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NOTES

Characterization of a Plasmid Determining Resistance to Erythromycin, Lincomycin, and Vernamycin B_α in a Strain of *Streptococcus pyogenes*

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A plasmid determining resistance to erythromycin, lincomycin, and vernamycin B_α was isolated from a strain of *Streptococcus pyogenes*. The plasmid has a molecular weight of approximately 17×10^6 and is present to the extent of one to two copies per chromosomal genome equivalent.

Erythromycin and lincomycin are antibiotics used in the treatment of group A beta-hemolytic streptococcus (*Streptococcus pyogenes*) infections in patients for whom penicillin is contraindicated. *S. pyogenes* strains are usually susceptible to these agents, although resistant clinical isolates have been reported (7, 8, 10, 12, 13, 15). Whether resistance in this species is related to extrachromosomal (plasmid) determinants, as is often the case among drug-resistant staphylococci and enteric bacilli, has, to our knowledge, not been reported. In this communication we report on the isolation and characterization of a plasmid from a clinical isolate of *S. pyogenes* and show that it determines resistance to erythromycin, lincomycin, and vernamycin B_α (a streptogramin B-type antibiotic).

S. pyogenes strain 10535/72 (designated here as AC-1), serotype M22:T12, was originally isolated by J. Dixon in Alberta, Canada. This strain, which was isolated from the inflamed throat of a 17-year-old Cree Indian woman, is resistant to erythromycin and lincomycin (greater than 1 mg/ml in each case), as well as vernamycin B_α (no zone of inhibition with susceptibility disks containing 20 μg of drug). The patient had not been treated with antibiotics during the previous 12 months but had been in recent contact with a member of her family who was on erythromycin therapy.

Todd-Hewitt broth (THB; Difco) was used as a growth medium in both liquid and agar forms. Cell growth (at 37 C) was followed by measuring turbidity with a Klett-Summerson colorimeter. Materials and methods of plasmid analyses by dye-buoyant density and sucrose density centrifugation were as described in detail previously (3, 4), as were the methods of plasmid elimination with intercalating dyes ("curing") and electron microscopy analysis (5). Extraction of deoxyribonucleic acid (DNA) was performed as follows. A 200-ml log phase culture isotopically labeled with 1 mCi of [*methyl*-^{3}H]-thymidine (22 Ci/mmol from Amersham/Searle) was washed with 15 ml of 0.03 M tris(hydroxymethyl)aminomethane-0.005 M Na_2 ethylenediaminetetraacetic acid (EDTA)-0.05 M NaCl, pH 8.0, (TES) and resuspended in 3.0 ml of 25% glucose (in TES). A 0.5-ml quantity of 0.25 M EDTA (pH 8.0) was added, followed by the addition of 1.0 ml of lysozyme (5 mg/ml in TES). The suspension was allowed to incubate for 3 h at 37 C, at which point 0.5 ml of Pronase (5 mg/ml in TES, preincubated at 37 C for 30 min) was added, followed by another 30-min period of incubation. The cells were then lysed by the addition of 2.0 ml of sodium dodecyl sulfate (a 2% solution in TES). $NaClO_4$ (from a 5 M solution) was added to make a final concentration of 1 M. The lysate was then twice extracted with an equal volume of TES-saturated phenol-chloroform (2:1). Two volumes of 95% ethanol were added to the separated aqueous phase, and the precipitated DNA was collected by centrifugation and resuspended in 5 ml of TES.

Figure 1A shows a dye-buoyant density profile of a sample of DNA extracted from *S. pyogenes* strain AC-1. A satellite component,

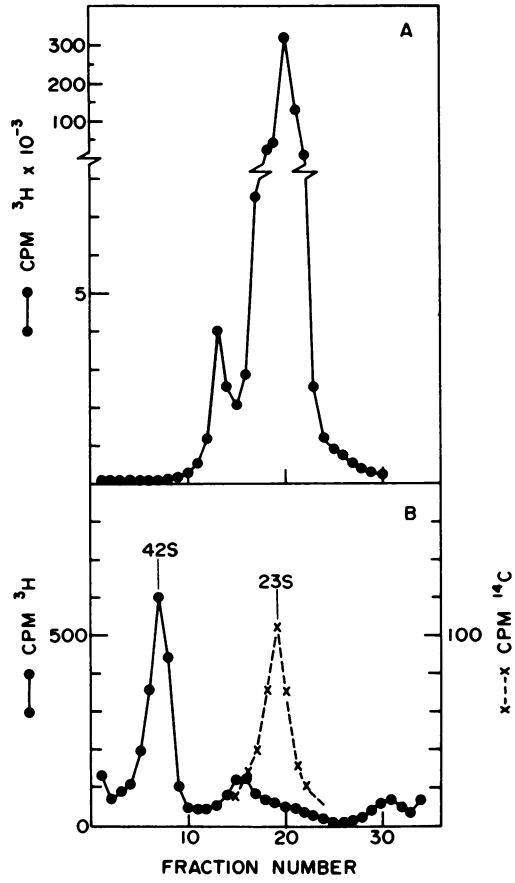

FIG. 1. *Analysis of plasmid DNA in S. pyogenes strain AC-1. (A) Dye-buoyant density centrifugation of extracted DNA. A 40-μg quantity of DNA was centrifuged to equilibrium (40,000 rpm in a Beckman 50Ti fixed-angle rotor for 60 h at 15 C) in a CsCl gradient containing ethidium bromide. The gradient was fractionated, and the amount of radioactivity determined in 0.05-ml samples of each fraction is plotted above. Density increases from right to left. (B) Sedimentation analysis of the CCC DNA component from the above dye-buoyant density gradient. Fractions 12 to 14 shown in panel A were pooled and dialyzed against 0.015 M NaCl-0.0015 M Na citrate, pH 7.4. ^{14}C-labeled 23S ColE1 DNA, serving as a marker, was mixed with the sample, and 0.2 ml was centrifuged through a 5 to 20% sucrose density gradient in an SW 50.1 rotor (15 C) at 48,000 rpm for 85 min. Sedimentation was from right to left.*

representing about 1.5% of the total DNA, is clearly evident in a position of higher density than the bulk DNA. This satellite component is absent in a CsCl gradient carried out in the absence of ethidium bromide, a characteristic of covalently closed circular (CCC) DNA. When fractions containing the satellite component were pooled and analyzed by sedimentation through a sucrose density gradient, in the presence of 23*S* ColE1 CCC marker DNA, a homogeneous substance with a calculated value of 42*S* was observed (Fig. 1B). The small amount of a 29*S* peak (fractions 14 to 17) probably represents the open circular configuration of 42*S* CCC DNA, because this material increases with a corresponding decrease in the 42*S* material upon storage of the satellite DNA in solution for several weeks. By using 42*S* for the value of Sccc in the equation Sccc = 0.034 $M^{0.428}$ (2), a molecular weight of 16.6 × 10^6 can be computed. Analysis of the satellite DNA by electron microscopy (Fig. 2) yielded an average contour-length measurement of 8.41 μm (± a standard deviation of 0.29) for 15 randomly selected molecules. Because 1.0 μm may be taken as 1.96 Mdaltons of DNA (14), the corresponding molecular weight is calculated to be 16.5 × 10^6, which agrees well with the value obtained from the velocity sedimentation data. On the basis of the molecular weight of the plasmid, the amount of plasmid DNA taken as a fraction of the chromosomal DNA, and the assumption that the genome size of *S. pyogenes* DNA is 1.27 × 10^9 daltons (1), it is computed that there must be a minimum of one to two copies of plasmid DNA per chromosomal genome equivalent in the cell.

"Curing" of erythromycin resistance could be accomplished by growing lawns (48 h of growth in a candle jar) on THB agar containing 15 μg of acridine orange per ml. Cells were removed from the plates with THB, and appropriate dilutions were plated out. Replica plating (on to THB agar containing 25 μg of erythromycin per ml) yielded erythromycin-susceptible variants at a frequency of 0.4% (a total of four cured strains were obtained). Spontaneous curing was not observed down to a level of 0.1%. Cured variants had minimum inhibitory concentrations for both erythromycin and lincomycin of 1 to 2 μg/ml when determined in liquid THB. Large zones of inhibition were produced by susceptibility disks containing 20 μg of vernamycin B_α. Colony morphology (on THB agar) and serotype (with regard to M and T antigens) remained unchanged. Beta-hemolysis on blood agar was not affected. Reversion to erythromycin resistance was not observed (no colonies appeared on erythromycin plates seeded with 10^8 cells).

A cured derivative AC-1C4 was labeled as described above except that 0.1 mCi of [*methyl*-^{14}C]thymidine (51.5 mCi/mmol; New England Nuclear Corp.) was used to label the DNA. The culture, in log phase, was chilled on ice and

FIG. 2. *Electron micrograph of two circular molecules from a dye-buoyant density gradient preparation of plasmid DNA. Relaxation of the molecules has occurred due to spontaneous nicking during storage for several weeks in solution.*

mixed with a chilled, similarly prepared ^{3}H-labeled culture of AC-1. DNA was extracted and banded in a dye-buoyant density gradient. The results are shown in Fig. 3. A ^{14}C-labeled satellite component is absent, indicating that this component in the parent strain is related to resistance. A similar observation was made by using another independently cured derivative.

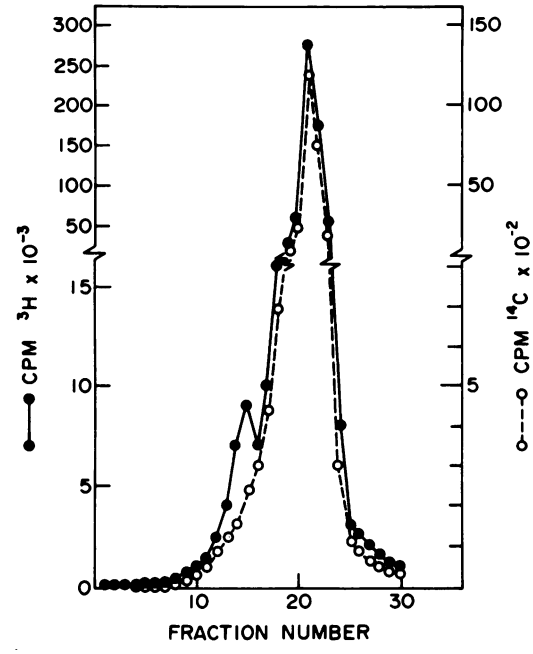

FIG. 3. *Dye-buoyant density centrifugation of cellular DNA (about 70 μg) extracted from the mixed lysates of strain AC-1 labeled with [^{3}H]thymidine and the erythromycin-susceptible derivative AC-1C4 labeled with [^{14}C]thymidine. Centrifugation was as described in Fig. 1A.*

Direct demonstrations of plasmids in the genus *Streptococcus* have only recently been reported (5, 6, 9). Work in our laboratory has recently demonstrated the presence of plasmid DNA in two other streptococcal species—*S. mutans* strain LM-7 (9) and *S. faecalis* strain DS-5 (5). With regard to the latter strain, it is interesting that a plasmid determining resistance to erythromycin and lincomycin (as well as vernamycin B_{α} [unpublished observation]) was found to have a molecular weight very similar (about 17×10^{6}) to that reported here for *S. pyogenes* strain AC-1.

The pattern of co-resistance reported here has been reported previously in *S. faecalis* by Courvalin et al. (6) and in *S. pyogenes* by Lai et al. (11). In the work of Courvalin et al. (6), resistance to pristinamycin I, a streptogramin B-type antibiotic similar to vernamycin B_{α}, paralleled resistance to erythromycin and lincomycin. A similar resistant phenotype has also been found in *Staphylococcus aureus*, and biochemical studies demonstrated that the expression of resistance is a result of specific N^{6}-dimethylation of adenine in 23*S* ribosomal ribonucleic acid, as a consequence of which erythromycin

and lincomycin are unable to bind to their receptor on the 50S ribosomal subunit (11).

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