in strains that are physiologically unique, based on the criterion of bacteriocin production.

Our current studies are aimed at further attempting to elucidate the function of these plasmid elements. Irrespective of whether or not such phenotypic function(s) is(are) immediately established, these plasmids can be exploited in experiments designed to genetically transpose chromosomal gene sequences onto these extrachromosomal gene pools; accomplishment of this goal could result in the amplification of genes (and gene products), which would greatly facilitate the biochemical and genetic study of metabolic properties that contribute to this organism's ability to form dental plaque and to promote caries.

Recently, we began examining strains of *S. mutans* from ecological niches other than human dental plaque. A limited number of strains

of *S. mutans* isolated from infected human blood (14) and from the teeth of wild rats (9) have been investigated in this regard and plasmid-containing strains have been identified in both groups (S. S. Virgili and F. L. Macrina, manuscript in preparation). These plasmids should greatly facilitate comparative molecular and physiological studies. In addition, discovery of these plasmids (all of which are of molecular size $<5 \times 10^6$ daltons) serves to establish the presence of an extrachromosomal gene pool in these unique *S. mutans* isolates.

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LITERATURE CITED

1. **Bøvre, K., and W. Szybalski.** 1971. Multistep DNA-RNA hybridization techniques. Methods Enzymol. 21:350–383.
2. **Bradley, S. G.** 1973. Relationship among mycobacteria and nocardiae based on deoxyribonucleic acid association. J. Bacteriol. 113:645–651.
3. **Bratthall, D.** 1970. Demonstration of five serological strains rsembling *Streptococcus mutans*. Odontol. Revy 21:142–143.
4. **Clewell, D. B., and A. E. Franke.** 1974. Characterization of a plasmid determining resistance to erythromycin, lincomycin, and vernamycin B_α in a strain of *Streptococcus pyogenes*. Antimicrob. Agents Chemother. 5:534–537.
5. **Clewell, D. B., Y. Yagi, and B. Bauer.** 1975. Plasmid determined tetracycline resistance in *S. faecalis*: evidence for gene amplification during growth in presence of tetracycline. Proc. Natl. Acad. Sci. U.S.A. 72:1720–1724.
6. **Clewell, D. B., D. R. Oliver, G. M. Dunny, A. E. Franke, Y. Yagi, J. van Houte, and B. L. Brown.** 1976. Plasmids in cariogenic streptococci, p. 713–724. *In* H. M. Stiles, W. J. Loesche, and T. C. O'Brien (ed.), Microbial aspects of dental caries. Special supplement to microbiological abstracts, vol. 3. Information Retrieval Inc., Washington, D.C.
7. **Courvalin, P. M., C. Carlier, and Y. A. Chabbert.** 1972. Plasmid-linked tetracycline and erythromycin resistance in group D "*Streptococcus*." Ann. Inst. Pasteur Paris 123:755–759.
8. **Coykendall, A. L.** 1974. Four types of *S. mutans* based on their genetic, antigenic and biochemical characteristics. J. Gen. Microbiol. 83:327–338.
9. **Coykendall, A. L., P. A. Specht, and H. H. Samol.** 1974. *Streptococcus mutans* in a wild, sucrose-eating rat population. Infect. Immun. 10:216–219.
10. **Delisle, A. L.** 1975. Production of bacteriocins in liquid medium by *Streptococcus mutans*. Antimicrob. Agents Chemother. 8:707–712.
11. **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.
12. **Dunny, G. M., and D. B. Clewell.** 1975. Transmissible toxin (hemolysin) plasmid in *Streptococcus faecalis* and its mobilization of a noninfectious drug resistance plasmid. J. Bacteriol. 124:784–790.
13. **Efstathiou, J. D., and L. L. McKay.** 1976. Plasmids in *Streptococcus lactis*: evidence that lactose metabolism and proteinase activity are plasmid linked. Appl. Environ. Microbiol. 32:38–44.
14. **Facklam, R. R.** 1974. Characteristics of *Streptococcus mutans* isolated from human dental plaque and blood. Int. J. Syst. Bacteriol. 24:313–319.
15. **Falkow, S.** 1975. Infectious multiple drug resistance. Pion Limited, London.
16. **Gibbons, R. J., and J. van Houte.** 1975. Dental caries. Annu. Rev. Med. 26:121–136.
17. **Gibbons, R. J., and J. van Houte.** 1975. Bacterial adherence in oral microbial ecology. Annu. Rev. Microbiol. 29:19–44.
18. **Gillis, M., J. DeLey, and M. DeCleene.** 1970. The determination of molecular weight of bacterial genome DNA from renaturation rates. Eur. J. Biochem. 12:143–153.
19. **Hamada, S., and T. Ooshima.** 1975. Production and properties of bacteriocins from *S. mutans*. Arch. Oral Biol. 20:641–648.
20. **Holdeman, L. V., and W. E. C. Moore (ed.).** 1972. Anaerobe laboratory manual, 2nd ed. Virginia Polytechnic Institute and Anaerobe Laboratory, Blacksburg.
21. **Horodniceanu, T., D. H. Vouanchaud, G. Bieth, and Y. A. Chabbert.** 1976. R plasmids in *Streptococcus agalactiae* (group B). Antimicrob. Agents Chemother. 10:795–801.
22. **Jacob, A. E., and S. J. Hobbs.** 1974. Conjugal transfer of plasmid-borne multiple antibiotic resistance in *Streptococcus faecalis*. var. *zymogenes*. J. Bacteriol. 117:360–372.
23. **Kleinschmidt, A. K., D. Lang, D. Jacherts, and R. K. Zahn.** 1962. Preparation and length measurements of the total deoxyribonucleic acid content of T2 bacteriophages. Biochim. Biophys. Acta 61:857–864.
24. **Kontomichalou, P., M. Mitani, and R. C. Clowes.** 1970. Circular R-factor molecules controlling penicillinase synthesis, replicating in *Escherichia coli* under either relaxed or stringent control. J. Bacteriol. 104:34–44.
25. **Kopecko, D. J., J. Brevet, and S. N. Cohen.** 1976. Involvement of multiple translocating DNA segments and recombinational hotspots in the structural evolution of bacterial plasmids. J. Mol. Biol. 107:333–360.
26. **Kupersztoch, Y., and D. R. Helinski.** 1973. A catenated DNA molecule as an intermediate in the replication of the resistance transfer factor R6K in *E. coli*. Biochem. Biophys. Res. Commun. 54:1451–1459.
27. **Lancefield, R. C.** 1933. A serological differentiation of human and other groups of hemolytic streptococci. J. Exp. Med. 57:571–595.
28. **Lethback, A., C. Christiansen, and A. Stenderup.** 1970. Bacterial genome sizes determined by DNA renaturation studies. J. Gen. Microbiol. 64:377–380.
29. **Macrina, F. L., G. G. Weatherly, and R. Curtiss III.** 1974. R6K plasmid replication: influence of chromosomal genotype in minicell-producing strains of *Escherichia coli* K-12. J. Bacteriol. 120:1387–1400.
30. **Novick, R. P., R. C. Clowes, S. N. Cohen, R. Curtiss III, N. Datta, and S. Falkow.** 1976. Uniform nomenclature for bacterial plasmids: a proposal. Bacteriol. Rev. 40:168–198.
31. **Novick, R. P., K. Smith, R. J. Sheehy, and E. Murphy.** 1973. A catenated intermediate in plasmid replica-

tion. Biochem. Biophys. Res. Commun. 54:1460–1469.
32. **Reider, J. L., and F. L. Macrina.** 1976. Plasmid DNA isolation in *S. mutans*: glycine-enhanced cell lysis, p. 725–736. *In* H. M. Stiles, W. J. Loesche, and T. C. O'Brien (ed.), Proceedings microbial aspects of dental caries. Special supplement to microbiological abstracts, vol. 3. Information Retrieval Inc., Washington, D.C.
33. **Rogers, A. H.** 1976. Bacteriocin patterns of strains belonging to various serotypes of *S. mutans*. Arch Oral Biol. 21:243–249.
34. **Shklair, I. L., and H. J. Keene.** 1974. A biochemical scheme for the separation of the five varieties of *S. mutans*. Arch. Oral Biol. 19:1079–1081.
35. **Tinnell, W. H., and F. L. Macrina.** 1976. Extrachromosomal elements in a variety of strains representing the *Bacteroides fragilis* group of organisms. Infect. Immun. 14:955–964.
36. **Yagi, Y., and D. B. Clewell.** 1976. Plasmid determined tetracycline resistance in *Streptococcus faecalis*: tandemly repeated resistance determinants in amplified forms of pAMα1 DNA. J. Mol. Biol. 102:583–600.