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Rapid Screening Procedure for Detection of Plasmids in Streptococci

DONALD J. LEBLANC* AND LINDA N. LEE

Laboratory of Microbiology and Immunology, National Institute of Dental Research, Bethesda, Maryland 20205

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An enrichment procedure, yielding plasmid deoxyribonucleic acid preparations normally containing less than 5% chromosomal contamination, has been devised for the isolation of plasmids from virtually all species of streptococci. The procedure is rapid, reproducible, and inexpensive, requiring no radioisotopes or density gradient centrifugation. The procedure can be used for routine screening of several hundred isolates in a short period of time, and plasmids obtained from 10- to 20-ml cultures can readily be visualized in agarose gels.

The existence of plasmids in the genus *Streptococcus* was first reported in 1972 (4), and interest in the study of plasmids in this important group of bacteria has intensified ever since. However, very little work appears to have been done with species of streptococci common to the human oral cavity. To our knowledge, with the exception of one strain of *S. sanguis* (17) and a few isolates of *S. mutans* which apparently all harbor the same plasmid (6, 14, 15), no reports have appeared on the occurrence of extrachromosomal elements among the oral streptococci. In the past 3 years we have isolated numerous strains of streptococci from human dental plaque and have been attempting to study the plasmid DNA pool in these representatives of the oral flora.

Plasmid isolation techniques requiring dye-buoyant density gradient centrifugation were too cumbersome, expensive, and time consuming for preliminary screening of hundreds of isolates. Enrichment techniques which permit detection of plasmids directly on agarose gels, although appropriate for other bacterial genera (5, 8-10) or for certain strains of group N streptococci (11), were inadequate for plasmid screening among the oral streptococci. The major problems associated with these procedures, with regard to the oral streptococci, included (i) inadequate removal of chromosomal DNA, leading to considerable smearing on gels; (ii) residual RNA species preventing visualization of small (1 to 3 megadaltons [Mdal]) plasmid species; and (iii) poor cell lysis in some cases. By selecting critical steps from three published enrichment procedures (5, 8, 9) and incorporating techniques which enhance the lysis of oral streptococci, we have developed a rapid screening procedure for the isolation and detection of plasmid DNA in nearly all species of streptococci. Several of our isolates previously thought to be devoid of plasmid DNA, following analysis by procedures involving dye-buoyant density gradient centrifugation, were shown by our enrichment technique to harbor one or more plasmid species. The procedure has been used to screen more than 150 strains of streptococci, including nine different species of viridans streptococci isolated from clinical sources and known plasmid-containing laboratory strains representing Lancefield groups A, D, F, and N streptococci. Plasmids have been demonstrated in members of all species tested except one, *S. salivarius*.

Routine plasmid screening was performed on either exponential or stationary phase cultures grown in 20 ml of complex medium (16) containing 20 mM glucose and 10 mM L-threonine (1). Cells were harvested by centrifugation and washed with 20 ml of 10 mM sodium phosphate buffer, pH 7.0. Washed cells were suspended in 0.73 ml of 50 mM Tris-hydrochloride (pH 8.0) followed by the addition of 0.72 ml of lysozyme (40 mg/ml in Tris-hydrochloride). After 20 min of incubation at 37°C, or 5 min at ambient temperature for *S. lactis* strains (11), 0.5 ml of 0.25 M EDTA (pH 8.0) was added. The cell suspension was chilled for 5 min in ice water, and lysis was achieved by the addition of 0.5 ml of 20% sodium dodecyl sulfate (SDS) in TE buffer (50 mM Tris-hydrochloride-10 mM EDTA, pH 8.0). Cell lysis was enhanced by 15-s incubations at 55°C followed by five gentle inversions in 15 s to mix, repeated eight times (9). DNA in the lysate was denatured by the addition of 0.25 ml of 3 N NaOH (made fresh daily from a 10 N stock solution, Fisher certified), and 20 gentle inversions per min for 3 min. Unless the NaOH solution is standardized on

the day of use, the volume of alkali required may vary from day to day. Thus, it was critical to check at least one sample on any given day to be certain that after the gentle mixing the pH was between 12.25 and 12.40, as suggested by Currier and Nester (5). After alkali denaturation, the volume of the lysate was raised to 2.95 ml by the addition of water. The lysate was rapidly neutralized by the addition of 0.5 ml of 2 M Tris-hydrochloride (pH 7.0) and mixed by 10 gentle inversions in 30 s. An additional 0.5 ml of 2 M Tris-hydrochloride was added, and the lysate was mixed again as described above. High-molecular-weight chromosomal DNA was removed by SDS-NaCl precipitation (8) by adding 0.65 ml of 20% SDS in TE, followed rapidly by the addition of 1.25 ml of 5 M NaCl and immediate mixing by 20 inversions in 1 min (9). The sample was stored overnight at 4°C, and the supernatant fraction was collected after centrifugation for 30 min at 17,000 × *g*, 4°C, in an SS34 rotor (Sorvall). At this stage the preparation still contained a considerable amount of chromosomal DNA, much of which was presumably small single-stranded fragments, as well as RNA.

The single-stranded DNA and contaminating RNA were removed as follows. The volume of the supernatant fraction was doubled by the addition of water, and RNase (2 mg/ml in water, heated at 100°C for 15 min) was added to a final concentration of 100 μg/ml. After a 60-min incubation at 37°C, single-stranded DNA was removed by extraction with an equal volume of NaCl-saturated phenol (5). The aqueous phase was then extracted with an equal volume of chloroform-isoamyl alcohol (24:1). The DNA in the aqueous phase was separated from degraded RNA by ethanol precipitation. A 1/20 volume of 3 M sodium acetate and 2 volumes of 95% ethanol were added and, after mixing, the sample was stored at −20°C for 4 h or overnight. Precipitated DNA was recovered by centrifugation at 17,000 × *g* for 30 min, 4°C, in an SS34 rotor. The DNA was suspended in 100 μl of TES buffer (10 mM Tris-hydrochloride–10 mM EDTA–50 mM NaCl, pH 8.0), and 20 μl was examined by agarose gel electrophoresis (13).

Eleven species of streptococci, comprising seven isolates from human dental plaque, one strain from the NIDR collection, and three laboratory strains whose plasmid composition had been previously described, were chosen to illustrate the plasmid screening procedure described here. Relevant properties of these strains are

TABLE 1. *Properties of Streptococcus strains used to illustrate plasmid screening procedure*

Strain	Species[a]	Previously reported plasmids (Mdal)	Plasmid bands observed by new procedure (Mdal)[b]	Source of strain
A	*S. acidominimus*	None	24.0, 14.0, 12.0, 9.6, 9.3, 7.0, 3.7	Dental plaque
B	*S. sanguis* (type II)	None	13.5, 9.8, 4.2	Dental plaque
C	*S. mitis*	None	9.0, 3.2, 2.4	Dental plaque
D (DR 1501)	*S. lactis*	32.0, 29.0, 23.5, 19.0, 3.0, 1.5; LeBlanc et al. (12)[c]	32.0, 29.0, 23.5, 19.0, 17.5, 10.2, 3.0, 1.5	ATCC 11454
E	*S. mutans*	None	10.4, 3.3	Dental plaque
F	*S. sanguis* (type I)	None	23.5, 9.1	Dental plaque
G	*S. morbillorum*	None	13.0, 4.4	Dental plaque
H (DS5)	*S. faecalis*	34.0, 17.0, 6.0; Clewell et al. (3)[c]	35.0, 18.0, 12.0, 6.1, 3.1	D. B. Clewell
I	*S. MG-intermedius*	None	18.5, 17.5, 7.2	Dental plaque
J (AC1)	*S. pyogenes*	17.0; Clewell and Franke (2)[c]	39.0, 15.5	D. B. Clewell
K	*S. anginosus*	None	26.0, 12.2, 9.6	NIDR[d] collection

[a] Streptococci isolated from human dental plaque were identified according to the method of Facklam (7).

[b] Plasmid sizes were estimated from relative mobilities of the bands in the agarose gel illustrated in Fig. 1, assuming each band to represent a CCC plasmid molecule. However, in certain cases a band may represent a relaxed form or a multimer of a smaller CCC plasmid molecule.

[c] The plasmids from strains D(12), H(3), and J(2) were previously isolated from dye-buoyant density gradients, and plasmid molecular weights were determined by agarose gel electrophoresis (strain D), neutral sucrose gradient centrifugation (strains H and J), and contour length measurements (strains D, H, and J). Bands corresponding to the plasmids previously described in each of these three strains were observed in agarose gels after isolation by the enrichment procedure, although the calculated molecular weights from Fig. 1 differed slightly from those previously reported for strains H and J (2, 3). Additionally, previously unreported bands were also observed in preparations from all three strains after the enrichment procedure.

[d] NIDR, National Institute of Dental Research.

shown in Table 1. Cultures of each of these strains were enriched for plasmid DNA as described above and then examined by agarose gel electrophoresis (Fig. 1). Residual chromosomal DNA banded in the region of 25-Mdal covalently closed circular (CCC) plasmid DNA in the agarose gel illustrated in Fig. 1. We have found that the location of the chromosome band may vary from gel to gel, between the region of 20- to 25-Mdal CCC DNA, depending on the condition of electrophoresis—in particular, the electrophoresis voltage, agarose concentration, and variations in ambient temperature. Based on band intensities, as determined from densitometric tracings of the gel (data not shown), it appeared that less than 5% of the DNA remaining at the end of the procedure was of chromosomal origin. The efficiency with which the isolation procedure removed contaminating chromosomal DNA was further demonstrated by subjecting plasmid-enriched preparations, derived from cultures which had been labeled with [^{3}H]thymidine, to ethidium bromide-cesium chloride density gradient analyses. A typical result is illustrated in Fig. 2. In most instances between 80 and 90% of the labeled DNA banded in the region of CCC plasmid DNA (fractions 10 to 15). In addition, when the DNA from the "chromosome" band in dye-buoyant density gradients was examined by electron microscopy, although many of the molecules were obviously linear, a significant proportion consisted of open circles of distinct sizes (data not shown). In at least two instances the sizes calculated from contour length measurements of open circles corresponded to agarose gel bands exhibiting mobilities expected of nicked plasmids of the sizes estimated from the contour lengths.

The results presented here show that the plasmid screening procedure yields a preparation which is generally contaminated with less than 5% chromosomal DNA. The combined dye-buoyant density gradient and electron microscopic analyses provided evidence that the bands visualized in agarose gels represented distinct species of either CCC or open circular plasmid DNA.

The enrichment procedure can be used for routine screening of several bacterial strains at a time, and visualization of plasmid bands in agarose gels can usually be achieved by applying

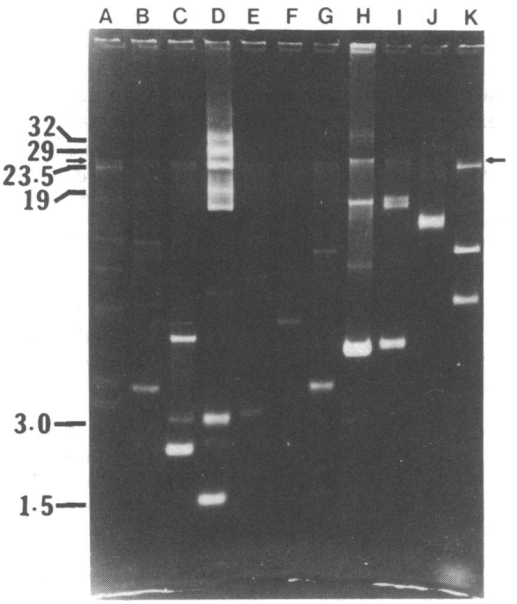

FIG. 1. *Agarose gel electrophoresis of DNA from plasmid-enriched streptococcus cultures. (A to K) Strains described in Table 1. Ethanol-precipitated DNA (20 μl), obtained as described in the text, was electrophoresed in Tris-acetate-buffered (13) 0.7% agarose for 5 h at 60 mA, constant current. DNA was obtained from stationary-phase cultures in all cases except S. lactis (D), which was in mid-exponential growth. Arrows point to region of residual chromosomal DNA. In some cases (E and K), distinct chromosome bands were not observed. Numbers correspond to bands in (D) for plasmid sizes (in Mdal) previously confirmed by electron microscopy (12) and are correlated with the migration of the plasmids in the CCC plasmid configuration.*

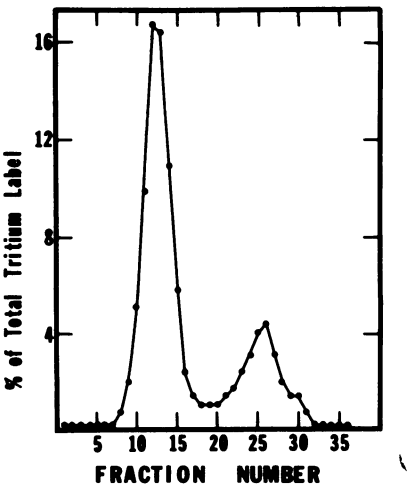

FIG. 2. *Dye-CsCl-buoyant density gradient centrifugation of DNA from plasmid-enriched S. anginosus. [^{3}H]thymidine-labeled culture of strain K described in Table 1 was used as the cell source for plasmid enrichment as described in the text. Total ^{3}H counts per minute in gradient = 27,000. Left of figure corresponds to the bottom and the right corresponds to the top.*

sample derived from the equivalent of 4 ml of culture, or less. The procedure is rapid, reproducible, and inexpensive, requiring neither radioisotopes nor density gradients for preliminary screening purposes. We have successfully employed the enrichment procedure for preparative purposes, starting with cells from 400 to 800 ml of culture. With appropriate minor modifications, the procedure has also been used for the isolation of plasmids from gram-negative and other gram-positive bacteria.

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