Electron Microscopy of Plasmid Deoxyribonucleic Acid from Neisseria gonorrhoeae

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Received for publication 3 December 1974

The existence of plasmids in all four types of colony morphology of Neisseria gonorrhoeae has been established by electron microscopy of the plasmid deoxyribonucleic acid (DNA) fractions separated by density equilibrium centrifugation on ethidium bromide-cesium chloride solutions. Cells of each colony type (T1, T2, T3, and T4) were lysed with detergent, and most of the chromosomal DNA was removed by centrifugation and alkali denaturation. A simplified procedure avoiding the latter has also been developed for N. gonorrhoeae plasmid isolation. The heavier bands containing the covalently closed, circular DNA plasmid molecules were collected and converted to the open circular form for electron microscopy. Contour lengths of the plasmid DNA isolated from all four colony types measured 1.5 μm, with a calculated molecular weight of 2.9 × 10⁶. An internal standard of colicin E1 DNA was used in our determinations. All four isolates used were further characterized for the presence of pili (found only in T1 and T2) and for their sensitivities to several antibiotics. The functions of these gonococcal plasmids are unknown at the present time.

Evidence for the presence of extrachromosomal deoxyribonucleic acid (DNA) in colony type T1 of Neisseria gonorrhoeae has been presented by Engelkirk and Schoenhard (5) and by Maness and Sparling (11). In both instances, however, the plasmids were not visualized by electron microscopy nor were they physically characterized. We decided accordingly to examine the plasmid fractions from all four colony types of Kellogg et al. (9, 10) using a modification of the procedure described by Palchaudhuri et al. (14) for the isolation of plasmids and their molecular weight determinations. We wish to report the presence of plasmids in all four colony morphology types of independently isolated strains carefully examined for their state of piliation and for their antibiotic sensitivities. Moreover, we report molecular weight determinations based on the inclusion of an internal standard (colicin E1 DNA) to obviate some of the variables inherent in determining contour lengths by electron microscopy as discussed by Davidson and Szymbalski (4). Thus our results also establish the existence of plasmids in piliated isolates of colony types T1 and T2 and in nonpiliated types T3 and T4 of N. gonorrhoeae strains possessing very similar sensitivities to selected antibiotics.

During the preparation of this manuscript, the publication of the investigations of Mayer et al. (13) appeared and confirmed the existence of plasmid DNA in types 1, 3, and 4 of N. gonorrhoeae. The contour lengths of the plasmids were determined by electron microscopy, and the mean molecular weights were calculated to be about 2.4 × 10⁶.

MATERIALS AND METHODS

Bacterial isolates. Fresh clinical isolates were kindly provided by Yvonne C. Faur of the General and Special Bacteriology Laboratory of the Public Health Laboratory Services, New York City Department of Health, New York. The organisms were received on NYC medium described by Faur et al. (6). Colonies conforming to Kellogg's (9, 10) types T1 and T2 were selected and transferred to NYC medium agar plates, and colonial types T3 and T4 were subsequently selected. Colonial morphology was constantly checked on the NYC medium by the procedure described by Kellogg et al. (10). The NYC agar medium (6) proved to be particularly valuable and reliable in monitoring the colony morphology. All four colony types have been transferred selectively on the NYC agar medium (6), usually several times weekly as required, and they have been maintained in the Revco at −70°C.

All four types were checked periodically for the presence of absence of pili by selecting numerous colonies of each type from the NYC agar medium, suspending in buffer, and examining in the electron microscope cells which had been negatively stained with ammonium molybdate.

Medium and growth conditions. The medium was prepared essentially as described by Faur et al. (6)
except that plasma used was drawn off from outdated bottles of sterile, citrated horse blood after the erythrocytes had settled out, and the latter were used for preparation of the 3% (vol/vol) hemoglobin solution. The yeast dialysate was prepared from Fleischman's active dry yeast (402 g in 1,250 ml) essentially as described by Faur et al. (6). No antibiotics (6) were included in the media used for growing the cells for plasmid isolation.

Petri dishes of the NY agar medium were inoculated with selectively transferred inocula of the appropriate colony types. Plates were incubated in candle jars at 36°C, and the confluent growth from the surface was harvested after 24 h by emulsification of the bacteria with proteose-peptone broth: suspensions were pooled and centrifuged, and the cells were washed once with 0.05 M tris(hydroxymethyl)-aminomethane (Tris)-hydrochloride buffer, pH 7.5. The packed cells were weighed for wet weight determinations and were then used for plasmid isolation as described below. Approximately 25 agar plates yielded sufficient cells (about 1 to 1.5 g [wet weight]) for the preparation of lysates. Samples from the pooled suspensions were generally streaked out on fresh plates to determine homogeneity of colonial morphology.

Antibiotic sensitivity tests. Antibiotics were incorporated directly into the NY agar medium (6) to give suitable concentration ranges for each antibiotic. The agar surfaces were inoculated by streaking suspensions of each colonial type, and the plates were incubated as described above and read for the minimal inhibitory concentrations after 24 and 48 h in accordance with recommended procedures (17).

Isolation of plasmid DNA for electron microscopy. About 1 g (wet weight) of cells harvested from NY agar medium (6) as described above was suspended in 15 ml of 25% sucrose in 0.05 M Tris, pH 8.0, and frozen at −20°C. After thawing, 2 ml of lysozyme (5 mg/ml) in 0.25 M Tris-hydrochloride, pH 8.0) and 2 ml of 0.2 M NaCl ethylenediaminetetraacetic acid were added gently and incubated for 10 min at room temperature. Spheroplasts thus obtained were lysed at room temperature by adding 20 ml of 0.2% sodium lauryl sarcosine containing 1.5 M NaCl and 0.05 M Tris. The viscous lysate was chilled and then centrifuged at 48,000 × g for 20 min at 2°C to remove most of the chromosomal DNA as a viscous sediment. The clear supernatant was titrated to pH 12.0 by slowly adding 4 N NaOH and then adjusted to pH 8.0 with 2 M Tris-hydrochloride (pH 5.0). To remove alkali-denatured DNA, 20 g of nitrocellulose (Her- cules, 0.25-s cubed) which had been washed four times with 0.3 M sodium chloride-0.03 M sodium citrate solution was stirred with the DNA solution for 30 min at 4°C. The nitrocellulose was removed by centrifugation, and the supernatant containing the DNA was then layered onto a cushion of 2.5 ml of saturated CsCl and centrifuged for 16 h at 15,000 rpm in a fixed-angle rotor 30 for concentration of the DNA. The lower 9-ml portion from each rotor 30 tube was collected and pooled, the refractive index was adjusted to 1.398 with crystals of CsCl, and 0.15 ml of ethidium bromide (10 mg/ml) was finally added and then centrifuged as described previously (14). The heavier bands were collected and pooled and were again run on CsCl-ethidium bromide gradients to ensure the removal of any remaining chromosomal DNA fragments.

Electron microscopy of plasmid DNA and measurement. Fractions from CsCl-ethidium bromide density gradient centrifugation that contained the fast sedimenting species of DNA (covalently closed circles) were dialyzed against 50 ml of 0.8 M NaCl, 0.05 M Tris, 0.01 ethylenediaminetetraacetic acid, pH 8.5, containing 10 g of Bio-Rad AG 50W-X8 (100 to 200 mesh) for 20 h at 4°C. The DNA solution was further dialyzed against 1 liter of buffer (0.05 M NaCl, 0.05 M Tris-hydrochloride, 0.01 M ethylenediaminetetraacetic acid, pH 8.5), and thus most of the CsCl and ethidium bromide were removed. Supertwisted molecules were converted to open circular forms by X-ray doses ranging from 0.5 to 2.0 krads at room temperature (24 to 25°C). The DNA was prepared for electron microscopy by a modification of Kleinschmidt's technique (3). DNA was diluted to 0.5 μg/ml in 0.5 M ammonium acetate, and cytochrome c was added to a final concentration of 0.1 mg/ml. A quantity of 50 μl of the above solution was spread over a hypophase of 0.25 M ammonium acetate (pH 7.5); films were picked up immediately on parlodion-coated copper grids, stained for 30 s in the freshly prepared 5 × 10−4 M uranyl acetate in 95% ethanol, and rinsed for 10 s in isopentane. The preparations were shadowed with platinum-palladium (80:20) at an angle of 7° on a rotary table. All molecules were photographed in a Siemens Elmiskop 101 which was calibrated for a set of exposures at the same operational magnifications by a grating replica (2,160 lines/mm; Fulham, Inc.), and their lengths were obtained by making enlarged prints and measuring their contours with a map measurer. Usually colicin factor E1 DNA (1) was added to each preparation to serve as an internal length standard. At least 50 molecules having well-defined open contours were measured to determine the average length of a particular class of plasmid.

RESULTS

The appearance of the plasmid fraction isolated from N. gonorrhoeae type T1 cells is illustrated in Fig. 1, which shows the presence of the major open circle form seen in all preparations (including types T2, T3, and T4) together with some supertwisted forms and several open circles having the appearance of dimers and tetramers.

The sizes of the plasmids were determined by electron microscopy in two independent ways. Contour lengths of the gonococcal plasmids and colicin E1 DNA were determined from length measurements and magnifications as described in Materials and Methods. The molecular weights were calculated from the linear density value of 1.94 × 10⁶ daltons/μm for duplex DNA (7). These data for the four colony types of N. gonorrhoeae and colicin E1 DNA are presented in Table 1. The molecular weight of 4.28 × 10⁶ for the colicin E1 DNA determined from our
measurements is therefore in excellent agreement with the published values (1). Since the colicin E1 DNA could be distinguished from the gonococcal plasmids (Fig. 2), it was selected in preference to φX174 DNA as an internal standard for the determination of the ratios of contour lengths of the two circular forms of DNA. These ratios are summarized in Table 2, and the molecular weights of the gonococcal plasmids gave a value of $2.9 \times 10^6$, assuming a molecular weight of $4.2 \times 10^6$ for the colicin E1 DNA. Thus there is excellent agreement between the contour length ratio determinations and those based on the linear density and average molecular lengths (Table 1). These results establish that the plasmids of all four colony types of the gonococcus are practically identical in size.

It should be noted that the isolation and measurements of the plasmids were reproducible, since there was excellent agreement between individual batches of cells of all four types. A minimum of two individual preparations of each colonial type was examined. Moreover, plasmid DNA fractions isolated from cells harvested after 48 and 72 h of incubation under the growth conditions described in Materials and Methods showed identical properties to those of the 24-h cell cultures. Although the plasmid fractions from the 72-h cells were in every respect indistinguishable from those isolated from 24- and 48-h cells, the 72-h cultures appeared to be more resistant to the lytic procedures.

In subsequent experiments it was found that

<table>
<thead>
<tr>
<th>Source of plasmid DNA (colony type)</th>
<th>No. of molecules measured</th>
<th>Contour lengths (µm)</th>
<th>Standard deviation (µm)</th>
<th>Mol wt* ($\times 10^6$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T1</td>
<td>50</td>
<td>1.50</td>
<td>±0.04</td>
<td>2.91</td>
</tr>
<tr>
<td>T2</td>
<td>50</td>
<td>1.50</td>
<td>±0.04</td>
<td>2.91</td>
</tr>
<tr>
<td>T3</td>
<td>50</td>
<td>1.49</td>
<td>±0.06</td>
<td>2.89</td>
</tr>
<tr>
<td>T4</td>
<td>50</td>
<td>1.50</td>
<td>±0.03</td>
<td>2.91</td>
</tr>
<tr>
<td>Colicin E1</td>
<td>25</td>
<td>2.21</td>
<td>±0.04</td>
<td>4.28</td>
</tr>
</tbody>
</table>

* Molecular weights of the plasmids and colicin E1 DNA were calculated from their average contour lengths and the value of $1.94 \times 10^6$ daltons/µm (7) for the linear density of duplex DNA.
the plasmid isolation could be simplified considerably. The modifications of the methods are worthy of note since they have given measurements of the *N. gonorrhoeae* plasmids identical to those summarized in Tables 1 and 2. Cell pellets (approximately 1.5 g [wet weight]) were suspended in 10 ml of 25% (wt/vol) sucrose and lysed as described above, but adjusting the volume to 20 ml. Both the more dilute lysates of *N. gonorrhoeae* used in the procedure described above and the more concentrated ones used in the modified procedure did not require shearing to reduce the viscosity prior to centrifugation to remove chromosomal DNA. The *N. gonorrhoeae* lysates were less viscous than those obtained from similar weights of cells of *Escherichia coli* or *Pseudomonas* species. After removal of chromosomal DNA by centrifugation, the clear supernatants from the gonococcal lysates could thus be centrifuged directly on CsCl-ethidium bromide gradients. In this way it was possible to avoid the use of alkali, limit the volume of *N. gonorrhoeae* lysate, and thus eliminate the time otherwise required for the concentration of the plasmid DNA on the CsCl cushion as described in the first procedure. The plasmid DNA was then recovered as described in the initial procedure. Another feature of the isolation of *N. gonorrhoeae* plasmids which should be noted is that this has to be performed within a short period of harvesting, since cells which had been stored in 25% sucrose for 48 h or more at -20 C

![Figure 2. Electron micrograph typical of the results obtained with type T1 *N. gonorrhoeae* plasmid DNA fraction and colicin E1 DNA added as an internal standard. Arrows indicate the colicin E1 DNA. Bar, 1 μm.](image)

<table>
<thead>
<tr>
<th>Source of plasmid DNA (colony type)</th>
<th>No. of molecules measured</th>
<th>Contour length ratios of <em>N. gonorrhoeae</em> plasmids to colicin E1 DNA*</th>
<th>Standard deviation (μm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T1</td>
<td>100</td>
<td>0.68</td>
<td>±0.02</td>
</tr>
<tr>
<td>T2</td>
<td>54</td>
<td>0.69</td>
<td>±0.03</td>
</tr>
<tr>
<td>T3</td>
<td>55</td>
<td>0.69</td>
<td>±0.03</td>
</tr>
<tr>
<td>T4</td>
<td>76</td>
<td>0.69</td>
<td>±0.03</td>
</tr>
</tbody>
</table>

* Measurements were performed on 25 colicin E1 DNA molecules as internal standard for each gonococcal type. Assuming a molecular weight of $4.2 \times 10^8$ for colicin E1 DNA (1), the molecular weight of all four plasmids calculated from the above ratios was $2.9 \times 10^8$. 
prior to lysis exhibited loss of almost all of the covalently closed circular plasmids (i.e., the heavy bands on CsCl-ethidium bromide indicative of circular DNA plasmids were absent).

In addition to the characterization of the plasmid DNA fractions of the four colony types used in our experiments we have confirmed by electron microscopy that the types T1 and T2 are indeed pilated as expected from previous reports and that the types T3 and T4 are nonpiliated (8, 16). We have also determined for the sake of completeness the antibiotic sensitivities of the four colony types which we selected originally from a type T1 clinical isolate. The four colony types T1, T2, T3, and T4 showed no significant differences in their sensitivities to the following antibiotics, as indicated by the minimal inhibitory concentrations. The following minimal inhibitory concentrations were found for all four colony types: penicillin G, 0.6 \( \mu g/ml \); ampicillin, 0.3 \( \mu g/ml \); methicillin, approximately 0.24 \( \mu g/ml \); tetracycline, 0.7 \( \mu g/ml \); chloramphenicol, 10 \( \mu g/ml \); gentamycin, 5 \( \mu g/ml \); streptomycin, resistant, \( >1,600 \mu g/ml \). Although there is at the present time no suggestion that the existence of plasmids in the gonococcus is related to antibiotic sensitivity, it is our feeling that the simultaneous characterization of plasmids in strains of specified antibiotic sensitivities will provide valuable basic information for the full documentation of the properties of the large reservoir of \( N. \) gonorrhoeae isolates.

DISCUSSION

The preliminary evidence for the existence of extrachromosomal DNA in the gonococcus (5, 10) has been confirmed by Mayer et al. (13) and by the investigations presented in this paper. The plasmids have been detected in independent isolates of colony types T1, T3, and T4 by Mayer et al. (13) and in all four colony types (T1, T2, T3, and T4) in our study. Although there are differences in the contour lengths and, therefore, molecular weights of the plasmids between the two independent investigations, in each study the plasmid size was virtually identical for those colony types examined. Thus the plasmids in our studies have molecular weights of \( 2.9 \times 10^4 \), whereas those of Mayer et al. (13) averaged 2.37 \( \times 10^4 \). The differences between the two sets of data appear to exceed the levels expected for experimental error. The basis for this difference will undoubtedly be resolved by further studies of a wider range of gonococcal strains. It should be noted that the measurements on the four colony types used in our study have been based upon the use of colicin E1 DNA as an internal standard, and the observed molecular weight of this standard agrees within 5% of the published data for this molecule (1).

All four colony types used in our experiments were carefully checked for the presence of pili, and in agreement with the previous reports (8, 16) only those of colonial types T1 and T2 showed the presence of these structures. The correlation of the piliated T1 and T2 types with virulence (8, 9, 16) and the absence of pili in strains believed to be avirulent (8, 9, 16) have created much interest in establishing a basis for the pathogenicity of the gonococcus. Although the presence of plasmids of identical sizes in colonial types of the gonococcus does not appear to be directly correlated with the possession of pili and the virulence of the pilated strains, nor with the fact that types T1 and T2 are more readily transformable than types T3 and T4 (2, 15), further evidence on the identity of the DNA sequences in these plasmids will be needed to substantiate this conclusion.

At the present time, therefore, the functions of these plasmids in all four colonial morphologies of the gonococcus are unknown. The antibiotic sensitivities of the four cultures used in our experiments were found to be very similar for a range of antibiotics, including several penicillins and aminoglycosides. It will thus be of interest to determine the characteristics of the plasmids in strains of the gonococcus exhibiting wider differences in antibiotic sensitivity. There is, however, no suggestion at the present time that the plasmids are related to R factors (12). The characterization of plasmids in cultures of all four types of \( N. \) gonorrhoeae provides yet another feature of this pathogen, and, although no function can be assigned to this extrachromosomal DNA at present, the data provide another parameter which may prove valuable in future studies of understanding the biochemical properties related to pathogenicity and possible antibiotic resistance mechanisms of this important pathogen.

ACKNOWLEDGMENTS

We wish to thank J. Seibert for X-irradiation of DNA samples, M. Oishi for the gift of colicin E1 DNA, and Kwang S. Kim for checking cells of the various colony types for pili in the electron microscope. We are grateful to Werner K. Maas for his interest and helpful suggestions.

This work was supported by grant no. U 2235 from the Health Research Council of the City of New York to Milton R. J. Salton and support from Public Health Service grant AI 9079 from the National Institute of Allergy and Infectious Diseases to Werner K. Maas.

LITERATURE CITED


