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Isolation of Plasmids from Mycobacteria

JACK T. CRAWFORD[1,2]* AND JOSEPH H. BATES[2,3,4]

Medical Research[1] and Medical Service[3], Veterans Administration Medical Center, Little Rock, Arkansas 72206, and Department of Microbiology and Immunology[2] and Department of Medicine,[4] University of Arkansas for Medical Sciences, Little Rock, Arkansas 72201

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A procedure for preparing sodium dodecyl sulfate-salt-cleared lysates of mycobacteria was developed. Standard techniques were employed to demonstrate covalently closed circular deoxyribonucleic acid in lysates of nine strains of *Mycobacterium avium-intracellulare*.

This paper describes the isolation of plasmid DNA from several strains of *Mycobacterium avium-intracellulare* and, to the best of our knowledge, is the first report of the isolation of plasmids from mycobacteria. The *M. avium-intracellulare* complex is a heterogeneous assemblage of slowly growing non-photochromogenic strains. These organisms are common in soil and water, and some strains are capable of causing serious infections in humans. Twenty strains were used in this study. Nine strains thought to be human pathogens were obtained from the clinical laboratory at this hospital. Eleven laboratory strains of known serotypes were obtained from the collection at the National Jewish Hospital, Denver, Colorado.

The major obstacle to the isolation of deoxyribonucleic acid (DNA) from mycobacteria is the lack of a gentle lysis procedure. All of the strains that we tested were resistant to lysozyme and were not lysed by 4% sodium dodecyl sulfate (SDS) at 60°C. However, we found that treatment of actively growing cells with cycloserine (7) rendered them sensitive to SDS. Cells were grown in 200 ml of Middlebrook 7H9 broth (Difco) containing 0.05% Tween 80 in 500-ml baffled side-arm flasks. Cultures were incubated at 37°C on a rotary shaker, and a Klett colorimeter with a no. 54 (green) filter was used to monitor the optical density. Filter-sterilized D-cycloserine (Sigma) was added to cultures in late exponential phase (about 100 Klett units) to give a final concentration of 1 mg/ml, and the cultures were incubated for an additional 8 to 16 h. Usually there was a slight drop in optical density at the end of this time. The cells were harvested by centrifugation and suspended in lysis buffer (2 ml per 200-ml culture) containing 25% sucrose, 0.05 M ethylenediaminetetraacetic acid and 0.05 M tris(hydroxymethyl)aminomethane (pH 8.0). The cells from a total of 600 to 1,000 ml of culture were pooled for each lysate. Lysozyme (1 mg/ml) was added, and the suspension was warmed to 37°C. SDS (20%) was added to give a concentration of 4%. Usually lysis occurred within 10 min at 37°C, but sometimes it was necessary to warm the mixture to 55°C for 10 min to obtain lysis. In addition to the *M. avium-intracellulare* strains, we have used this procedure to lyse *M. tuberculosis* $H_{37}Rv$ and *M. smegmatis* ATCC 607. With *M. smegmatis*, incubation for 2 h after the addition of cycloserine was sufficient. The extent of lysis varied considerably from batch to batch and was probably dependent upon the effectiveness of the cycloserine treatment. The slow and somewhat erratic growth of the cultures made it difficult to standardize the treatment.

The salt precipitation method (1) was used to prepare cleared lysates. NaCl at 5 M was added to give a final concentration of 1 M. The lysate was refrigerated overnight and then centrifuged at 12,000 rpm for 45 min. Lysates were analyzed by cesium chloride-ethidium bromide equilibrium density gradient centrifugation. Dry cesium chloride was dissolved in the lysate (50% wt/wt) in a polyallomer tube, ethidium bromide (0.3 mg/ml) was added, and the sample was centrifuged at 40,000 rpm for 40 h at 15°C in a Beckman SW65Ti or SW50.1 rotor. Bands were detected by illumination with ultraviolet light. Satellite (heavy) DNA bands, indicating covalently closed circular DNA, were observed with lysates from 9 of the 20 strains of *M. avium-intracellulare*. Four of these were laboratory strains of serotypes 2 (an *M. avium* type), 4, 12, and 18 (*M. intracellulare* types). The remaining five were wild strains. In addition, we observed a satellite band in a gradient of a lysate from *M. tuberculosis* $H_{37}Rv$. Better results were obtained using a large-capacity, fixed-angle rotor. Gradients (20 ml) containing 47% CsCl (wt/wt) in thick-walled polycarbonate tubes were centrifuged in a Beckman type 50.2 rotor at 35,000 rpm for 40 h at 15°C.

Crude plasmid DNA was prepared by extrac-

tion with redistilled phenol (Bethesda Research Labs) and ethanol precipitation (6) or by precipitation with polyethylene glycol 6000 (3). Samples were analyzed by agarose gel electrophoresis as described by Meyers et al. (6). Plasmids of known molecular weights were isolated from *Escherichia coli* and used as markers. Results for one of the wild strains (LR25) are shown in Fig. 1. This was the most readily lysed strain, and it gave the most consistent yields of plasmid DNA. Two plasmid bands were observed (Fig. 1A and B). The lower band represents a plasmid with a molecular weight of approximately 6×10^6. The upper band represents a very large plasmid which migrated considerably more slowly than the largest marker plasmid tested (Fig. 1E). The size of such large plasmids cannot be accurately determined from these gels (2). The yield of the large plasmid was low, and it was not detected in some samples. Analysis of DNA from the heavy band of cesium chloride-ethidium bromide gradients (Fig. 1F) confirmed our conclusion that the two bands represent covalently closed circular DNA. Identical results were observed with one of the other wild strains. Small plasmids similar in size to the one carried by LR25 were detected in the other seven strains. The failure to detect plasmids in the other 11 strains may be due to shortcomings in the technique.

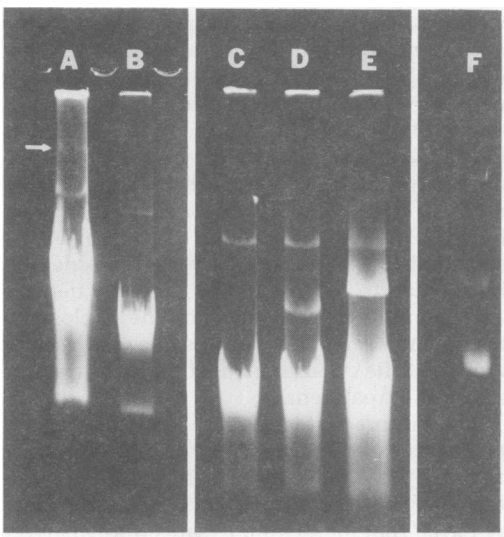

FIG. 1. *Agarose gel electrophoresis of plasmid DNA isolated from M. intracellulare LR25. DNA was subjected to electrophoresis in 0.7% agarose dissolved in buffer containing 40 mM tris(hydroxymethyl)-aminomethane base, 5 mM sodium acetate, and 1 mM ethylenediaminetetraacetic acid (pH 8.0) (5). Gel dimensions were 10 by 20 by 0.5 cm. Gels were run at 100 V for 4 to 8 h in a vertical slab gel apparatus and then stained overnight with ethidium bromide. Gels were illuminated with short-wavelength ultraviolet light for photographing. Migration was from top to bottom. (A and B) 75 μl and 25 μl of crude plasmid DNA prepared by polyethylene glycol 6000 precipitation. Upper and lower bands are covalently closed circular plasmid DNA. Middle diffuse band is chromosomal DNA. Faint band at top (arrow) probably represents the open circular form of the large plasmid. (C, D, and E) Crude plasmid DNA prepared by phenol extraction. (C) Strain LR25. (D) Strain LR25 plus plasmid RPI (molecular weight 40×10^6). (E) Strain LR25 plus plasmid R_{222} (molecular weight 70×10^6). (F) Plasmid DNA purified by cesium chloride-ethidium bromide centrifugation. Upper and lower bands correspond to plasmid DNA bands shown in (B).*

Our results indicate that covalently closed circular DNA can be isolated from lysates of mycobacteria using standard techniques. The cycloserine technique is probably applicable to a variety of mycobacteria. Identifying the functions of the plasmids may be difficult since techniques for genetic analysis of mycobacteria, especially the slowly growing strains, are poorly developed. Results suggesting the existence of an R-factor coding for streptomycin resistance in *M. smegmatis* have been reported (4). Mating of *M. smegmatis* has been reported, but no evidence of a transmissible sex factor was found (8). Certainly the very large plasmids demonstrated in two of our strains would be expected to make a major contribution to the phenotype of the cells. There is considerable interest in the *M. avium-intracellulare* complex because of the widespread distribution of the organisms in nature and their recognized importance for causing disease in man. Although there has been reasonable success in classifying the organisms by biochemical and serological means (9), there are no laboratory criteria for identifying strains which are virulent for man. The demonstration of plasmids in at least 9 of 20 strains suggests that plasmids may be common in this group, and the plasmid content of the strains may be an important marker for classification.

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