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Characterization of Plasmid Deoxyribonucleic Acid from *Neisseria gonorrhoeae*

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Five of six independent gonococcal isolates have been found to harbor a plasmid pool of covalently closed-circular deoxyribonucleic acid about 2.4×10^6 daltons in size. The plasmid species were 0.5-mol fraction of guanine plus cytosine and comprised 6 to 8% of the total gonococcal deoxyribonucleic acid equivalent, to approximately 24 to 32 copies per cell. There was no apparent correlation between the presence of these plasmid species and the in vitro clonal variation of gonococci associated with the loss of pili.

Kellogg et al. (12) described four colony types of *Neisseria gonorrhoeae* and showed a correlation between these types and virulence for human volunteers. Two types, cells forming type T1 and T2 colonies, were most often cultured from infected material and were capable of causing infections in volunteers. Type T1 and T2 colonies could be maintained during repeated transfer in vitro only by selective cloning. During nonselective transfer, type T1 and T2 were noted to rapidly give rise to colonial variants designated type T3 and type T4. The transition of type T1 colony-forming cells to cells capable of forming the avirulent colonial types was often apparent after 30 to 36 h of incubation, and it was concluded that type T1 colony-forming cells were unstable for some genetic trait. After 38 in vitro selective transfers, T3 and T4 cells retained infectivity for human volunteers and retained the capability to produce T1 colonies. However, 69 in vitro selective transfers produced colony type T4 cells, which proved to be avirulent. Fifteen months of selective transfer of T4 cells in vitro produced T4 colonies which did not revert to the parental type T1 colonial morphology at a detectable rate.

Within the past few years, there have been much data accumulated to show that virulent type T1 and T2 cells possess pili, whereas the avirulent cells which give rise to type T3 and T4 colonies do not exhibit this cellular appendage (11, 20). The correlation between colonial morphology, piliation, and virulence has led to the conclusion that pili play an important role in the pathogenesis of gonococcal infection. Piliated cells not only show increased ability to attach to tissue culture cells (19) and mammalian erythrocytes (17), but also are more resistant to phagocytosis than T3 or T4 cells (17, 21). Additionally, cells from type T1 and T2 colonies of *N. gonorrhoeae* are more capable than T3 or T4 cells of prolonged survival in subcutaneous chambers (7); they have greater infectivity and lethality for the chicken embryo (1, 2), and only T1 and T2 cells appear competent for transformation (18).

One strain of colony type T1 gonococci has been shown to possess extrachromosomal deoxyribonucleic acid (DNA), although no characterization of this putative plasmid was presented (6). The rapid transition from piliated type T1 and T2 cells to nonpiliated avirulent cells, the eventual irreversable loss of virulence of T4 cells, and the loss of the capacity to form T1 cells, together with the knowledge that some pili are encoded by bacterial plasmids (16), has led to speculation that the genetic determinants for colonial morphology and possibly other genetic determinants of virulence might be plasmid mediated. In this paper, we report the characterization of plasmid DNA from several gonococcal isolates. No association between colonial type and the presence of a plasmid was observed.

MATERIALS AND METHODS

Bacterial strains. The strains and sources of *N. gonorrhoeae* used in this study are listed in Table 1. Cultures were verified by gram stain, oxidase reaction, and carbohydrate fermentation. The strains of *Escherichia coli* used were SF184, a thymine auxotroph derived from W1485, and a K-12 carrying R144 of Hedges and Datta (10).

Media. The solid medium used for gonococci was a

TABLE 1. *Strains and sources of N. gonorrhoeae*

Stock no.	Colony type[a]	Site of isolation	Source
KH1432	DWF	Throat	K. K. Holmes
KH1911	T1	Cervix	K. K. Holmes
KH1947	T3	Synovial fluid	K. K. Holmes
KH1990	T1	Cervix	K. K. Holmes
KH4190FL	T1	Urethra	K. K. Holmes
F62T1	T1	Urethra	D. Kellogg (11)
F62T4	T4[b]		D. Kellogg (11)
SFG 390	T3		Derived from KH1911
SFG 391	T3		Derived from KH1990
SFG 392	T4		Derived from KH1990
SFG 393	T3		Derived from KH4190FL
SFG 394	T4		Derived from KH4190FL

[a] Colonial types according to Kellogg (11). DWF, Dwarf colony type (does not fit into Kellogg's scheme). They are 0.1 mm or less in diameter, light brown in color, and translucent. They have low convex elevation and are glistening and friable, with slightly crenated or irregular edges which sometimes appear as if part of the colony had flown into a trough cut in the agar while streaking.

[b] Passed in vitro for 35 months by Kellogg.

modification of the GCB2DS described by Kellogg (13), which consisted of Difco GC medium base plus a supplement composed of glucose (40 g), L-glutamine (0.5 g), ferric nitrate (50 mg), thiamine pyrophosphate (1 ml of a 0.2% solution), and distilled water (100 ml). The supplement was filter sterilized (Nalgene 0.45-μm membrane), and 1% was added to cooled molten agar immediately prior to pouring plates.

Broth medium for gonococci was similar in composition to GC medium base without agar, except *N*-2-hydroxyethyl-piperazine-*N'*-2'-ethanesulfonic acid was used (7.15 g/liter) as buffer instead of phosphate, and 0.5 g of soluble starch per liter was used instead of corn starch. The pH of the broth was adjusted to 7.2 with NaOH or HCl before autoclaving. Before using, the broth was prewarmed to 36 C, and 1% of the supplement was added.

The medium used for *E. coli* was previously described (9).

Materials. Reagents and sources were as described previously (9) and as follows: *N*-2-hydroxyethyl-piperazine-*N'*-2'-ethanesulfonic acid and thiamine pyrophosphate chloride from Calbiochem, [2-^{3}H]adenine (6 to 23 Ci/mmol), and [*methyl*-^{14}C]thymine (47 mCi/mmol) from New England Nuclear Corp.

Labeling of cells. Broth (10 to 30 ml) with 0.1 to 0.3 mCi of [^{3}H]adenine was inoculated with several colonies of the appropriate colonial morphology from an overnight plate. The flask was placed in a 5% CO_2 incubator for several minutes and then removed. The screw cap was immediately tightened and the flask was incubated 12 to 16 h in a shaker-water bath at 36.5 C.

E. coli cultures were labeled as previously described (9).

Isolation of plasmid DNA. Cleared lysates and sarkosyl lysates were prepared, and dye-buoyant density centrifugation was done as described previously (9). After suspension, the cells were frozen overnight and thawed just before lysis (6). [^{14}C]Thymine-labeled SF184 DNA was used in all gradients as a linear marker.

More than 90% of the precipitable counts after labeling with [^{3}H]adenine were present in ribonucleic acid, as is found in *N. meningitidis* (15). Alternate fractions were, therefore, alkaline hydrolyzed with 0.5 ml of 1 N NaOH at 37 C overnight and then were neutralized with 0.5 ml of 1 N HCl before precipitating with 5% trichloroacetic acid.

On several occasions, plasmid DNA was prepared for electron microscopy by making a cleared lysate of cells from a 30- to 100-ml unlabeled culture, which was submitted to dye-buoyant centrifugation with ^{14}C-labeled *E. coli* chromosomal DNA as marker, and fractionated. The alternate fractions were precipitated and counted; the fractions pooled were between 14 to 18 fractions corresponding to the more dense side of the ^{14}C-labeled linear marker, as was consistently observed with labeled cultures.

Plasmid DNA was also prepared for dye-buoyant centrifugation by the preferential precipitation method (9).

Characterization of plasmid DNA. The mole fraction of guanine plus cytosine (G+C) of purified plasmid DNA was determined in neutral cesium chloride (CsCl) density gradients; electron microscopy of plasmid DNA and contour length measurements were done as previously described (9). The number of copies of plasmid DNA per chromosome equivalent was determined in whole cell sarkosyl lysates centrifuged to equilibrium in CsCl-ethidium bromide gradients as described earlier (3).

RESULTS

The demonstration of plasmids in N. gonorrhoeae. Six strains of *N. gonorrhoeae* were initially examined to determine if they contained plasmid DNA. Cleared lysates from [^{3}H]adenine-labeled cells were prepared and centrifuged in CsCl-ethidium bromide. Five of six strains showed a small dense DNA component characteristic of covalently closed-circular (CCC) DNA molecules (Fig. 1). The only strain that did not show a DNA fraction which banded in the characteristic position of CCC molecules was the strain KH1432, an unusual strain, forming dwarf colonies, that was isolated from the throat of a patient with disseminated gonococcal infection.

The material from the dense CCC fractions was pooled, extracted with isopropyl alcohol, dialyzed, and examined in the electron microscope. Electron microscopy revealed that these fractions from each of the five strains contained circular DNA. Upon initial examination, the majority of the molecules were in the CCC form. After storage at 4 C for 1 week, most of the molecules had relaxed to the open-circular form (Fig. 2). Contour length measurements of these circular species were approximately the same for each isolate, ranging from 2.34 to 2.45 $\times$ 10^6 daltons in size with an average for the five strains of 2.37 $\times$ 10^6 daltons (Table 2). The DNA plasmid pool of each strain occasionally showed circular species somewhat larger than the vast majority of molecular species present in the preparation. These larger species represented 0.1% or less of the total plasmid DNA and were invariably some multiple (usually twice the size) of the basic monomeric form. We presume that these molecules represent oligomeric species (dimers) of the basic DNA plas-

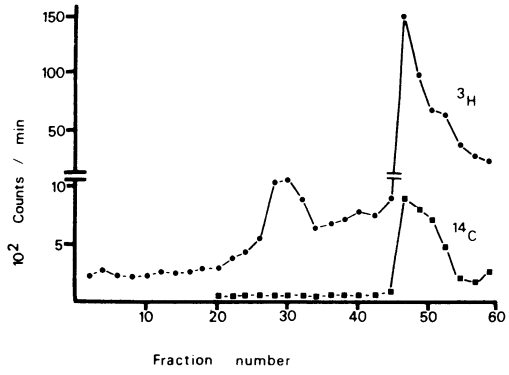

FIG. 1. *Separation of gonococcal plasmid DNA from chromosomal DNA by dye-buoyant density centrifugation. Symbols:* ●, *[^3H]Adenine-labeled gonococcal DNA;* ■, *[^{14}C]thymine-labeled E. coli DNA employed as a linear marker.*

TABLE 2. *Contour length measurements of circular species*

Strain no.	No. of expt	No. of molecules measured	Mean mol wt
KH1911	2	73	2.45 ± 0.15
KH1990	4	387	2.34 ± 0.11
KH1947	1	62	2.43 ± 0.13
KH4190FL	1	43	2.40 ± 0.09
F62T1	1	109	2.39 ± 0.04

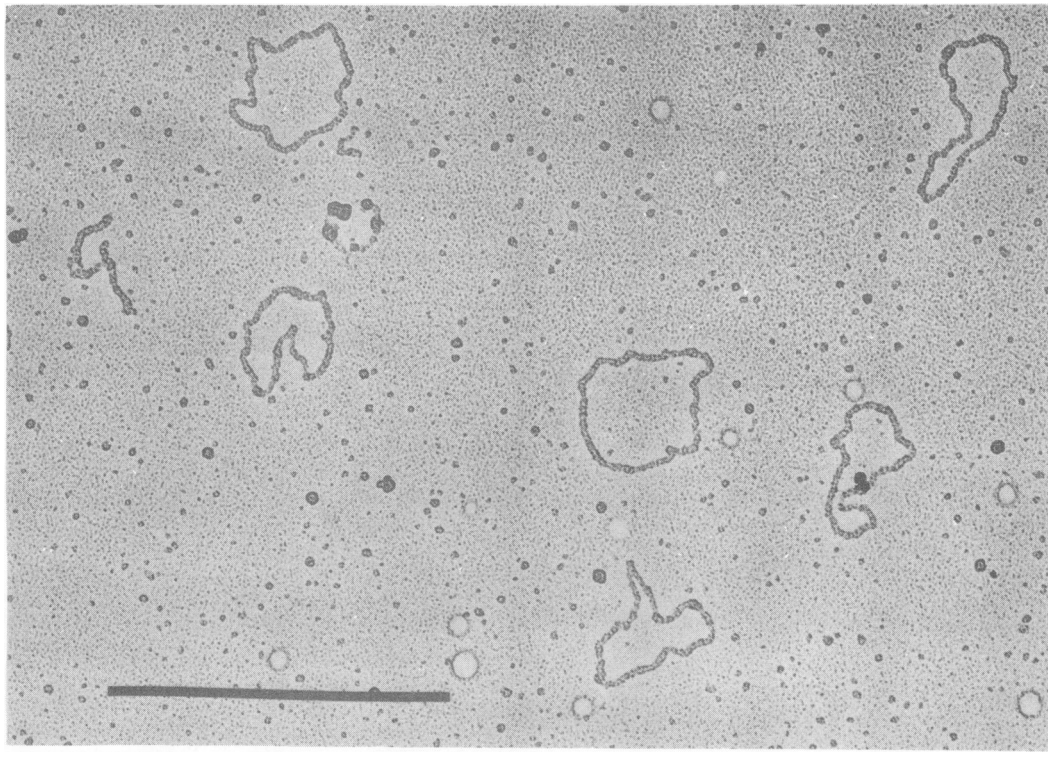

FIG. 2. *Electron micrograph of KH1911 plasmid DNA isolated by dye-buoyant density centrifugation. Bar equals 1 μm.*

mid species similar to the oligomeric species of small plasmid observed in enteric organisms (3).

Since the plasmid species in the gonococcal isolates were uniformly of a relatively small size, we were interested in the possibility that the methodology employed, or some form of nuclease activity, was precluding the isolation of larger plasmid species from the cultures. Consequently, *E. coli* K-12 cells known to contain the 65×10^6 dalton R-plasmid R144 (8) were mixed with cells of strain KH1990. The mixture was lysed by the cleared lysate procedure, submitted to dye-buoyant density centrifugation, precipitated, and counted. There was no evidence of the loss of CCC forms of the R-plasmid by our procedures, suggesting that larger gonococcal plasmids, if present, would have been detected by our methods.

Plasmid base composition (G+C) determination. Plasmid DNA from the dye-buoyant density centrifugation procedure was centrifuged to equilibrium in neutral CsCl density gradients to determine its buoyant density relative to added ^{14}C-labeled *E. coli* DNA (1.710 g/cm^3, 0.50-mol fraction of G+C). The plasmid DNA and the *E. coli* marker DNA banded in identical positions in the gradient (Fig. 3). The mole fraction G+C of the gonococcal plasmid species from KH1990, KH4190FL, and F62T1 gave identical results, and we concluded that these plasmid species have the same G+C content of 0.50, essentially identical to that of the *N. gonorrhoeae* chromosomal DNA (5).

Determination of copy pool size. It has been well documented in enteric species that plasmids may be divided into two distinct classes on the basis of whether their replication is under stringent or relaxed control. In part, this distinction has been that larger plasmids ($>40 \times 10^6$ daltons) replicate under stringent control and are present as but a few copies per cell. In contrast, small plasmids ($<20 \times 10^6$ daltons) most often replicate randomly as a multicopy pool (3). [^{3}H]Adenine-labeled cells of several gonococcal isolates were lysed with sarkosyl, centrifuged to equilibrium in CsCl-ethidium bromide, fractionated, hydrolyzed with NaOH, and counted. The fraction of the plasmid DNA was estimated from the proportion of [^{3}H]adenine counts in the CCC fraction relative to the total ^{3}H-labeled DNA counts. The average proportion of CCC plasmid DNA in the isolates examined ranged from 6 to 8%. On the basis of an average of 2.5×10^6 daltons for a single plasmid and 1×10^9 daltons for the chromosome of the gonococcus (14), it may be calculated that the average number of plasmids per chromosome equivalent was between 24 and 32.

Plasmid complement of colonial variants of N. gonorrhoeae. Types T3 and T4 colonial variants of strains KH1911, KH1990, and KH4190FL were isolated as described by Kellogg (13) and were streaked daily for at least 70 single clone transfers. Additionally, a type T4 colonial variant of strain F62 passed in vitro for 35 months by Kellogg was obtained. All of these colonial variants were found to be nonpiliated by electron microscopy, to be avirulent in the chicken embryo model, and to possess the typical colony morphology corresponding to the Kellogg classification scheme. Plasmid DNA isolated from these colonial variants were not found to be significantly different from their counterparts in type T1 colony-forming cells from each of the strains in terms of molecular size, mole fraction G+C, and average number of plasmid copies per chromosome equivalent. Comparative data are shown in Table 3. The relative distribution of plasmid size in type T1 and type T4 colony-forming cells is shown in Fig. 4, where it can be seen that there was no evidence of an alteration of any plasmid subclass during the type T1 to type T4 transition.

DISCUSSION

Five of six gonococcal isolates examined have been found to harbor a multicopy plasmid pool of small CCC DNA species that have an average G+C content similar to that of the gonococcal chromosome. These plasmid species bear a superficial resemblance in terms of molecular size and in terms of their presence as a multi-

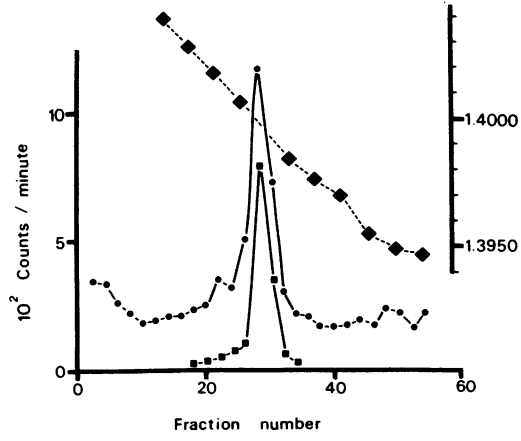

FIG. 3. *Mole fraction G+C determination of purified KH1990 plasmid DNA by neutral CsCl density centrifugation. Symbols:* ●, *[^{3}H]Adenine-labeled gonococcal plasmid DNA isolated by dye-buoyant density centrifugation;* ■, *[^{14}C]thymine-labeled E. coli DNA marker (1.710 g/cm^3, 0.50 mol fraction G + C);* ◆, *refractive index (N_D^{20}).*

TABLE 3. *Comparative data*

Strain no.	Colony type	No. of molecules measured	Mean mol wt	No. of plasmid copies per chromosome	Mole fraction G+C
KH1911	T1	33	2.43 ± 0.18		
SFG390	T3	23	2.40 ± 0.11		
KH1990	T1	190	2.36 ± 0.12	30	0.50
SFG391	T3	101	2.38 ± 0.15		
SFG392	T4	79	2.39 ± 0.05	27	0.50
KH4190FL	T1	43	2.40 ± 0.09		0.50
SFG393	T3	57	2.42 ± 0.18		
SFG394	T4	72	2.36 ± 0.13		0.50
F62T1	T1	109	2.39 ± 0.04	28	0.50
F62T4	T4	96	2.32 ± 0.10	29	0.50

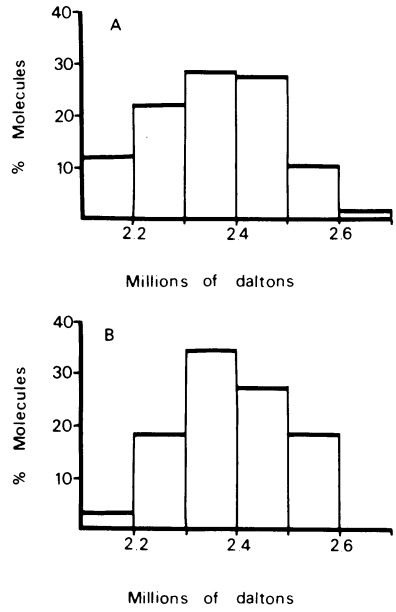

FIG. 4. *Distribution of molecular weights of KH1990 plasmid molecules measured. A, type T1 cells; B, type T4 cells.*

copy gene pool to the Col E1 and nonconjugative antibiotic resistance determinants of enteric bacteria (3). However, the gonococcal plasmid species remain cryptic in the phenotypic sense and do not appear to be related to the in vitro colonial variation of gonococci associated with the loss of pili.

The demonstration of plasmids in any bacterial species can no longer be considered as an exceptional finding. In the case of pathogenic bacterial species, however, plasmids often take on added significance, since a number of plasmids have been shown to encode for properties which directly contribute to the pathogenicity of the host cell or to permit the bacterial cell to persist within the animal host. We have no evidence to show that the plasmid species described in this investigation contribute in any way to the pathogenesis of gonococcal infection. The failure to demonstrate a plasmid in one gonococcal strain associated with disseminated gonococcal infection is not considered significant, since other disseminated gonococcal infection strains examined subsequently have been found to harbor the small plasmid species. Clearly, the amount of potential genetic information contained on a plasmid of 2.5×10^6 daltons would be expected to be limited in terms of its contribution to the host cell phenotype. At present, the results of this investigation (and unpublished observations with a number of other gonococcal isolates) merely point out that the multicopy pool of this small plasmid, whatever its phenotype, is a common addition to the gene pool of gonococci.

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ADDENDUM IN PROOF

We have recently characterized plasmid species of 0.6×10^6, 1.1×10^6, and 2.5×10^6 daltons from *N. flavescens* (ATCC 13120). We are currently attempting to determine whether these and other plasmids isolated from "nonpathogenic" *Neisseria* are related to gonococcal plasmid species.

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