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Simple Method for Demonstrating Small Plasmid Deoxyribonucleic Acid Molecules in Oral Streptococci

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A simple procedure for rapidly demonstrating small plasmids (<10 megadaltons) in oral streptococci is described. Logarithmic-phase, glycine-treated cells from 1.5-ml broth cultures were converted to osmotically fragile forms and lysed with sodium dodecyl sulfate. After the hydrodynamic shearing of host chromosomal deoxyribonucleic acid, such lysates were analyzed by low-voltage agarose gel electrophoresis. Small plasmids, migrating significantly faster than chromosomal deoxyribonucleic acid under such conditions, were readily visualized by ethidium bromide staining.

The clinical and ecological roles of traits conferred by extrachromosomal genetic elements (or plasmids) in bacteria and, in particular, the streptococci have been well established. Such traits include transmissible antibiotic resistance (3, 5, 13), hemolysin production (2), and bacteriocin production (2, 5). Streptococcal plasmids have also been implicated in various catabolic processes (7). Our interest has centered specifically on the occurrence and function of plasmids in the oral streptococci. Studies from our laboratory have revealed a paucity of plasmids in the oral streptococci. Further, we have been unable to ascribe any phenotypic function to the naturally occurring plasmids of *Streptococcus mutans* (10-12). This is in contrast to reports which claim the involvement of plasmids in certain aspects of sucrose metabolism associated with *S. mutans* (4, 6).

The physical demonstration of plasmid deoxyribonucleic acid (DNA) is a pivotal aspect of the study of extrachromosomally inherited traits in the oral streptococci. Recently, Weisblum et al. (16) reported the use of an agarose gel system for determining plasmid copy number in *Bacillus subtilis*. We have modified and adapted their basic methodology in developing a simple and rapid means for demonstrating the presence of small plasmids in *S. mutans*, *Streptococcus ferus* (formerly *S. mutans* subsp. *ferus*), and *Streptococcus sanguis*. This technique can be performed in clinical microbiology or research laboratories with readily available, relatively inexpensive equipment. As many as 36 separate bacterial stains can be examined with ease by a single individual within 24 h.

We chose five strains of plasmid-containing oral streptococci for use in the development of this plasmid screening assay. These strains are described in Table 1. Represented are oral streptococcal species that contain plasmids of both high and low copy number and that range in size from 2.4×10^6 to 17×10^6 daltons (2.4 to 17 megadaltons [Mdal]). Our assay procedure was as follows. Three to five similarly appearing colonies from solid complex medium (brain heart infusion agar or Todd-Hewitt agar, Difco Laboratories, Detroit, Mich.) were used to inoculate a 2-ml Todd-Hewitt broth contained in a culture tube (13 by 125 mm). This culture was grown at 37°C overnight (~16 h) anaerobically, using the GasPak System (BBL Microbiology Systems, Cockeysville, Md.). After incubation, the culture was adjusted with fresh Todd-Hewitt broth to an optical density at 660 nm of ~0.1 to 0.2 (Spectronic 20 spectrophotometer; Bausch & Lomb, Inc., Rochester, N.Y.) in a final volume of 4 ml. This was usually accomplished by simply adding 2 ml of prewarmed Todd-Hewitt broth to the culture tube. This culture was incubated in air at 37°C (using a standing water bath) for 30 to 60 min or until the culture density reached an optical density at 660 nm of 0.3 to 0.35. Nonsterile glycine (0.2 g) was added to the culture and mixed into solution, and incubation at 37°C in air was continued for 45 min. This glycine treatment has been shown to facilitate lysozyme-mediated spheroplast formation in *S. mutans* (15). It should be noted that we have found some oral streptococcal strains to be hypersensitive to glycine treatment. Such strains began lysing shortly after addition of the recommended 5% glycine. These strains should be treated with 3.0% (or less) glycine in this assay.

After glycine treatment, 1.5 ml of cells was harvested by centrifugation. For this purpose, we used an Eppendorf Microcentrifuge (model 5412; Brinkmann Instruments Inc., Westbury, N.Y.) at room temperature with disposable 1.5-ml polypropylene tubes. However, any readily

TABLE 1. *Bacterial strains*

Strain	Lab designation	Plasmid content				Comments and/or reference(s)
		Designation	Size (Mdal)	Copies per chromosome	Plasmid-conferred phenotype	
S. mutans	V318	pVA318	3.6	~20	Cryptic	10, 11
S. ferus	V380	pVA380	2.4	~30	Cryptic	12
		pVA380-1	2.8	~30	Cryptic	
S. sanguis	V486	pVA1	7.3	~10	Erythromycin resistance	Deletion mutant of pAMβ1 (Macrina et al., Plasmid, in press)
S. sanguis	V615	pAM77	4.6	~2	Erythromycin resistance	Naturally occurring R plasmid-containing *S. sanguis* strain (17)
S. sanguis	V656	pAMβ1	17	~1	Erythromycin resistance	pAMβ1 introduced by genetic transformation (Macrina et al., Plasmid, in press)

available clinical centrifuge could be used. The cells were washed once with TES buffer [0.05 M NaCl, 0.05 M tris(hydroxymethyl)aminomethane, 0.005 M ethylenediaminetetraacetate, pH 8]. After this washing step, the cell pellet was suspended (by pipetting with a micropipette) in 40 μl of 25% glucose in ET buffer [0.005 M ethylenediaminetetraacetate, 0.01 M tris(hydroxymethyl)aminomethane, pH 8.5]. Fifteen microliters of freshly prepared 15-mg/ml lysozyme (Sigma Chemical Co., St. Louis, Mo.) was added, followed by the addition of 5 μl of 5-mg/ml ribonuclease A (Sigma Chemical Co.) prepared in 50 mM sodium acetate, pH 5 (the ribonuclease solution was heated for 10 min at 80°C before use and could be stored for several weeks at −20°C). This enzyme-treated cell suspension was allowed to stand at room temperature (20 to 23°C) for 1 h, after which time 15 μl of 6-mg/ml protease (Sigma Chemical Co.) in TES was added. Protease preparations were always freshly made and allowed to self-digest at 37°C for 60 min before use. Incubation in the presence of protease was for 30 min at room temperature. This lysozyme-protease treatment renders the cells osmotically fragile (15).

Lysis of the osmotically fragile cells was effected by the addition of 75 μl of 2% (wt/vol) sodium dodecyl sulfate prepared in 0.01 M tris(hydroxymethyl)aminomethane, pH 8. The cell suspensions were allowed to stand at room temperature for 60 to 90 min in the presence of sodium dodecyl sulfate. These lysates were then placed at −35°C (or below) until frozen and then placed in a 60°C water bath for 5 min. The lysates then were agitated for two 30-s intervals at top speed with a Vortex mixer. This step resulted in the hydrodynamic shearing of the chromosomal DNA, rendering linear fragments of 10 to 20 Mdal in size.

A 25- to 35-μl amount of such a sheared lysate was directly analyzed by electrophoresis through 0.7% agarose with a tris(hydroxymethyl)aminomethane-borate buffer system (14). Larger amounts of lysate were found to result in the distortion of the DNA bands and in slight alterations in their migration rates through agarose. This presumably is due to the high solute concentration in the lysate. We have successfully used both vertical and horizontal slab gel units with path lengths of 10 to 20 cm. The running voltage was 2 to 3 V/cm of gel length; under such conditions, electrophoresis was allowed to proceed for 16 h (i.e., overnight) at room temperature. Gels were stained for 15 min in water containing 5 to 10 μg of ethidium bromide per ml. For best results, stained gels were placed in a running water bath for 30 min to effect destaining. Destained gels were photographed in front of shortwave ultraviolet light with a Polaroid MP4 camera and Polaroid type 55 P/N film. Exposures of 2.5 to 5 min usually were necessary to visualize the fluorescent plasmid bands. A Wratten no. 9 gelatin filter was used behind the camera lens. Obviously, depending on availability, a variety of camera systems could be easily substituted to document the gel.

Figure 1 is a photograph of a vertical agarose (0.7%) slab gel (10 cm long by 3.8 mm thick) upon which the strains described in Table 1 were analyzed. Lane A is a collection of closed circular plasmid size reference molecules (9). Lanes B, C, D, and E are dye-buoyant density (10) gradient-purified plasmid DNA preparations from strains V318, V380, V486, and V615, respectively. A small amount of contaminating chromosomal DNA is seen in each of these lanes (designated "chrom"). Faint bands (migrating behind major plasmid bands) correspond to open circular or linear forms (generated upon storage) of the

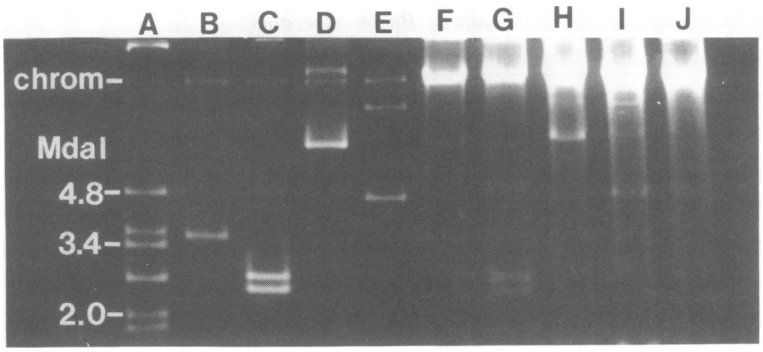

FIG. 1. *Electrophoretic analysis of streptococcal plasmids and crude lysates. (A) Size reference closed circular molecules obtained from Escherichia coli V517 (9). Sizes (Mdal) are given to the left of the lane. (B) pVA318, 3.6 Mdal. (C) pVA380, 2.4 Mdal, and pVA380-1, 2.8 Mdal. (D) pVA1, 7.3 Mdal. (E) pAM77, 4.6 Mdal. (F, G. H, I, and J) Crude, sheared lysates of V318 (pVA318), V380 (pVA380-pVA380-1), V486 (pVA1), V615 (pAM77), and V656 (pAMβ1). chrom, Chromosomal DNA fragments. Slower-migrating components are corresponding open circular or linear plasmid molecules generated upon storage (lanes B through E) or during the extraction procedure (lanes F through I).*

respective plasmid molecules. Crude lysates of V318, V380, V486, and V615 were analyzed in lanes F, G, H, and I. All show components which migrated consistently with the closed circular plasmid molecules known to be present in the respective strains (open circular forms are again seen as slowly migrating bands). An intensely staining chromosomal component was seen in each lysate. Note that analysis of strain V656 (lane J) containing the 17-Mdal pAMβ1 plasmid did not reveal a distinctly identifiable plasmid component. Plasmids in this size range (and larger) could not be detected due to masking by the slowly migrating chromosomal DNA. Based on the migration of reference markers, we calculate that plasmids larger than ~9 to 10 Mdal would remain undetected by this method.

Crude lysates analyzed by this method often, but not consistently, revealed brightly staining material in the lower 25% of the gel. This material corresponds to ribonucleic acid species, including 16S and 23S ribonucleic acids, which migrate as distinct bands.

Recently, LeBlanc and Lee (8) reported a procedure for plasmid screening in streptococci. Their method yields relatively chromosome-free plasmid preparations (size range = 1.5 to 39 Mdal) from 20-ml cultures. In contrast to our procedure, it includes a denaturation and high-salt precipitation step, organic solvent extractions, and ethanol precipitation of the plasmid DNA. We feel that the LeBlanc and Lee (8) technique and our method differ significantly in approach but can be used nicely to complement one another, depending on the needs of the investigator.

In conclusion, our technique provides the means for rapidly and reliably screening both clinical and laboratory isolates of oral streptococci. It could be readily adapted, with appropriate changes in the cell lysis protocol, for use with other gram-positive and gram-negative bacteria. It provides an immediate approach to the detection of plasmids which could be involved in drug resistance in the clinical setting. This is an area of increasing importance in the streptococci (13). Furthermore, it enables one to rapidly obtain a "plasmid fingerprint" for strains harboring small plasmids, providing microbiologists with a potential epidemiological parameter for augmenting bacteriocin, bacteriophage, or serological typing. Finally, the use of small plasmids as molecular cloning vectors in streptococcal recombinant DNA systems has been suggested (12). Such systems now appear imminent (1), and the analytic procedure described here will clearly facilitate the ready detection of recombinant plasmids (in the 2- to 10-Mdal range) from large numbers of transformed clones.

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