

Electrophoretic Comparison of Endonuclease-Digested Plasmids from *Neisseria gonorrhoeae*

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In order to associate virulence in *Neisseria gonorrhoeae* with an alteration of the nucleotide sequence of its small covalently closed plasmid, plasmid deoxyribonucleic acid was isolated from both virulent (T1) and avirulent (T3) morphological types for two strains. Electrophoretic and contour length measurements of intact plasmids indicated a homogeneous population with a molecular weight of approximately 2.6×10^6 . Digestion with two restriction endonucleases, Hinf I and Hpa II, generated distinct fragment patterns which in each case were identical for T1 and T3 plasmid molecules from the same strain. The analysis suggests no sequence differences between the plasmids from virulent and avirulent types. For both strains, however, a deletion or addition of about 1.5% of the total deoxyribonucleic acid appeared in the Hpa II C digestion fragment when patterns for gonococci serially passaged 300 times were compared to those for bacteria freshly established from frozen stocks. The significance of the plasmid instability remains undetermined.

Neisseria gonorrhoeae may grow in vitro as several distinct morphological types (8). Whereas type 1 (T1) and type 2 (T2) produce characteristic gonococcal urethritis in humans (7, 17), type 3 (T3) and type 4 (T4) isolated during nonselective subculture of T1 or T2 are avirulent (7). The transition from T1 and T2 to T3 and T4 is accompanied by loss of pili (6) and of competence for genetic transformation (16). Furthermore, this transition is reversible and occurs at a rate suggesting control by an extrachromosomal deoxyribonucleic acid (DNA) or plasmid.

The isolation of a plasmid DNA molecule from *N. gonorrhoeae* was reported by Maness and Sparling (10) and by Engelkirk and Schoenhard (2). Comparing plasmids from T1 and T3 gonococci, Mayer et al. found that both types contain 24 to 32 copies per cell of a small superhelical molecule of approximately 2.4×10^6 daltons, with 0.5-mol fraction of guanine plus cytosine (12). This observation has been confirmed by Palchaudhuri et al. (14) and by Stiffler et al. (18), who showed that these morphological types, whether from the same or from different clinical isolates, possessed a similar plasmid with molecular weights of approximately 2.9×10^6 and 2.8×10^6 , respectively. Stiffler also found a larger plasmid in both T1 and T3 gonococci from some but not all strains.

The studies, to date, have demonstrated a

physical similarity of the plasmids, regardless of the gonococcal type from which they were isolated. Therefore, in an attempt to support the theory of plasmid control of virulence factors, we have used the specificity of restriction endonuclease digestion to refine the physical analysis. Digestion with either Hinf I or Hpa II, however, revealed no molecular differences between T1 and T3 plasmids from two strains; i.e., the digestion products migrated identically during electrophoresis in agarose gels. For both strains a small heterogeneity did appear between plasmids isolated from multiply passaged T1 or T3 cultures and molecules from the same strain that had been newly established from a frozen stock. This fact suggests an instability in the molecule that is unrelated to virulence or morphology.

MATERIALS AND METHODS

Organisms. Two strains of *N. gonorrhoeae* were used. CDC 9, an isolate passed for several years in vitro, was provided by D. S. Kellogg, Center for Disease Control, Atlanta, Georgia, and 72H641, an isolate from the joint fluid of a patient with gonococcal arthritis, was provided by E. S. Murray, Department of Microbiology, Harvard School of Public Health. Colonies characteristic of morphological type 1 (T1) and type 3 (T3) were isolated from each strain and were independently maintained by over 300 consecutive daily subcultures on solid medium. For purposes of discussion, these strains and types are designated as 9 (M) T1, 9 (M) T3, 641 (M) T1, and

641 (M) T3. The (M) refers to a multiply passaged culture. Gonococci designated 9 T1, 9 T3, 641 T1, and 641 T3 were reestablished from frozen cultures (-70 C) and were subcultured a few times to establish morphological type prior to plasmid isolation.

Media. Daily subculture was made on GCA consisting of GC agar (Difco) supplemented with 1% glucose (final concentration) and 1% (vol/vol) Iso-VitaleX (Baltimore Biological Laboratories).

Liquid medium (GCB), prepared according to the method of Morse and Bartenstein (13), consisted of 1.5% (wt/vol) proteose peptone no. 3 (Difco), 0.4% K_2HPO_4 , 0.1% KH_2PO_4 , 0.5% NaCl, and 0.1% soluble starch (Difco). After autoclaving and immediately prior to use, 1% (vol/vol) glucose, 0.042% $NaHCO_3$, and 1% IsoVitaleX were added.

Cultivation of *N. gonorrhoeae*. Daily subculture of gonococci was on GCA at 37 C in a 5% CO_2 atmosphere. Colonies of each type were selected from an overnight culture on GCA, scraped into GCB, and vortexed to break up clumped gonococci. This suspension provided the inoculum for broth cultures that were grown at 37 C on a model G-76 gyratory water bath shaker (New Brunswick Scientific Co.) to late log phase. Samples were monitored for purity with respect to colony type.

Isolation of plasmid DNA. Only those broth cultures with less than 1% of the other colony type present were used for plasmid isolation. Gonococcal-cleared lysates from approximately 10^{11} colony-forming units were prepared by the method of Guerry et al. (3), except that sodium lauryl sulfate was replaced by sodium Sarkosyl (1% wt/vol), and the lysates were held at 4 C for 24 h. Superhelical plasmid DNA was further purified by dye-buoyant density centrifugation of lysate supernatants. The result was two sharp bands easily visualized under an ultraviolet lamp. The lower plasmid band was removed by cannula from the top of the tube. Ethidium bromide was extracted twice with an equal volume of isopropanol that had been saturated with cesium chloride (KBI). After extensive dialysis against tris(hydroxymethyl)aminomethane (Tris)-ethylenediaminetetraacetate (EDTA) buffer (0.01 M Tris-hydrochloride- 2.5×10^{-4} M EDTA, pH 8.0), the DNA was concentrated by Ficoll (Sigma) and redialyzed. The photographs shown in this paper all represent plasmids isolated simultaneously from cultures grown at the same time.

Restriction endonucleases and marker DNAs. This study was made possible by the very generous donation of the following enzymes and marker DNAs: Hinf I from *Haemophilus influenzae*, serotype f, by D. Jaffe, Department of Bacteriology and Immunology, University of North Carolina, Chapel Hill; Hpa II from *H. parainfluenzae* by J. Feunteun, Department of Microbiology, and R. Kolodner, Department of Biological Chemistry, Harvard Medical School, Boston, Mass.; ColE1 (an *Escherichia coli* plasmid DNA), λ plac5 DNA, and Hae III from *H. aegyptius* by D. Hamer, Department of Biological Chemistry, Harvard Medical School; and DNA from wild-type and defective mutants of simian virus 40 (SV40) by D. Davoli and D. Ganem, Department of Biological Chemistry, Harvard Medical School.

Digestion conditions. The reaction mixtures for endonuclease digestion of DNAs, 20 to 30 μ l in volume, included about 0.3 μ g of plasmid or SV40 DNA or about 1 μ g of λ plac5 DNA. These were made 6 mM with respect to Tris-hydrochloride, pH 7.6, $MgCl_2$, and β -mercaptoethanol, and a titrated amount of restriction endonuclease was added to give complete digestion in 90 min at 37 C. Under these conditions Hinf I and Hpa II produced characteristic patterns of digestion for SV40 DNA (Newbold, personal communication; 15) as Hae III produced for λ plac5 DNA (4).

Electrophoresis of DNAs. Agarose gels (6 by 95 mm) were prepared at several concentrations in electrophoresis buffer containing 40 mM Tris-acetic acid, pH 7.8, 5 mM sodium acetate, and 1 mM EDTA. Bromophenol blue and glycerol to a final concentration of 10% were added to reaction mixtures prior to loading. Electrophoresis was at 2 mA/gel until the bromophenol blue migrated near the bottom of the gels.

Horizontal slab gels 12 inches (ca. 30.48 cm) in length were constructed by the method of McDonnell, Simon, and Studier (personal communication) using the same buffer as for the cylindrical gels. The slabs were electrophoresed overnight at room temperature with 18 mA.

After electrophoresis, the gels were stained with 0.5 μ g of ethidium bromide per ml in distilled water for at least 15 min and were then photographed under ultraviolet light. Under these conditions 0.05 to 0.1 μ g of DNA in a band could be visualized. Some gels were scanned with a Zeiss PMQ-II spectrophotometer adapted as described by Manteuil et al. (11) to give a linear response to fluorescence.

Calculation of molecular weights from gels. The linear relation for the log molecular weight versus migration of DNA in agarose gels allows calculation of the molecular weight of an unknown DNA upon construction of a standard curve with DNAs of known molecular weight. A convenient standard for measuring the linear DNA products of restriction endonuclease digestion was provided by a λ plac5 DNA digestion with Hae III, which produced many fragments, the first six of which had the following molecular weights: 2.13×10^6 , 1.33×10^6 , 1.16×10^6 , 1.06×10^6 , 0.80×10^6 , and 0.60×10^6 (Hamer and Thomas, unpublished data). A mixture of wild-type and defective SV40 superhelical standards (D. Ganem et al., *J. Mol. Biol.*, in press) were used to determine the size of the superhelical gonococcal plasmid.

Calculation of molarity from fluorophotometric scans. Theoretically, the areas under the peaks in a scan are proportional to the molecular weight of the DNAs if the molecules are present in equimolar amounts. Since the molecular weights of the Hinf I and Hpa II digestion products could be determined (as described above), scans of the gels permitted estimation of fragment molarity. An area under the peak for a given fragment which is twice that expected on a molecular weight basis is taken to indicate that the DNA product is present in 2 M concentration.

Contour length measurements. Mixtures of gonococcal T1 or T3 plasmid and ColE1 plasmid (4.2×10^6

daltons) (5) were converted to the open circular form with β -mercaptoethanol (1) and were then spread on parlodian-coated electron microscope grids by the method of Kleinschmidt (9). From enlarged photomicrographs molecules were traced and measured. Mean molecular weights were calculated relative to the internal ColE1 standard.

RESULTS

Size determinations. Calculation of the size of the undigested plasmid from agarose gel elec-

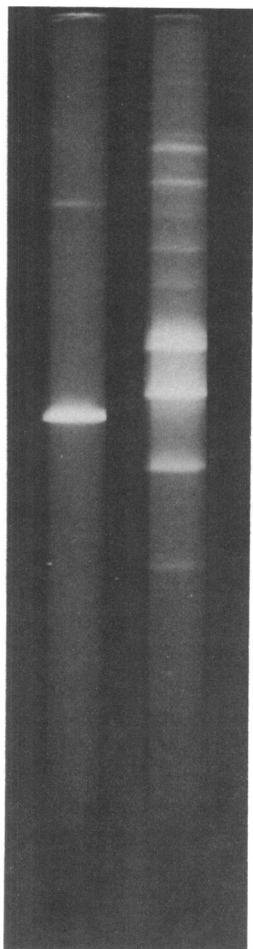


FIG. 1. Determination of plasmid size by electrophoresis. These 1.5% agarose gels show a typical gonococcal plasmid DNA preparation (left), in which the most prominent band represents superhelical DNA and the upper fainter band is the open circular form. The right gel contains both configurations of a mixture of wild-type and defective SV40 DNAs with molecular weights 3.3×10^6 , 2.9×10^6 , 2.3×10^6 , and 1.8×10^6 . From the relative migration of the superhelical DNAs, the bottom four bands, the molecular weight of the gonococcal plasmid is calculated to be 2.6×10^6 . Electrophoresis was at 2 mA/gel.

trophoresis gave a molecular weight of 2.6×10^6 (Fig. 1), which closely agrees with published observations (12, 14, 18). Contour length measurements of over fifty T1 and T3 plasmids each relative to the ColE1 marker gave a mean value of 2.7×10^6 for both types.

Comparison of T1 and T3 plasmids by digestion with restriction endonucleases. Both Hinf I and Hpa II specifically cleaved the plasmid at more than one recognition site. The agarose gel analysis of the resulting digestion products is represented in Fig. 2. The patterns for Hinf I and Hpa II digestion products are identical for plasmids from T1 and T3 of the strain 641. In fact, in numerous isolations of plasmids from this strain and strain CDC 9, no difference was found between the T1 and T3 molecules at any given subculture. For further discussion, gel bands will be designated A to E in descending order of molecular weight (i.e., from top to bottom of the gel).

For the Hinf I products A and B, the Hpa II products A, B, and C, and the larger of the Hinf I digestion products of SV40 DNA, A to E, fluorophotometric scans of the gels revealed the areas to be proportional to the calculated lengths, indicating the fragments were present in equimolar amounts. The calculated molecular weights of the plasmid products (Table 1) add to approximately 90% of the undigested plasmid size for both enzymes. The fraction of the DNA unaccounted for could not be detected on 2.4% agarose gels or on gels electrophoresed only a short time; presumably it contained Hinf I and Hpa II sites more closely spaced, producing fragments too small to be retained. In the Hpa II pattern the two faint bands, D and E, may be either true Hpa II sites or

TABLE 1. Sizing of Hinf I and Hpa II digestion products by agarose gel electrophoresis

Digestion	Fragment size ^a (daltons $\times 10^6$)					% of total recovered ^b
	A	B	B'	C	C'	
Hinf I						
641 T1 or T3	1.43		0.92			91
641 (M) T1 or T3	1.43	0.96				92
9 T1 or T3	1.43	0.96				92
9 (M) T1 or T3	1.43		0.96			91
Hpa II						
641 T1 or T3	0.99	0.67			0.62	88
641 (M) T1 or T3	0.99	0.67		0.66		89
9 T1 or T3	0.99	0.67		0.66		89
9 (M) T1 or T3	0.99	0.67			0.62	88

^a Mean molecular weight values within $\pm 1\%$ as calculated from fragments of λ plac5 digested with Hae III.

^b Based upon a molecular weight of 2.6×10^6 for the undigested plasmid.

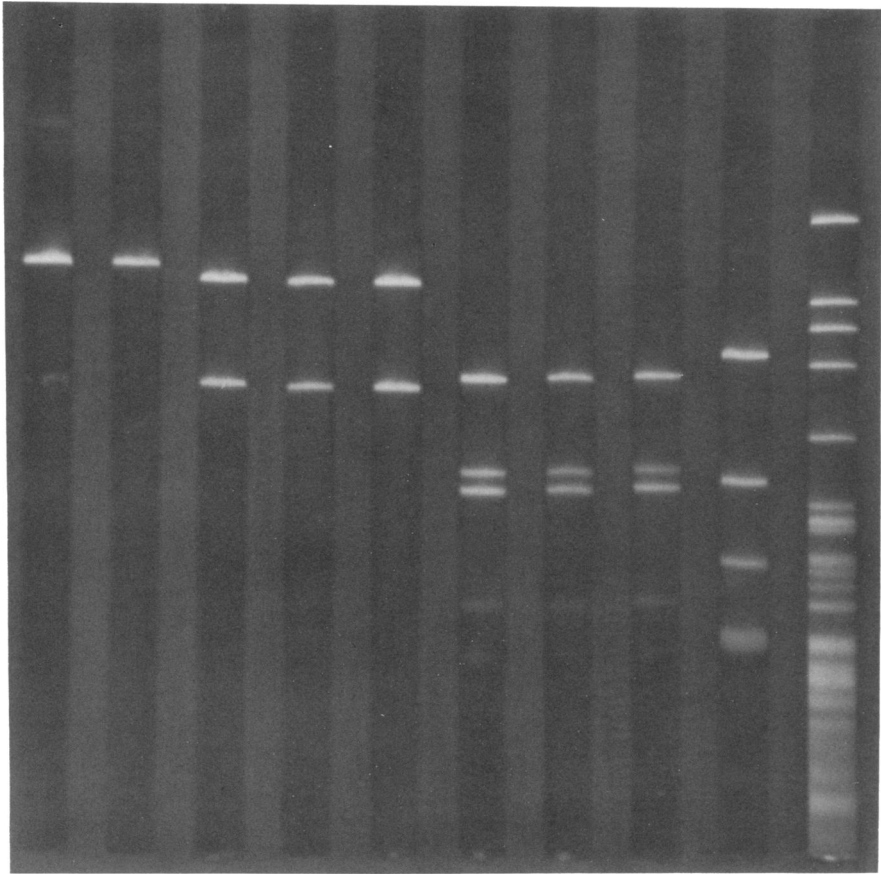


FIG. 2. Comparison of plasmid DNAs from gonococcal colony type 1 (T1) and type 3 (T3) from strain 641. Intact plasmids and those specifically cut with restriction endonucleases were electrophoresed on 1.5% agarose gels. The order of plasmids (left to right) is as follows: (1) undigested T1, (2) undigested T3, (3) T1 digested with *Hinf* I (T1/*Hinf* I), (4) T3/*Hinf* I, (5) (T1 + T3)/*Hinf* I, (6) T1/*Hpa* II, (7) T3/*Hpa* II, and (8) (T1 + T3)/*Hpa* II. Gel 9 contains wild-type SV40 DNA digested by *Hinf* I. The last gel (right) contains known linear marker fragments from λ plac5/*Hae* III for calculation of molecular weights of *Hinf* I and *Hpa* II digestion products. Electrophoresis was at 2 mA/gel.

due to slight contamination of this enzyme with the restriction endonuclease *Hpa* I from the same organism.

Analysis of plasmids from different passage levels. The only inconsistency observed in restriction patterns occurred within strains. For 641 and for CDC 9, the patterns changed to the same extent but in reverse order when a culture reestablished from frozen specimens (subcultured less than 10 times) was compared with the same strain passaged over 300 times.

Figure 3 shows the *Hinf* I restriction patterns in the following order (left to right): 641 T1, 641 T3, 641 (M) T1, 641 (M) T3, CDC 9 T1, CDC 9 T3, CDC 9 (M) T1, and CDC 9 (M) T3. Size markers are in the last gel (a *Hae* III digestion of λ plac5 DNA). Within the same passage for each strain, the patterns for T1 and T3 plas-

mids appear identical. However, an alteration appears in *Hinf* I band B when the patterns from few passaged T1 and T3 are compared with those from the multiply passaged counterparts T1 (M) and T3 (M). Band B (Table 1) for 641 (M) T1, 641 (M) T3, CDC 9 T1, and CDC 9 T3 is larger and more slowly migrating than *Hinf* I band B' in the plasmids from the remaining cultures. The difference, as demonstrated in Fig. 4, was measurable by slab gel electrophoresis at a lower agarose concentration, which permitted a better comparison against the λ plac5 DNA digested with *Hae* III standard pattern. The difference between 641 and 641 (M) and, also, CDC 9 and CDC 9 (M) plasmids was estimated to be about 4×10^4 daltons, or approximately 60 base pairs. This represents 1.5% of the total DNA (at the gel value of 2.6×10^6

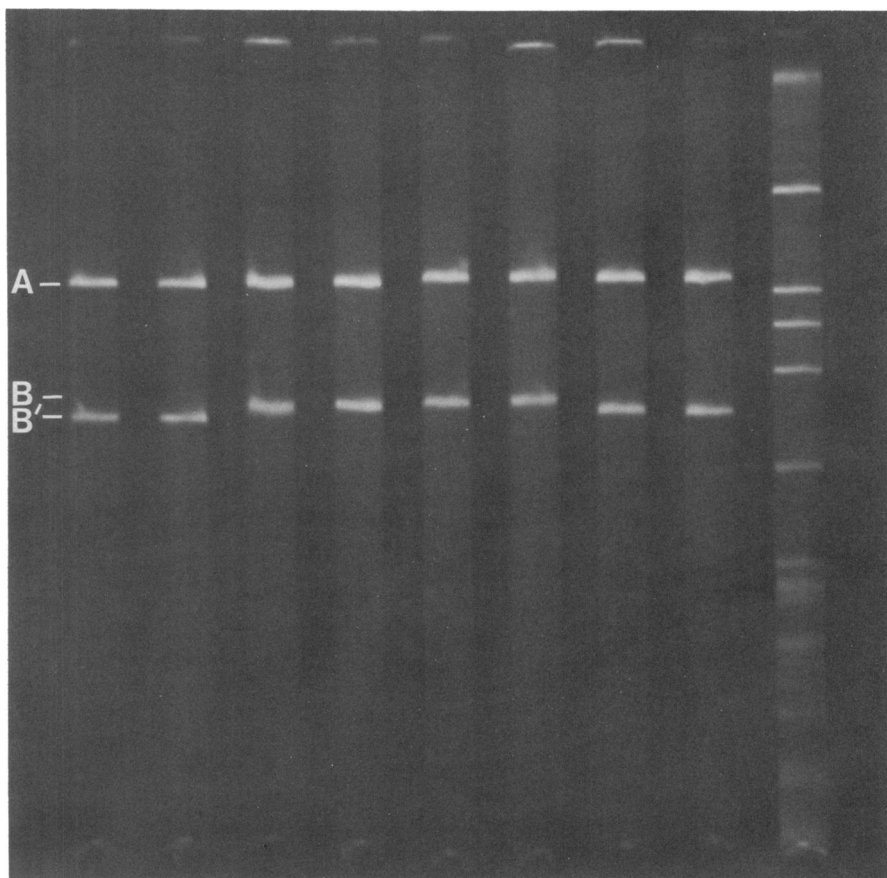


FIG. 3. Comparison of plasmid DNAs from different gonococcal strains by digestion with *Hinf*I. These 2% agarose gels contain (left to right) the products of *Hinf*I digestion of the following plasmids: (1) 641 T1, (2) 641 T3, (3) 641 (M) T1, (4) 641 (M) T3, (5) CDC 9 T1, (6) CDC 9 T3, (7) CDC 9 (M) T1, and (8) CDC 9 (M) T3. Gel 9 contains λ *plac5* digested by *Hae* III. Electrophoresis was at 2 mA/gel. Letters indicate digestion fragments.

daltons) (Table 1).

The heterogeneity in plasmids from different passage levels was again apparent in the *Hpa* II digestion products (Fig. 5). Scans revealed the second band in the gels for 641 (M) and CDC 9 to be a doublet that was resolved in a 1% agarose slab gel (gel not shown but data presented in Table 1) as *Hpa* II bands B and C. Band C was altered (to C') between few and multiply passaged organisms. The discrepancy was calculated from the slab gel to represent approximately 1.5% of the total DNA (Table 1). The faint bands D and E appeared to migrate to the same relative positions, regardless of the strain, type, or passage level.

DISCUSSION

The results of this study support previous observations of the physical similarity of plas-

mid DNAs from different strains of *N. gonorrhoeae*. Electrophoresis of intact plasmids concomitantly with superhelical standards gave a molecular weight of approximately 2.6×10^6 , or 3,900 base pairs, a value close to that determined from contour length measurements of T1 and T3 plasmids using the *E. coli* plasmid ColE1 as a standard and in good agreement with other estimates of the size of the small gonococcal plasmid (12, 14, 18). The sharp banding of the intact plasmid in agarose gels indicates this plasmid extracted from *N. gonorrhoeae* by conventional means is in fact a single species rather than a population of molecules of approximately the same size. Digestion of the plasmids with two restriction endonucleases provided a means for comparing them more closely. That the same patterns resulted from plasmids from diverse strains, a clinical isolate and a laboratory strain, suggests homogeneity

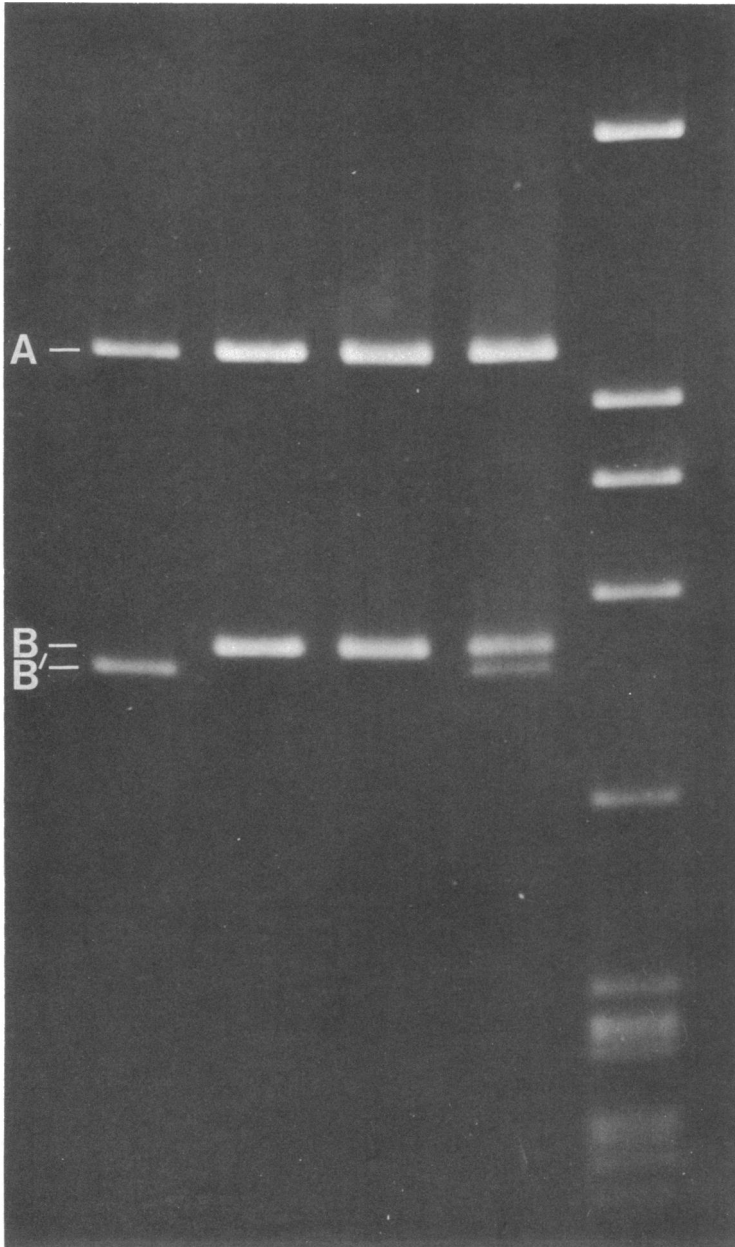


FIG. 4. Confirmation of plasmid differences between different gonococcal strains and different passages of the same strain. This 1% agarose slab gel contains (left to right) the following plasmid digestions: (1) 641 T3/HinfI, (2) 641 (M) T3/HinfI, (3) CDC 9 T3/HinfI, and (4) [641 T3 + 641 (M) T3]/HinfI. The pattern on the right is λ plac5/Hae III included as molecular weight markers. The letters (A, B, and B') denote separate digestion products. Electrophoresis was at 18 mA.

with respect to nucleotide sequence for plasmids in all strains.

Speculation that the critical difference between avirulence and virulence is coded for by the small plasmid remains unsupported. For

any strain and passage, the T1 and T3 plasmids appeared identical by our analysis. We have monitored two Hinf I sites and three Hpa II sites. This represents direct analysis of only 20 to 30 nucleotide pairs or less than 1% of the

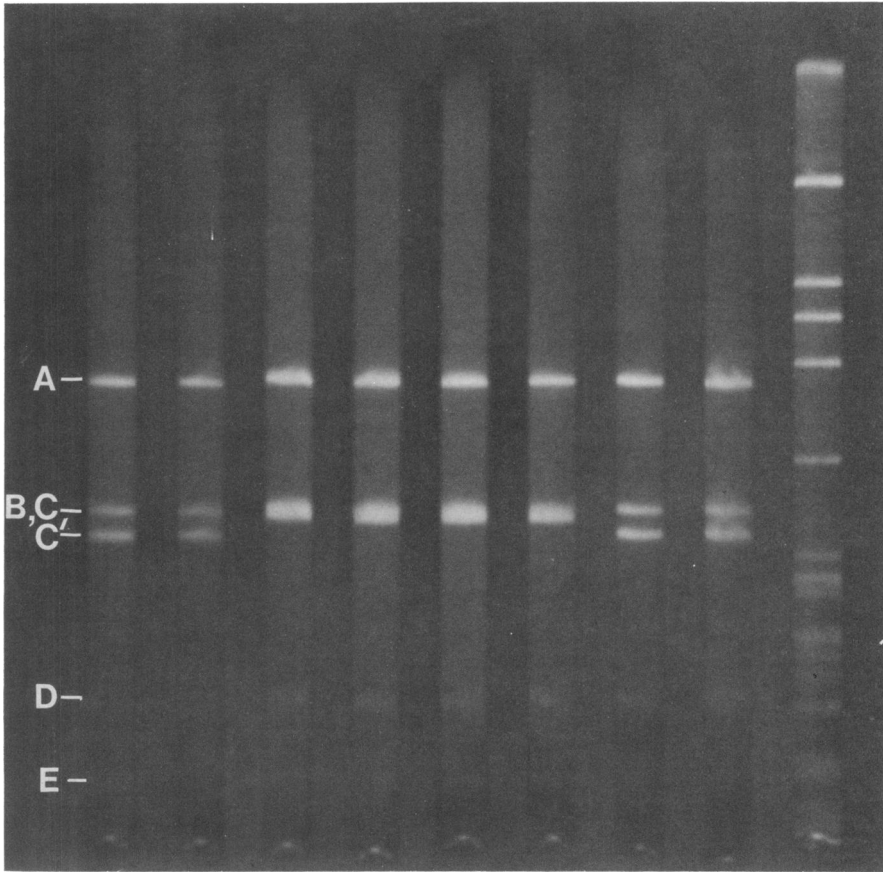


FIG. 5. Comparison of plasmid DNAs from different gonococcal strains by digestion with *Hpa* II. These 2% agarose gels contain (left to right) the products of *Hpa* II digestion of the following: (1) 641 T1, (2) 641 T3, (3) 641 (M) T1, (4) 641 (M) T3, (5) CDC 9 T1, (6) CDC 9 T3, (7) CDC 9 (M) T1, (8) CDC 9 (M) T3. Gel 9 contains λ *plac5* digested by *Hae* III. Electrophoresis was at 2 mA/gel. Letters indicate individual digestion products.

total sequence. However, the cleavage of the intact plasmid into a number of specifically defined fragments permits a more accurate comparison between molecules on the basis of electrophoresis. The digestion products appear to be the same size from gel migration. This does not rule out the possibility of small nucleotide sequence differences between T1 and T3 plasmids (we could not easily detect molecular weight differences much smaller than 60 base pairs, and we did not see approximately 10% of the total DNA). But if, as our analysis suggests, the plasmids are homogeneous, then a role in determining virulence or avirulence would have to result from differential expression or interaction with cofactors.

The observed differences between few and multiply passaged strains indicate that the molecular weight of the plasmid is not stable. The simplest explanation would be the occur-

rence of net insertions or deletions in a region of the molecule, which can undergo these changes without effect on phenotype. Perhaps even the insertion or deletion could result from plasmid DNA exchange with the chromosome.

In conclusion, analysis of gonococcal plasmids by restriction endonuclease cleavage has indicated that the plasmids from two strains and both types 1 and 3 are homogenous with respect to nucleotide sequence and are unstable with respect to passage level for about 1.5% of the total DNA. When further technology succeeds in assigning a function to this molecule, the restriction patterns will permit mapping of loci.

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