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PHYSICAL AND GENETIC ANALYSIS OF A CONJUGATIVE PLASMID OF NEISSERIA GONORRHOEAE AND ITS ROLE IN THE EVOLUTION OF GONOCOCCAL STRAINS

by

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and Research in partial fulfillment of
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ABSTRACT

In vitro conjugation using donor strains of *Neisseria gonorrhoeae* demonstrated mobilization of the gonococcal 7.2 kb and 5.1 kb beta-lactamase-encoding plasmids by a co-resident gonococcal conjugative plasmid. Mobilization of a novel 4.9 kb beta-lactamase-encoding plasmid and a 4.2 kb cryptic plasmid by a gonococcal conjugative plasmid was not observed.

The mobilization frequency of the 7.2 kb plasmid varied with the auxotype of the gonococcal recipient strain, but not according to the serovar. The mobilization frequency was consistently lower (ie. $<10^{-5}$ transconjugants/input donor cfu) to gonococcal recipient strains with multiple nutritional requirements, including proline-citrulline-uracil-requiring and ornithine-uracil-hypoxanthine-requiring strains, as compared to non-requiring and proline-requiring strains.

Mobilization of the 7.2 kb plasmid varied between gonococcal donor strains. Three gonococcal conjugative plasmids examined were structurally identical as ascertained from identical restriction patterns for the enzymes *Bgl*II, *Bcl*I, *Hind*II and *Pvu*I, suggesting that gonococcal conjugative plasmids represent a single group of related plasmids. Construction of a detailed restriction map of the gonococcal conjugative plasmid pJD38 and subsequent comparison with a previously reported map did demonstrate minor structural differences between gonococcal conjugative plasmids.

Extensive restriction endonuclease mapping and analysis by Southern blot and DNA hybridization suggested that the gonococcal conjugative plasmid may have as many as three separate regions encoding conjugative functions. Gonococcal conjugative plasmid pJD38 hybridized to a moderate degree to conjugative plasmids from *Haemophilus* and W Incompatibility group plasmid, pSa322A. Little or no hybridization was observed between pJD38 and plasmids representative of a variety of Incompatibility groups from *Enterobacteriaceae* and *Pseudomonaceae*. This suggests that gonococcal conjugative plasmids may represent a unique group of closely related plasmids.

RÉSUMÉ

La conjugaison in vitro, utilisant une souche donnatrice de *Neisseria gonorrhoeae*, a démontré la mobilisation des plasmides de 7.2 kb et de 5.1 kb, codant pour une bêta-lactamase, par l'intermédiaire d'un plasmide conjugatif co-résident d'origine gonococcique. La mobilisation d'un nouveau plasmide de 4.9 kb, codant aussi pour une bêta-lactamase, ou du plasmide cryptique de 4.2 kb par le plasmide conjugatif gonococcique n'a pas été observée.

La fréquence de mobilisation du plasmide de 7.2 kb variait avec l'auxotype de la souche réceptrice de *N. gonorrhoeae*, mais pas avec le sérotype. La fréquence de mobilisation était régulièrement plus faible (c.-à.-d.$<10^{-5}$ transconjugants/colonie du donneur) avec des souches réceptrices ayant de multiples besoins nutritifs (auxotypes proline-citrulline-uracile et ornithine-uracile-hypoxanthine), qu'avec des souches n'ayant aucun besoin nutritif ou d'auxotype proline.

La mobilisation du plasmide de 7.2 kb variait avec les souches gonococciques donnatrices. Trois plasmides conjugatifs gonococciques qui ont été caracterisés, se sont avérés identiques basé sur l'identité des patrons de bandes observés pour des digestions avec les enzymes de restrictions *Bgl*II, *Bcl*I, *Hin*dII et *Pvu*I. Ces données suggèrent que les

plasmides conjugatifs gonococciques représentent un seul groupe de plasmides apparentés. La construction d'une carte dessites de restriction du plasmide conjugatif gonococcique pJD38, et la comparaison de cette carte avec une carte précédemment rapportée dans la littérature, a démontré des différences structurales mineures.

La cartographie détaillée avec endonuclease de restriction ainsi que l'analyse par transfert de Southern et par hybridation d'ADN a suggéré que le plasmide conjugatif gonococcique pourrait avoir jusqu'à trois régions séparées codant pour les fonctions de conjugaison. Le plasmide conjugatif gonococcique pJD38 a hybridé modérément avec un plasmide conjugatif provenant d'Haemophilus ainsi qu'avec le plasmide pSa322A; peu ou pas d'hybridation a été observé avec des plasmides représentant une variété de groupe d'incompatibilité provenant des Enterobacteriaceae et des Pseudomonaceae. Ces données suggèrent que les plasmides conjugatifs gonococciques pourrait représenter un groupe unique de plasmides très apparentés.

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To Mark

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LIST OF ABBREVIATIONS

The genetic abbreviations are in accordance with the proposals of Novick et al. (1976) and Demerec et al. (1966).

-/+	Absence/presence of phenotype
A	Absorbance
Ap	Ampicillin
·C	Celcius
C	Citrulline
cfu	Colony forming unit
Cm	Chloramphenicol
DNA	Deoxyribonucleic acid
EDTA	Ethylene diamine tetra-acetate
GCMB	Gonococcal Medium Base agar
>	Greater than
h	Hours
H	Hypoxanthine
Hg	Mercury
Inc	Incompatibility
kb	Kilobase
Kn	Kanamycin
L	Liter
lac	Lactose-fermenting
<	Less than
M	Molar
MDa	Megadaltons
mg	Milligram
MIC	Minimum inhibitory concentration

min	Minutes
mL	Milliliter
Nal	Naladixic acid
Nm	Neomycin
NR	Non-requiring
O	Ornithine
%	Percent
P	Proline
r	Resistance
rec A	Defective homology-based recombination
res mod	Restriction modification
Rif	Rifampicin
rpm	Revolutions per minute
RT	Room temperature
SDS	Sodium dodycyl sulfate
Sm/Str	Streptomycin
Sp	Spectinomycin
Su	Sulfonamide
Tc	Tetracycline
Tn	Transposon
Tra	Conjugal transfer
TSA	Trypticase Soy agar
U	Uracil
µg	Microgram
v/v	Volume per unit volume
w/v	Weight per unit volume

INTRODUCTION

Antibiotic-resistance-encoding plasmids were first identified in Neisseria gonorrhoeae in 1976 (Elwell et al., 1977a). Subsequent epidemiological analysis demonstrated the rapid dissemination of antibiotic-resistance-encoding plasmids among gonococcal strains (Jephcott et al., 1978; Perine et al., 1977). Shortly afterwards, the gonococcal conjugal system, mediated by a gonococcal conjugative plasmid, was discovered (Baron et al., 1977; Eisenstein et al., 1977b; Kirven and Thornesberry, 1977; Roberts and Falkow, 1977). However, investigation into the genetics of the gonococcal conjugative plasmid, as well as the roles of gonococcal plasmids and conjugative host range in N. gonorrhoeae, as specified by auxotypes, has been limited. It is these aspects of the gonococcal conjugal system that this study addresses.

LITERATURE REVIEW

I CONJUGATION IN *N. gonorrhoeae*

a) Genetic Exchange in the Gonococcus

i) Conjugation

Stiffler *et al*. (1975) first described a class of large plasmids in *N. gonorrhoeae* lacking any detectable phenotype. These plasmids had a molecular weight of approximately 24.5 megadaltons (MDa) and varied about this mean plus or minus three MDa (Roberts *et al*., 1978; 1979; Sox *et al*., 1978; Stiffler *et al*., 1975). Although these plasmids may vary in size, the gonococcal conjugative plasmid has generally been referred to as the 24.5 MDa or 39.2 kilobase (kb) plasmid. Preliminary molecular analyses including hybridization, buoyant density and restriction endonuclease generated patterns suggested that these large molecules shared a common core of nucleotide sequence similarity [70 to 100 percent (%)] (Roberts *et al*., 1979; Sox *et al*., 1978). Shortly after the discovery of this large plasmid species, several workers demonstrated conjugative properties of the 39.2 kb plasmid (Baron *et al*., 1977; Eisenstein *et al*., 1977b; Kirven and Thornesberry, 1977; Roberts and Falkow, 1977).

'Conjugation' is the transfer of DNA which requires cell-to-cell contact and is mediated by a specific conjugative or transfer plasmid which encodes the necessary functions (Achtman and Skurray, 1977; Willets and Skurray, 1980; Willets and Wilkins, 1984). In addition to self-transfer, conjugative plasmids are often capable of transferring certain non-conjugative plasmids to suitable recipients in a process termed mobilization (Clark and Warren, 1979).

The property of mobilization was first used to detect conjugal activity of the large gonococcal plasmids since such plasmids used in early studies lacked any easily detectable phenotypic marker, making detection of self-transfer difficult. Several workers showed that the presence of the 39.2 kb plasmid in a donor gonococcal strain was required for mobilization of certain co-resident gonococcal beta-lactamase-encoding plasmids to a suitable recipient strain (Baron et al., 1977; Eisenstein et al., 1977b; Kirven and Thornesberry, 1977; Roberts and Falkow, 1977). Self-transmissability was subsequently detected by Roberts et al. (1979) and Sox et al. (1978) by demonstrating the presence of the 39.2 kb plasmid in gonococcal transconjugants that had acquired beta-lactamase production, following gel electrophoresis of transconjugant DNA.

Under certain conditions, when integrated into the chromosome, a conjugative plasmid can transfer the host chromosome from the donor cell to a recipient cell (Lederberg and Tatum, 1946). Initial results suggested that the gonococcal conjugative plasmid could transfer chromosomal genes during conjugation, since recombinants for a variety of chromosomal markers were observed in prolonged filter matings (Biswas et al., 1976; Roberts and Falkow, 1978). It is now known that these results were obtained not through conjugation, but by a DNase-resistant transformation mechanism (Biswas et al., 1980b; Norlander et al., 1979; Sox et al., 1978; Steinberg and Goldberg, 1980).

ii) Transformation

Gonococci have been shown to be naturally competent in genetic transformation (Catlin, 1974; Sparling, 1966). Norlander et al. (1979) suggested that the gonococcal conjugative plasmid enhanced transformation competence of recipient cells; however, other workers have not been able to repeat these experiments (Sparling et al., 1981).

The mechanism of transformation appears to involve sequence-specific uptake and likely accounts for chromosomal transfer from cell to cell (Biswas et al., 1980b; Graves et al., 1982; Norlander et al., 1979; Sox et al., 1978). On the other hand, uptake of gonococcal plasmids by transformation accounts for a minimal or rare level of gonococcal plasmid exchange (Biswas et al., 1980b). Plasmids usually undergo deletions on transformation unless the recipient contains an homologous plasmid (Biswas et al., 1980b; Sox et al., 1979).

iii) Transduction

Despite several attempts, no one has demonstrated bacteriophage specific for the gonococcus, suggesting that either transduction of genetic material among gonococcal strains does not occur or is a rare event (Goldberg et al., 1978). The absence of transduction and the rare event of plasmid exchange by transformation in the absence of homologous uptake sequences in the recipient, suggests that conjugation is the major mode of plasmid exchange in the gonococcus (Roberts et al., 1978).

b) Properties of the Gonococcal Conjugative System

i) Self-transmissability of the Gonococcal Plasmid

Simple and sensitive analysis of the self-transmissability and host range of the gonococcal conjugative plasmid was hindered in early studies by the lack of an easily detectable phenotypic marker. However, a group of workers recently isolated a naturally-occurring gonococcal conjugative plasmid (40.3 kb) harboring the tetracycline-resistance determinant *tet*M, a transposon-borne determinant initially found in the genus *Streptococcus* (Morse et al., 1986). The 40.3 kb tetracycline-resistance-encoding conjugative plasmid was also detected in natural isolates of *Kingella denitrificans* and *Eikenella corredens*. *In vitro* transfer of the 40.3 kb plasmid from a gonococcal donor strain was detected to a variety of *Neisseria* species including strains of *N. gonorrhoeae*, *N. meningitidis*, *N. cinerea*, *N. flava*, *N. perflava*-*N. sicca*, *N. subflava*, *N. flavascens*, *N. lactamica* and *N. mucosa*, but not strains of *Branhamella catarrhalis* (Roberts and Knapp, 1988). In contrast, the host range of the 39.2 kb gonococcal conjugative plasmid is narrower, with transfer detected only to strains of *N. gonorrhoeae* and *N. cinerea* (Flett *et al*., 1981; Genco *et al*., 1984; Roberts and Knapp, 1988; Sox *et al*., 1978). In such host range studies there was not sufficient information to determine whether the host range of the gonococcal conjugative plasmid was limited either by its transfer and/or replication functions, or as a result of other barriers to conjugation (ie. restriction barriers).

Host range studies using the 40.3 kb conjugative plasmid encoding tetracycline-resistance could not demonstrate the stable transfer into restriction deficient (res^-) *Escherichia coli* recipients (Morse *et al*., 1986). Similarly, the 39.2 kb gonococcal conjugative plasmid does not appear to replicate stably in certain recipient strains, including res^- *E. coli* and *Haemophilus influenzae*. However, mobilization of a gonococcal beta-lactamase-encoding plasmid resident in an intermediate res$^-$ E. coli donor strain by a 39.2 kb conjugative plasmid resident in a gonococcal donor strain to an E. coli recipient strain has been observed in triparental matings. This suggested that the gonococcal conjugative plasmid may survive transiently in the intermediate res$^-$ E. coli donor strain long enough to provide the functions necessary for the mobilization of the resident non-conjugative plasmid (Flett et al., 1981; Morse et al., 1986; Sox et al., 1978).

ii) Mobilization of Plasmids

The gonococcal conjugative plasmid can mobilize a variety of non-conjugative plasmids resident in res$^-$ E. coli strains derived from E. coli and H. influenzae (Flett et al., 1981). Plasmids mobilized by the conjugative plasmid included NTP5 and ColE1.Tn501 of Enterobacteriaceae origin, but not NTP1 or NTP1y; and RSF0885 from Parainfluenzae and pJB1 from Haemophilus ducreyi were also mobilized.

The mobilization of a class of small gonococcal plasmids known as cryptic plasmids (4.2 kb) has not been demonstrated (Flett et al., 1981; Roberts and Falkow, 1977). These cryptic plasmids were first observed by Maness and Sparling (1972) and subsequently characterized and genetically analyzed by several workers (Davies and Normark, 1980; Foster and Foster, 1976; Mayer et al., 1974; Palchaudhuri et al., 1975; Roy et al., 1988). The structure of these plasmids is highly conserved but small insertions, deletions and differences in restriction patterns have been reported (Davies and Normark, 1980; Foster and Foster, 1976; Roberts et al., 1977; Roy et al., 1988).

Mobilization of certain gonococcal non-conjugative plasmids that encode a TEM-1 type beta-lactamase has been demonstrated by a number of workers (Baron et al., 1977; Eisenstein et al., 1977b; Flett et al., 1981; Genco et al., 1984; Kirven and Thornesberry, 1977; Roberts and Falkow, 1977). These plasmids vary in size by virtue of various DNA deletions but are closely related structurally (Roberts et al., 1977; Sox et al., 1979; Yeung et al., 1986). The most common beta-lactamase-encoding plasmids found in clinical isolates of gonococci are the 7.2 kb and 5.1 kb plasmids (Perine et al., 1977; Roberts et al., 1979).

Mobilization can occur by one of two proposed mechanisms, 'conduction' and 'donation' (Broome-Smith, 1980; Clark and Warren, 1979). Some plasmids can exploit both mechanisms. Conduction is the process whereby a transfer plasmid causes transmission of a non-conjugative plasmid by physical association with it to form a cointegrate (Broome-Smith, 1980). Donation is the process whereby a non-conjugative plasmid is transferred via effective contact determined by a conjugative plasmid without physical association of the two plasmids (Clark and Warren, 1979).

Donation appears to require at least one cis-acting (bom) and one trans-acting (mob) gene on the donated plasmid (Broome-Smith, 1980; Clark and Warren, 1979; Finnegan and Willets, 1972). In general, the donated plasmid is transferred as efficiently as the conjugative plasmid. On the other hand the transfer frequency of a conducted plasmid is much lower than that of a donated plasmid probably because recombination between two plasmids to form a cointegrate could be rare (Clark and Warren, 1979).

In the gonococcus there is no evidence of stable cointegrate formation between the conjugative plasmid and the 7.2 kb beta-lactamase-encoding plasmid (Sox et al., 1978). Such studies could not rule out the possibility of transient cointegrate formation. However, the gonococcal beta-lactamase-encoding plasmid is tranferred at high frequency (Biswas et al., 1980a), thereby supporting the donation model for the mobilization of the beta-lactamase-encoding plasmid by the gonococcal conjugative plasmid (Sox et al., 1978).

The frequency of mobilization of the 7.2 kb beta-lactamase-encoding plasmid has been reported to vary from 10^{-5} to 10^{-3} (Baron et al., 1977; Eisenstein et al., 1977b; Kirven and Thornesberry, 1977; Roberts and Falkow, 1977; Sox et al., 1978). Despite the reported variation, the 7.2 kb plasmid has been mobilized at a very high frequency; up to 10^{-1} transconjugants/input donor cfu (colony forming unit) in matings with isogenic strains of gonococci (Biswas et al., 1980a). Molecular variations in beta-lactamase-encoding plasmids however can affect the mobilization frequency. Tenover *et al*. (1985) noted that a composite gonococcal plasmid lacking a 1.9 kb *Hin*fI fragment of the 7.2 kb plasmid could not be mobilized to either gonococcal or res$^-$ *E. coli* recipient strains (Tenover *et al*., 1985).

The host range for the *in vitro* mobilization of gonococcal beta-lactamase-encoding plasmids is much broader than for the 39.2 kb conjugative plasmid alone, but comparable to that of the 40.3 kb tetracycline-resistance-encoding conjugative plasmid (Eisenstein *et al*., 1977b; Genco *et al*., 1984; Ikeda *et al*., 1986; Roberts and Falkow, 1977; Roberts and Knapp, 1988; Sox *et al*., 1978). The 7.2 kb beta-lactamase-encoding plasmid is mobilized by the 39.2 kb gonococcal conjugative plasmid to a number of *Neisseria* species, including *N. gonorrhoeae*, *N. flava*, *N. flavascens*, *N. subflava*, *N. mucosa*, *N. perflava*-*N. sicca* and *N. cinerea*. Transfer of the 7.2 kb plasmid was not detected to all recipient strains of those species tested (Genco *et al*., 1984). Furthermore, mobilization of the 7.2 kb plasmid was not detected to either *N. lactamica*, *B. catarrhalis* or *N. meningitidis* (Genco *et al*., 1984). However, Dillon *et al*. (1983) documented the spread of the 7.2 kb plasmid to *N. meningitidis*, a result confirmed by the *in vitro* conjugation experiments of Ikeda *et al*. (1986). The conjugative plasmid also successfully mobilized the 7.2 kb plasmid to strains of

H. influenzae and res$^-$ *E. coli* (Eisenstein *et al*., 1977b; Flett *et al*., 1981; Sox *et al*., 1978).

Although the host range of the 39.2 kb gonococcal conjugative plasmid is narrow, it is apparent that some non-conjugative plasmids efficiently exploit the mobilization functions of the conjugative plasmid and can be mobilized to an even broader host range than the conjugative plasmid. This suggests that transient vectors like the gonococcal conjugative plasmid might be important in nature permitting penetration of resistance and other genes into gene pools and the subsequent dissemination into new host species (Flett *et al*., 1981).

c) Conjugation and the Ecology and Distribution of Gonococcal Plasmids

A number of workers have correlated plasmid content, auxotype and serovar in gonococcal strains (Danielsson *et al*., 1983; Dillon *et al*., 1981; 1986; 1987; Dillon and Pauze, 1981b; 1984; Sompolinsky *et al*., 1985; van Embden *et al*., 1985). In order to demonstrate auxotype, plasmid and serological relationships gonococci can initially be subdivided according to the presence or absence of beta-lactamase-encoding plasmids. Gonococcal strains harboring plasmids encoding beta-lactamase are termed penicillinase-producing *N. gonorrhoeae* or "PPNG" (Elwell *et al*., 1977a).

The PPNG and non-PPNG categories can then be further characterized according to predominant serogroups/serovars, as well as auxotype. In the most recent study of non-PPNG strains in Canada by Dillon et al. (1987), the majority (67%) were found to belong to the serogroup WII/III. This serogroup was found to comprise eighteen serovars and fifteen auxotypes; however, the most predominant auxotypes were the non-requiring (NR) and proline-citrulline-uracil-requiring (PCU^-). Thirty-three percent of the non-PPNG strains were plasmid-free and the majority of plasmid-free strains were PCU^-. Conversely, PCU^- strains were always plasmid-free, a finding confirmed by previous studies (Dillon and Pauze, 1981b). Moreover, the presence of the gonococcal conjugative plasmid correlated "appreciably" with the NR or proline-requiring (P^-) auxotypes.

The remaining WI serogroup non-PPNG strains comprised eight serovars and ten auxotypes; the most predominant auxotype being arginine-uracil-hypoxanthine-requiring (AUH^-). The arginine requirement may be due to citrulline and/or ornithine requirements since both of these intermediates are precursors in the arginine biosynthetic pathway (Catlin and Nash, 1978). The majority (98%) of these strains harboured only the 4.2 kb cryptic plasmid. The presence of the 4.2 kb plasmid was not correlated with the presence or absence of any other plasmid(s) or with any particular auxotype; however isolates containing a 4.2 kb plasmid but lacking a beta-lactamase-encoding plasmid were distributed evenly between NR and P$^-$ strains (Dillon and Pauze, 1984; Roberts <u>et</u> <u>al</u>.; 1979; Stiffler <u>et</u> <u>al</u>., 1975).

Historically, PPNG strains were distinguished as "African" or "Asian" types based on such criteria as demographics, plasmid content, susceptibility to antimicrobial agents and auxotype (Elwell and Falkow, 1977; Perine <u>et</u> <u>al</u>., 1979; Riou and Courvalin, 1985). "Asian-type" strains carried a 7.2 kb beta-lactamase-encoding plasmid with or without a conjugative plasmid, were auxanographically typed as being either NR or P$^-$, and were resistant to antibiotics such as tetracycline. "African-type" strains harboured a 5.1 kb beta-lactamase-encoding plasmid, rarely harboured conjugative plasmids and were ornithine-requiring (O$^-$).

The majority of Canadian PPNG strains tested by Dillon <u>et</u> <u>al</u>. (1987) comprised the WI serogroup. Several workers have observed that serogroup W1 PPNG strains that were typed auxanographically as NR or P$^-$ or A$^-$ could be traced to African origins, whereas certain WII/III serovars were characteristic of PPNG isolated on the Asian mainland (Bygdeman, 1981; Bygdeman <u>et</u> <u>al</u>., 1981; 1983; Dillon <u>et</u> <u>al</u>., 1987; Odugbemi <u>et</u> <u>al</u>., 1983). Moreover, the conjugative plasmid has been isolated predominantly from NR and P$^-$ auxotypes consistent with the "Asian-type" strain profile (Dillon and Pauze, 1981b). The spread of the conjugative plasmid and beta-lactamase-encoding plasmids into gonococcal strains of other auxotypes in natural isolates has been reported (Ansink-Schipper *et al.*, 1982; Dillon and Pauze, 1981a; 1984; Dillon *et al.*, 1986; Jephcott *et al.*, 1978; Johnston and Kolator, 1982; Odugbemi *et al.*, 1983; van Embden *et al.*, 1985). Thus, the "African-type" and "Asian-type" categories for PPNG strains are no longer sufficient to describe the distribution of gonococcal plasmids relative to strain auxotype.

Observations of the distribution of gonococcal plasmids among various auxotypes and serogroups suggests that the appearance of new combinations of gonococcal plasmids, auxotypes and serogroups occurred by conjugation in nature. Previous studies, however, of conjugation in the gonococcus, of the mobilization of gonococcal plasmids and of the potential role of conjugation in the spread of gonococcal plasmids has been hindered by the general lack of/or inadequate genetic characterization (eg. auxotype) of gonococcal strains used in previous studies (Eisenstein *et al.*, 1977b; Flett *et al.*, 1981; Kirven and Thornesberry, 1977; Sox *et al.*, 1978).

II GENETIC CHARACTERIZATION AND CLASSIFICATION OF GONOCOCCAL CONJUGATIVE PLASMIDS

a) Relationships Between Non-gonococcal Conjugative Plasmids

Several criteria have been used to establish relationships between conjugative plasmids. These included incompatibility, host range, fertility inhibition, properties of plasmid-encoded products such as pili or surface exclusion, genetic complementation of transfer genes (*tra*) and nucleic acid sequence analysis of *tra*.

i) Incompatibility and Host Range

Incompatibility and host range have been the basis for the primary classification of conjugative plasmids (Jacob *et al*., 1977). Incompatibility is functionally defined as the inability of two distinct plasmids to be stably co-inherited in a single clone of dividing bacteria, usually *E. coli*, in the absence of continued selective pressure for both plasmid types (Timmis, 1979). In general, if two plasmids can stably replicate in the same cell, then they have different replication systems and belong to different Incompatibility (Inc) groups (Timmis, 1979). Hence, conjugative plasmids in the same Inc group tend to share a common replication system. Moreover, conjugative plasmids in the same Inc group usually have similar conjugation systems (Datta, 1975; 1979). At least thirty Inc groups are recognized in *E. coli* K-12 (Datta, 1975; 1979; Jacob *et al.*, 1977).

Host range is the second indication of replication specificity. Plasmids may be able to replicate in one cell type but not another. In general, plasmids of a given Inc group show similar host ranges (Datta, 1979; Jacob *et al*, 1977). For example, conjugative plasmids of IncC, IncN, IncP, IncQ and IncW have broad host ranges and replicate stably in a wide variety of Gram-negative bacteria (Jacob *et al.*, 1977). On the other hand, plasmids of IncFII do not replicate in *Pseudomonas*; plasmids of IncIalpha do not replicate in *Pseudomonas* or *Proteus* species, plasmids of IncV are unstable in *E. coli*, and plasmids of IncP-2 do not replicate in *E. coli* (Jacob *et al.*, 1977). However, many host-plasmid systems have not been tested and there are a number of factors other than replication ability that may affect host range (eg. restriction barriers).

ii) Genetic Complementation of Plasmids

Genetic complementation between transfer genes has been used as criteria for relationships between conjugative systems (Foster and Willets, 1976; 1977; Ohtsubo *et al.*, 1970). For example, most conjugative plasmids code for surface appendages called pili, which can play a significant role in the initial contact between potential donor and recipient cells (Datta and Hedges, 1971). Similarity of pili encoded by plasmids of the same Inc group has been established by determining whether non-piliated mutants of one plasmid can be transferred by the conjugation system of a second (Willets, 1970). However, minor variations in the genes specifying the pilin subunit protein of pili in IncF plasmids have been identified (Alfaro and Willets, 1972; Lawn and Meynell, 1970; Willets, 1971). Moreover, it has been found that IncI conjugative plasmids will not complement transfer-deficient mutants of F, although in some cases an F-factor can utilize the I-factor transfer system (Cooke _et al_., 1970; Willets, 1970). Such studies raise the question as to what degree of complementation represents plasmid relatedness and is thus required to establish Inc groups or subgroups.

iii) Plasmid Properties

There is evidence for the correlation of Inc group with properties such as molecular weight, percent guanine plus cytosine content and copy number (Datta, 1979; Hedges and Datta, 1972; 1973; Meynell _et al_., 1986; Willets and Maule, 1979). A brief survey of these properties for various Inc groups however, shows overlapping in many of these characteristics (Jacob _et al_., 1977) and for this reason these criteria alone are often not sufficient for establishing the Inc group of a conjugative plasmid.

iv) Nucleic Acid Sequence Analysis

Nucleic acid sequence analysis by hybridization studies between plasmids of a variety of Inc groups has shown several important trends. In general, plasmids of the same Inc group tend to share a large degree of nucleotide sequence similarity (40 to 80%), while plasmids in different Inc groups do not (<10%) (Falkow et al., 1974; Grindley et al., 1973; Ingram, 1973; Roussell and Chabbert, 1978; Sharp et al., 1973). The location of nucleotide sequence similarity in plasmids belonging to the same Inc group is largely localized in that region(s) of the plasmid encoding the functions necessary for conjugation (Falkow et al., 1974; Grindly et al., 1973; Roussel and Chabbert, 1978). There are, however, exceptions to this general rule of sequence similarity, where much hybridization is observed between compatible plasmids, or little hybridization is observed between incompatible plasmids (Datta, 1975).

Usually, but not always, incompatibility indicates relatedness, but compatible plasmids can also be related (Broda, 1979). Despite apparent differences in phylogenetic origin, virtually all plasmids show some minimal level of nucleotide sequence similarity (Grindley et al., 1973;

Ingram, 1973; Roussell and Chabbert, 1978). Roussel and Chabbert (1978) studied plasmids from ten different Inc groups (C, M, FI, FII, W, N, I1, H1, H2, H). Generally, less than 15% reassociation occurred between plasmids from different Inc groups. However, a general trend of intermediate reassociation (10 to 17%) was observed between plasmids of IncFI, FII, C and M groups. Similarly, Grindley _et al_. (1973) noted that plasmids of the IncB and IncI1 groups had as much as 25% nucleotide sequence similarity. The significance of this intermediate nucleotide sequence similarity is not known; it seems too large to represent fortuitous matching and thus, may reflect a low level of genetic exhange between heterogenic plasmids or evolutionary threads of conserved nucleotide sequences (Falkow, 1975). On the other hand, it may reflect co-evolution of similar sequences.

Recent studies have attempted to identify conserved nucleotide sequences in plasmids from different Inc groups (Golub and Low, 1985; 1986). Golub and Low (1986) found nucleotide sequences similar to the F conjugative plasmid leading region containing the _ssb_ gene, which encodes a single-strand DNA binding protein, in plasmids belonging to the IncIalpha, Y, 9, B and K groups. Studies of this kind raise questions as to the degree of nucleotide sequence similarity that is required to establish Inc groups when relatively small or intermediate amounts of hybridization are observed. In general, information regarding not only the degree of nucleotide sequence similarity but also the relative location of similar nucleotide sequences in plasmids of various Inc groups, may provide useful information for establishing Inc groups of conjugative plasmids and identifying evolutionary conserved tra sequences in plasmids from different Inc groups, as well as locating tra regions in conjugative plasmids.

Another less direct approach to classifying conjugative plasmids has been to suppose that in related regions of plasmids, target sites for site-specific endonucleases will be conserved. Complete digestion of two related plasmids will yield a number of restriction fragments of identical sizes, recognizable by gel electrophoresis. This was shown to be the case with a series of IncF plasmids (Thompson and Achtman, 1979). Chabbert et al. (1979) found similar EcoRI restriction patterns for plasmids from IncC, M and I1 suggesting that plasmids within these Inc groups shared similar structural "cores" and that the structural arrangements of these "cores" are different from one Inc group to another. On the other hand, EcoRI restriction patterns of eight IncN plasmids showed very little structural similarity (Chabbert et al., 1979). These differences could be due to transposon insertions in multiple sites. However, in a more recent study by Konarska-Kozlowska and Iyer (1983), the majority of IncN plasmids examined showed conservation of distribution of *BglII* and *KpnI* restriction sites. Moreover, restriction endonuclease fragments of similar electrophoretic mobility encoded the same genetic function as determined by hybridization of restriction fragments with DNA probes coding for known phenotypic traits (Konarska-Kozlowska and Iyer, 1983).

In general, the "core" of structural regularity is related to the organization of genes involved in conjugation in the plasmid. Conjugation genes are often organized into a single contiguous region (Brown and Willets, 1981; Hartskeerl *et al*., 1984; Thatte and Iyer, 1983; Ward and Grinsted, 1982; Willets and Skurray, 1980). Some plasmids may have more than one conjugation or *tra* region; for example three *tra* regions have been identified in the IncP plasmid RP4 (Depicker *et al*., 1977). Correlation of restriction and genetic maps of conjugative plasmids indicates that fewer restriction endonuclease recognition sites for enzymes with hexanucleotide specificity are located within the *tra* region. Most of the restriction sites are clustered within 'nonessential' regions often occupied by antibiotic genes.

III A MOLECULAR APPROACH TO THE GENETIC ANALYSIS OF THE GONOCOCCAL CONJUGATIVE PLASMID

This study initiates a genetic analysis of the gonococcal conjugative plasmid in order to identify the tra region(s) and examine potential nucleic acid sequence relationships with conjugative plasmids of various Inc groups. The characteristic structural regularities observed in conjugative plasmids including the conservation of restriction endonuclease recognition sites in plasmids from the same Inc group and the general absence of sites in tra region(s) of conjugative plasmids provides a useful approach for the localization of the putative tra region(s) in the gonococcal plasmid.

In order to examine structural regularities in the gonococcal conjugative plasmid a variety of restriction endonucleases were first screened for the presence of enzyme restriction sites on the conjugative plasmid. Restriction endonucleases found to cleave the gonococcal conjugative plasmid a limited number of times were selected for restriction mapping, since the complexity of restriction mapping increases with the number of restriction fragments to be mapped. The subsequent restriction maps generated for various enzymes were examined for trends or regularities in restriction site distribution. Conservation of restriction sites in conjugative plasmids isolated from different gonococcal strains was also examined.

Moreover, assuming plasmids with similar conjugation systems demonstrate nucleotide sequence similarities, then restriction endonuclease analysis coupled with Southern blot and nucleic acid hybridization analysis between the gonococcal conjugative plasmid and conjugative plasmids representative of a variety of Inc groups may provide an indication of not only the phylogenetic relationships between the gonococcal conjugative plasmid and other conjugative plasmids, but also the genetic organization of the gonococcal conjugative plasmid.

This study also explores the ultimate role of the gonococcal conjugative plasmid in the evolution of novel gonococcal strains. Mobilization of various classes of gonococcal plasmids is examined with particular emphasis on the mobilization of the 7.2 kb gonococcal beta-lactamase-encoding plasmid from gonococcal donor strains to gonococcal recipient strains varying in plasmid content and auxotype. The genetic characteristics of gonococcal strains constructed _in vitro_ by intraspecific matings can then be compared to current trends in the genetic properties of naturally-occurring clinical isolates of _N_. _gonorrhoeae_ to ascertain the role of conjugation in the generation and evolution of gonococcal strains _in vivo_.

MATERIALS AND METHODS

I MEDIA, ENZYMES, CHEMICALS AND REAGENTS

a) Media

Trypticase Soy agar (TSA) and Trypticase Soy broth (TSB) were purchased from Oxoid Limited (Nepean, Ont., Canada). Other media, including: Mueller Hinton agar, Noble agar, MacConkey agar, Gonococcal Medium Base agar (GCMB), Proteose #3 agar, Brain Heart Infusion broth and Cystine Trypticase agar Base Medium, as well as carbohydrates (glucose, lactose, maltose, sucrose, fructose, mannitol) were purchased from Difco Laboratories (Detroit, Mich., U.S.A.). New York City agar was purchased from Bioclass (Sherbrooke, Que., Canada). Casamino acids, Skim Milk medium and hemoglobin were supplied by Difco Laboratories, and Isovitalex supplement by Becton Dickinson and Company (Mississauga, Ont., Canada).

b) Antibiotics

Naladixic acid, rifampicin and streptomycin sulfate were purchased from the Sigma Chemical Company (St. Louis, Miss., U.S.A.). Ampicillin was purchased from Ayerst Laboratories (Saint-Laurent, Que., Canada). Tetracycline was purchased from Bristol-Meyers Pharmaceutical Group (Candiac, Que., Canada). Antibiotic susceptibility disks were purchased from Warner Lambert Incorporated (Morris Plains, N.J., U.S.A.).

c) Enzymes

The majority of restriction endonucleases used in this study were purchased from Boehringer Mannheim Company (Dorval, Que., Canada). They include the following enzymes: *Acc*I, *Aha*III, *Ava*I, *Ava*II, *Alu*I, *Bst*EII, *Bam*HI, *Bgl*I, *Bgl*II, *Dpn*I, *Eco*RI, *Hae*II, *Hae*III, *Hin*dIII, *Hin*fI, *Mbo*I, *Mbo*II, *Msp*I, *Nci*I, *Pst*I, *Pvu*I, *Rsa*I, *Sac*I, *Sau*3A, *Sst*II, *Stu*I, *Xba*I, *Xho*I. Additional enzymes; such as, *Kpn*I, *Hin*dII, *Hha*I, *Hpa*II, *Mlu*I and *Sal*I were obtained from Amersham International (Oakville, Ont., Canada), and *Bcl*I was purchased from Pharmacia Limited (Dorval, Que., Canada).

Specialized enzymes such as lysozyme, proteinase K and DNase I were obtained from Boehringer Mannheim Company, whereas DNA polymerase I (Kornberg and Klenow fragment) was purchased from Amersham International.

d) Chemicals and Reagents

Chemicals and reagents used in agarose and polyacrylamide gel electrophoresis (ie. ultrapure agarose, bis-acrylamide, ammonium persulfate and N,N,N',N'-tetramethylethylene diamine-TEMED) were purchased from Bio-Rad Laboratories (Mississauga, Ont., Canada). Acrylamide and crude agarose were purchased from Schwarz/Mann Biotech (Cambridge, Mass., U.S.A.). All vitamins and amino acids used were obtained from the Sigma Chemical Company.

Special chemicals and reagents were purchased from a variety of suppliers. Cesium chloride was obtained from Terochem Laboratories Limited (Toronto, Ont., Canada), Sephadex G-50 from Pharmacia, bovine serum albumin from the Sigma Chemical Company, lambda DNA, herring sperm DNA and deoxyribonucleotides (dGTP, dCTP, dTTP, dATP) from Bethesda Research Laboratories (Burlington, Ont., Canada), pBR322 DNA and dithiothreitol from Boehringer Mannheim Company and radiolabelled [alpha-^{32}P] dATP from Amersham International. All other chemicals and reagents in this study were purchased either from Fisher Scientific Company (Don Mills, Ont., Canada), Sigma Chemical Company or British Drug House (Toronto, Ont., Canada).

II BACTERIAL STRAINS AND PLASMIDS

All strains of N. gonorrhoeae used in these studies are listed in Table 1. The strains, all naturally-occurring isolates, were selected from the culture collection of the Antimicrobials and Molecular Biology Division, Laboratory Centre for Disease Control, Ottawa, Canada. E. coli strains used in this study are listed in Table 2.

Plasmids used in these studies are listed in Table 3. Dr. V.N. Iyer (Dept. of Biology, Carleton University) kindly supplied a large number of E. coli plasmid-containing strains. Two plasmid-containing strains of H. influenzae were gifts from Dr. P. Mendelman (Children's Orthopedic Hospital and Medical Center, Seattle, Wash., U.S.A.). Dr. W.L. Albritton (Dept. of Microbiology, University of Saskatchewan) kindly donated H. influenzae strain R217 containing a modified conjugative plasmid (pHD147.Tn2) from H. ducreyi. All plasmid DNA was isolated and purified during these studies (see Section V).

TABLE 1. Bacterial strains: *N. gonorrhoeae* and *N. cinerea*.

Strain^a	Auxotype^b	Ampicillin^c MIC (mg/L)	Antibiotic resistance^d Host	Antibiotic resistance^d Plasmid	Plasmid content (kb)^e	Serovar^f
N. gonorrhoeae						
CH992	P$^-$	0.25			38.9	Aedih
1-182	P$^-$	128		Ap	39.2 7.2 4.2	Bcegjk
1-881	NR	>256		Ap	39.2 7.2 4.2	Back
1-159	NR	>256		Ap	39.2 5.1 4.2	Ae
1-530	NR	>256		Ap	39.2 4.9 4.2	Bacjk
1-554	P$^-$	>256		Ap	7.2 4.2	Bcgjk
1-627	PCU$^-$	0.25	Nal			Bacejk
1-835	PCU$^-$	0.42	Rif			Bacejk
1-183	NR	0.125	Rif		4.2	Bahjk
CH811	NR	0.5	Rif			Aedih
A9	OUH$^-$	0.016	Str		4.2	Aedgkih
H12593	P$^-$	0.032	Rif		4.2	Bacejk
N. cinerea						
1-485	POUH$^-$	ND^g	Nal			ND

[a] Spontaneous antibiotic-resistant mutants of _N. gonorrhoeae_ and _N. cinerea_ were isolated and characterized in this study (see Section IIId).

[b] Bacterial auxotypes were determined in this study (see Section IIIc). The symbols P, C, O, U, H define nutritional requirements for proline, citrulline, ornithine, uracil and hypoxanthine, respectively. NR denotes a non-requiring or wild-type strain.

[c] Ampicillin minimum inhibitory concentrations were determined in this study (see Section IIIe).

[d] Antibiotic-resistance determined by host cell chromosome or plasmid was determined in this study (see Section IIIc). Antibiotic abbreviations denote ampicillin (Ap), naladixic acid (Nal), rifampicin (Rif) and streptomycin (Str). Chromosomal-encoded antibiotic-resistance is indicated by three letter abbreviation, in contrast to two letter antibiotic abbreviations for plasmid-encoded antibiotic-resistance.

[e] The size of the plasmid in strain CH992 was determined in this study (see Results, Section IIIf). Other plasmid sizes were based on the similar electrophoretic mobility to plasmid controls defined by this laboratory.

[f] Serological characterization was performed in this study (see Section IIIf). All serovars beginning with A belonged to serogroup WI and those beginning with B were WII/III.

[g] ND indicates not determined.

TABLE 2. Bacterial strains: *E. coli*.

Strain	Phenotype[a]	Source[b]	Reference
DH1	Nal^r lac^+ res^-mod^+ rec^-	LCDC	Low (1968)
DT591	Nal^r lac^- res^-mod^+	D. Taylor	Taylor *et al.* (1981)
C600	Nal^r lac^- res^-mod^-	LCDC	Appleyard (1954)
1100	Rif^r Str^r lac^+ res^+mod^+ rec^-	LCDC	Durwald and Hoffman-Berling (1968)
HB101	Rif^r Str^r lac^- res^-mod^-	LCDC	Boyer and Roulland-Dussoix (1969)

[a] Abbreviations for chromosomal-encoded antibiotic-resistance include naladixic acid (Nal), rifampicin (Rif) and streptomycin (Str). Restriction modification is indicated by res mod and lactose fermentation by lac. Proficiency and deficiency are indicated by + and - , respectively. rec^- denotes defective homology-based recombination.

[b] LCDC represents the Laboratory Centre for Disease Control.

TABLE 3. Bacterial plasmids.

Plasmid	Plasmid size (kb)	Relevant marker[a]	Source	Reference
pJD7	4.9	Ap^r	K.-H. Yeung	Yeung *et al.* (1986)
pCU101	24.5	Cm^r Tra^+	V.N. Iyer	Thatte and Iyer (1983)
pSa322	19.4	Ap^r Tra^+	V.N. Iyer	Tait *et al.* (1983)
RP4	54.4	Tc^r Kn^r Ap^r Tra^+	V.N. Iyer	Datta and Hedges (1971)
R6K	40.0	Sm^r Ap^r Tra^+	V.N. Iyer	Hedges and Datta (1973)
R112	19.4	Nm^r Kn^r Tra^+	V.N. Iyer	Scavizzi (1974)
pDT87	320.0	Ap^r Cm^r Tc^r Su^r Tra^+	D. Taylor	Whiteley and Taylor (1983)
R100	112.0	Cm^r Sm^r Sp^r Su^r Tc^r Hg^r Tra^+	V.N. Iyer	Datta and Hedges (1972)
pBR322	4.4	Ap^r Tc^r	Boehringer Mannheim Co.	Bolivar *et al.* (1977)
R849	57.6	Tc^r Tra^+	P. Mendelman	Stuy (1980)
RSF007	48.0	Ap^r Tra^+	P. Mendelman	De Graaf *et al.* (1976)
pHD147.Tn2	35.0	Ap^r Tra^+	W.L. Albritton	McNicol *et al.* (1986b)

[a] Relevant markers denote ampicillin (Ap), chloramphenicol (Cm), kanamycin (Kn), tetracycline (Tc), streptomycin (Sm), neomycin (Nm), sulfonamide (Su), spectinomycin (Sp) and mercury (Hg) plasmid-encoded resistance, and conjugal transfer proficiency (Tra^+).

III GROWTH, IDENTIFICATION AND GENETIC AND BIOLOGICAL CHARACTERIZATION OF STRAINS

a) Growth and Identification of Bacterial Strains

Strains of N. gonorrhoeae were subcultured on GCMB agar supplemented with 1.0% Kellogg's defined supplement at 35 degrees celcius (·C) in a humid environment containing 5.0% carbon dioxide (Kellogg et al., 1963). Antibiotic-resistant strains were subcultured on GCMB containing combinations of the following antibiotics: 5.0 milligram per litre (mg/L) ampicillin, 1000.0 mg/L streptomycin, 50.0 mg/L naladixic acid, 50.0 mg/L rifampicin.

Strains of N. gonorrhoeae were identified by Gram stain, oxidase test and carbohydrate utilization using the cystine tryptic agar sugar utilization method (Morello et al., 1985; Reyn, 1980; Vera, 1948). Strains of N. gonorrhoeae were purified on selective New York City agar and additionally characterized by colony morphology on GCMB agar (Kellogg et al., 1963; Martin et al., 1974; Swanson et al., 1971). In addition, auxotyping (see Section IIIc) and plasmid profile analysis were used to further characterize strains of Neisseria species.

H. influenzae strains were subcultured on Chocolate agar (Proteose #3 agar supplemented with 1.0% hemoglobin and 1.0% Isovitalex) and incubated at 35·C in a humid environment containing 5.0% carbon dioxide. Alternately, H. influenzae strains were grown in 3.5% Brain Heart Infusion broth supplemented with 10.0 mg/L histidine, 10.0 mg/L hemin and 2.0 mg/L nicotinamide adenine dinucleotide phosphate at 35°C. Strains of H. influenzae were identified by Gram stain and an obligatory requirement for Factor X (hemin) and Factor V [beta-nicotinamide adenine dinucleotide (NAD)] when incubated at 37°C in room air (Kilian, 1980). Strains were also screened for plasmid content (see Section V) and antimicrobial susceptibilities were tested by Kirby-Bauer Single Disk Diffusion test (see Section IIIe).

E. coli strains were subcultured on TSA at 37°C or grown in TSB with aeration at 90 oscillations/minute in a shaker bath (Fisher Scientific Co.) at 37°C. Antibiotic-resistant strains were grown on TSA containing one of the following antibiotics: 100.0 mg/L ampicillin, 1000.0 mg/L streptomycin, 40.0 mg/L naladixic acid or 40.0 mg/L rifampicin. E. coli strains were identified by Gram stain, the inability to oxidize dimethylparaphenylene diamine, and a variety of biochemical tests including the ability to use acetate but not citrate as a sole carbon source, as well as production of acid from the fermentation of glucose and other carbohydrates (Cowen, 1980; Martin and Washington, 1980). E. coli strains were tested for antimicrobial susceptibility by the Kirby-Bauer Single Disk Diffusion test [National Committee for Clinical Laboratory Standards (NCCLS), 1985] (see Section

IIIe) and lactose-fermenting organisms were differentiated from lactose-non-fermenting strains on MacConkey agar.

b) Storage of Bacterial Strains

E. coli strains were stored either at room temperature (RT) on Dorsett egg slants [750 milliliters (mL) whole eggs, 250 mL 0.85% NaCl] with wax sealed caps or stored frozen at -70°C in small aliquots of TSB plus 40.0% glycerol (Dillon, 1983). *N. gonorrhoeae* and *H. influenzae* strains were stored either in freezing solution [GC medium broth, pH 7.2, 5.0 weight per unit volume (w/v) bovine serum albumin] at -70°C or by lyophilization in 2.0% Skim Milk medium (Dillon, 1983).

c) Determination of Nutritional Requirements (Auxotype)

The auxotyping medium used was developed by Hendry and Stewart (1979) and further modified by Hendry (Dillon, 1983). Auxotypes were determined by noting requirements for one or a combination of proline, citrulline, citrulline replaceable by ornithine, uracil and hypoxanthine as detected by individual chemical deletions from the defined medium. The basic auxotyping medium consisted of purified double strength Noble agar combined with double strength basic liquid medium, pH 7.2 as described by Dillon (1983). 0.5% volume per unit volume (v/v) of each of the stock solution containing a single nutritional marker [0.21 molar (M) L-citrulline, 0.21M L-ornithine, 0.8M L-proline, 0.2M uracil, 0.14M hypoxanthine) was added to each petri dish containing basic auxotyping media, except where a requirement for a specific nutritional marker was being tested. In that case, the nutritional marker was omitted from the mixture. Each auxotyping plate also contained 0.5% (v/v) amino acids mixture (0.75M cysteine HCl, 0.25M L-cystine) except for the Negative control plate, since cysteine (or cystine) is required by all strains of gonococci (Catlin, 1973).

Auxotyping plates were inoculated in either one of two ways. A master plate of colonies was grown overnight in a matrix fashion on GCMB agar (Dillon, 1983). A Steers replicator was used to pick up colonies from the master plate and deliver the cells into auxotyping buffer [10.0% (v/v) Salts solution, 0.005% (v/v) glycerol-lactate solution, phosphate buffer, pH 7.0] (Steers _et_ _al_., 1959). Auxotyping media were subsequently inoculated with the Steers replicator. In the second inoculation technique colonies from the master plate were transferred to a sterile velvet cloth and the velvet was used to inoculate the auxotyping media, thus transferring a replica of the colonies from the master plate to each auxotyping plate (Lederberg and Lederberg, 1952). This was useful for determining the auxotype of large numbers of _N_. _gonorrhoeae_ strains simultaneously. All inoculated plates were incubated under conditions described previously (see Section IIIa), and the plates were scored for growth after 24 and 48 hours (h) of incubation.

d) Selection of Antibiotic-resistant Strains

To isolate spontaneous antibiotic-resistant mutants of Neisseria species, a generous loopful of 18 to 24 h bacterial growth was suspended in 1.0 mL of 0.7% casamino acids and 0.5 mL of the cell suspension was used to inoculate a Chocolate agar plate containing 5.0 to 10.0 mg/L of a particular antibiotic (ie. rifampicin, naladixic acid or streptomycin). Plates were incubated for 48 h under conditions described previously (see Section IIIa).

Single colonies were picked and subcultured once on Chocolate agar containing 5.0 to 10.0 mg/L of the same antibiotic, followed by subculture on Chocolate agar containing successively higher antibiotic concentrations, until colonies resistant to 50.0 mg/L rifampicin, 50.0 mg/L naladixic acid or 1000.0 mg/L streptomycin were obtained.

The auxotype of the spontaneous antibiotic-resistant strains was verified by selecting a single mutant colony and streaking it out on media to isolate single colonies. Fifty to one-hundred colonies were picked and auxotyped (see Section IIIc). Of these, one colony was selected and streaked to obtain isolated colonies. Again, fifty to one-hundred of these colonies were selected and auxotyped. Of those tested, one colony was selected and subcultured for subsequent experiments.

e) Antimicrobial Susceptibility Testing

The agar dilution method was used to determine the minimum inhibitory concentration (MIC) for a variety of antibiotics (NCCLS, 1985). For testing N. gonorrhoeae strains GCMB agar was used in MIC determinations (Dillon, 1983). Dilutions of filter-sterilized antibiotic stock solutions (10,000.0 mg/L) were added to molten media to give two-fold serial dilutions of antibiotic with final concentrations ranging from 0.002 mg/L to 256.0 mg/L. Specific concentration ranges tested varied with each antimicrobial agent.

Isolates were tested for susceptibility to ampicillin (0.002-256.0 mg/L) and tetracycline (0.063-2.0 mg/L). A master plate containing bacterial colonies grown in a matrix fashion was used as a template to inoculate media for MIC determination using a Steers replicator (see Section IIIc). The inoculated plates were incubated under conditions described previously (see Section IIIa) and plates were read after 24 and 48 h to determine that antibiotic concentration necessary to inhibit bacterial growth or which allowed the growth of only one colony.

The *in vitro* susceptibility of *E. coli* and *H. influenzae* strains to antimicrobial agents was tested by the Single Disk Diffusion test (NCCLS, 1985). To standardize the inoculum density, a 0.5 McFarland nephelometer barium sulfate standard was used. The standard was prepared by adding 0.5 mL of 1.0% (w/v) barium chloride solution to 99.5 mL of 1.0% (v/v) sulfuric acid solution. Bacterial cells from a 20 h culture were suspended in 0.7% casamino acids to obtain a turbidity visually comparable to that of the McFarland standard. A sterile cotton swab was dipped in the suspension and used to inoculate the entire surface of either a Chocolate agar plate (*Haemophilus* species), or a Mueller-Hinton agar plate (*E. coli* strains).

The antimicrobial disks [ampicillin 10.0 micrograms (ug), chloramphenicol 30.0 ug, kanamycin 30.0 ug, naladixic acid 30.0 ug, neomycin 30.0 ug, streptomycin 10.0 ug, tetracycline 30.0 ug] were placed on the surface of the inoculated agar plate and gently pressed onto the agar surface with sterile forceps. The plates were inverted and incubated at 35°C. After 16 to 18 h of incubation, the diameters of the zones of inhibition were measured including the disk diameter. The susceptibility of the strain to the antimicrobial agent was determined by comparing the size of the zones of inhibition with interpretive zone sizes (NCCLS, 1985).

f) Serological Characterization

Strains were serologically characterized by co-agglutination using a panel of monoclonal antibodies to protein Pl as described by Tam et al. (1982). Serogroups were subdivided into different serovars using monoclonal antibodies specific for protein 1A or 1B. All serovars beginning with A belonged to serogroup WI, whereas all beginning with B were serogroup WII/WIII. Monoclonal antibodies were purchased from S. Bygdeman of Huddinge University Hospital, Sweden.

IV BACTERIAL CONJUGATION

a) Determination of Conjugation Frequency

A general filter mating method was used for all conjugation experiments (Sox et al., 1978). Matings consisted of a 24 h incubation of donor and recipient in a 1:1 ratio for intraspecific gonococcal matings and a 10:1 ratio for intergeneric matings, except in triparental matings where the ratio of donor:intermediate donor:recipient was 10:10:1.

Both donor and recipient strains were suspended in 1.0 mL of 0.7% casamino acids to an approximate bacterial density of 10^8 cells/mL as estimated by nephelometry (see Section llle). Approximately 10^7 colony forming units (cfu) of both donor and recipient were added to a sterile Metricel membrane filter (13 millimeter diameter, 0.45 micrometer pore size, Gelman Sciences Incorporated, Dorval, Que., Canada) resting on an agar plate suitable for supporting growth of both donor and recipient strains.

The number of cfu/mL was confirmed by viable cell counts. Serial ten-fold dilutions of the donor and recipient bacterial suspensions were made in 0.7% casamino acids, and 0.1 mL of each dilution was plated on supplemented GCMB or TSA with a sterile bent glass rod and incubated under conditions previously described (see Section IIIa). After 48 h incubation, the colonies were counted and the total number of donor and recipient cfu placed on the mating filter was calculated.

The mating filter was incubated 24 h under conditions described in Section IIIa. The filter was carefully transferred with sterile forceps from the agar plate to a sterile tube containing 2.0 mL 0.7% casamino acids. The tube was vortexed for 20 seconds to disrupt mating pairs and to remove cells from the filter. The cells were pelleted for 10 minutes (min) at 2,000 revolutions per minute (rpm) in an IEC-7-Centra centrifuge (Fisher Scientific Company). The pellet was washed twice by resuspending the cells in 2.0 mL of 0.7% casamino acids and re-centrifuging under the same conditions. The pellet was then resuspended in 1.0 mL 0.7% casamino acids. Ten-fold serial dilutions of the mating cell suspension were made and 0.1 mL of the original suspension and each dilution were plated onto media selective for beta-lactamase-producing transconjugants. Cells were incubated under conditions described previously (see Section IIIa).

N. gonorrhoeae transconjugants were selected on GCMB agar containing ampicillin (5.0 mg/L), and either rifampicin (20.0 mg/L), naladixic acid (40.0 mg/L) or streptomycin (50.0 mg/L) depending on the phenotype of the recipient strain. E. coli transconjugants were selected on TSA containing ampicillin (100.0 mg/L) and either naladixic acid (40.0 mg/L), rifampicin (20.0 mg/L) or streptomycin (50.0 mg/L) depending on the phenotype of the recipient strain. Antibiotic plates were incubated under conditions described previously (see Section IIIa) until colonies were visible. The number of colonies on selective medium were counted at each dilution and the mobilization frequency was expressed as the total number of transconjugants/input donor cfu (Sox et al., 1978). Fifty to one-hundred putative transconjugant colonies were selected and purified twice on selective medium.

Control experiments to ascertain the frequency of spontaneous mutation included incubating donor and recipient strains separately on filters. Control experiments to exclude transformation included incubating the recipient strain with an equal volume of a cell-free lysate of the donor strain followed by plating on appropriate selective media. The donor strain was suspended in 1.0 mL of 0.7% casamino acids to a density of 10^8 cells/mL and left at RT overnight. Gonococci are highly autolytic and freely release biologically active transforming DNA into their environment (Sarubbi and Sparling, 1974). The suspension was then centrifuged in an IEC-7-Centra centrifuge at 2000 rpm for 10 min to remove cellular debris. The mating suspensions were incubated for 24 h on filters and plated on appropriate selective media. An equal volume of cell lysate was plated on GCMB to verify the lysate was free of viable cells. In addition, cells from Type 4 transparent colonies of N. gonorrhoeae were used in matings since they are essentially non-competent for transformation (Sparling, 1966). A control to exclude transduction as a mode of genetic transfer consisted of incubating 0.1 mL of the donor strain suspension filtered through a 13 millimeter diameter, 0.45 micrometer pore size filter (Gelman Sciences Incorporated) with 0.1 mL of the recipient strain suspension.

V ISOLATION AND VISUALIZATION OF PLASMID DNA

a) Boiling Method

A boiling method was used to screen cells for plasmid content. The procedure is an adaptation by Dillon et al. (1985) of the method developed by Holmes and Quigley (1981). Organic extractions of cell lysates cleared of debris by centrifugation were used to inactivate and remove proteins. The DNA suspension was mixed with an equal volume of TE-saturated phenol (TE: 0.05M Tris-HCl, pH 8.0, 0.05M disodium EDTA, 0.005M NaCl) and centrifuged in a minifuge at maximum speed for 3 min at 4°C. The upper aqueous layer was transferred to a clean tube and extracted with an equal volume of a phenol-chloroform mixture (1:1), followed by a third extraction of the aqueous phase with an equal volume of chloroform [24:1 (v/v) chloroform and isoamyl alcohol] (Maniatis et al., 1982). The aqueous phase was then transferred to a clean tube and the DNA was recovered by precipitation with ethanol as described below.

Precipitation of DNA with ethanol was completed as described by Dillon et al. (1985). The salt concentration of the DNA suspension was first adjusted to 0.3M sodium acetate by adding an appropriate volume of 3.0M stock solution. Two volumes of ethanol at RT were then added to the solution. After mixing by gentle inversion the tube was stored either at -20·C for 2 h or at -70·C for at least 20 min. DNA was pelleted in a Brinkman minifuge (Toronto, Ont., Canada) at full speed for 20 min at 4·C. The supernatant was poured off and the tube inverted to air dry the DNA pellet. DNA was stored by either suspending the DNA in sterile distilled water and freezing at -70·C or storing in ethanol at -20·C.

b) SDS Alkaline Method

Strains of *E. coli* and *Haemophilus* species were lysed by a modified alkaline-detergent method (Dillon *et al.*, 1985) derived from three groups (Birnboim and Doly, 1979; Casse *et al.*, 1979; Kado and Liu, 1981). A loopful of cells scraped from agar plates of an 18 to 24 h culture, or cells from 3.0 mL of an 18 to 24 h broth culture (see Section IIIa) were used for lysis. Cells grown in broth were pelleted in a Sorvall RC5C centrifuge (Du Pont Canada Inc., Lachine, Que., Canada) at 10,000 rpm for 10 min at 4·C prior to lysis. When lysing *Haemophilus* strains it was necessary to place cell lysates in a boiling water bath for 1 min to decrease the amount of gelatinous material often recovered after lysis. Organic extractions of cell lysates (see Section Va), cleared by centrifugation, were carried out prior to precipitation of the DNA with ethanol (see Section Va).

c) Isolation of DNA by Cesium Chloride-Ethidium Bromide Ultracentrifugation

The cesium chloride-ethidium bromide ultracentrifugation procedure was used primarily for the isolation of the gonococcal conjugative plasmid and for the isolation of the conjugative plasmids from a number of *E. coli* and *H. influenzae* strains used in this study (Dillon *et al.*, 1985). This method is a modification of the method developed by Clewell and Helinski (1969).

The density of the cells lysed varied according to the genus to be lysed. 40.0 mL of an overnight *E. coli* broth culture were diluted into 400.0 mL of fresh TSB and incubated with aeration at 37°C until midlog phase was achieved. Cells were harvested by centrifugation in a Sorvall RC5C centrifuge (Du Pont Canada Inc.) at 10,000 rpm at 4°C for 15 min. Alternatively, cells scraped with a sterile loop from ten plates of 18 h growth of *N. gonorrhoeae* or *H. influenzae* provided suitable cell density for subsequent lysis.

Cell lysates were cleared of debris by centrifugation and the supernatant containing nucleic acid was transferred to a clean plastic tube. The volume was adjusted to 8.0 mL with TES buffer (0.03M Tris-HCl, pH 8.0, 0.05M EDTA, 0.005M NaCl). 7.3 grams of cesium chloride was added to the tube along with 0.2 mL ethidium bromide (0.015 mg/L). The DNA solution was transferred to an ultracentrifuge tube (Beckman Instruments

Canada Inc., Mississauga, Ont., Canada) and the remaining space in the tube was filled with paraffin oil. The sealed tubes were centrifuged in a Beckman TY65 fixed angle rotor at 55,000 rpm at 10°C in a Beckman L8-M ultracentrifuge for 21 h.

The plasmid band was visualized using a longwave (300 nanometers) ultraviolet handheld source (Fisher Scientific). The top of the tube was punctured to open the system to atmospheric pressure. The tube was then punctured at the side, just below the plasmid band, with an 18-gauge needle and fifteen to twenty-five drops were collected through the end of the needle.

To remove ethidium bromide from DNA suspensions, one volume of water-saturated isobutanol was added, the mixture was gently inverted several times and the phases were allowed to separate (Dillon et al., 1985). Ethidium bromide was extracted into the upper organic phase which was removed and discarded. This procedure was repeated at least three times. Two volumes of sterile distilled water were added to dilute the cesium chloride in the sample and the DNA was precipitated with ethanol (see Section Va). DNA was further purified by a proteinase K treatment (Maniatis et al., 1982) followed by precipitation with ethanol (see Section Va).

d) Determination of DNA Concentration

Two methods were used to determine DNA concentration. In the first, the optical density of an aliquot of a purified DNA suspension was determined at 260 nanometers (A_{260}) using a Bausch and Lomb spectrophotometer (Ottawa, Ont., Canada). The concentration of DNA was then determined based on the relationship that a suspension of double-stranded nucleic acid at a concentration of 50.0 mg/L has an optical density of 1.0 at A_{260} (Rodriguez and Tait, 1983). The purity of the DNA preparation was evaluated by determining the ratio of optical density A_{260}:A_{280} (Dillon *et al.*, 1985).

In the second method, an aliquot of DNA of unknown concentration was electrophoresed on a 1.0% agarose gel along with various known quantitites of purified lambda DNA. The DNA concentration was approximated by comparing the fluorescence to that of the lambda DNA standard following staining of the gel in ethidium bromide (see Section Vg).

e) Agarose and Polyacrylamide Gel Electrophoresis

i) Agarose Gel Electrophoresis

A solution of molten agarose was prepared by adding the appropriate amount of agarose powder (depending on the desired agarose concentration) to 1X electrophoresis buffer

(10X Tris-acetate: 0.4M Tris-base, 0.2M sodium acetate, 0.01M disodium EDTA, pH 7.8). The agarose was completely dissolved by bringing the solution to a boil and the gel was cast in a horizontal mold. The solidified gel was immersed horizontally in 1X Tris-acetate buffer in an electrophoresis chamber with the sample wells at the cathode end.

DNA samples were resuspended in TE buffer, pH 7.8 up to a volume of 0.025 mL. Loading buffer [0.05M disodium EDTA, 4.0M urea, 50.0% (w/v) sucrose, 0.1% bromophenol blue, pH 7.0] was added to the DNA suspension in a ratio of 1:3 (v/v). The samples were loaded into the wells, under the buffer, using a 0.2 mL micropipette. Electrophoresis was completed at RT between 50 to 75 volts/centimeter until the bromophenol blue tracking dye had either migrated 3/4 the length of the gel or had run off the gel.

ii) Polyacrylamide Gel Electrophoresis

5.0% vertical polyacrylamide gels were prepared as described by (Dillon et al., 1985). DNA samples for electrophoresis were prepared as described for agarose gels. The samples were loaded into the wells under buffer and electrophoresed at 50 volts/centimeter for approximately 3 h at RT.

f) Staining, Visualization and Photography of Gels

The method used to stain DNA was a modification of the procedure of Aaij and Borst (1972). Gels were placed in a solution of ethidium bromide (0.001 mg/L) for 15 min in the dark. The staining solution was poured off and the gel was destained for 15 min in water. Gels were illuminated by a 300 nanometers light source emitted from a Fisher transilluminator. The illuminated gel was photographed with a Polaroid MP4 camera (Toronto, Ont., Canada) placed directly over the gel. Gels were photographed through a red filter with Polaroid type 55 film and type 545 film holder. The film was exposed for approximately 1 min and the film negative was developed in a solution of 18.0% (w/v) sodium sulfite, followed by a wash in cold water and was air dried.

g) Electroelution of DNA from Agarose Gels

DNA was isolated from agarose gel slices by electroelution into dialysis tubing (11.5 millimeter, Spectrum Medical Industries Incorporated, Los Angeles, Calif., U.S.A.) as described by Maniatis *et al*. (1982). Following electroelution, the buffer containing DNA was extracted twice with phenol-chloroform (see Section Va). Ethidium bromide was removed from the aqueous layer (see

Section Vc) and the DNA was precipitated in ethanol (see Section Va).

VI RADIOACTIVE LABELLING OF DNA

a) Nick Translation

DNA molecules labelled with radiolabelled nucleotides were used as radioactive probes. The procedure used for uniformly labelling DNA molecules using DNase I and *E. coli* DNA polymerase I (Kornberg) is a modification by Maniatis *et al*. (1982) of the original method developed by Rigby *et al*. (1977). Unincorporated nucleotides were separated from labelled DNA by passing 0.1 mL of the mixture twice through spun columns of Sephadex G-50 (Maniatis *et al*., 1982). DNA was precipitated with ethanol twice (see Section Va) to remove any trace unincorporated labelled nucleotides.

b) Labelling the 3' Ends of DNA with DNA Polymerase I

The recessed 3' ends of DNA fragments, generated by restriction endonucleases producing 5' overhangs were radioactively labelled using the Klenow fragment of DNA polymerase I as described by Maniatis *et al*. (1982). The labelled DNA was extracted three times with phenol-chloroform (see Section Va). The volume was adjusted to 0.1 mL with TE buffer, pH 7.8 and the mixture was passed through spun columns as described above (see Section VIa). DNA was precipitated with ethanol as described previously (see Section Va).

VlI RESTRICTION ENDONUCLEASE DIGESTION OF DNA

a) Single and Multiple Digestions

Purified DNA was suspended in 0.05 mL of the appropriate restriction buffer to a concentration of 2.0 to 3.0 mg/L. The restriction buffer varied depending on the enzyme tested and according to the recommendations of Maniatis et al. (1982) and the manufacturers. Standard restriction buffers (0.01M Tris-HCl, pH 7.5, 0.01M $MgCl_2$, 0.001M dithiothreitol) differed mainly in salt concentration; ranging from no salt, to medium (0.05M NaCl) and high salt (0.1M NaCl) concentrations.

A list of restriction enzymes used in this study and the enzyme suppliers are indicated in Section Ic. The volume of restriction endonuclease added to a DNA suspension was no more than 0.1% (v/v). Digestion reactions were incubated for 2 to 3 h at 37·C, except for the restriction endonuclease BstEII which was incubated at 60·C. To terminate the reaction, the mixture was incubated at 70°C for 10 min, followed by two extractions with phenol-chloroform (see Section Va) and DNA was precipitated with ethanol (see Section Va). Controls consisted of incubating purified DNA in the restriction buffer under identical conditions without the restriction enzyme(s).

For multiple digestions with two or more restriction endonucleases having different buffer conditions, the reaction was terminated after cleaving with one enzyme. The DNA, after precipitation with ethanol, was resuspended in a second restriction buffer and the second enzyme was added. For multiple digestions in which the recommended restriction buffer conditions were the same for each of the restriction endonucleases, the DNA was cleaved with the enzymes simultaneously.

Restriction fragment sizes were determined by standard curve analysis, based on the relationship that molecules of linear, duplex DNA, travel through gel matrices at rates that are inversely proportional to the $\log_{10}$ of the molecular weights (Helling et al., 1974). Lambda DNA cleaved with HindIII (Murray & Murray, 1975) was electrophoresed on the same gel as the restriction mixtures and a standard curve was constructed by plotting the distance, in centimeters, of migration of each of the lambda HindIII restriction fragments from the bottom of the well (origin) against the $\log_{10}$ size (kb) of the corresponding fragment. Unknown restriction fragment sizes were then determined from the standard curve based on the migration distance of each fragment from the origin.

b) Restriction Mapping by Partial DNA Digestion

Partial digestion mapping of DNA was carried out as described by Smith and Birnstiel (1976). The plasmid to be mapped was first linearized with a restriction endonuclease which produced 5' protruding ends. The 3' recessed ends were end-labelled using DNA polymerase (see Section Vlb). The linear molecule was cleaved with a second enzyme to give two fragments, each labelled at a single terminus. The two end-labelled fragments were separated by electrophoresis on a 0.7% agarose gel followed by electroelution (see Section Vg).

To carry out the partial digestion reaction, the labelled fragment was resuspended in appropriate restriction buffer. Lambda carrier DNA was added so that the total DNA present in the reaction vessel was approximately 1.0 ug and the final reaction volume was 0.1 mL. One unit of restriction endonuclease was added and the mixture was incubated at 37·C.

To obtain partial digestion products, aliquots of the reaction mixture were taken at successive time intervals and added to 0.005 mL of loading buffer. The partial digestion products were incubated at 70·C for 10 min then electrophoresed on agarose or polyacrylamide gels (see Sections Ve). Gels were supported on a rectangular piece of BioRad filter paper backing and dried on a BioRad 1125B slab dryer vacuum suction at 60°C for 1 to 2 h. The dried gel was wrapped completely in Saran wrap and autoradiographed as described below.

Radiolabelled DNA was visualized by autoradiography (Dillon et al., 1985). X-ray film (Du Pont Canada Inc.) was exposed to the support containing the radiolabelled DNA at -70°C in an x-ray exposure cassette lined with image intensifying screen (Du Pont Canada Inc.). After 24 h exposure the film was allowed to equilibriate to RT and then developed by washing in liquid x-ray developer (Eastman Kodak Co., Rochester, N.Y., U.S.A.) for 1 min, followed by a brief rinse in 5.0% acetic acid solution and a 3 min wash in Kodak fixer. The films were rinsed in cold water and air dried. Longer exposures were carried out under the same conditions.

VIII DNA HYBRIDIZATION

a) Southern Blots

A Southern blot procedure was used to transfer denatured DNA from agarose gels to nitrocellulose paper (Amersham International) (Southern, 1975). Blotting was completed after 24 h and the filter was allowed to air dry and then baked 2 h at 80°C under vacuum in order to fix the DNA to the membrane. Filters were stored at RT.

b) DNA Hybridization

Nick translated DNA was used to hybridize with DNA in Southern blots. Southern blots were prehybridized by incubating the nitrocellulose filter with 10.0 mL of prehybridization buffer consisting of 5X SSPE (0.05M NaH_2PO_4, 0.005M disodium EDTA, 0.9M NaCl, pH 7.0), 1.0% SDS, and 100.0 mg/L herring sperm DNA for 2 h at 70°C (Dillon et al., 1985).

The hybridization reaction was classified as either low, medium or high stringency. The nick-translated DNA probe with a specific activity of approximately 10^8 counts per minute/ug DNA, as determined by liquid scintillation counting in 1217 Rackbeta liquid scintillation counter (LKB-ProdukterAB, Bramma, Sweden) was suspended in 0.5 mL TE buffer, pH 7.5, boiled for 5 min to denature double-stranded DNA and rapidly cooled on ice. For DNA hybridizations, the denatured radiolabelled DNA probe was added to 10.0 mL of hybridization buffer (50.0% formamide, 5X Denhardt's, 5X SSPE, 0.1% SDS, 100.0 mg/L denatured herring sperm DNA) and added to the prehybridized filter in a plastic bag. The filter and probe were hybridized 24 h at 42°C.

Following hybridization the probe was removed and the filters washed. Essentially, the temperature and salt concentration of the post-hybridization wash solution determined the degree of stringency (Meinkoth and Wahl, 1984). For low stringency the filter was washed for 2 min at RT in 1X SSPE plus 0.1% SDS followed by autoradiography. If too much background level radiation remained after the first wash, the stringency was increased so that the subsequent wash was carried out at 42·C in 0.1X SSPE and 0.1% SDS for 5 min. The most stringent wash consisted of a low and medium stringency wash followed by a 5 min wash at 65·C in 0.1X SSPE and 0.1% SDS.

RESULTS

I INTRASPECIFIC GONOCOCCAL MATINGS

a) Selection of Antibiotic-resistant Recipient Strains

The ability of gonococcal strains to act as conjugal recipients was established by performing intraspecific matings with genetically characterized recipient strains differing in auxotype and plasmid profile. Antibiotic-resistant mutants of recipient strains were constructed (see Materials and Methods, Section IIId) to facilitate selection of transconjugants in mating experiments. Mutant strains are described in Table 1 (see Materials and Methods, Section II). Most strains yielded mutants resistant to rifampicin including strains CH811, 1-183, 1-835 and H12593. However, only streptomycin-resistant mutants of strain A9 and naladixic acid-resistant mutants of strain 1-627 could be isolated.

The auxotypes of all mutant gonococcal antibiotic-resistant strains were stable with the exception of an OUH$^-$ strain A9. When 120 colonies of streptomycin-resistant strain A9 were auxotyped 9 colonies were POUH$^-$ and the remaining 111 were OUH$^-$. When a single POUH$^-$ colony was subsequently subpassaged and individual colonies auxotyped, 2 of 25 colonies were OUH^- and the remaining 23 colonies were $POUH^-$. This phenomenon, however, has been described by previous workers (Hendry and Stewart, 1979) and was attributed to variable $CaCl_2$ content in the auxotyping media.

b) Intraspecific Matings

Putative gonococcal transconjugant colonies derived from intraspecific matings were visible on selective media after 48 h incubation. When such colonies were subpassaged on non-selective media and subsequently auxotyped and examined for plasmid content, colonies having donor or recipient strain characteristics were often detected. However, it was observed that only real transconjugants (ie. those recipient cells that acquired beta-lactamase production) and not donor or recipient colonies could be propogated on a second subpassage on selective media. Spontaneous mutation or transformation could not account for the appearance of these colonies as ascertained from controls in this study.

Biswas et al. (1980a) and Sox et al. (1978) have also reported the presence of recipient colonies on media selective for gonococcal transconjugants. Kirven and Thornesberry (1977) proposed that plating mixed cell cultures on selective media at high density results in an overlapping of cells, so that each cell having a particular antibiotic resistance buffers the environment for growth of antibiotic sensitive cells. This could explain the observation that single colonies of false transconjugants did not grow following a second subpassage, where cells were plated at a lower cell density. Thus, putative transconjugant colonies from all matings were subcultured twice on selective media before subsequent auxotype verification and plasmid analysis.

A total of 236 transconjugants derived from intraspecific matings between gonococcal donor strains 1-182 and 1-881 and five gonococcal recipient strains, including CH811 (NR), 1-183 (NR), H12593 (P^-), A9 (OUH^-) and 1-835 (PCU^-) were examined for plasmid content. The total number of transconjugants examined for plasmid content included 50 P^-, 56 PCU^-, 10 OUH^- and 120 NR. The typical plasmid profile of the gonococcal transconjugants obtained is shown in Figure 1. All transconjugants examined had acquired a 7.2 kb beta-lactamase-encoding plasmid. Co-transfer of the 4.2 kb cryptic plasmid with the 7.2 kb plasmid was never observed. Co-transfer of the conjugative plasmid with the 7.2 kb plasmid was only detected following agarose gel electrophoresis in a single mating to recipient strain H12593 (P^-) (Figure 1, Lane F).

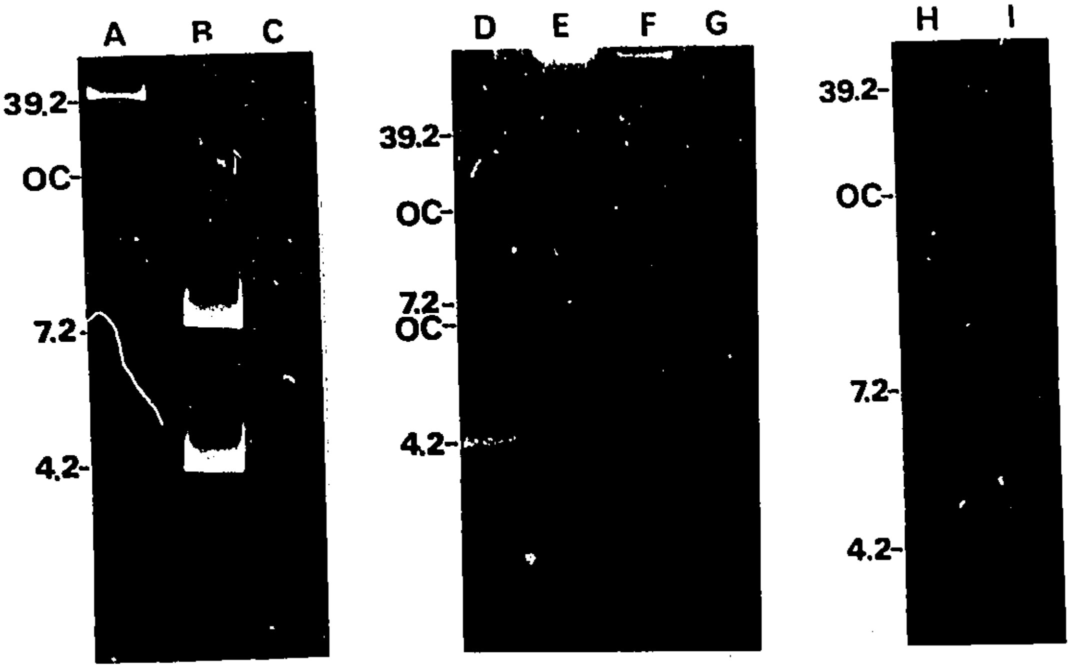


FIG. 1. Plasmid profiles of gonococcal transconjugants derived from intraspecific matings with gonococcal donor strain 1-182. Gonococcal strains are described in Table 1 (see Materials and Methods, Section II). 1.0% agarose gel electrophoresis of DNA isolated from gonococcal donor strain 1-182 (Lanes A, D and H). Transconjugant strains 183-1 (Lane B), CH811-1 (Lane C), H12593-1 (Lane E), H12593-2 (Lane F), A9-1 (Lane G) and 835-1 (Lane I). Numbers in margins indicate covalently-closed-circular forms of the 39.2 kb, 7.2 kb and 4.2 kb gonococcal plasmids. The unmarked band below the 39.2 kb plasmid represents chromosomal DNA. OC indicates the open-circular form of the 7.2 kb plasmid. The open-circular form of the 4.2 kb plasmid is indicated in Lanes D, E, and F below the 7.2 kb plasmid band.

Mobilization of the 7.2 kb plasmid from gonococcal donor strain 1-182 (P^-) was observed to every recipient auxotype including NR, P^-, OUH^- and PCU^- strains (Table 4). The 7.2 kb plasmid was efficiently mobilized from donor strain 1-182 (P^-) to NR recipient strains 1-183 and CH811 with a similar average mobilization frequency and comparable to the average mobilization frequency observed to the P^- recipient strain H12593 (Table 4, Mating #1, 2 and 3). Interestingly, the mobilization frequency of the 7.2 kb plasmid from donor strain 1-182 to each recipient strain 1-183, CH811 and H12593 exhibited intratest variation of approximately 10^{-4} to 10^{-6} transconjugants/input donor cfu for each set of matings.

In general, the mobilization frequency of the 7.2 kb plasmid from gonococcal donor strain 1-182 (P^-) was at least ten-fold lower to recipient strains having multiple nutritional requirements (ie. PCU^-, OUH^-). Moreover, such matings also demonstrated intratest variation in mobilization frequency. Mobilization of the 7.2 kb plasmid from donor strain 1-182 (P^-) to an OUH^- recipient strain A9 was detected in only one of eight matings at a mobilization frequency of 7.6×10^{-6} transconjugants/input donor cfu (Table 4, Mating #4). The lowest mobilization frequency that could have been detected in these matings was on the order of 10^{-6} transconjugants/input donor cfu, given that approximately 10^{7} cfu of each of the donor and recipient strains were mated and that 0.1 mL of the 1.0 mL mating suspension was tested for transconjugants.

Mobilization of the 7.2 kb beta-lactamase-encoding plasmid from donor strain 1-182 was detected to the rifampicin-resistant PCU$^-$ strain 1-835 in three of six matings at an average mobilization frequency of 6.2x10^{-6} transconjugants/input donor cfu (Table 4, Mating #5). Mobilization of the 7.2 kb plasmid from donor strain 1-182 (P$^-$) was not detected (<2.5x10^{-7} transconjugants/input donor cfu) to a naladixic acid-resistant PCU$^-$ recipient strain 1-627 in ten mating attempts (Table 4, Mating #6).

c) Comparison of Conjugation Efficiency of Gonococcal Donor Strains

Gonococcal donor strains 1-182 (P$^-$) and 1-881 (NR) were compared for the ability of their conjugative plasmids to mobilize a resident 7.2 kb beta-lactamase-encoding plasmid to recipient strains H12593 (P$^-$) and CH811 (NR). Donor strain 1-881 (NR) mobilized a resident 7.2 kb plasmid at a higher frequency than donor strain 1-182 (P$^-$) to both recipient strains (Table 5). Intratest variation in mobilization frequency was also observed in matings using donor strain 1-881. An approximately ten-fold range in mobilization frequency was observed for the mobilization of the 7.2 kb plasmid from donor strain 1-881 (P$^-$) to recipient strains

TABLE 4. Intraspecific mobilization of beta-lactamase-encoding plasmids from gonococcal donor strain 1-182 to gonococcal recipient strains varying in auxotype and plasmid profile.[a]

Mating no.	Recipient/ Auxotype/ Serogroup	Mobilization frequency[b]		No. Matings[c]
		Range[d]	Average[e]	
1	1-183/NR WII/III	$1.7\mathrm{x}10^{-4}$, $8.6\mathrm{x}10^{-6}$	$7.1\mathrm{x}10^{-5}$	3/3
2	CH811/NR WI	$1.7\mathrm{x}10^{-4}$, $5.9\mathrm{x}10^{-6}$	$6.6\mathrm{x}10^{-5}$	3/3
3	H12593/P^{-} WII/III	$1.5\mathrm{x}10^{-4}$, $1.0\mathrm{x}10^{-6}$	$5.4\mathrm{x}10^{-5}$	3/3
4	A9/OUH^{-} WI	$7.6\mathrm{x}10^{-6}$	–[f]	1/8
5	1-835/PCU^{-} WII/III	$9.9\mathrm{x}10^{-6}$, $2.9\mathrm{x}10^{-6}$	$6.2\mathrm{x}10^{-6}$	3/6
6	1-627/PCU^{-} WII/III	$<2.5\mathrm{x}10^{-7}$	-	0/10

[a] Donor and recipient strains were mated in a 1:1 ratio in an overnight filter mating. Transconjugants were subpassaged twice on selective media prior to auxotype determination and plasmid analysis. Gonococcal strains are described in Table 1 (see Materials and Methods, Section II).

[b] Mobilization frequency is defined as the number of recipient cells that acquired beta-lactamase production/input donor cfu.

[c] The number of matings indicates the number of times mobilization was detected/total number of matings.

[d] The range of mobilization frequency identifies the lowest and highest mobilization frequencies detected.

[e] The average moblization frequency was determined from at least two matings where mobilization was detected.

[f] -Indicates an average mobilization frequency could not be calculated due to insufficient data.

TABLE 5. Comparison of gonococcal donor strains in the intraspecific mobilization of beta-lactamase-encoding plasmids.[a]

		Mobilization frequency[b]		
Donor	Recipient/ Auxotype	Range[d]	Average[e]	No. matings[c]
1-182	H12593/P^{-}	1.5×10^{-4}, 1.0×10^{-6}	5.4×10^{-5}	3/3
1-182	CH811/NR	1.7×10^{-4}, 5.9×10^{-6}	6.6×10^{-5}	3/3
1-881	H12593/P^{-}	2.5×10^{-2}, 2.6×10^{-1}	1.4×10^{-1}	2/12
1-881	CH811/NR	4.7×10^{-2}, 6.9×10^{-1}	3.7×10^{-1}	2/12

[a] Donor and recipient strains were mated in a 1:1 ratio in an overnight filter mating. Transconjugants were subpassaged twice on selective media prior to auxotype determination and plasmid analysis. Gonococcal strains are described in Table 1 (see Materials and Methods, Section II).

[b] Mobilization frequency is defined as the number of recipient cells that acquired beta-lactamase production/input donor cfu.

[c] The number of matings indicates the number of matings in which mobilization was detected/total number of matings.

[d] The range of mobilization frequency identifies the lowest and highest mobilization frequencies detected.

[e] The average mobilization frequency was determined from at least two matings where mobilization was detected.

H12593 (P^-) and CH811 (NR). Moreover, mobilization of the 7.2 kb plasmid from donor strain 1-881 to H12593 (P^-) or CH811 (NR) recipient strains was not detected ($<10^{-6}$ transconjugants/input donor cfu) in ten of twelve matings in each case.

II INTERGENERIC MATINGS: CONJUGATION BETWEEN N. GONORRHOEAE AND E. COLI

Mobilization of gonococcal plasmids from gonococcal donor strain 1-182 to four E. coli recipient strains was examined. The highest average mobilization frequency of a 7.2 kb beta-lactamase-encoding plasmid by a co-resident conjugative plasmid was to E. coli recipient strain HB101, followed by C600 and DT591 (Table 6). Mobilization of the 7.2 kb plasmid was not detected directly to res^+ strain 1100 ($<2.2\times10^{-6}$ transconjugants/input donor cfu) (Table 6). However, the 7.2 kb could be mobilized to a res^+ E. coli recipient strain from a res^-mod^+ intermediate donor strain (DT591) (Table 7, Mating #7).

A total of 349 transconjugants derived from the intergeneric matings between gonococcal donor strain 1-182 and three E. coli recipient strains (HB101, C600, DT591) were examined for plasmid content. The 7.2 kb plasmid was observed in all the transconjugants. The co-transfer of

TABLE 6. Intergeneric mobilization of a beta-lactamase-encoding plasmid from gonococcal donor strain 1-182 to *E. coli* recipient strains.[a]

E. coli recipient	Mobilization frequency[b]	
	Range[c]	Average[d]
HB101	$2.3x10^{-2}$, $3.6x10^{-3}$	$1.3x10^{-2}$
C600	$4.9x10^{-3}$	-[e]
DT591	$1.3x10^{-4}$, $8.5x10^{-4}$	$4.9x10^{-4}$
1100	$<2.2x10^{-6}$	-

[a] Donor and recipient strains were mated in a 10:1 ratio in an overnight filter mating. Transconjugants were subpassaged twice on selective media prior to analysis. *E. coli* strains are described in Table 2 (see Materials and Methods, Section II).

[b] Mobilization frequency is defined as the number of recipient cells that acquired beta-lactamase production/input donor cfu.

[c] The mobilization frequency range identifies the lowest and highest mobilization frequencies detected. A single value indicates the mating was performed once.

[d] The average mobilization frequency was calculated from two matings where mobilization was detected.

[e] -Indicates that an average mobilization frequency could not be calculated due to insufficient data.

TABLE 7. Mobilization frequency of gonococcal beta-lactamase-encoding plasmids to *E. coli* recipient strains.[a]

Mating no.	Donor	Intermediate Donor	Recipient	Mobilization frequency[b]		
				Range[c]	Average[d]	Plasmid[e]
1	1-182		DT591	$1.3\text{x}10^{-4}$, $8.6\text{x}10^{-4}$	$4.9\text{x}10^{-4}$	7.2
2	1-881		DT591	$9.2\text{x}10^{-2}$	-[f]	7.2
3	1-159		DT591	$2.6\text{x}10^{-4}$, $3.9\text{x}10^{-5}$	$1.5\text{x}10^{-4}$	5.1
4	1-530		DT591	$<1.5\text{x}10^{-7}$	-	
5	CH992	1-554	DT591	$3.9\text{x}10^{-2}$, $4.9\text{x}10^{-4}$	$1.9\text{x}10^{-2}$	7.2
6	1-554		DT591	$<1.2\text{x}10^{-7}$	-	
7	CH992	DT591 (pJD4)	1100	$4.3\text{x}10^{-3}$, $5.0\text{x}10^{-4}$	$2.4\text{x}10^{-3}$	7.2
8	CH992	DH1 (pJD7)	1100	$<3.0\text{x}10^{-7}$	-	

[a] Strains were mated in either a 1:1 ratio of donor:recipient or a 10:1:1 ratio of donor:intermediate donor:recipient in an overnight filter mating. Transconjugants were subpassaged twice on selective media prior to analysis. Gonococcal strains are described in Table 1 and *E. coli* strains in Table 2 (see Materials and Methods, Section II).

[b] Mobilization frequency is defined as the number of recipient cells that acquired beta-lactamase production/input donor cfu.

[c] Mobilization frequency range identifies the lowest and highest mobilization frequencies detected. A single value indicates the mating was performed once.

[d] The average mobilization frequency was calculated from at least two matings where mobilization was detected.

[e] Plasmids observed in transconjugants were determined by agarose gel electrophoresis of transconjugant DNA. No plasmid indicates that plasmids were not observed in transconjugants.

[f] -Indicates the average mobilization frequency could not be calculated due to insufficient data.

either the 4.2 kb cryptic plasmid and/or the conjugative plasmid with the 7.2 kb plasmid was never observed. The typical plasmid profile of E. coli transconjugants is shown in Figure 2 (Lane B).

Table 7 lists the average and the range of mobilization frequency for various gonococcal beta-lactamase-encoding plasmids to E. coli recipient strains. The 7.2 kb beta-lactamase-encoding plasmid in gonococcal strain 1-554 was not mobilized in the absence of a gonococcal conjugative plasmid (Table 7, Mating #6), but could be mobilized when a conjugative plasmid was provided by gonococcal donor strain CH992 in a triparental mating (Table 7, Mating #5, Figure 2, Lanes D and E). Mobilization of the 5.1 kb gonococcal beta-lactamase-encoding plasmid by a co-resident conjugative plasmid from a gonococcal donor strain to a res$^-$ E. coli recipient strain was also detected (Table 7, Mating #3, Figure 2, Lanes G and H). Mobilization of the 4.9 kb beta-lactamase-encoding plasmid (pJD7) from gonococcal donor strain 1-530 to a res$^-$ E. coli recipient strain was not detected (Table 7, Mating #4). Moreover, when this plasmid had been transformed into a res$^-$mod$^+$ E. coli strain (DH1), it still could not be mobilized in a triparental mating with a gonococcal conjugative plasmid known to be functional (Table 7, Mating #8).

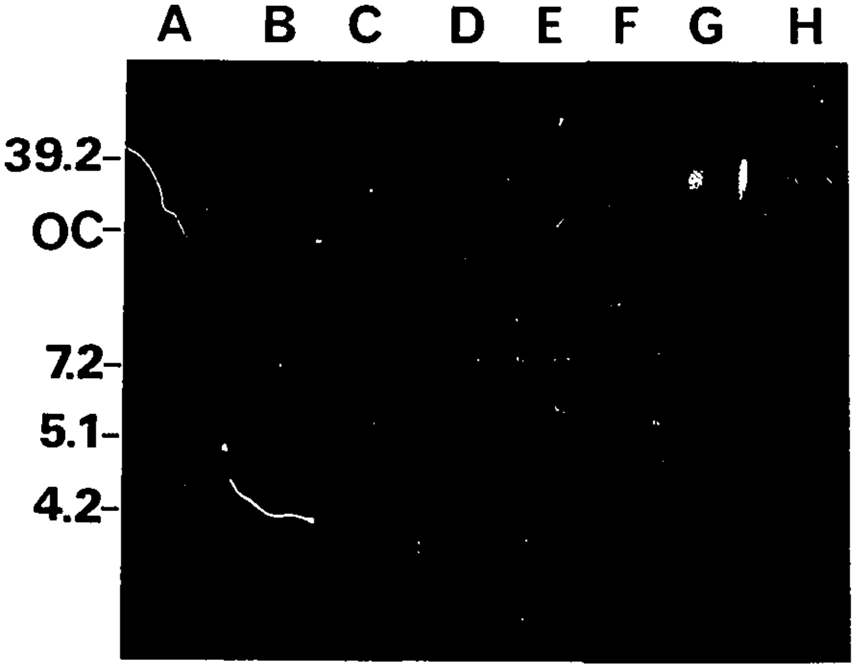


FIG. 2. Plasmid profiles of *E. coli* transconjugant strains derived from intergeneric matings with gonococcal donor strains. *E. coli* and gonococcal strains are described in Table 1 and 2 (see Materials and Methods, Section II). 1.0% agarose gel electrophoresis of DNA from donor strains 1-182 (Lane A), 1-159 (Lane F) and from transconjugant strains, including 1-182/DT591 (Lanes B and C), CH992/ 1-554/DT591 (Lanes D and E) and 1-159/DT591 (Lanes G and H). Values in the margin represent covalently-closed-circular form of the 39.2 kb, 7.2 kb, 5.1 kb and 4.2 kb plasmids. The unmarked band below the 39.2 kb plasmid represents chromosomal DNA, below which migrates the open-circular form (OC) of the 7.2 kb plasmid.

The average mobilization frequency of the 7.2 kb beta-lactamase-encoding plasmid to an *E. coli* recipient strain (DT591) varied between gonococcal donor strains. Gonococcal donor strain 1-881 mobilized the 7.2 kb plasmid at the highest frequency, followed by donor strain 1-182 (Table 7, Mating #1 and 2). However, the second highest mobilization frequency of the 7.2 kb plasmid was observed in a triparental mating with gonococcal donor strain CH992 (Table 7, Mating #5). The 5.1 kb beta-lactamase-encoding plasmid in donor strain 1-159 was mobilized at an average frequency similar to that for the 7.2 kb plasmid in donor strain 1-182 (Table 7, Mating #3). Intratest variation in the range of mobilization frequency was also observed in intergeneric matings. Up to one-hundred-fold variation was detected in matings between the same donor and recipient strains (Table 7, Mating #3, 5 and 7).

III RESTRICTION ENDONUCLEASE MAPPING

a) Screening the Gonococcal Conjugative Plasmid with Restriction Endonucleases

Gonococcal strain CH992 was selected as the source of conjugative plasmid for physical and genetic analysis since this strain had demonstrated conjugal properties (Table 7, Mating #5) and harbored only the conjugative plasmid, thus facilitating its isolation. Isolation of gonococcal conjugative plasmids from strains containing other plasmids was complicated by low plasmid yield, plasmid linearization and presence of endonuclease inhibitors following isolation by electroelution from agarose gels. The conjugative plasmid of strain CH992 was designated pJD38.

Thirty-six type II restriction endonucleases were tested for their ability to cleave pJD38 (Table 8). Eleven enzymes including *Alu*I, *Ava*I, *Hha*I, *Hin*fI, *Hpa*II, *Mbo*I, *Mbo*II, *Msp*I, *Nci*I, *Rsa*I and *Sau*3A produced ten or more restriction fragments of pJD38 as observed following gel electrophoresis. Since the complexity of restriction mapping increases with the number of restriction fragments to be mapped, none of the enzymes generating ten or more restriction fragments of pJD38 were selected for further analysis.

Several restriction enzymes generated fewer than ten restriction fragments of pJD38, including *Eco*RI with four, *Bgl*II and *Pvu*I with five, *Hin*dII with eight and *Bcl*I, *Bgl*I and *Cla*I with nine restriction fragments (Figure 3). All of these enzymes, except *Bgl*I and *Cla*I were selected for further restriction mapping.

TABLE 8. Restriction endonuclease digestions of pJD38.[a]

Restriction endonucleases	No. of restriction fragments[b]
*Hin*dIII, *Mlu*I, *Sac*I, *Xho*I	1
*Eco*RI	4
*Bgl*II, *Pvu*I	5
*Hin*dII	8
*Bcl*I, *Bgl*I, *Cla*I	9
*Acc*I, *Aha*III, *Ava*II, *Bam*HI, *Bst*EII, *Dpn*I, *Hae*II, *Hae*III, *Kpn*I, *Pst*I, *Sal*I, *Sst*II, *Stu*I, *Xba*I	0
*Alu*I, *Ava*I, *Hha*I, *Hin*fI, *Hpa*II, *Mbo*I, *Mbo*II, *Msp*I, *Nci*I, *Rsa*I, *Sau*3A	>10

[a] pJD38 was cleaved with the enzyme indicated and electrophoresed on agarose gels. Enzymes which did not cleave pJD38 or cleaved at a single site were detected by double digestions of pJD38 (see text for details).

[b] The number of restriction fragments from each digestion was determined by counting restriction fragments observed following gel electrophoresis and by restriction mapping (see text for details).

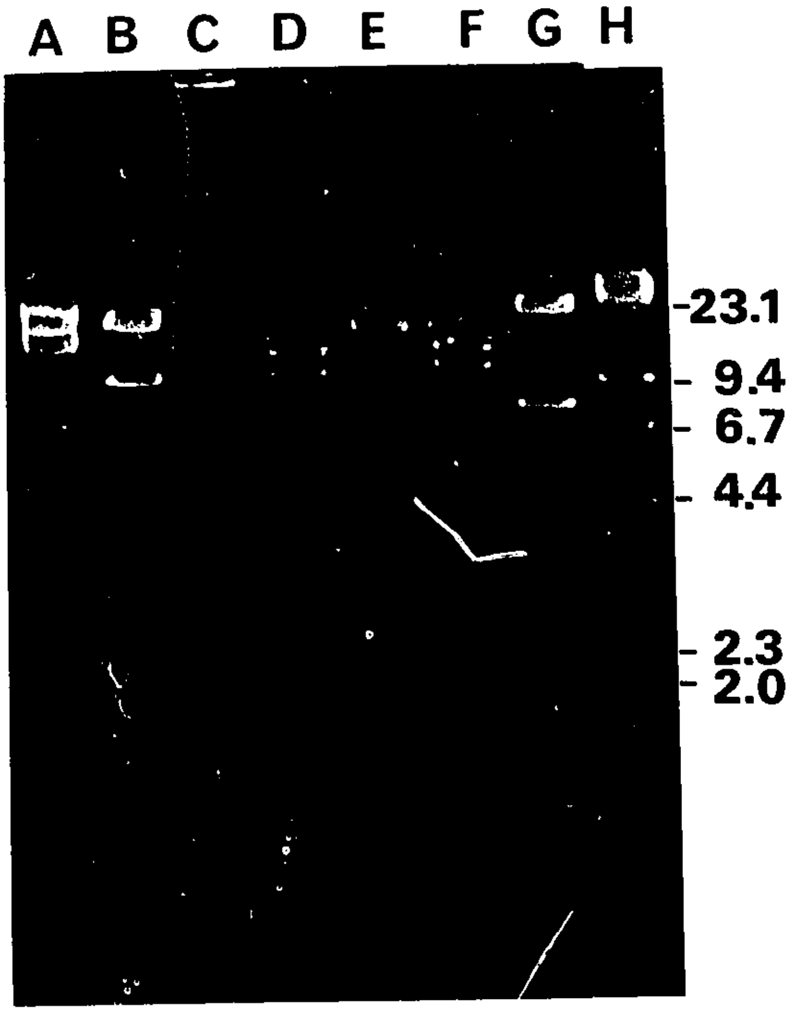


FIG. 3. Single restriction endonuclease digestions of pJD38. 1.0% agarose gel electrophoresis of pJD38 cleaved with *EcoRI* (Lane A), *PvuI* (Lane B), *BclI* (Lane C), *HindII* (Lane D), *ClaI* (Lane E), *BglI* (Lane F) and *BglII* (Lane G). Restriction fragment size markers lambda+*Hin*dIII (23.1, 9.4, 6.7, 4.4, 2.3, 2.0 kb) (Lane H).

Restriction enzymes that either did not cleave pJD38 or cleaved only at a single site were more difficult to identify. Following plasmid purification and gel electrophoresis, pJD38 could be observed in any of three possible forms, including covalently closed circular, the slowest migrating form, open circular, and linear, the fastest migrating form which migrated very close to chromosomal DNA on a 0.7% to 1.0% agarose gel. Following isolation of pJD38 from cesium chloride-ethidium bromide density gradients covalently closed circular plasmid DNA was obtained, open circular plasmid form was occasionally observed, however plasmid DNA was usually linearized during isolation manipulations. Thus, if only linear pJD38 was available for screening with restriction enzymes, the absence of digestion of pJD38 or cleavage at a single site by a given restriction endonuclease could not be ascertained following gel electrophoresis.

Double digestions of pJD38 were necessary to distinguish those restriction enzymes that cleaved pJD38 at a single site from those that did not cleave at all. Such double digestions included the test enzyme in addition to a control enzyme, such as EcoRI or HindII, known to cleave pJD38 a limited number of times. Restriction fragment patterns obtained from double digestions of pJD38 were compared to the restriction pattern of pJD38 cleaved with the control enzyme. Using this approach several enzymes were found not to cleave pJD38, including *Acc*I, *Aha*II, *Ava*II, *Bam*HI, *Bst*EII, *Dpn*I, *Hae*II, *Hae*III, *Kpn*I, *Pst*I, *Sal*I, *Sst*II, *Stu*I and *Xba*I. Lack of enzyme cleavage was confirmed by retesting these enzymes in double digestions of pJD38 with the control enzymes *Eco*RI and *Hin*dII. Four enzymes, including *Hin*dIII, *Mlu*I, *Sac*I and *Xho*I were found to cleave pJD38 at a single site using double digestions of pJD38 with *Eco*RI.

b) Methylation Specificity of Gonococcal Strain CH992

The methylation specificity of gonococcal strain CH992, harboring pJD38, was determined by digestion of pJD38 with restriction endonucleases which recognized a similar nucleotide sequence, but whose activity was affected by methylation of specific nucleotides. *Dpn*I, *Mbo*I and *Sau*3A enzymes recognize the nucleotide sequence -GATC-, while *Bam*HI recognizes a similar nucleotide sequence -GGATCC-. Cleavage of the nucleotide sequence -GATC- by *Dpn*I requires methylation of the adenine residue (Lacks and Greenberg, 1975; Streeck, 1980). However, methylation of this adenine residue protects the nucleotide sequence from cleavage by *Mbo*I (McClelland, 1983). Both *Sau*3A and *Bam*HI can cleave the unmethylated and methylated nucleotide sequence (Maniatis *et al.*, 1982).

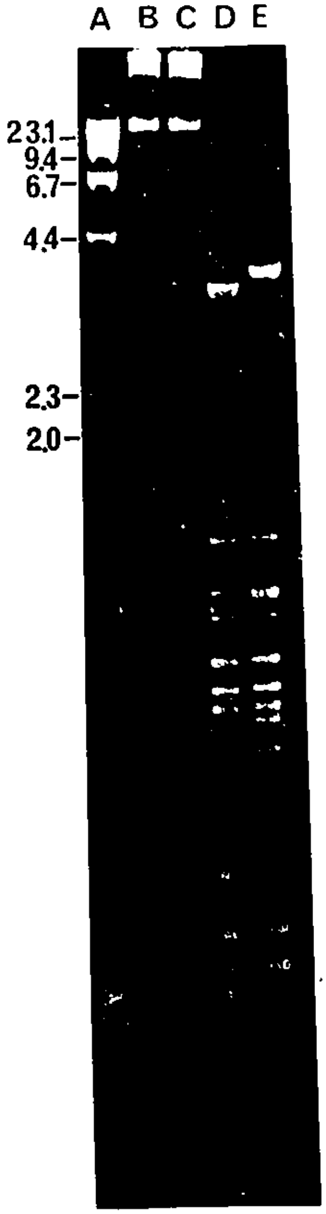


FIG. 4. Methylation specificity of gonococcal strain CH992. 1.5% agarose gel electrophoresis of pJD38 cleaved with BamHI (Lane B), DpnI (Lane C), MboI (Lane D) and Sau3A (Lane E). Restriction fragment size markers lambda+HindIII (23.1, 9.4, 6.7, 4.4, 2.3, 2.0 kb) (Lane A).

pJD38 was cleaved by MboI and Sau3A a large number of times, but was not cleaved by DpnI (Figure 4, Lanes, D, E, and C, respectively). This suggested that the adenine residue in the nucleotide recognition sequence -GATC- on pJD38 was not methylated. BamHI did not cleave pJD38, however, methylation of the cytosine residue in the fifth position of the nucleotide sequence -GGATCC- protects this sequence from cleavage by BamHI (Hattman et al., 1978).

c) Mapping Enzymes with a Single Restriction Site on pJD38

The single HindIII restriction site on pJD38 was selected, arbitrarily, as the reference point for mapping the restriction sites for each of the enzymes MluI, SacI and XhoI. Each of these enzyme restriction sites were located within 11.0 kb of the HindIII restriction site as determined from the average restriction fragment sizes observed following gel electrophoresis of double digestions of pJD38 (Table 9, Figure 5). The positions of the restriction sites for the enzymes MluI and XhoI relative to both the HindIII and SacI restriction sites were determined from the sizes of restriction fragments obtained from multiple digestions of pJD38 (ie. HindIII+SacI+MluI or HindIII+SacI+XhoI) (Table 9, Figure 5, Lanes E and F, respectively). The relative orientation of the restriction sites for the enzymes MluI, SacI, XhoI and HindIII was verified by digestion of pJD38

TABLE 9. Mapping restriction endonucleases cleaving pJD38 at a single site.[a]

Restriction endonucleaseS	Restriction fragment size (kb)[b]
*Hin*dIII+*Sac*I	(28.2)[c] 10.7
*Hin*dIII+*Mlu*I	(30.9) 8.0
*Hin*dIII+*Xho*I	(37.5) 1.4
*Hin*dIII+*Sac*I+*Xho*I	(28.0) 9.5 1.4
*Hin*dIII+*Sac*I+*Mlu*I	(28.4) 8.0 2.5
*Hin*dIII+*Mlu*I+*Xho*I	(30.7) 6.8 1.4
*Hin*dIII+*Sac*I+*Mlu*I+*Xho*I	(28.2) 6.8 2.5 1.4

[a] pJD38 was cleaved with the enzymes indicated and electrophoresed on agarose gels (see text for details).

[b] Restriction fragment sizes were the average of the sizes from at least two digestions.

[c] Restriction fragment sizes in parenthesis were calculated by subtracting the sum of the other restriction fragments from the total estimated size of pJD38.

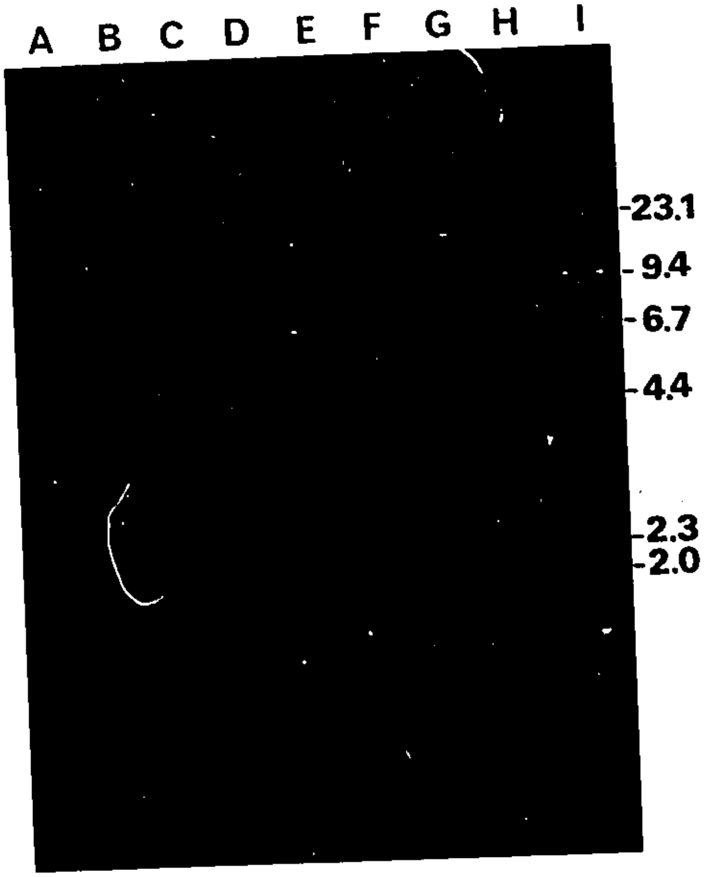


FIG. 5. Mapping restriction endonucleases having a single restriction site on pJD38. 1.0% agarose gel electrophoresis of pJD38 (Lane A) and pJD38 cleaved with HindIII+SacI (Lane B), HindIII+MluI (Lane C), HindIII+XhoI (Lane D), HindIII+SacI+MluI (Lane E), HindIII+SacI+XhoI (Lane F), HindIII+MluI+XhoI (Lane G) and HindIII+SacI+MluI+XhoI (Lane H). Restriction fragment size markers lambda+HindIII (23.1, 9.4, 6.7, 4.4, 2.3, 2.0 kb) (Lane I).

with HindIII+MluI+XhoI or HindIII+SacI+MluI+XhoI (Table 9, Figure 5, Lanes G and H, respectively). The restriction fragment sizes observed following gel electrophoresis (Table 9) corresponded to the sizes predicted by the proposed restriction map for enzymes cleaving pJD38 at a single site (Figure 6).

d) Mapping with EcoRI

EcoRI digestion of pJD38 generated four restriction fragments of 18.7 kb, 14.0 kb, 4.9 kb and 1.3 kb (Table 10a, Figure 7, Lane C). The size of the 4.9 kb and 1.3 kb EcoRI restriction fragments represented the average of the sizes observed in six digestions. The 14.0 kb EcoRI restriction fragment size was too large to be accurately determined from standard curve analysis and was based on the average of the restriction fragment sizes determined from mapping the HindIII (Figure 6), PvuI (see Appendix, Section Ia, Figure 19), BglII (see Appendix, Section Ib, Figure 22) and HindII (see Appendix, Section Ic, Figure 23) restriction sites in the 14.0 kb EcoRI restriction fragment. The 18.7 kb EcoRI restriction fragment size was calculated by mapping the enzymes PvuI (see Appendix, Section Ia, Figure 19) and HindII (see Appendix, Section Ic, Figure 23) relative to the EcoRI restriction sites.

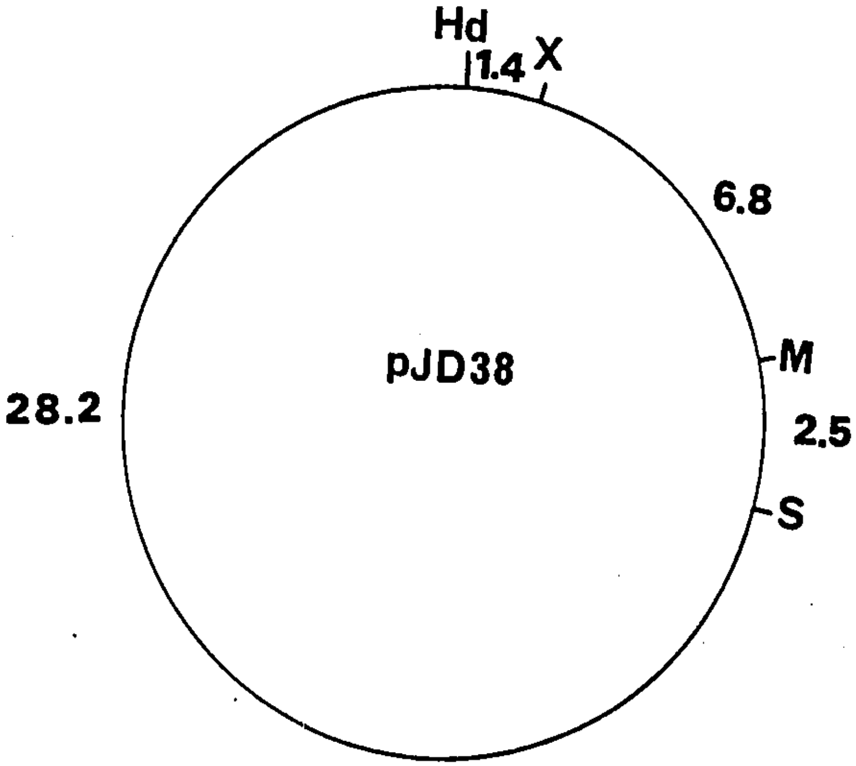


FIG. 6. Restriction map of endonucleases cleaving pJD38 at a single site. Restriction endonucleases *HindIII* (Hd), *MluI* (M), *SacI* (S) and *XhoI* (X) were mapped by single and multiple digestions of pJD38 (see text for details). Distances are given in kilobases.

TABLE 10. Mapping EcoRI restriction endonuclease sites on pJD38.

a) Double endonuclease digestions.[a]
b) Partial endonuclease digestions.[b]

	Restriction endonuclease(s)	Restriction fragment size (kb)[c]
a)	EcoRI	(18.7)[d] (14.0) 4.9 1.3
	EcoRI+HindIII	(18.7) 9.5 4.9 4.5 1.2
	EcoRI+SacI	(18.7) (14.0) 3.9 1.2
b)	EcoRI+28.2 kb HindIII/SacI	(28.2) 5.3 4.3
	EcoRI+10.7 kb HindIII/SacI	(10.7) 9.0

---

[a] pJD38 was cleaved with the enzyme indicated and electrophoresed on polyacrylamide and agarose gels (see Figure 7).

[b] End-labelled 28.2 kb and 10.7 kb HindIII/SacI restriction fragments of pJD38 were cleaved with EcoRI under partial digestion conditions and fragment sizes were determined following electrophoreses on agarose and polyacrylamide gels (see text for details).

[c] Restriction fragment sizes were the average of the sizes from at least two digestions.

[d] Restriction fragment sizes in parenthesis were too large to be accurately determined by standard curve analysis (see text for details).

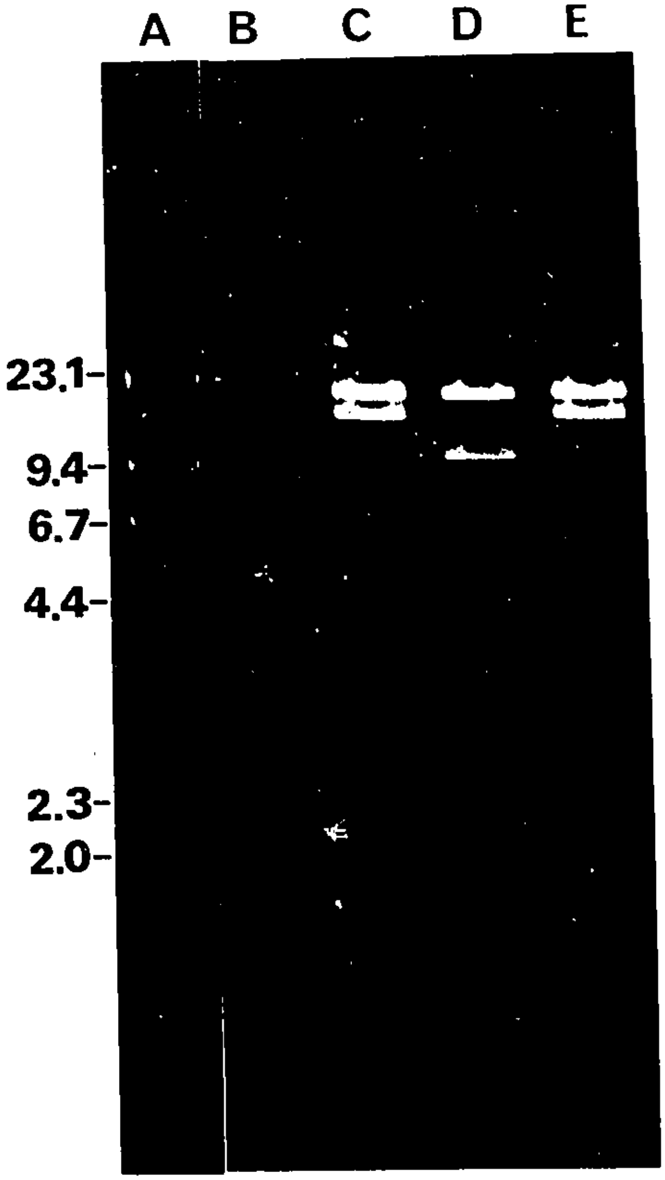


FIG. 7. Mapping <u>EcoRI</u> restriction sites on pJD38. 1.0% agarose gel electrophoresis of pJD38 (Lane B) and pJD38 cleaved with <u>EcoRI</u> (Lane C), <u>EcoRI</u>+<u>HindIII</u> (Lane D) and <u>EcoRI</u>+<u>SacI</u> (Lane E). Restriction fragment size markers lambda+<u>HindIII</u> (23.1, 9.4, 6.7, 4.4, 2.3, 2.0 kb) (Lane A).

The 14.0 kb and 4.9 kb *Eco*RI restriction fragments were oriented by double digestions of pJD38 with *Eco*RI+*Hin*dIII and *Eco*RI+*Sac*I (Table 10a, Figure 7). *Hin*dIII cleaved the 14.0 kb *Eco*RI restriction fragment to yield 9.5 kb and 4.5 kb restriction fragments (Figure 7, Lane D). *Sac*I cleaved the 4.9 kb *Eco*RI restriction fragment to give restriction fragments of 3.9 kb and 1.2 kb (Figure 7, Lane E). The 1.2 kb restriction fragment generated by *Sac*I digestion of the 4.9 kb *Eco*RI restriction fragment of pJD38 co-migrated with the 1.3 kb *Eco*RI restriction fragment, thus only four restriction fragments of pJD38 cleaved with *Eco*RI+*Sac*I were observed following gel electrophoresis (Figure 7, Lane E). The location of the *Sac*I restriction site in the 4.9 kb *Eco*RI restriction fragment was verified by *Sac*I digestion of the 4.9 kb end-labelled *Eco*RI restriction fragment (data not shown). Thus, based on the proposed orientation of the *Hin*dIII and *Sac*I restriction sites on pJD38, the 14.0 kb and 4.9 kb *Eco*RI restriction fragments were located adjacent to one another (Figure 8a).

The orientation of the 18.7 kb and 1.3 kb *Eco*RI restriction fragments of pJD38 was determined by partial digestion mapping. Partial *Eco*RI restriction digestion of a 28.2 kb *Hin*dIII/*Sac*I restriction fragment of pJD38, end-labelled at the *Hin*dIII terminus, generated four distinct restriction fragments (10.7 kb, 9.0 kb, 5.3 kb and 4.3 kb) (see Appendix, Section Ib, Figure 21, Lane B). The uncleaved end-labelled 28.2 kb restriction fragment was visible following gel electophoresis. Thus, the partial mapping data predicted four EcoRI restriction sites in the 28.2 kb end-labelled HindIII/Sacl fragment, at 4.3 kb, 5.3 kb 9.0 kb and 10.7 kb from the HindIII terminus. One restriction fragment of 9.0 kb was observed following gel electrophoresis of a partial EcoRI digestion of the 10.7 kb end-labelled HindIII/SacI restriction fragment (Table 10b, see Appendix, Section Ib, Figure 21, Lane C). The uncleaved, end-labelled 10.7 kb fragment was also visible following gel electrophoresis.

Thus, the partial mapping data predicted a total of five EcoRI restriction sites in pJD38, which contradicted the observation that EcoRI digestion of pJD38 generated four restriction fragments. This dilemma was resolved when it was observed that the 10.7 kb and 9.0 kb partial EcoRI digestion products of the 28.2 kb end-labelled HindIII/SacI restriction fragment were 'ghost bands', corresponding to the 10.7 kb and 9.0 kb end-labelled fragments observed following partial EcoRI digestion of the end-labelled 10.7 kb HindIII/SacI restriction fragment (see Appendix, Section Ib, Figure 21, Lanes B and C).

These 'ghost bands' likely arose as a result of contamination of the 28.2 kb HindIII/SacI restriction fragment with linear end-labelled plasmid during isolation. To isolate the end-labelled HindIII/SacI restriction fragments, pJD38 was cleaved with the enzyme HindIII and end-labelled. The resulting mixture was cleaved with the enzyme SacI, electrophoresed on a 0.7% preparative agarose gel and the restriction fragments were isolated by electroelution. It is likely that linear, end-labelled pJD38 was not cleaved to completion with the enzyme SacI. Thus, since linear plasmid DNA migrated closely to the 28.2 kb HindIII/SacI restriction fragment of pJD38 and since a large amount of DNA was applied to the preparative agarose gel further masking any separation between linear pJD38 and the 28.2 kb restriction fragment, the contaminating linear end-labelled plasmid was electroeluted along with the 28.2 kb end-labelled restriction fragment.

Incomplete digestion of gonococcal plasmid DNA was a recurrent problem. It was observed that gonococcal plasmid purified by electroelution from agarose gels or density gradient ultracentrifugation was often refractory to complete restriction enzyme digestion under optimal conditions specified by the restriction enzyme manufacturers. Gonococcal DNA may have been refractory to complete enzyme digestion as a result of intrinsic properties (ie. methylation). On the other hand, incomplete enzyme digestion of gonococcal DNA may have been due to the presence of inhibitors such as agaropectin (Dillon et al., 1985) in the agarose that were carried into the electroeluted DNA suspension. However, attempts to eliminate potential inhibitors, including use of ultrapure agarose, phenol-chloroform extractions, proteinase K treatment, dialysis (Maniatis et al., 1982), electroelution onto diethylaminoethyl paper (Dillon et al., 1985), dissolution of agarose gel slices with potassium iodide followed by hydroxyapatite extraction of DNA and a "Gene-Clean" kit (Bio101 Inc., LaJolla, Calif., U.S.A.) were not successful in eliminating the problems leading to partial digestion of gonococcal DNA following electroelution from agarose gels. Complete DNA digestion coupled with improved electrophoretic separation of restriction fragments of similar size by recent technologies such as pulsed field gel electrophoresis (Smith and Cantor, 1986) could help eliminate the problems of 'ghost bands'. Once the 'ghost bands' were identified, the distance of the EcoRI restriction sites from the end-labelled HindIII terminus on the 28.2 kb HindIII/SacI restriction fragment (Table 10b) were consistent with both the observed size of the EcoRI restriction fragments of pJD38 and the proposed orientation of the 14.0 kb and 4.9 kb EcoRI restriction fragments. The location of the EcoRI restriction sites relative to the HindIII restriction site, as determined by partial digestion mapping is shown in Figure 8b. The two EcoRI restriction sites were located 5.3 kb and 4.3 kb from the HindIII terminus of the 28.2 kb end-labelled HindIII/SacI

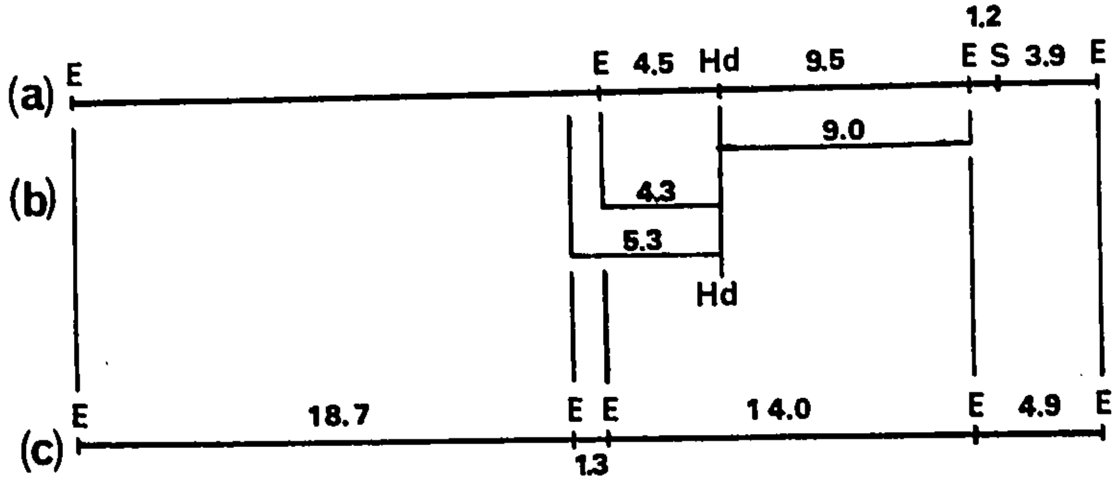


FIG. 8. *EcoRI* restriction endonuclease map of pJD38. Restriction sizes are expressed in kilobases. (a) *EcoRI* restriction sites mapped by multiple digestions of pJD38 with *EcoRI* (E), *HindIII* (Hd) and *SacI* (S). (b) *EcoRI* (E) restriction sites mapped relative to the *HindIII* (Hd) site by partial digestion mapping. (c) *EcoRI* restriction map of pJD38. *EcoRI* restriction fragment sizes have been adjusted to correspond to the average restriction fragment sizes observed (see Table 10b).

restriction fragment and were separated by a 1.0 kb segment, which corresponded approximately to the size of the smallest (1.3 kb) *Eco*RI restriction fragment of pJD38. This 1.3 kb segment was located between the 18.7 kb and 14.0 kb *Eco*RI restriction fragments. The orientation of the 1.3 kb and 14.0 kb *Eco*RI restriction fragments was verified by the presence of an *Eco*RI restriction site 9.0 kb from the *Hin*dIII terminus of the 10.7 kb end-labelled *Hin*dIII/*Sac*I restriction fragment. The proposed *Eco*RI restriction map of pJD38 is shown in Figure 8c.

e) Mapping PvuI, BglII, HindII and BclI Sites

i) *Pvu*I

*Pvu*I digestion of pJD38 generated five restriction fragments of 18.1 kb, 10.7 kb, 4.7 kb, 3.6 kb and 1.8 kb (Figure 3, Lane B, Table 11), which represented the average sizes from six digestions. The size of the 18.1 kb *Pvu*I restriction fragment was estimated by mapping the *Pvu*I restriction sites relative to *Eco*RI restriction sites on pJD38 (see Appendix, Section Ia).

*Pvu*I restriction sites were mapped by complete digestions of end-labelled *Eco*RI restriction fragments of pJD38 and by double digestions of pJD38 with *Pvu*I+*Hin*dII

TABLE 11. Restriction endonuclease digestion of pJD38 with *Pvu*I, *Bgl*II, *Hin*dII and *Bcl*I.[a]

Restriction endonuclease	Restriction fragment size (kb)[b]
*Pvu*I	(18.1)[c]
	(10.7)
	4.7
	3.6
	1.8
*Bgl*II	(21.2)
	8.0
	7.7
	1.1
	0.9
*Hin*dII	(12.9)
	9.4
	5.1
	4.2
	2.7
	1.9
	1.8
	0.7
*Bcl*I	6.6
	6.6[d]
	5.8
	5.3
	4.0
	3.3
	2.8
	1.1
	0.86

[a] pJD38 was cleaved with the enzyme indicated and electrophoresed on agarose gels (see Figure 3).

[b] Restriction fragment sizes were the average of sizes, from at least five digestions.

[c] Restriction fragment sizes in parenthesis were too large to be accurately determined by standard curve analysis (see text for details).

[d] Restriction fragments of similar size were identified by restriction mapping (see text for details).

(see Appendix, Section Ia). The proposed *PvuI* restriction map of pJD38 is shown in Figure 9.

ii) *BglII*

BglII digestion of pJD38 generated five restriction fragments of 21.2 kb, 8.0 kb, 7.7 kb, 1.1 kb and 0.9 kb (Figure 3, Lane G, Table 11). However, only four restriction fragments were visible following electrophoresis on a 1.0% agarose gel, since the 8.0 kb and 7.7 kb restriction fragments migrated closely together (Figure 3, Lane G). The estimated size of the largest 21.2 kb *BglII* restriction fragment was based on the size of the 18.7 kb *EcoRI* restriction fragment and the position of the *BglII* restriction sites of the 21.2 kb segment relative to these *EcoRI* restriction sites (see Appendix, Section Ib).

BglII restriction sites were mapped by complete digestion of end-labelled *EcoRI* restriction fragments of pJD38, as well as partial digestion mapping of the end-labelled 28.2 kb and 10.7 kb *HindIII*/*SacI* restriction fragments of pJD38 and confirmed by double digestions of pJD38 with *BglII*+*EcoRI* (see Appendix, Section Ib). The proposed *BglII* restriction map of pJD38 is shown in Figure 9.

iii) HindII

HindII digestion of pJD38 generated eight restriction fragments of average size 12.9 kb, 9.4 kb, 5.1 kb, 4.2 kb, 2.7 kb, 1.9 kb, 1.8 kb and 0.7 kb, as determined from six digestions (Figure 3, Lane D, Table 11).

HindII restriction sites were mapped by complete digestion of end-labelled EcoRI restriction fragments of pJD38, as well as partial digestion mapping of end-labelled 28.2 kb and 10.7 kb HindIII/SacI restriction fragments of pJD38 and confirmed by double digestions of pJD38 with HindII+EcoRI (see Appendix, Section Ic). The proposed HindII restriction map of pJD38 is shown in Figure 9.

iv) BclI

BclI digestion of pJD38 generated nine restriction fragments of 6.6 kb, 6.6 kb, 5.8 kb, 5.3 kb, 4.0 kb, 3.3 kb, 2.8 kb, 1.1 kb and 0.86 kb as determined from the average of seven digestions (Figure 3, Lane C, Table 11). However, since the two largest 6.6 kb BclI restriction fragments co-migrated following gel electrophoresis (Figure 3, Lane C), the presence of these fragments was ascertained from subsequent mapping data (see Appendix).

BclI restriction sites were mapped by complete digestion of end-labelled EcoRI restriction fragments of pJD38, as well

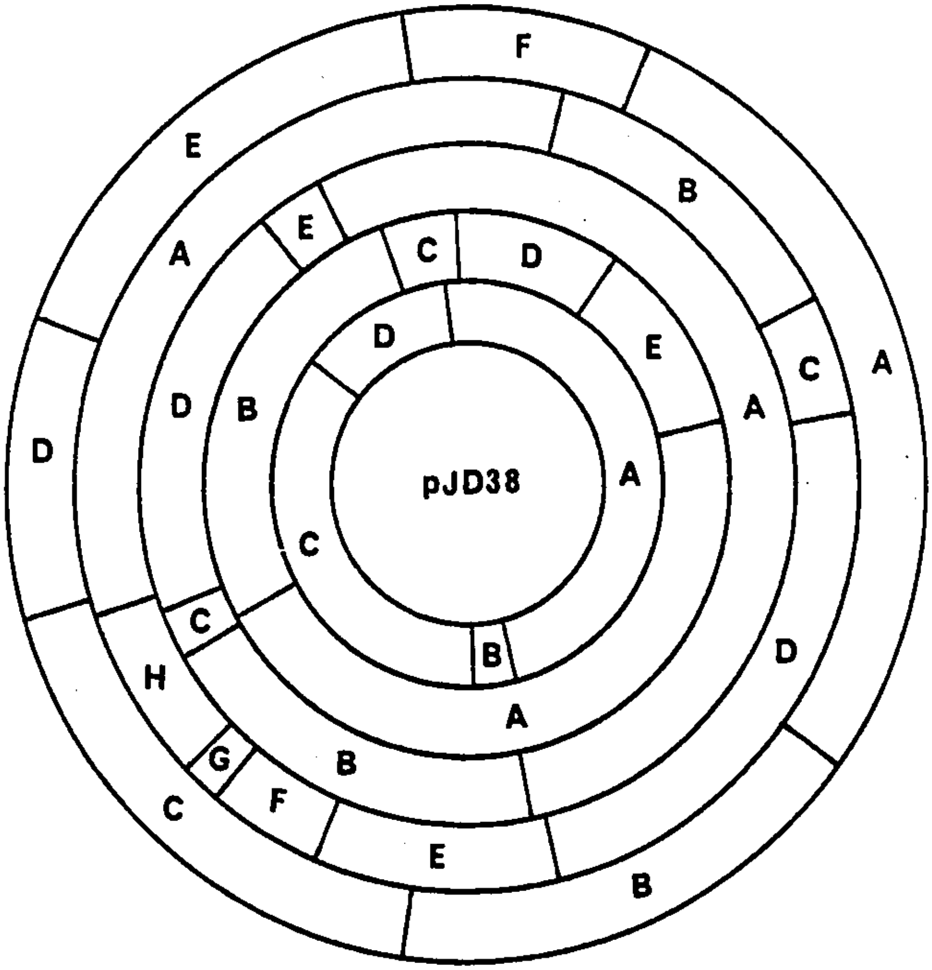


FIG. 9. Restriction maps of pJD38 for the enzymes EcoRI, PvuI, BglII, HindII and BclI. Restriction endonuclease maps include EcoRI, PvuI, BglII, HindII and BclI as ordered in the direction from innermost to outermost circle, respectively. Letters represent restriction fragment sizes calculated for EcoRI [18.7 (A), 1.3 (B), 14.0 (C), 4.9 (D) kb], PvuI [18.1 (A), 10.7 (B), 1.8 (C), 3.6 (D), 4.7 (E) kb], BglII [21.2 (A), 7.7 (B), 0.9 (C), 8.0 (D), 1.1 (E) kb], HindII [12.9 (A), 5.1 (B), 1.8 (C), 9.4 (D) 4.2 (E), 1.9 (F), 0.7 (G), 2.7 (H) kb] and BclI [12.6 (A), 6.6 (B), 5.8 (C), 4.0 (D), 6.6 (E), 3.3 (F) kb].

as partial digestion mapping of end-labelled 28.2 kb and 10.7 kb Hindlll/Sacl restriction fragments of pJD38 (see Appendix, Section Id). However, such approaches left an approximately 12.6 kb region of pJD38 unmapped. The tentative Bcll restriction map of pJD38 is shown in Figure 9.

f) Calculating the Size of pJD38

Since EcoRl was used as a basis for much of the restriction enzyme mapping of pJD38, the sum of the four EcoRl restriction fragments was used to estimate the size of pJD38. The size of the two smallest EcoRl restriction fragments was 4.9+/-0.2 kb and 1.3+/-0.2 kb as determined by calculating the mean and standard deviation of restriction fragment sizes determined from six digestions. The 14.0 kb EcoRl restriction fragment was too large to be accurately determined from standard curve analysis. Its size was estimated to be 14.1 kb from Hindll mapping (see Appendix, Section Ic, Figure 23), 14.0 kb from Pvul mapping (see Appendix, Section Ia, Figure 19) and 14.0 from Hindlll mapping (Figure 6), which gave a mean size of 14.0+/-0.07 kb. The size of the 18.7 kb EcoRl restriction fragment was also too large to be accurately determined from standard curve analysis. Its size was estimated to be 19.1 kb from Pvul mapping (see Appendix, Section Ia, Figure 19) and 18.3 kb from Hindll mapping (see Appendix, Section Ic, Figure 23) to give a mean size of 18.7+/-0.6 kb. Thus, the estimated size of pJD38 was 38.9+/-1.5 kb.

g) Physical Comparison of Gonococcal Conjugative Plasmids

Gonococcal strains 1-182 and 1-881 both harbor the conjugative plasmid, the 7.2 kb beta-lactamase-encoding plasmid and the 4.2 kb cryptic plasmid, whereas strain CH992 harbors only the conjugative plasmid (Figure 10, Lanes A, B and C, respectively). Total DNA from each gonococcal strain was cleaved with either BclI, BglII, HindII or PvuI and electrophoresed on agarose gels (Figure 10).

The BclI restriction fragment patterns of the conjugative plasmid from strains 1-182, 1-881 and CH992 were identical (Figure 10, Lane E, F and G, respectively). Neither the 7.2 kb or 4.2 kb plasmids were cleaved by BclI and neither of these plasmids overlapped with the BclI restriction fragment pattern of the conjugative plasmids. The 1.1 kb and 0.5 kb BclI restriction fragments are not visible in Figure 10, however they were visible on the photo negative.

The BglII restriction fragment patterns of the conjugative plasmids from the three gonococcal strains were also identical (Figure 10, Lanes H, I and J). BglII did not cleave either the 7.2 kb or 4.2 kb plasmids. The 1.1 kb and 0.9 kb BglII restriction fragments of the conjugative plasmid

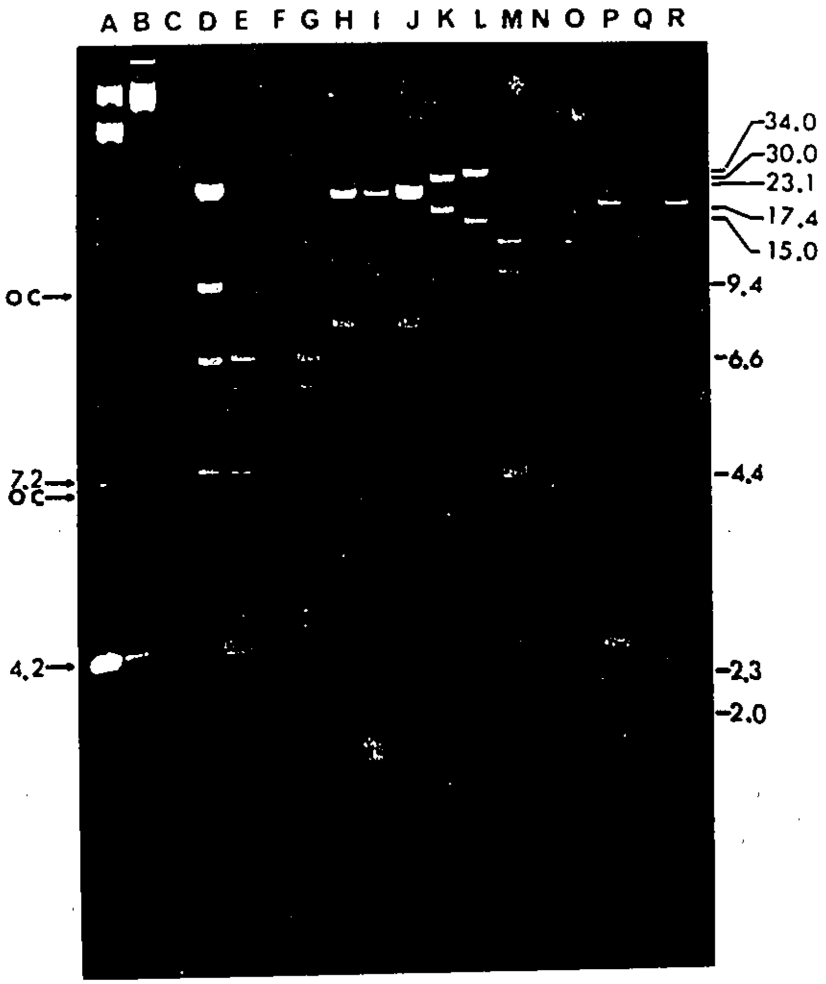


FIG. 10. Comparison of restriction endonuclease-generated patterns of conjugative plasmids from gonococcal strains 1-182, 1-881 and CH992. 0.7% agarose gel electrophoresis of conjugative plasmids from gonococcal strains 1-182, 1-881 and CH992 (pJD38) (Lanes A, B and C, respectively) and 1-182, 1-881 and CH992 cleaved with *Bcl*I (Lanes E, F and G, respectively), *Bgl*II (Lanes H, I and J, respectively), *Hin*dII (Lanes M, N and O, respectively) and *Pvu*I (Lanes P, Q and R, respectively). Restriction fragment size markers include lambda+*Hin*dIII (23.1, 9.4, 6.7, 4.4, 2.3, 2.0 kb) (Lane D), lambda+*Kpn*I (30.0, 17.4 kb) (Lane K) and lambda+*Xho*I (34.0, 15.0 kb) (Lane L). Size markers for covalently-closed-circular plasmid DNA are indicated in the left hand margin for the 7.2 kb and 4.2 kb plasmids. OC indicates the open-circular forms of the 7.2 kb and 4.2 kb plasmids.

were only faintly visible in Figure 10, however they were present in all three digestions in the photo negative.

Both PvuI and HindII cleave at a single site on the 7.2 kb plasmid (Yeung *et al*., 1986), however complete digestion of this plasmid was not achieved as observed by the presence of covalently closed circular and open circular forms of the 7.2 kb plasmid in Figure 10 (Lanes M, N, O, P, Q and R). However, HindII restriction patterns for the three conjugative plasmids were identical (Figure 10, Lanes M, N and O), as were the PvuI restriction-generated patterns (Figure 10, Lanes P, Q and R).

IV HYBRIDIZATION ANALYSIS OF THE GONOCOCCAL CONJUGATIVE PLASMID pJD38 WITH OTHER CONJUGATIVE PLASMIDS

a) Hybridization Analysis

pJD38 hybridized, at high stringency to all the *Haemophilus* conjugative plasmids examined, including RSF007 and R849, both derived from *H. influenzae* and pHD147.Tn2 derived from *H. ducreyi* (Figure 11). The degree of nucleotide sequence similarity could not be directly ascertained from this study since only part of the plasmids needed to share nucleotide sequence similarity for hybridization to be observed. However, relative to the pJD38

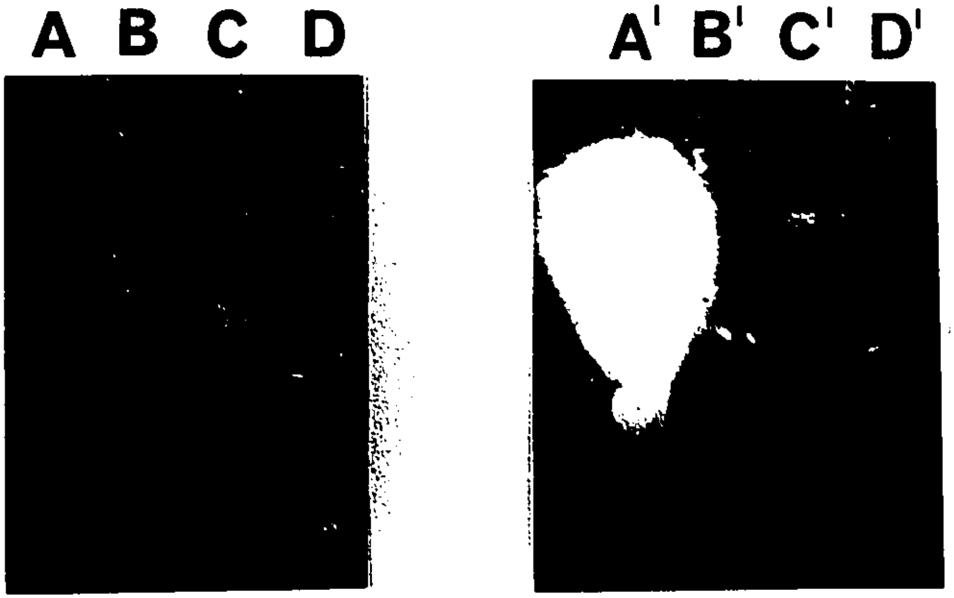


FIG. 11. Hybridization of pJD38 to conjugative plasmids of *H. ducreyi* and *H. influenzae*. 1.0% agarose gel electrophoresis of pJD38 (Lane A), pHD147.Tn2 (Lane B), RSF007 (Lane C) and R849 (Lane D). Hybridization of pJD38 under high stringency conditions to Southern blot of pJD38 (Lane A'), pHD147.Tn2 (Lane B'), RSF007 (Lane C') and R849 (Lane D').

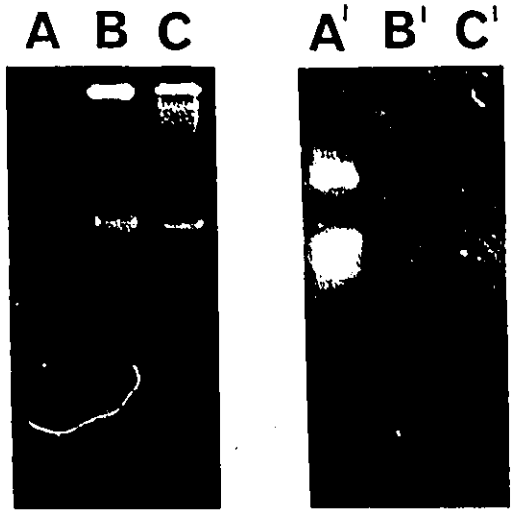
control (Figure 11, Lane A'), the intensity of the hybridization signal between pJD38 and the Haemophilus conjugative plasmids was much lower, indicating that the plasmids shared only a small to moderate degree of nucleotide sequence similarity.

Hybridization of pJD38 to conjugative plasmids representative of a number of Inc groups, including pCU101 (IncN), R112 (IncIalpha), pDT87 (IncH1) and R100 (IncF) was not observed following low stringency hybridization conditions. Such studies included hybridization with intact plasmid, as well as endonuclease cleaved preparations of pCU101 (HinfI, BglII), R112 (HaeII, EcoRI) and pDT87 (EcoRI).

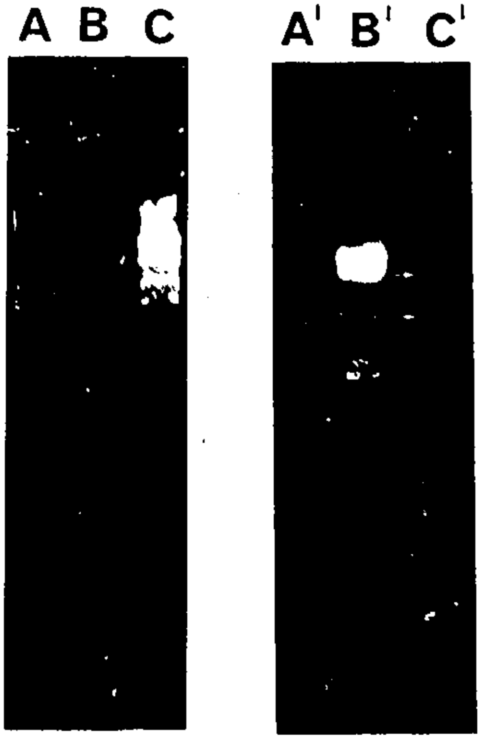
pJD38 hybridized to a number of conjugative plasmids originating from different Inc groups, including R6K (IncX), RP4 (IncP) and pSa322A (IncW). Hybridization of pJD38 to RP4 was only observed at low stringency with either intact plasmid DNA (two blots tested) (Figure 12a, Lane C') or PstI restriction fragments of RP4; where PstI cleaved RP4 six times (data not shown). However, pJD38 did not appear to hybridize to any HaeII restriction fragments of RP4 (four blots tested), although HaeII generated more than fifteen restriction fragments. pJD38 also hybridized to R6K at low stringency. However, pJD38 was only observed to hybridize to intact R6K plasmid (two blots tested) (Figure 12a, Lane B') and to the two EcoRI restriction fragments of R6K (two blot tested) (data not shown), but not to any of the twelve or

FIG. 12. Hybridization of pJD38 to IncP, X and W conjugative plasmids. (a) Hybridization of pJD38 to intact IncX (R6K) and P (RP4) plasmids. 1.0% agarose gel electrophoresis of pJD38 (Lane A), R6K (Lane B) and RP4 (Lane C). Hybridization of pJD38 under low stringency conditions to Southern blot of pJD38 (Lane A'), R6K (Lane B') and RP4 (Lane C'). (b) Hybridization of pJD38 to IncW (pSa322A) plasmid. 1.0% agarose gel elctrophoresis of restriction fragment size markers lambda+*Hin*dIII (23.1, 9.4, 6.7, 4.4, 2.3, 2.0 kb) (Lane A), pJD38+*Eco*RI (Lane B), and pSa322A+*Pvu*I (Lane C). Hybridization of pJD38 under high stringency conditions to Southern blot of lambda+*Hin*dIII (Lane A'), pJD38+*Eco*RI (Lane B') and pSa322A+*Pvu*I (Lane C').

(a)



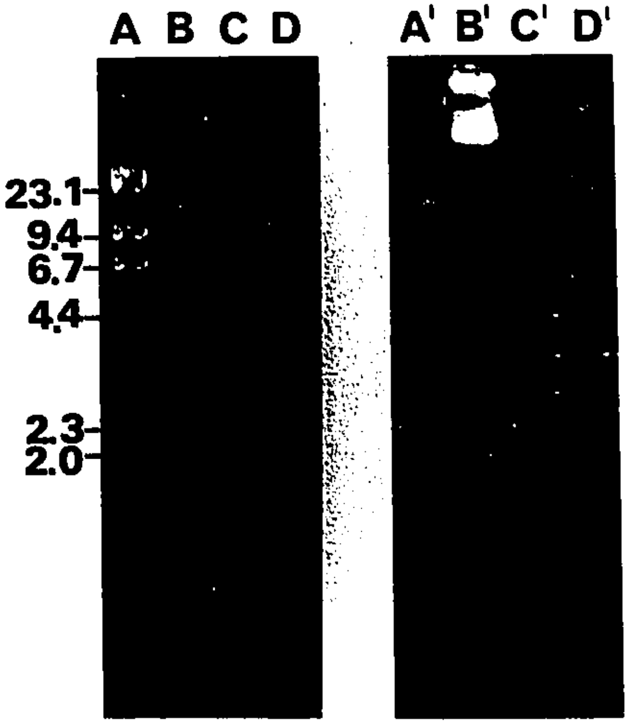
(b)

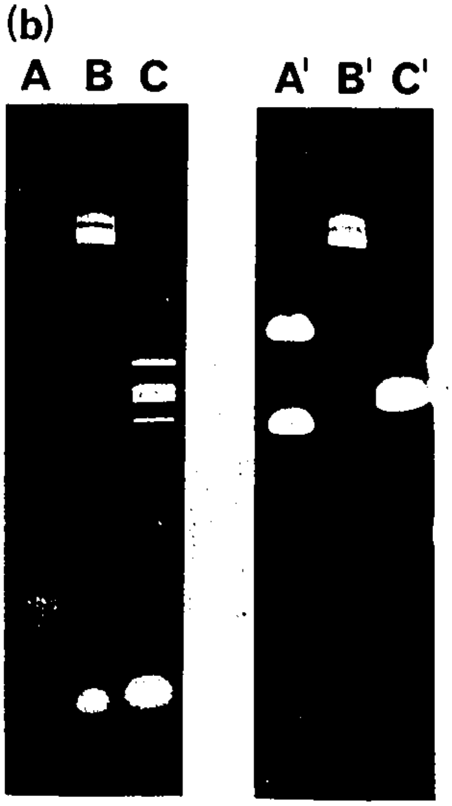


more HaeII restriction fragments of R6K (one blot tested). pJD38 hybridized most consistently at high stringency to pSa322A, including intact plasmid (two blots tested) (data not shown), and PvuI (one blot tested) (Figure 12b, Lane C'), HaeII (one blot tested) (data not shown) and HindII (two blots tested) (Figure 13a, Lane C') digestions of pSa322A. pJD38 hybridized to two PvuI restriction fragments of pSa322A of approximately 20.0 kb and 8.8 kb under high stringency conditions (Figure 12b, Lane C') and to at least three HindII restriction fragments of pSa322A restriction fragments of 4.5 kb, 2.9 kb and 3.8, 3.5 kb and/or 3.4 kb (these two restriction fragments migrated very closely) under high stringency conditions (Figure 13a, Lane C'). However, in both cases, the hybridization signal between pJD38 and pSa322A was not as strong as the control signal indicating only a moderate to low level of nucleotide sequence similarity between pJD38 and pSa322A.

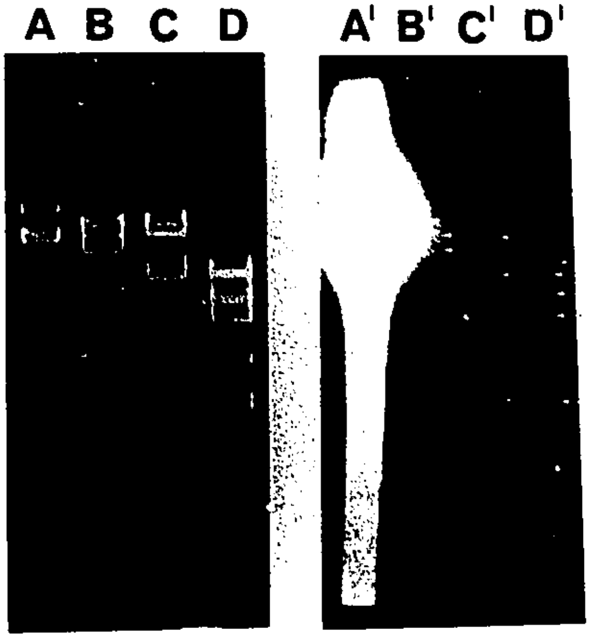
FIG. 13. Identification of pSa322A restriction endonuclease fragments hybridizing to pJD38. (a) Hybridization of pJD38 to pSa322A and pBR322. 1.0% agarose gel electrophoresis of restriction fragment size markers lambda+*Hin*dIII (23.1, 9.4, 6.7, 4.4, 2.3, 2.0 kb) (Lane A), pJD38 (Lane B), pSa322A+*Hin*dII (Lane C) and pBR322+*Hin*dII (Lane D). Hybridization of pSa322A under high stringency conditions to Southern blot of lambda+*Hin*dIII (Lane A'), pJD38 (Lane B'), pSa322A+*Hin*dII (Lane C'), and pBR322+*Hin*dII (Lane D'). (b) Hybridization of pBR322 to pSa322A. 1.0% agarose gel electrophoresis of pBR322 (Lane A), pSa322A (Lane B) and pSa322A+*Hin*dII (Lane C). Hybridization of pBR322 under high stringency conditions to a Southern blot of pBR322 (Lane A'), pSa322A (Lane B') and pSa322A+*Hin*dII (Lane C'). (c) Hybridization of pSa322A to pJD38. 1.0% agarose gel electrophoresis of pSa322A (Lane A), pJD38+ *Eco*RI (Lane B), pJD38+*Pvu*I (Lane C) and pJD38+*Eco*RI+*Pvu*I (Lane D). Hybridization of pSa322A under high stringency conditions to a Southern blot of pSa322A (Lane A'), pJD38+*Eco*RI (Lane B'), pJD38+*Pvu*I (Lane C') and pJD38+*Eco*RI+*Pvu*I (Lane D').

(a)




(b)
A
B
C
A'
B'
C'

(c)



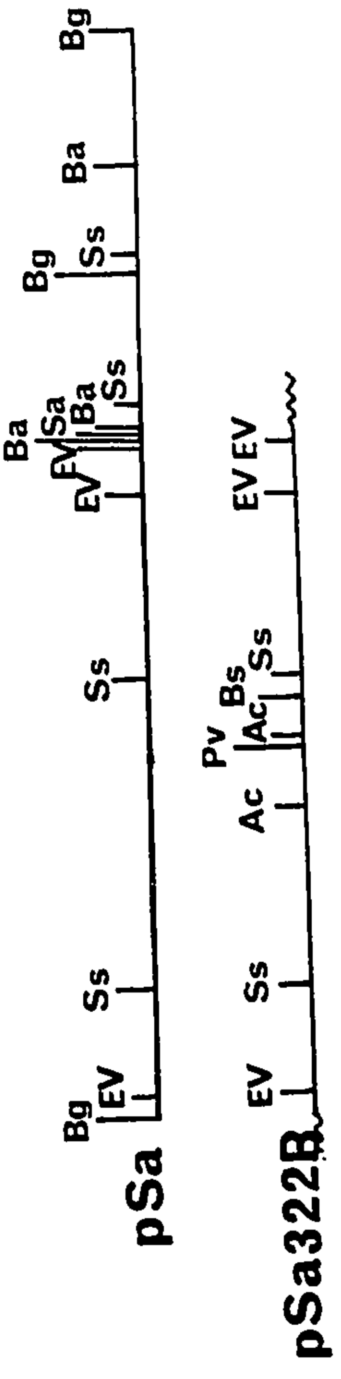
b) Localization of Nucleotide Sequence Similarity of pSa322A and pJD38

A number of difficulties were encountered when trying to determine whether pSa322A restriction fragments hybridizing to pJD38 were tra related. Tait et al. (1982) originally constructed and correlated the physical and genetic map of the 29.6 kb IncW plasmid pSa. Subsequently, Tait et al. (1983) constructed pSa322 by cloning a 15.0 kb BamHI/BglII restriction fragment of pSa into the BamHI restriction site of pBR322. Tait et al. (1983) claimed that the composite plasmid pSa322 was 19.0 kb in size, Tra$^+$ and that the pSa tra region was located in a 10.0 kb pSa322 region flanked by SstII and BamHI restriction sites.

pSa322A, used in this study (donated by Dr. V.N. Iyer) was tested for conjugative properties and found to transfer to res$^-$ E. coli recipient strain DH1. However, repeated attempts in this study to cleave pSa with SstII yielded a restriction fragment (approximately 24.0 kb) larger than the size of either of the two restriction fragments (10.0 kb and 19.0 kb) predicted by the restriction map of Tait et al. (1982). It was later discovered (from a personal communication with Dr. C.I. Kado, University of California, Calif., U.S.A., May 27, 1987) that during construction of the restriction map of pSa by Tait et al. (1982) a restriction fragment of approximately 10.0 kb had been omitted from the map. However, as of that time it was not certain as to where this restriction fragment was located relative to the proposed genetic map of pSa322A (personal communication with Dr. C.I. Kado, University of California, Calif., U.S.A., May 27, 1987).

In the mean time, uncertain of the composition of pSa322A used in the hybridization with pJD38, a second strain containing pSa322B, estimated to be 28.8 kb, was supplied by Dr. V.N. Iyer as well as a restriction map of pSa and a preliminary identification of a 12.2 kb tra region of pSa as determined by transposon mutagenesis and construction of deletion derivatives (Figure 14). Preliminary restriction anaylsis in this laboratory of both pSa322A and pSa322B generated identical restriction fragment patterns for both plasmids with various enzymes (Figure 15), however the sizes of these restriction fragments were not in complete accordance with the restriction map of pSa322B. BstEII did cleave pSa322A and pSa322B at a single site as predicted (Figure 15, Lanes B and F, respectively), however SstII only generated a single restriction fragment instead of the predicted two restriction fragments of 11.0 kb and 17.8 kb (Figure 15, Lanes D and H, respectively). Moreover PvuI digestion of pSa322A and pSa322B generated two restriction fragments of 9.1 kb and 16.0 kb (Figure 15, Lanes C and G, respectively) which did not correspond to the sizes predicted

FIG. 14. Restriction endonuclease map of pSa and pSa322B. pSa was mapped with the enzymes *Bam*HI (Ba), *Bgl*II (Bg), *Eco*RV (EV), *Sal*I (Sa) and *Sst*II (Ss). The 12.2 kb region inferred to be required for conjugative transfer of pSa is shown in the map of pSa322B. The restriction endonuclease maps were supplied courtesy of Dr. V.N. Iyer (Depart. of Biology, Carleton University, Ottawa, Canada).


pSa
Bg
EV
Ss
Ss
EV
EV
Ba
Sa
Ba
Ss
Bg
Ss
Ba
Bg
pSa322B
EV
Ss
Ac
Pv
Ac
Bs
Ss
EV
EV
EV

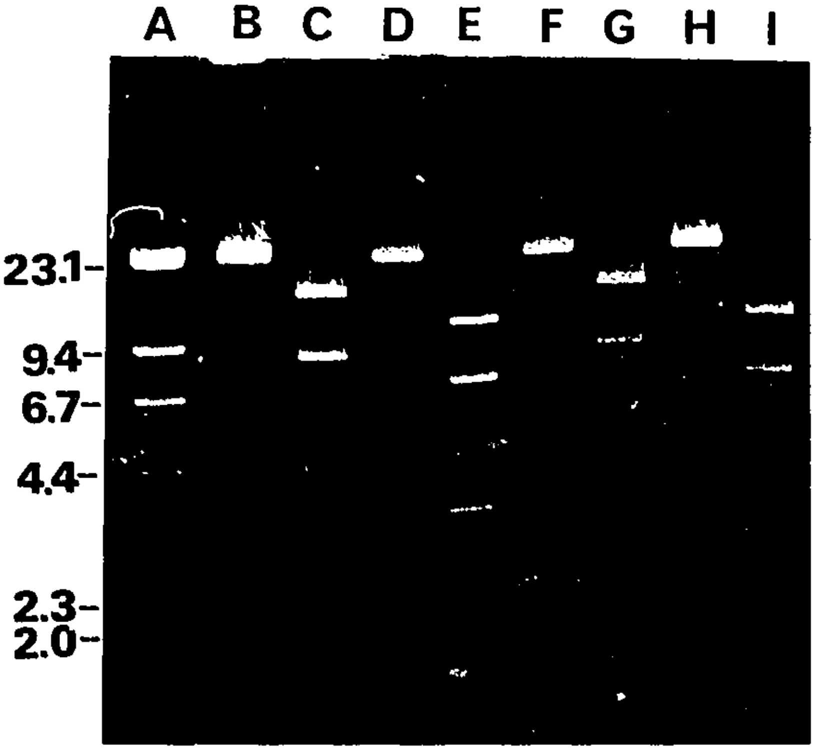


FIG. 15. Restriction endonuclease analysis of pSa322A and pSa322B. 1.0% agarose gel electrophoresis of restriction fragment size markers lambda+*Hin*dIII(23.1, 9.4, 6.7, 4.4, 2.3, 2.0 kb) (Lane A), pSa322A cleaved with *Bst*EII (Lane B), *Pvu*I (Lane C), *Sst*II (Lane D) and *Acc*I (Lane E) and pSa322B cleaved with *Bst*EII (Lane F), *Pvu*I (Lane G), *Sst*II (Lane H) and *Acc*I (Lane I).

by the restriction map of pSa322B. Thus, the mapped location of the PvuI restriction site was not a reliable marker of the pSa322 tra region.

AccI digestion of pSa322A and pSa322B generated four restriction fragments of 1.75 kb, 3.30 kb, 7.6 kb and approximately 12.5 kb in size (Figure 15, Lanes E and I, respectively) which did not correspond to the sizes predicted by the restriction map of pSa322B. Thus, AccI restriction sites were not reliable indicators of the pSa322 tra region. Thus, it was not known with certainty how the physical and genetic maps of pSa322 are correlated. However, to narrow down the region(s) of nucleotide sequence similarity, pJD38 was also hybridized to HindII restriction fragments of pSa322A. pJD38 hybridized to the 4.5 kb, 2.9 kb and the 3.8 kb, 3.5 and/or 3.4 kb HindII restriction fragments of pSa322A, but not the 1.4, 1.2, 0.9, 0.76 or 0.56 kb HindII restriction fragments (Figure 13a, Lane C'). Since the 3.8 kb, 3.5 kb and 3.4 kb restriction fragments migrated so closely it could not determined which had hybridized with pJD38.

To complicate matters, pJD38 also hybridized with a 3.4 kb HindII restriction fragment of pBR322 (Figure 13a, Lane D'). Ten HindII restriction fragments of pSa322A were hybridized with pBR322 to determine which contained components of pBR322. pBR322 hybridized to the 3.5/3.4 kb restriction fragment band and 0.76 kb HindII restriction fragments of pSa322A (Figure 13b, Lane C'). Thus, by elimination, it could be concluded that the pSa components of pSa322A that shared nucleotide sequence similarity with pJD38 were contained within the 4.5 kb, 3.8 kb and/or 3.5 kb and 2.9 kb HindII restriction fragments. It could not be ascertained whether pJD38 shared nucleotide sequence similarity with the 3.8 kb pSa322A HindII restriction fragment or the 3.5 kb pBR322 restriction fragment since they migrated so closely.

Hybridization of pJD38 to pSa322A was confirmed by hybridizing a nick translated pSa322A probe to EcoRI, PvuI and PvuI+EcoRI restriction fragments of pJD38 (Figure 13c). pSa322A hybridized under high stringency conditions to the 18.7 kb and 14.0 kb EcoRI restriction fragments (Figure 13c, Lane B'), the 18.1 kb and 10.7 kb PvuI restriction fragments (Figure 13c, Lane C') and the 10.0 kb, 7.7 kb, 6.6 kb and one or both of the 3.6 kb co-migrating PvuI+EcoRI restriction fragments (Figure 13c, Lane D') of pJD38. Moreover, the hybridization signal was not as strong as the control signal indicating a moderate to low level of nucleotide sequence similarity. However, a control hybridization of pBR322 to pJD38 restriction fragments was not completed.

V OTHER EXPERIMENTS

This section describes a number of strategies that were attempted in order to tag the gonococcal conjugative plasmid with a marker having an easily detectable phenotype. Several attempts to clone restriction fragments of pJD38 into suitable cloning vector pBR322 by a standard cloning procedure (Maniatis *et al.*, 1985) were unsuccessful. Attempts were made to ligate *Pvu*I restriction fragments of pJD38 into the single *Pvu*I restriction site of pBR322, located in the beta-lactamase-encoding gene, and transform competent cells of *E. coli* strain DT591. No ampicillin-sensitive, tetracycline-resistant transformants were obtained as would be expected as a result of insertional inactivation of the beta-lactamase-encoding gene by cloned *Pvu*I restriction fragments of pJD38.

Another cloning approach was to ligate *Bgl*II restriction fragments of pJD38 to *Bam*HI cleaved pBR322, where the *Bam*HI site was located in the gene encoding tetracycline-resistance. No ampicillin-resistant, tetracycline-sensitive transformants were obtained.

An alternate strategy for tagging the gonococcal conjugative plasmid consisted of ligating a *Bgl*II digestion mixture of pJD38 with a purifed 2.4 kb *Bam*HI restriction fragment containing the beta-lactamase-encoding gene of the 7.2 kb gonococcal plasmid, and reportedly containing the origin of replication (McNicol et al., 1984). No ampicillin-resistant transformants were obtained following transformation of competent cells of E. coli strain DT591 with the ligation mixture. However, there was no real evidence as to whether a gonococcal conjugative plasmid tagged with such a marker would replicate in an E. coli host strain, since it was subsequently shown that this 2.4 kb BamHI fragment, when religated, could not replicate on its own in E. coli (Yeung and Dillon, 1988). Thus, a new system employing a host background, compatible with the replication of the gonococcal conjugative plasmid was selected for tagging the conjugative plasmid.

Attempts were made to establish a transposon mutagenesis system in N. cinerea host background since the gonococcal conjugative plasmid could be conjugated to and replicate in N. cinerea and N. cinerea could act as a conjugal intermediate between the gonococcus and other species (Genco et al., 1984; McNicol et al., 1986a). The Tra^{+} suicide vector pUW942 harboring the mercury transposon (Tn501) was obtained from Dr. T. Nicas (Faculty of Medicine, University of Ottawa). pUW942 was only maintained in E. coli but could conjugate to a variety of hosts. Once inside a host, other than E. coli, pUW942 could not be maintained thus forcing the transposition of Tn501. Gonococcal donor strain 1-881 was mated with a naladixic acid-resistant N. cinerea recipient strain isolated in this study and transconjugants harboring both the conjugative plasmid and the 7.2 kb beta-lactamase-encoding plasmid were identified and selected for further manipulations. The N. cinerea transconjugant was incubated in a filter mating with naladixic acid-sensitive HB101 donor strain harboring pUW942. The mating suspension was plated on TSA media containing successive concentrations of mercury ranging from 5.0 to 50.0 mg/L, since the optimal level of mercury was a critical step and varied with bacteria and media. Mercury-resistant transconjugants were to be screened for an increased conjugative plasmid size due to transposon insertion and if this correlated to transposon mutagenesis of the conjugative plasmid it could be transferred into another suitable recipient if desired. However, no mercury-resistant transconjugants were observed in any of these matings.

A second vector R68.Tn501 was a Tra^+, broad host range, temperature-sensitve vector which would not replicate at 42·C. Such a temperature-sensitive vector in N. cinerea could possibly aid in establishing optimal mercury levels for selecting mercury-resistance in N. cinerea in addition to its use in transposon mutagenesis. However, N. cinerea transconjugants did not grow following overnight incubation at 42·C. It may have been possible to find an optimal incubation time for growing N. cinerea at 42·C without killing the cells yet forcing the transposition of Tn501 and the subsequent loss of R68.Tn501. However, no mercury-resistant transconjugants were obtained in any of these mating attempts.

DISCUSSION

I EVOLUTION OF GONOCOCCAL STRAINS

This study addresses the roles of gonococcal conjugative plasmids, non-conjugative plasmids and gonococcal conjugal recipient cells in the evolution of gonococcal strains. Conjugation is the primary mode of plasmid exchange in the gonococcus (Roberts *et al*., 1978). Using animal models Roberts and Falkow (1979) demonstrated that the gonococcal conjugative plasmid promotes transfer of the 7.2 kb beta-lactamase-encoding plasmid *in vivo*. Moreover, infections with multiple strains of gonococci are common (Handsfield *et al*., 1980) and thereby provide an opportunity for plasmid exchange between different gonococcal strains *in vivo*.

a) Role of Recipient Cells in Conjugation

The role of recipient gonococcal strains in conjugation was examined in this study. Intraspecific matings with gonococcal recipient strains of various nutritional and plasmid profiles demonstrated several examples of the *in vitro* generation of novel gonococcal strains. Mobilization of the 7.2 kb beta-lactamase-encoding plasmid by a co-resident conjugative plasmid to NR and P$^-$ gonococcal recipient strains was demonstrated. In natural isolates of gonococci, NR and P$^-$ auxotypes frequently harbor a beta-lactamase-encoding plasmid and a cryptic plasmid with or without a conjugative plasmid (Bygdeman, 1981; Bygdeman et al., 1981; Dillon and Pauze, 1981b; Odugbemi et al., 1983). Thus, the relatively high mobilization frequency of the 7.2 kb plasmid observed in vitro to NR and P$^-$ gonococcal recipient strains is consistent with the high frequency of occurrence of this plasmid in NR and P$^-$ natural isolates.

The most significant finding was the mobilization of the 7.2 kb plasmid to a NR recipient strain (CH811) lacking any plasmid. To date, no natural isolates of gonococci, of any auxotype, have been observed to harbor only a beta-lactamase-encoding plasmid. However, this trend may be a result of the large prevalence of the 4.2 kb cryptic plasmid in strains with auxotypes, other than PCU$^-$ (Roberts et al., 1979).

A novel observation was the in vitro mobilization of the 7.2 kb beta-lactamase-encoding plasmid to both PCU$^-$ and OUH$^-$ gonococcal recipient strains. To date, all naturally-occurring gonococcal PCU$^-$ isolates examined for plasmid content have been plasmid-free, while naturally-occurring OUH$^-$ isolates frequently harbor the 4.2 kb cryptic plasmid (Dillon and Pauze, 1981b; Dillon et al., 1987). The low mobilization frequency of the 7.2 kb plasmid to PCU$^-$ recipient strains 1-835 and 1-627, and OUH$^-$ recipient strain

A9 is consistent with the low frequency or complete absence of the 7.2 kb plasmid in these auxotypes in natural isolates.

Thus, in vitro mobilization data predicts the appearance of novel plasmid combinations in gonococcal strains with various nutritional requirements. Although in vitro controls were negative for spontaneous mutation, a role of mutation either alone or in conjunction with mobilization in the generation of such novel strains cannot be ruled out completely. Gonococcal nutritional requirements can be altered by spontaneous genetic defects in genes coding for important components in biosynthetic pathways and by complementation of inactivated genes by transformation with gonococcal DNA (Catlin, 1974; Copley, 1987). Thus, genetic examination of natural isolates may not be sufficient to predict whether a novel strain arose by conjugal transfer of plasmids into a strain with a given auxotype or by mutation of a given auxotype in a strain already containing plasmid(s).

The exact role of the gonococcal recipient cell in the regulation of mobilization is not known. Recipient cells could affect conjugation at two levels; (1) the formation of stable mating aggregates and (2) the establishment of mobilized DNA, including those events leading up to the stable replication of transferred DNA (Willetts and Skurray, 1980). To be conjugationally competent, a recipient cell must have receptor(s) for a specific site(s) on the donor cell, and must also possess a cell envelope able to allow passage of the transferred DNA (Willets and Skurray, 1980). An _E_. _coli_ recipient lacking an outer membrane protein, designated 3A, was found to be defective with F-like plasmid donors (Achtman _et_ _al_., 1978; Manning and Reeves, 1976). Thus, outer membrane proteins of a recipient cell (and donor cell) may play a significant role in the formation of stable mating aggregates (Willets and Skurray, 1980).

_N_. _gonorrhoeae_ produces at least three classes of outer membrane proteins, designated PI, PII and PIII. The PIII outer membrane proteins (60 MDa) are important in resistance to human serum (Heckels and Everson, 1978), however no correlation between Plll proteins and auxotype could be demonstrated (Eisenstein _et_ _al_., 1977a). PII outer membrane proteins (28-29 MDa), known as opacity proteins, are responsible for colony forms of different coloration and aggregation characteristics (Swanson, 1977; 1982). Biswas _et_ _al_. (1980a) noted a five- to ten-fold increase in mobilization frequency in isogenic crosses with transparent strains having lower levels of opacity proteins than in dark, opaque strains. Biswas _et_ _al_. (1980a) proposed that the presence of these outer-membrane proteins may reduce the ability of the gonococcus to form stable mating aggregates and in this way affect the conjugation frequency. However, in this study, Type 4 transparent colonies of each gonococcal recipient strain were selected specifically for matings.

PI comprises the principal outer membrane proteins (32-39 MDa) and are the major contributor to serological specificity of N. gonorrhoeae (Johnston and Gotschlich, 1974). These are the only gonococcal proteins known to correlate with specific auxotypes (Dillon et al., 1987; Knapp and Holmes, 1975). Sera for serological characterization of gonococcal strains used in this study was not available until after completion of this study, thus strains representative of a variety of serovars could not be selected for such conjugal host range studies. However, no correlation was observed between serovar of those gonococcal strains examined in this study and recipient conjugal efficiency.

Recipient strains can also affect conjugation at the stage of establishment of DNA (Willets and Skurray, 1980). One of the most obvious barriers at this stage is restriction barriers. Mobilizaton of the 7.2 kb gonococcal plasmid was not detected directly to res$^+$ E. coli recipient strain from a gonococcal donor strain unless it was first present in a mod$^+$ E. coli intermediate donor strain. This suggested that plasmid DNA resident in the gonococcal donor strain was not modified (methylated) adequately to be protected from the restriction system of the res$^+$ E. coli recipient strain.

As many as five restriction endonucleases and eleven methylases from strains of N. gonorrhoeae have been identified (Norlander et al., 1981; Stein et al., 1988). It has been proposed that every gonococcal strain produces at least one endonuclease (Stein et al., 1988). Stein et al. (1988) demonstrated that restriction modification variations between gonococcal donor and recipient strains was not sufficient to completely inhibit conjugation, but did decrease the conjugation frequency by approximately ten-fold. However, Stein et al. (1988) only examined the effect of restriction of unmethylated *Ngo*II restriction sites on conjugation of the composite plasmid and thus, did not exclude the possibility of a larger role of other gonococcal restriction/modification systems in reduction of conjugation frequencies. Correlation between auxotype and specific restriction/modification systems has not been examined in the gonococcus. Furthermore, no information regarding specificity of restriction/modification systems of the gonococcal recipient strains used in this study is available. It would be interesting to know if recipient auxotypes which act as poor conjugal recipients behave similarly in a conjugal donor capacity.

b) Non-conjugative Plasmids and Evolution of Gonococcal Strains

Generation of gonococcal strains with novel combinations of genetic properties and plasmids will depend, in part, on the successful mobilization of non-conjugative plasmids. *In vitro* mobilization of both the 7.2 kb and 5.1 kb non-conjugative beta-lactamase-encoding plasmids from gonococcal donor strains to res$^-$ E. coli recipient strains, as well as mobilization of the 7.2 kb plasmid to gonococcal recipient strains was demonstrated in this study and confirmed the general observations of previous workers (Baron et al., 1977; Eisenstein et al., 1977b; Kirven and Thornesberry, 1977; Roberts and Falkow, 1977; Sox et al., 1978).

A general mobilization frequency of the 7.2 kb plasmid was not calculated since the mobilization frequency was observed to vary not only with different gonococcal donor strains, but also with recipient strains of E. coli or N. gonorrhoeae. Such observations may explain the discrepencies in previously reported mobilization frequencies of the 7.2 kb plasmid. For example, in this study the 7.2 kb plasmid from gonococcal donor strain 1-182 was mobilized at a frequency of $4.9x10^{-3}$ transconjugants/input donor cfu to E. coli recipient strain C600, while Eisenstein et al. (1977b) and Sox et al. (1978) reported a mobilization frequency of 10^{-4} and 10^{-5} transconjugants/input donor cfu to C600, respectively. Sox et al. (1978) have also demonstrated functional differences in the ability of gonococcal conjugative plasmids from different strains to mobilize the 7.2 kb plasmid.

An unusual observation was that of intratest variation in mobilization frequency of the 7.2 kb plasmid in mating trials using the same donor and recipient strains, including gonococcal and E. coli strains. Such variation made calculation of an average mobilization frequency difficult since the observed frequency varied ten-fold or more; where mobilization was not detected in some matings (ie. <10^{-6} transconjugants/input donor cfu). Intratest variation in mobilization frequency has not been reported by previous workers.

It is not known whether this intratest variation is related to regulatory controls exerted on mobilization (ie. by donor or recipient cells and/or the conjugative plasmid) or the result of mutation in recipient cells. If mechanisms regulating mobilization were active, it is not known in what way conjugation could be repressed in a large population of donor cells, in an overnight mating, such that mobilization is not detected in some tests, while the frequency may be as high as 10^{-1} transconjugants/input donor cfu with the same strains in other matings. On the other hand, studies of mutation of gonococci _in vitro_ have shown that gonococci can undergo spontaneous mutation and back-mutation at a low frequency (Maness and Sparling, 1972). It is not known whether such mutations are linked to regulation of conjugation in the gonococcus.

Mobilization of a novel 4.9 kb gonococcal beta-lactamase-encoding plasmid was not detected in this study. Similarly, a 4.6 kb beta-lactamase-encoding plasmid described by van Embden _et al_. (1985) was not successfully mobilized. The 4.6 kb plasmid was similar to the 4.9 kb plasmid described by Yeung et al. (1986) and used in this study, except for a 100 base pair deletion on the large Hindlll/BamHl fragment that was not observed on the 4.9 kb plasmid. Both plasmids had a 2.3 kb fragment deleted from the 7.2 kb gonococcal beta-lactamase-encoding plasmid which corresponds to part of the nucleotide sequence encoding a protein necessary for mobilization (Tenover et al., 1985).

Co-transfer of the 4.2 kb cryptic plasmid with the 7.2 kb beta-lactamase-encoding plasmid from gonococcal donor strains to res$^-$ E. coli recipient strains was not observed in the 349 transconjugants selected for the acquisition of beta-lactamase production and analyzed for plasmid content. Although no information regarding stability of the 4.2 kb plasmid in E. coli is available, it is possible that the 4.2 kb plasmid may not replicate in this species. The 4.2 kb plasmid was not observed to co-transfer with the 7.2 kb plasmid to gonococcal recipient strains lacking a cryptic plasmid, suggesting it is not mobilizable. Moreover, the absence of the 4.2 kb cryptic plasmid in the donor strain did not inhibit mobilization of the 7.2 kb beta-lactamase-encoding plasmid suggesting it was not required for conjugation.

Thus, mobilization studies of gonococcal non-conjugative plasmids suggest that mobilization of the 7.2 kb and 5.1 kb beta-lactamase-encoding plasmids would play the most significant role in generation of novel gonococcal strains harboring these plasmids. However, generation of new strains harboring beta-lactamase-encoding plasmids, such as the 4.9 kb plasmid, will depend on whether mobilization functions are present on the plasmid. In contrast, the natural distribution of non-mobilizable plasmids, such as the 4.2 kb cryptic plasmid, would account for the appearance of such plasmids in gonococcal strains with novel genetic properties.

c) Role of the Conjugative Plasmid in the Evolution of Gonococcal Strains

Direct investigation of the role of the gonococcal conjugative plasmid in the evolution of gonococcal strains has generally been hindered by the absence of an easily detectable phenotypic marker. Although indirect examination has provided some insight into its function in conjugation. Intergeneric matings in this study demonstrated that the gonococcal conjugative plasmid was required for mobilization of the 7.2 kb beta-lacatamase-encoding plasmid. The gonococcal conjugative plasmid was never observed in _E. coli_ transconjugants selected for acquisition of beta-lactamase production. Although the conjugative plasmid could not be physically isolated from _E. coli_ transconjugants, it did appear to be transiently stable in _E. coli_, mobilizing a 7.2 kb plasmid out of an intermediate donor in triparental matings, thereby confirming the observations of others (Flett et al., 1981; Morse et al., 1986; Sox et al., 1978). Detection of the conjugative plasmid in gonococcal transconjugants in intraspecific matings in this study confirmed its self-transmissable property.

Co-transfer of the conjugative plasmid with the 7.2 kb plasmid to a gonococcal recipient strain was detected in only one mating in this study. A frequency of co-transfer could not be reliably calculated due to the insufficient number of gonococcal transconjugants examined for plasmid in that mating. Another approach to determine the frequency of co-transfer by colony-hybridization of transconjugant colonies with a conjugative plasmid probe was complicated by the apparent contamination of plasmid preparations with chromosomal DNA. However, Sox et al. (1978) has reported a low frequency of co-transfer, where the conjugative plasmid was observed in only six of sixty-four NR gonococcal transconjugants. Similarly, Roberts and Knapp (1988) have reported that transfer of the beta-lactamase-encoding plasmid was more frequent than that of the 39.2 kb conjugative plasmid, however, it was also observed that recipient gonococcal strains acquired the 40.3 kb tetracycline-resistance-encoding conjugative plasmid at frequencies ten- to one-hundred-fold higher than the beta-lactamase-encoding plasmids.

Mobilization frequencies of the 7.2 kb beta-lactamase-encoding plasmid varied with different gonococcal donor strains. These observations suggest that functional differences exist between conjugative plasmids from different strains. Unfortunately, comparison of plasmid function in matings with isogenic strains was hindered by the lack of stable transfer of the conjugative plasmid from donor strains to various recipient strains, including N. gonorrhoeae, N. cinerea and E. coli. Thus, such studies in which mobilization frequencies varied between different gonococcal donor strains could not exclude the possibility of functional differences in resident 7.2 kb plasmids and/or host chromosome which could also affect mobilization frequency (Silverman, 1985; Sox et al., 1978). However, in a study employing isogenic strains, Sox et al. (1978) did observe differences in both self-transfer and mobilization efficiency. Although conjugative plasmids were reported to differ in frequency of self-transfer, some conjugative plasmids which had similar self-transfer frequencies were observed to mobilize the same 7.2 kb plasmid at different frequencies, suggesting that gonococcal conjugative plasmids can also vary in their ability to initiate mobilization.

Thus, the gonococcal conjugative plasmid likely plays a significant role in the generation of novel strains largely by its capacity to efficiently mobilize specific gonococcal plasmids. Whereas, self-transfer of the 39.2 kb conjugative plasmid likely plays a relatively minor role in its spread to new strains. In natural isolates the distribution of the conjugative plasmid is limited to the NR and P$^-$ auxotypes (Dillon *et al.*, 1987). Its spread into an OUH$^-$ requiring gonococcal strain has been reported (Dillon and Pauze, 1981b; van Embden *et al.*, 1985). However, it is not known whether these novel strains arose by conjugation or by mutation of another auxotype already harboring the conjugative plasmid. The relationship between the frequency of self-transfer of the gonococcal conjugative plasmid and its distribution in natural isolates has yet to be fully examined.

II PHYSICAL AND GENETIC ANALYSIS OF THE GONOCOCCAL CONJUGATIVE PLASMID

The study of the conjugal system of *N. gonorrhoeae* is still in its infancy. Moreover, the basic issue of whether a single conjugal plasmid species is present in *N. gonorrhoeae* remains to be fully addressed, not only from a genetic perspective, but also at the structural level.

A gonococcal conjugative plasmid, pJD38, has been characterized in structural detail in this study. The size of pJD38 was estimated to be 38.9+/-1.5 kb, similar to the size of the 39.6 kb gonococcal conjugative plasmid reported by Tenover *et al.* (1980) and falling within the previously reported range of 39.2+/-4.8 kb (Roberts *et al.*, 1978; 1979;

Sox *et al*., 1978; Stiffler *et al*., 1975). pJD38 shared structural similarity with other conjugative plasmids isolated from gonococcal strains, having different mobilization frequencies, as determined by identical restriction fragment patterns of the three plasmids for each of the enzymes *Bcl*I, *Bgl*II, *Hin*dII and *Pvu*I. Similarly, Roberts *et al*. (1979) reported identical *Eco*RI and *Bam*HI restriction fragment patterns for conjugative plasmids isolated from three gonococcal strains. Tenover *et al*. (1980) also demonstrated identical *Eco*RI, *Bgl*II and *Hin*dII restriction fragment patterns for conjugative plasmids from two gonococcal strains.

Structural differences in conjugative plasmids from different gonococcal strains have also been observed. Sox *et al*. (1978) reported a gonococcal conjugative plasmid having a different *Bam*HI restriction fragment pattern as compared to conjugative plasmids isolated from two different gonococcal strains, yet all three plasmids reportedly shared similar restriction fragment patterns for *Eco*RI and seventeen resolvable *Hpa*II restriction fragments. Morse *et al*. (1986) have described a 40.3 kb naturally-occurring gonococcal conjugative plasmid containing a *tet*M sequence, whose *Sma*I and *Hin*dII restriction fragment patterns differed significantly from a 39.2 kb gonococcal conjugative plasmid examined at that time. Moreover, the *Hin*dII restriction fragment pattern of this same 39.2 kb gonococcal conjugative plasmid demonstrated an 8.0 kb *Hin*dII restriction fragment not previously observed in gonococcal conjugative plasmids examined in this study or by Tenover *et al*. (1980).

In general, such comparison of restriction fragment patterns between groups has not always been possible for several reasons. Often figures of restriction fragment patterns and/or actual restriction fragment sizes have been omitted from publications. Moreover, use of gonococcal strains with different nucleotide modification specificities have also confused comparisons. For example, Sox *et al*. (1978) and Roberts *et al*. (1979) reported cleavage of a gonococcal conjugative plasmid with *Bam*HI, however *Bam*HI did not cleave pJD38 in this study or pLE2451 in a study by Tenover *et al*. (1980). Such differences can be explained by the observation that methylation of the cytosine residue in the fifth position of the *Bam*HI nucleotide recognition sequence -GGATCC-, protects this sequence from digestion by *Bam*HI (Hattman *et al*., 1978), and methylation of the cytosine and/or adenine residues in the nucleotide sequence -GATC- occurs in some, but not all gonococci (Norlander *et al*., 1981; Young *et al*., 1980).

Finally, several shortcomings are present in the actual approach of comparing restriction fragment electrophoretic mobilities to assess structural similarity of plasmids. For example, minor differences in sizes of large restriction fragments may not be detected depending on the resolution of a given gel system. Furthermore, the presence of an extra restriction fragment(s) may be masked if it co-migrates with another restriction fragment of identical size or if it is too small to be resolved on a given gel system. Differences in restriction fragment patterns are most noticeable if a large number of restriction fragments are generated and resolved on a given gel system, and restriction enzymes having widely distributed restriction sites are used. In general, these criteria have not been addressed in previous reports where restriction enzyme patterns of conjugative plasmids from different gonococcal strains were compared.

A more precise approach to examine structural regularities is to determine the actual size and orientation of restriction fragments by restriction endonuclease mapping. Restriction maps of pJD38 for the enzymes BclI, BglII, EcoRl, HindII, HindIII, MluI, PvuI, SacI and XhoI were constructed in this study. These restriction maps were compared to a previously reported restriction map of the gonococcal conjugative plasmid pLE2451 by Tenover et al. (1980) (Figure 16).

Tenover et al. (1980) reported the absence of cleavage of pLE2451 by HindIII, XhoI and SacI, whereas each of these enzymes cleaved pJD38 at a single site. There is evidence to suggest that methylation differences exist in gonococcal strain CH992 harboring pJD38 and strain KH45 harboring pLE2451. pLE2451 was cleaved by Sau3A but not by the enzymes

MboI or BamHI, while pJD38 was cleaved by MboI, but not BamHI or DpnI. This suggests that gonococcal strain CH992 does not methylate the adenine residue in the nucleotide sequence -GATC-, whereas strain KH45 does. Both gonococcal strains either lack the nucleotide recognition sequence for the enzyme BamHI or methylate cytosine residues, since BamHI cleavage was not observed. Thus, the methylation specificity with respect to adenine residues, of gonococcal strain CH992, harboring pJD38, is different from that of KH45, harboring pLE2451. Thus, methylation differences in strains KH45 and CH992 may account for the observation that HindIII, SacI and XhoI cleaved pJD38 but not pLE2451.

Restriction maps of pJD38 and pLE2451 for the enzymes BglII, EcoRI and HindII were similar (Figure 16). The number and size of restriction fragments generated by the enzymes BglII, EcoRI and HindII were similar for both pJD38 and pLE2451. However, the relative positions of the 1.3 kb EcoRI restriction fragment and the 0.9 kb BglII restriction fragment were not in agreement. On the other hand, a preliminary restriction map of a 39.2 kb gonococcal conjugative plasmid by another group (J. Knapp, personal communication, 1988, Centre for Disease Control, Atlanta, Ga., U.S.A.), had five BglII restriction fragments, of similiar size to those for pJD38, and oriented identically to that determined for pJD38 in this study. Significant differences in the orientation of the eight HindII restriction fragments in pLE2451 and pJD38 were apparent (Figure 16).

Although the general restriction maps of pJD38 and pLE2451 were very similar, suggesting these plasmids were structurally alike with minor differences, the question remains whether such minor structural differences are responsible for genetic differences observed in gonococcal conjugative plasmids. Such a question cannot be readily answered until the genetics of the gonococcal conjugative plasmid are better understood.

Genetic analysis of the gonococcal conjugative plasmid pJD38 was initiated in this study to identify the tra region(s). Thomas et al. (1980) proposed that conjugative plasmids, particularly broad host range plasmids, in response to host range restriction activities evolve away from the carriage of many restriction enzyme sites, especially in regions essential for conjugation. Although the gonococcal conjugative plasmid may have a narrow host range (Flett et al., 1981; Genco et al., 1984; Sox et al., 1978)), it may still encounter a variety of restriction activities in intraspecific matings since most gonococcal strains likely produce at least one of five gonococcal restriction endonucleases identified (Norlander et al., 1981; Young et al., 1980). These restriction activities could possibly

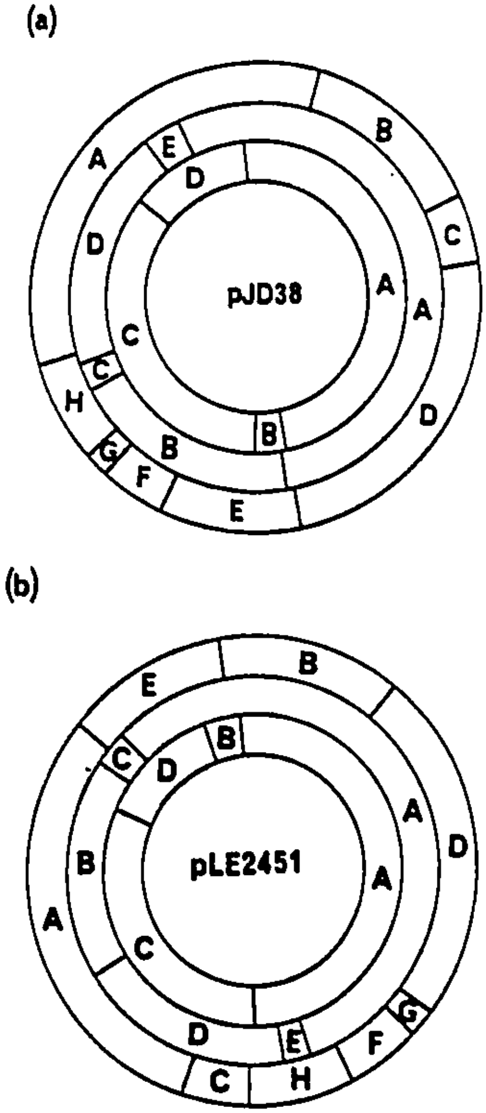


FIG. 16. Comparison of restriction endonuclease maps of pJD38 and pLE2451. a) Restriction map of pJD38. _Eco_RI (inner circle), _Bgl_II (middle circle) and _Hin_dII (outer circle) restriction maps of pJD38 were constructed in this study. b) Restriction map of pLE2451. _Eco_RI (inner circle), _Bgl_II (middle circle) and _Hin_dII (outer circle) restriction maps of pLE2451 were reproduced from that of Tenover _et_ _al_. (1980).

provide the selective force to reduce the number of restriction enzyme sites in the gonococcal _tra_.

The distribution of recognition sites for restriction enzymes mapped in this study was assymetric (Figure 17). An approximately 18.7 kb region of pJD38 corresponding to the largest _Eco_RI restriction fragment, contained only six restriction endonuclease sites for those enzymes examined, in contrast to the eighteen restriction sites mapped in the remaining 20.5 kb of pJD38. _Bcl_I restriction sites were not examined for distribution symmetry since the restriction map was incomplete.

The assymetric distribution of restriction sites in pJD38 suggest that important conjugation genes may be present in the 18.7 kb _Eco_RI restriction fragment according to the argument of Thomas _et al_. (1980). However, the general argument regarding correlation between the location of _tra_ and the distribution of restriction sites falls short when one considers that conjugative plasmids, in general, still have many sites for restriction endonucleases which recognize penta- or tetra-nucleotide sequences and thus, the resultant number of restriction sites may be a balance between the need to evolve away from the carriage of many restriction sites and the restraints on the coding sequence (Ward and Grinsted, 1982).

A second approach to localize the _tra_ region(s) of the gonococcal conjugative plasmid pJD38 was based on hybridization of pJD38 with a conjugative plasmid sharing related _tra_ sequences. The composite plasmid pSa322A, containing the _tra_ region of the IncW plasmid pSa cloned into pBR322, hybridized to several pJD38 restriction fragments. These fragments and the regions where they overlap are indicated on the restriction map of pJD38 in Figure 17. Three putative _tra_ regions of 14.0 kb, 10.0 kb and 3.6 kb were identified in this way. However, the sizes of these regions are maximum estimates since the entire fragment did not have to hybridize to the probe to be detected. The 10.0 kb putative _tra_ region was located in the 18.7 kb _Eco_RI restriction fragment, which had demonstrated a limited number of restriction sites. A single _Hin_dII restriction site was located in this 10.0 kb region. The association of the 14.0 kb region, as identified by hybridization analysis, with _tra_ functions is further supported by the observation of McNicol _et_ _al_. (1986a, 1986b) that a 5.6 kb restriction fragment derived from the hypothesized _tra_ region of a _H_. _ducreyi_ conjugative plasmid (pHD147) hybridized to a restriction fragment of a gonococcal conjugative plasmid corresponding to the putative 14.0 kb _tra_ region of pJD38. Furthermore, Young _et_ _al_. (1980) reported the loss of conjugal activity when a 6.2 kb beta-lactamase-encoding gene was inserted into a 11.0 kb _Hin_dII restriction fragment of a gonococcal conjugative plasmid. The size of this _Hin_dII restriction fragment corresponded to that which mapped approximately in the 14.0

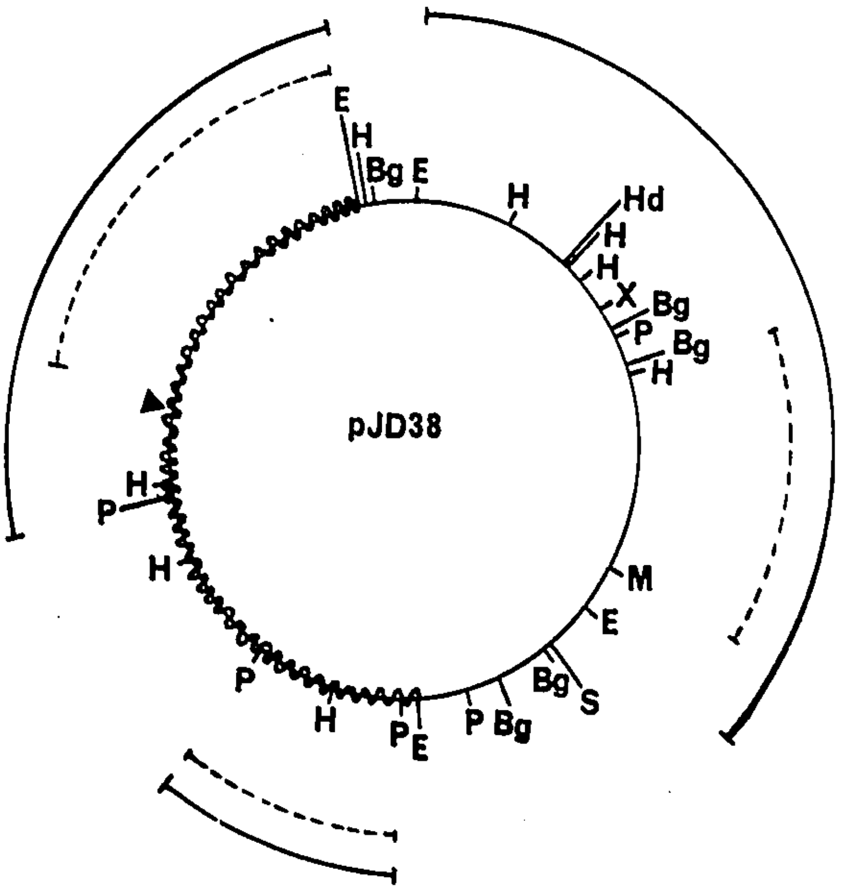


FIG. 17. Distribution of restriction endonuclease sites on pJD38. Restriction maps for the enzymes *Bgl*II (Bg), *Eco*RI (E), *Hin*dII (H), *Hin*dIII (Hd), *Mlu*I (M), *Pvu*I (P), *Sac*I (S) and *Xho*l (X) were constructed in this study (see Results, Section III). ---Indicates the 18.7 kb *Eco*RI fragment of pJD38. Three putative *tra* regions identified by hybridization are indicated by the solid line on the outer circle (see text for details). ---Indicates putative *tra* regions identified by both restriction site distribution and hybridization (see text for details). >Indicates the putative insertion site of *tet*M (see text for details).

kb putative tra region of pJD38 and thus supports the hypothesis that important tra genes are located in this region. However, much of this region, with the exception of a 6.5 kb region contained a large number of restriction sites, suggesting that tra functions may be limited to the smaller 6.5 kb region (Figure 17).

Often tra genes do not occupy the entire plasmid. The tra region of a 39.0 kb IncN plasmid, pCU1 has been localized within a 19.4 kb region (Thatte and Iyer, 1983); the tra genes and origin of transfer of the 100 kb F plasmid are located on a 33.0 kb segment (Johnson and Willets, 1980; Willets and Maule, 1979; 1980; Willets and McIntire, 1978); a continuous tra region of approximately 32.0 kb was proposed for the 107.2 kb IncI plasmid R114 (Hartskeerl et al., 1984); and tra genes are clustered in two or three distinct regions of the IncP plasmid, RP4, which add up to no more than 12 kb (Barth, 1979; Barth et al., 1978).

Morse et al. (1986) reported that the naturally-occurring gonococcal conjugative plasmid, inserted with the tetM transposon was still capable of self-transfer in conjugation. This suggested that the transposon inserted into a non-essential site, which was located approximately 7.8 kb from a BglII restriction site (J. Knapp, personal communication, 1988, Centre for Disease Control, Atlanta, Ga., U.S.A.). Moreover, the insertion site of tetM was located within the putative 10.0 kb tra region in pJD38

(Figure 17). This suggests that tra functions may occupy a region less than the entire 10.0 kb segment. Thus, the restriction endonuclease site distribution and hybridization data suggest that more than one region essential for conjugation is present in pJD38. Additional approaches to further verify and localize the putative tra region of pJD38 were complicated in this study largely by lack of success to either tag the gonococcal conjugative plasmid with an easily detectable phenotypic marker or to clone restriction fragments of pJD38.

III CLASSIFICATION OF THE GONOCOCCAL CONJUGATIVE PLASMID

Classification of the gonococcal conjugative plasmid by conventional incompatibility and genetic complementation approaches have not been attempted due to the instability of this plasmid in most bacterial species (Flett et al., 1981; Genco et al., 1984; Sox et al., 1978) and to the absence, until recently, of an easily detectable phenotypic marker. Moreover, the gonococcal conjugative plasmid does not specify conjugative pili or surface exclusion (Cannon and Sparling, 1984; Sox et al., 1978). Thus, these fertility-associated phenotypes are not useful for classifying the gonococcal conjugative plasmid by the phenotype approach (Willets, 1972).

Where information is available on plasmid properties of various Inc groups, the gonococcal conjugative plasmid is not significantly similar to any previously described (Jacob et al., 1977). For example, the size of the gonococcal conjugative plasmid is consistent with IncW whose members represent the smallest naturally-occurring conjugative plasmids in Gram-negative bacteria (Datta, 1975; Datta and Hedges, 1971; Roberts et al., 1978; Ward and Grinsted, 1982). On the other hand, when examining plasmid properties of size, percent guanine plus cytosine content and copy number, the gonococcal conjugative plasmid is most similar to the IncN plasmids, having 50.0% guanine plus cytosine (compared to 48.0% for gonococcal conjugative plasmids), a low copy number and size ranging from 48.0 kb to 64.0 kb (Jacob et al., 1977; Roberts et al., 1979; Sox et al., 1978). However, the gonococcal conjugative plasmid does not share the broad host range that IncN plasmids are known for (Datta and Hedges, 1972; Jacob et al., 1977; Tardiff and Grant, 1980). Moreover, the absence of both fertility-associated phenotypes of pili and surface exclusion in the gonococcal conjugative plasmid are not consistent with any known Inc group. The IncX plasmid R6K is the only other conjugative plasmid in Gram-negative bacteria that does not specify conjugative pili (Kontimichalou et al., 1970). The absence of surface exclusion specified by the gonococcal conjugative plasmid (Sox et al., 1978) it is not a unique finding, however, most conjugative plasmids encode surface exclusion (Falkow, 1975).

The most direct methods of assessing relationships between plasmids is by comparing nucleotide sequences. The nucleotide sequence similarity observed by Southern blot and DNA hybridization between the gonococcal conjugative plasmid pJD38 and the IncP plasmid RP4 and the IncX plasmid R6K did not provide sufficient evidence to classify pJD38 into either of these Inc groups. Such hybridization likely represented only a very low level of nucleotide sequence similarity as indicated not only by the low hybridization signal intensity following low stringency hybridization, but also by the observation that the hybridization signal was no longer observed when the number of restriction fragments of RP4 and R6K increased prior to hybridization, thereby diluting out the actual sequence(s) that hybridized with pJD38. The low level of nucelotide sequence similarity between pJD38 and R6K and RP4 was however consistent with the observations of others that most conjugative plasmids share some low level of nucleotide sequence similarity (Golub and Low, 1985; 1986; Grindley et al., 1973; Ingram, 1973; Roussel and Chabbert, 1978).

On the other hand, an intermediate level of nucleotide sequence similarity between pJD38 and pSa322A, derived from the IncW conjugative plasmid pSa, was observed. Unfortunately, due to inconsistencies in the physical and genetic maps of pSa322 (see Results, Section IVb) it could not be concluded with certainty that pJD38 hybridized to actual *tra* related sequences of pSa322A. Thus, hybridization of pJD38 under high stringency conditions to pSa322A was not sufficient evidence to classify it into the IncW group.

It is possible that the gonococcal conjugative plasmid may share nucleotide sequence similarity with plasmids originating from other species such as *Streptococcus* or *Bacillus*, although these possibilities were not explored. Indirect evidence suggested that the gonococcal conjugative plasmid may be more related to *Haemophilus* conjugative plasmids since previous workers demonstrated that the 7.2 kb gonococcal beta-lactamase-encoding plasmid originated from the genus *Haemophilus* (Albritton *et al.*, 1982; Brunton *et al.*, 1979; 1982; 1986; McNicol *et al.*, 1984).

The gonococcal conjugative plasmid pJD38 was observed in this study to hybridize to a moderate degree, under high stringency conditions, with a variety of *Haemophilus* conjugative plasmids, including the 48.0 kb extra-chromosomal plasmid, RSF007, and 57.6 kb chromosomal integrated plasmid, R849 both originating from *H. influenzae* and 35.0 kb plasmid, pHD147.Tn2 originating from *H. ducreyi*. A number of workers have demonstrated that extrachromosomal conjugative plasmids larger than 45.0 kb from *H. influenzae* share a large degree of nucleotide sequence similarity (Elwell *et al.*, 1977b; Jahn *et al.*, 1979; Laufs and Kaulfers, 1977; van Klingeren *et al.*, 1977). Although no one has examined nucleotide sequence relationships between extrachromosomal and chromosomally-integrated conjugative plasmids of _H. influenzae_. Albritton and Arnold (1983) have demonstrated that the conjugative plasmid of _H. ducreyi_ does share a considerable degree of nucleotide sequence similarity with extrachromosomal _H. influenzae_ conjugative plasmids as ascertained by similar, but not identical restriction fragment patterns. Similarity of these plasmids explains the observation that pJD38 hybridized to the conjugative plasmids from both _H. influenzae_ and _H. ducreyi_. McNicol _et al_. (1986a, 1986b) also reported hybridization of a gonococcal conjugative plasmid with a 5.6 kb _tra_ fragment of the _H. ducreyi_ conjugative plasmid, pHD147, suggesting that nucleotide sequence similarity between pJD38 and _Haemophilus_ conjugative plasmids, although limited, was due to similar _tra_ sequences.

Thus, the gonococcal conjugative plasmids appear to constitute a single group of structurally similar but not identical plasmids as determined by comparison of restriction fragment patterns and restriction maps. Moreover, minor structural differences may account for significant functional differences of gonococcal conjugative plasmids. Based on hybridization analysis, results suggest that gonococcal conjugative plasmids share some nucleotide sequence similarity but are not closely related to _Haemophilus_ plasmids or conjugative plasmids representative of a number of Inc groups of Enterobacteriaceae (eg. IncHl, Ialpha, F, N, W and X) and Pseudomonaceae (eg. IncP). Thus, gonococcal conjugative plasmids may represent their own unique group of closely related conjugative plasmids.

CONCLUSIONS

Conjugation in the gonococcus appears to be mediated by a group of closely related conjugative plasmids. Moreover, conjugation appears to play an important role in the generation of novel gonococcal strains, especially as a result of the capacity of the conjugative plasmid to mobilize certain non-conjugative gonococcal plasmids, since the host range of the gonococcal conjugative plasmid appears to be limited. However, the role of mutation in the generation of novel strains *in vivo* has not been fully addressed. Initial genetic analysis of the gonococcal conjugative plasmid suggests that more than one distinct region for conjugation may be present. However, further genetic studies are required to confirm the unique nature of the conjugative system of *N. gonorrhoeae*.

APPENDIX

I Mapping pJD38 with PvuI, BglII, HindII and BclI

a) PvuI

PvuI restriction sites were mapped by digestion of end-labelled EcoRI restriction fragments of pJD38 (Table 12, Figure 18a, Lanes A, B and C). The location of PvuI restriction sites relative to the EcoRI restriction sites of pJD38 are shown in Figure 19a. PvuI cleaved the end-labelled 18.7 kb EcoRI restriction fragment to generate 10.0 kb and 0.8 kb restriction fragments (Figure 18a, Lane A). 'Ghost bands' were observed in PvuI digestions of the 18.7 kb EcoRI restriction fragment (Figure 18a, Lane A), so identified as only a maximum of two end-labelled restriction fragments should be observed if PvuI cleaved the end-labelled EcoRI restriction fragment at least once and since these bands corresponded to the restriction fragments observed after digestion of the 14.0 kb and 4.9 kb EcoRI restriction fragments (Figure 18a, Lanes B and C, respectively). These 'ghost bands' likely arose as a result of electroelution of partially EcoRI cleaved end-labelled plasmid which co-migrated with the 18.7 kb end-labelled EcoRI restriction

TABLE 12. Restriction endonuclease digestion of end-labelled *EcoRI* restriction fragments of pJD38.[a]

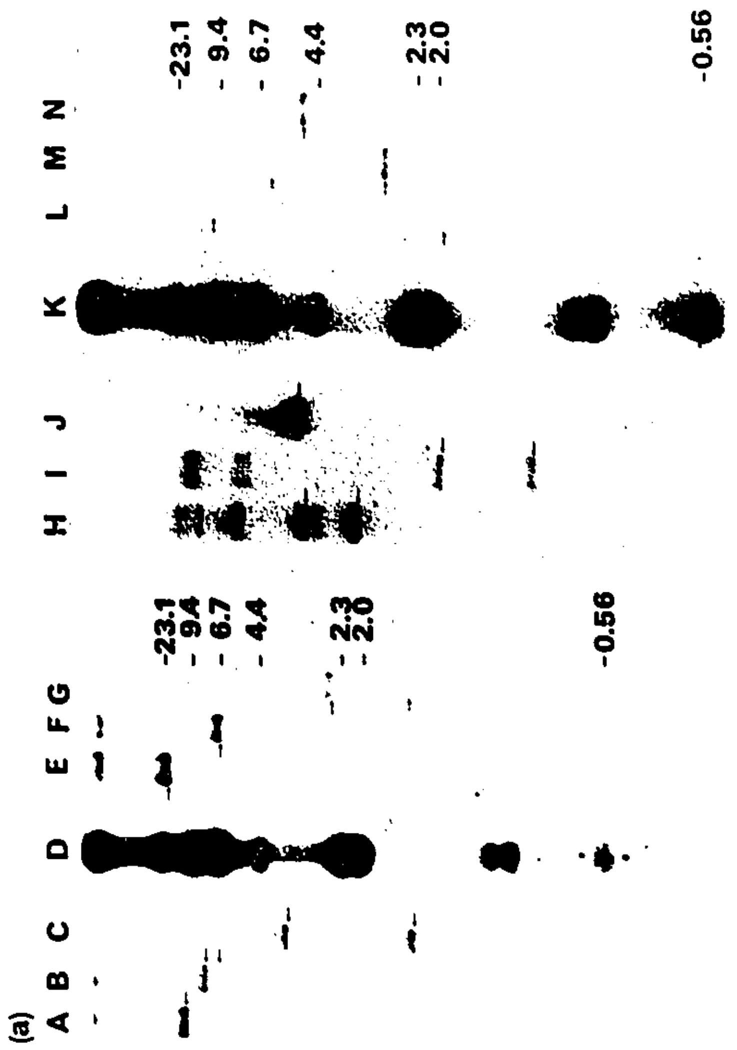
Restriction endonuclease	Restriction fragment size (kb) after digestion of:[b] 18.7	14.0	4.9	1.3
PvuI	10.0	7.2	3.5	1.3
	0.8	6.8	1.3	
BglII	18.7	6.9	2.5	1.06
			1.3	0.31
HindII	9.6	6.2	4.9	1.3
	1.8	2.8		
BclI	4.5	1.9	4.9	1.3
	3.4	1.0		

---

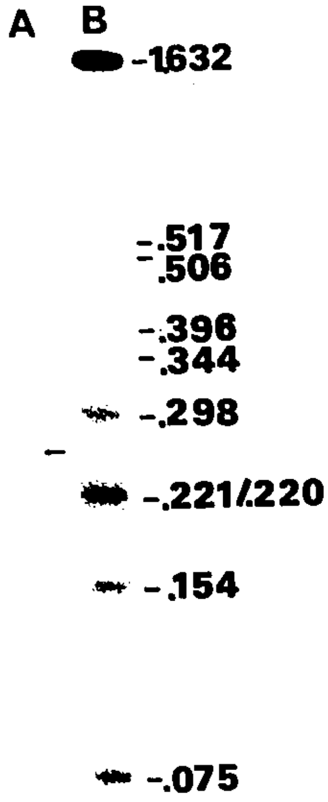
[a] *EcoRI* restriction fragments of pJD38 (18.7 kb, 14.0 kb, 4.9 kb, 1.3 kb) were end-labelled and isolated by electroelution from agarose gels. Purified end-labelled *EcoRI* restriction fragments were cleaved with the enzyme indicated and electrophoresed on agarose and polyacrylamide gels (see Figure 18).

[b] Restriction fragment sizes were the average of the sizes from at least two digestions.

FIG. 18. Restriction endonuclease digestion of end-labelled *Eco*RI restriction fragments of pJD38. (a) Endonuclease digestion of the 18.7 kb, 14.0 kb and 4.9 kb end-labelled *Eco*RI restriction fragments. 1.0% agarose gel electrophoresis of restriction endonuclease digestions of purified end-labelled *Eco*RI restriction fragments visualized by autoradiography. 18.7 kb, 14.0 kb and 4.9 kb end-labelled *Eco*RI restriction fragments of pJD38 cleaved with *Pvu*I (Lanes A, B and C, respectively), *Bgl*II (Lanes E, F and G, respectively), *Bcl*I (Lanes H, I and J, respectively) and *Hin*dII (Lanes L, M and N, respectively). Restriction fragment size markers lambda+*Hin*dIII (23.1, 9.4, 6.7, 4.4, 2.3, 2.0, 0.56 kb) (Lanes D and K). (b) Endonuclease digestion of the 1.3 kb end-labelled *Eco*RI restriction fragment. 5.0% polyacrylamide gel electrophoresis of the 1.3 kb end-labelled *Eco*RI restriction fragment of pJD38 cleaved with *Bgl*II (Lane A) and visualized by autoradiography. Restriction fragment size markers pBR322+*Hin*fI (1.632, 0.517, 0.506, 0.396, 0.344, 0.298, 0.221, 0.220, 0.154, 0.075 kb) (Lane B).


(a)
A B C D E F G
-23.1
- 9.4
- 6.7
- 4.4
- 2.3
- 2.0
-0.56
H I J K L M N
-23.1
- 9.4
- 6.7
- 4.4
- 2.3
- 2.0
-0.56

(b)



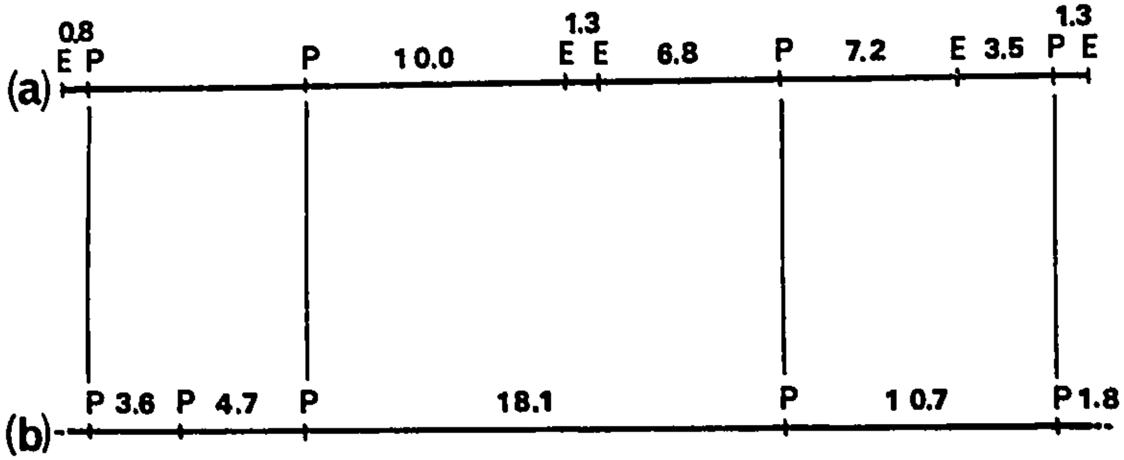


FIG. 19. PvuI restriction endonuclease mapping of pJD38. Restriction fragment sizes are expressed in kilobases. (a) PvuI sites mapped by digestion of end-labelled EcoRI restriction fragments. Distance of PvuI (P) restriction sites from EcoRI (E) restriction sites as determined from PvuI digestion of end-labelled EcoRI restriction fragments of pJD38. (b) PvuI restriction map of pJD38. The location and orientation of the 3.6 kb and 4.7 kb PvuI restriction fragments were determined from double enzyme digestion of pJD38 with PvuI (P) and HindII (H). --- Indicates that the 1.8 kb PvuI restriction fragment comprised the 1.3 kb and 0.8 kb EcoRI/PvuI restriction fragments. PvuI restriction fragment sizes have been adjusted to correspond to the average restriction fragment sizes observed (see Table 11).

fragment during gel electrophoresis. Moreover, the 'ghost bands' were not as predominant as the major digestion products.

PvuI cleaved the end-labelled 14.0 kb EcoRI restriction fragment at a single site to generate 6.8 kb and 7.2 kb restriction fragments (Figure 18a, Lane B) and also cleaved the end-labelled 4.9 kb EcoRI restriction fragment at a single site to generate 3.5 kb and 1.3 kb restriction fragments (Figure 18a, Lane C). PvuI did not cleave the end-labelled 1.3 kb EcoRI restriction fragment as determined by the absence of restriction fragments smaller than 1.3 kb (or larger than 0.075 kb) following electrophoresis on a 5.0% polyacrylamide gel (data not shown).

The location of the PvuI restriction sites relative to the EcoRI restriction sites was verified by the correlation of the observed restriction fragment sizes of pJD38 cleaved with PvuI+EcoRI or PvuI+HindII (Table 13, Figure 20, Lanes B and C, respectively), with the restriction fragment sizes predicted from the proposed PvuI (Figure 19b) and HindII (Figure 23c) restriction maps.

TABLE 13. Comparison of restriction fragment sizes predicted or observed in multiple restriction endonuclease digestions of pJD38.[a]

Restriction endonucleases	Restriction fragment size (kb)	
	Observed[b]	Predicted[c]
*Pvu*I+*Eco*RI	10.0	10.0
	7.7	7.2
	6.6	6.8
	4.6	4.7
	3.5	3.6
	-[d]	3.5
	1.4	1.3
	1.3	1.3
	0.4	0.8
*Pvu*I+*Hin*dII	9.6	9.7
	9.1	9.6
	4.1	4.2
	2.9	3.3
	2.1	1.9
	1.7	1.8
	-	1.8
	1.6	1.5
	1.5	1.4
	-	1.4
	-	1.3
	0.6	0.7
	0.5	0.4
*Bgl*II+*Eco*RI	(19.9)[e]	18.7
	7.1	6.9
	6.2	6.7
	2.4	2.5
	1.3	1.3
	-	1.2
	0.8	1.0
	-	0.9
	0.3	0.3

*Hin*dII+*Eco*RI	9.5	9.4
	5.8	6.2
	-	5.1
	4.8	4.9
	2.8	2.9
	2.6	2.7
	1.8	1.9
	1.6	1.8
	-	1.8
	1.2	1.3
	0.7	0.7

---

[a] pJD38 was cleaved with the enzymes indicated and electrophoresed on agarose and polyacrylamide gels (see Figure 20).

[b] Observed restriction fragment sizes were calculated from the average sizes from at least two digestions.

[c] Predicted restriction fragment sizes were based on the proposed restriction maps of the enzymes indicated (see text for details).

[d] -Indicates a restriction fragment of size corresponding to the predicted size was not detected by gel electrophoresis, either due to co-migration of different restriction fragments of similar size co-migrated or the restriction fragment was too small to be resolved on a given gel system.

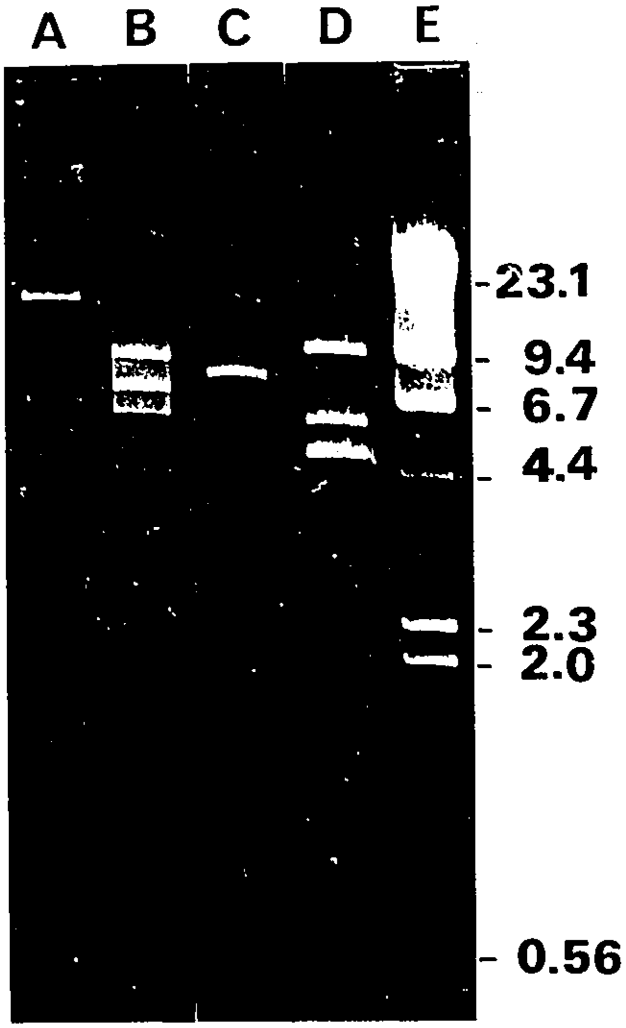


Fig. 20. Double restriction endonuclease digestion of pJD38. 1.0% agarose gel electrophoresis of pJD38 cleaved with EcoRI+BglII (Lane A), EcoRI+PvuI (Lane B), PvuI+HindII (Lane C) and EcoRI+HindII (Lane D). Restriction fragment size markers lambda+HindIII (23.1, 9.4, 6.7, 4.4, 2.3, 2.0, 0.56 kb) (Lane E).

b) BglII

Orientation of most BglII restriction sites was achieved by BglII digestion of end-labelled EcoRI restriction fragments of pJD38 (Table 12, Figure 18a, Lanes E, F and G). The location of BglII restriction sites relative to EcoRI restriction sites on pJD38 is shown in Figure 22a. BglII did not cleave the end-labelled 18.7 kb EcoRI restriction fragment as determined by the absence of restriction fragments smaller than 18.7 kb (or larger than 0.075 kb) following electrophoresis on a 1.0% agarose gel (Figure 18a, Lane E) or a 5.0% polyacrylamide gel (data not shown).

BglII cleaved the end-labelled 14.0 kb EcoRI restriction fragment at least once to generate a 6.9 kb restriction fragment (Figure 18a, Lane F). BglII cleaved the end-labelled 4.9 kb EcoRI restriction fragment to generate 2.5 kb and 1.3 kb restriction fragments (Figure 18a, Lane G). These two restriction fragments accounted for 3.8 kb of the 4.9 kb EcoRI restriction fragment, thus an additional 1.1 kb BglII restriction fragment was present within the 4.9 kb EcoRI restriction fragment. BglII cleaved the end-labelled 1.3 kb EcoRI restriction fragment to yield restriction fragments of approximately 1.06 kb and 0.31 kb (Figure 18b, Lane A).

Partial BglII digestion of the 28.2 kb and 10.7 kb end-labelled HindIII/SacI restriction fragments of pJD38 mapped the remaining BglII restriction sites (Table 14, Figure 21).

TABLE 14. Partial endonuclease digestion of end-labelled *HindIII*/*SacI* restriction fragments of pJD38.[a]

Restriction endonuclease	Restriction fragment size (kb) after digestion of:[b] 28.2	10.7
EcoRI	28.2 5.3 4.3	10.7 9.0
BglII	28.2 5.3	10.7 3.4 2.5
HindII	28.2 5.7 1.4	10.7 3.7 1.4 0.8
BclI	28.2 10.0 3.4	10.7 6.8 2.8

[a] End-labelled 28.2 kb and 10.7 kb *HindIII*/*SacI* restriction fragments of pJD38 were cleaved with the enzyme indicated under partial digestion conditions and electrophoresed on agarose and polyacrylamide gels (see Figure 21).

[b] Restriction fragment sizes were averaged from at least two digestions.

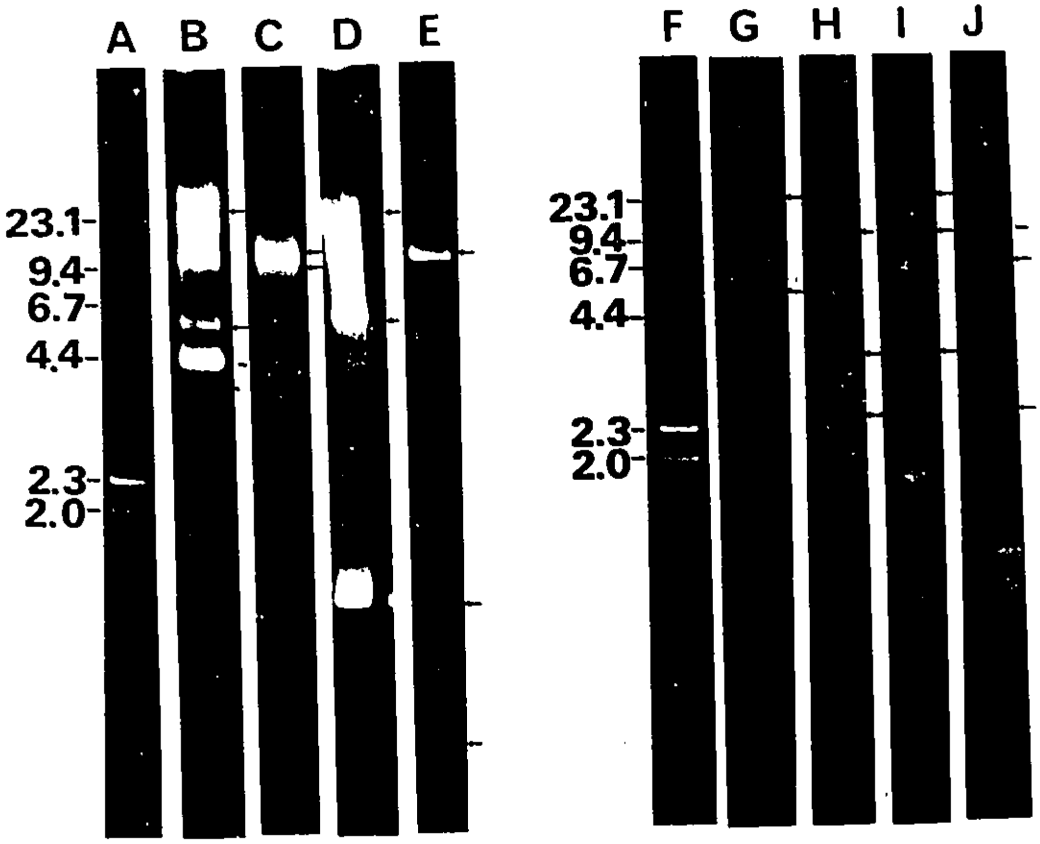


FIG. 21. Partial restriction endonuclease digestion of end-labelled *Hin*dIII/*Sac*I fragments of pJD38. 1.0% agarose gel electrophoresis of the 28.2 kb and 10.7 kb end-labelled *Hin*dIII/*Sac*I restriction fragments of pJD38 cleaved with *Eco*RI (Lanes B and C, respectively), *Hin*dII (Lanes D and E, respectively), *Bgl*II (Lanes G and H, respectively) and *Bcl*I (Lanes I and J, respectively). Restriction fragment size markers lambda+*Hin*dIII (23.1, 9.4, 6.7, 4.4, 2.3, 2.0 kb) (Lanes A and F).

Figure 22b shows the proposed location of the *Bgl*II restriction sites as ascertained from partial digestion mapping. *Bgl*II digestion of the 28.2 kb end-labelled *Hin*dIII/*Sac*I restriction fragment generated a 5.3 kb restriction fragment (Figure 21, Lane G), and represented a complete digestion product as determined by the absence of restriction fragments smaller than 5.3 kb (or larger than 0.075 kb) on a 1.0% agarose gel (Figure 21, Lane G) or 5.0% polyacrylamide gel (data not shown).

However, a 'ghost band' of 2.5 kb was also observed (Figure 21, Lane G). If the 2.5 kb restriction fragment was a legitimate restriction fragment then a *Bgl*II restriction site would be expected 2.5 kb from the *Hin*dIII restriction site, corresponding to 2.0 kb from the *Eco*RI restriction site in the 14.0 kb *Eco*RI restriction fragment (Figure 22a). However, *Bgl*II digestion of the end-labelled 14.0 kb *Eco*RI restriction fragment did not generate a 2.0 kb restriction fragment. Thus, the 2.5 kb restriction fragment observed following partial *Bgl*II digestion of the 28.2 kb end-labelled *Hin*dIII/*Sac*I restriction fragment was a 'ghost band'.

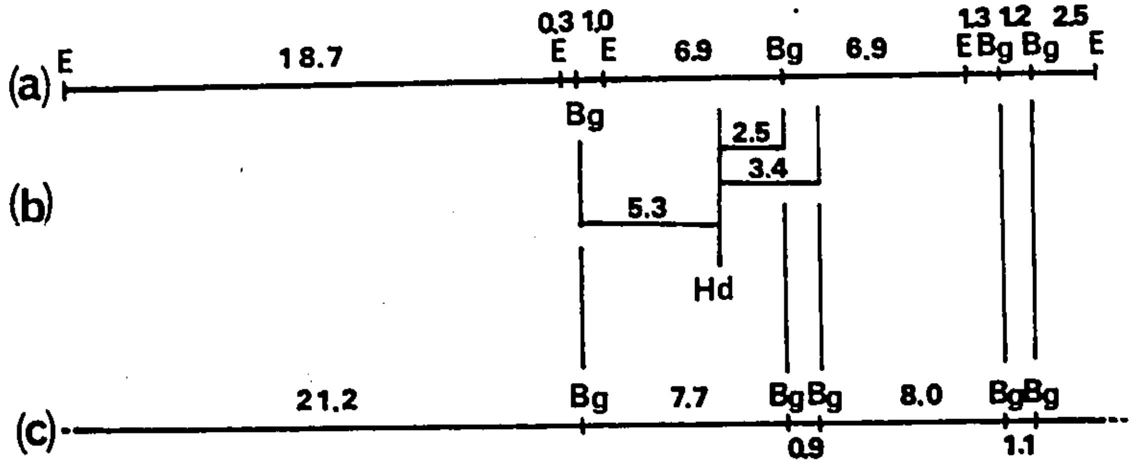


FIG. 22. BglII restriction endonuclease mapping of pJD38. Restriction fragment sizes are expressed in kilobases. (a) BglII sites mapped by digestion of end-labelled EcoRI restriction fragments. Distance of BglII (Bg) restriction sites from EcoRI (E) restriction sites as determined from BglII digestion of end-labelled EcoRI restriction fragments of pJD38. (b) BglII sites mapped by partial digestion mapping. BglII (Bg) restriction sites mapped relative to the HindIII (Hd) restriction site by partial digestion mapping of pJD38. (c) BglII restriction map of pJD38. BglII (Bg) restriction fragment sizes have been adjusted to correspond to the average restriction fragment sizes observed following BglII digestion of pJD38 (see Table 11). ---Indicates the 21.2 kb BglII restriction fragment includes the 2.5 kb EcoRI/BglII restriction fragment.

Thus, a *BglII* restriction site was located 5.3 kb from the *HindIII* terminus which corresponded to and thereby confirmed the presence of the *BglII* restriction site in the 1.3 kb *EcoRI* restriction fragment (Figure 22a, b). Partial *BglII* digestion of the 10.7 kb end-labelled *HindIII*/*SacI* restriction fragment indicated *BglII* restriction sites 2.5 kb and 3.4 kb from the *HindIII* terminus (Figure 21, Lane H). The positions of the *BglII* restriction sites were further confirmed by the correlation of the restriction fragment sizes generated from digestion of pJD38 with *EcoRI*+*BglII* (Figure 20, Lane A), with those predicted by the proposed restriction map of *EcoRI* (see Results, Section IIIc, Figure 8) and *BglII* (Figure 22c). The proposed *BglII* restriction map of pJD38 is shown in Figure 22c.

c) *HindII*

Mapping of *HindII* restriction sites was initiated by complete *HindII* digestion of end-labelled *EcoRI* restriction fragments of pJD38 (Table 12, Figure 18, Lanes L, M and N). Figure 23a shows the preliminary map of the *HindII* restriction sites relative to the *EcoRI* restriction sites on pJD38. *HindII* cleaved the end-labelled 18.7 kb *EcoRI* restriction fragment to generate 9.6 kb and 1.8 kb restriction fragments (Figure 18, Lane L). However, 'ghost bands' were observed. These were so identified since complete

*Hin*dII digestion of the 18.7 kb *Eco*RI restriction fragment should not generate more than two end-labelled restriction fragments. This is supported by the observation that these 'ghost bands' although fainter than the true end products correspond to those observed following *Hin*dII digestion of the 14.0 kb and 4.9 kb end-labelled *Eco*RI restriction fragments (Figure 18, Lanes M and N, respectively).

*Hin*dII digestion of the end-labelled 14.0 kb *Eco*RI restriction fragment generated 6.2 kb and 2.8 kb restriction fragments (Figure 18a, Lane M). No *Hin*dII restriction sites were observed in either the 4.9 kb (Figure 21, Lane D) or 1.3 kb (data not shown) end-labelled *Eco*RI restriction fragments as determined by the absence of restriction fragments smaller than either 4.9 kb or 1.3 kb (or larger than 0.075 kb) on a 1.0% agarose gel or 5.0% polyacrylamide gel.

The remaining *Hin*dII restriction sites were mapped by partial *Hin*dII digestion of the 28.2 kb and 10.7 kb end-labelled *Hin*dIII/*Sac*I restriction fragments of pJD38 (Figure 21, Lanes D and E, respectively). The preliminary *Hin*dII restriction map derived from partial digestion mapping is shown in Figure 23b. Partial *Hin*dII digestion of the 28.2 kb end-labelled *Hin*dIII/*Sac*I fragment generated 10.7 kb, 5.7 kb, 1.4 kb and 0.80 kb restriction fragments (Figure 2_, Lane D). The uncleaved end-labelled 28.2 kb fragment was also visible following gel electrophoresis. The 10.7 kb and 0.8 kb restriction fragments represented 'ghost bands' as they were also observed following partial *Hin*dII digestion of the 10.7 kb end-labelled *Hin*dIII/*Sac*I restriction fragment (Figure 21, Lane E). Moreover, if a *Hin*dII restriction site was located 0.8 kb from the *Hin*dIII terminus then *Hin*dII digestion of the end-labelled 14.0 kb *Eco*RI restriction fragment should have generated a 2.0 kb restriction fragment (Figure 23a), but this was not observed. Similarly, if a *Hin*dII restriction site was located 10.7 kb from the *Hin*dIII terminus, then *Hin*dII digestion of the end-labelled 18.7 kb *Eco*RI restriction fragment should have yielded a 6.6 kb restriction fragment (Figure 23a, b), but this was not observed. Thus, it was concluded that the 10.7 kb and 0.8 kb restriction fragments were 'ghost bands'.

The 1.4 kb restriction fragment represented the complete *Hin*dII digestion product of the 28.2 kb end-labelled *Hin*dIII/*Sac*I fragment. This *Hin*dII restriction site 1.4 kb from the *Hin*dIII site corresponded to the *Hin*dII restriction site observed 2.8 kb from the *Eco*RI restriction site and 4.3 kb from the *Hin*dIII restriction site in the 14.0 kb *Eco*RI restriction fragment (Figure 23a). The *Hin*dII restriction site located 5.7 kb from the *Hin*dIII terminus, on the 28.2 kb end-labelled *Hin*dIII/*Sac*I restriction fragment, mapped within the 1.3 kb *Eco*RI restriction fragment of pJD38 (Figure 23b). However, no restriction fragments larger than 0.075 kb were observed following *Hin*dII digestion of the 1.3 kb end-labelled *Eco*RI restriction fragment (data not shown). This suggested that the *Hin*dII restriction site was within 0.075 kb of the *Eco*RI restriction site on the 1.3 kb segment. Thus, *Hin*dII restriction sites were located 5.7 kb and 1.4 kb from the *Hin*dIII terminus on the 28.2 kb end-labelled *Hin*dIII/*Sac*I restriction fragment.

Partial *Hin*dII digestion of the 10.7 kb end-labelled *Hin*dIII/*Sac*I restriction fragment of pJD38 yielded three restriction fragments of 3.7 kb, 1.4 kb and 0.8 kb as averaged from four digestions (Figure 21, Lane E). The uncleaved 10.7 kb end-labelled *Hin*dIII/*Sac*I fragment was also present. The 3.7 kb and 1.4 kb restriction fragments represented partial digestion products, however the 3.7 kb restriction fragment was not as predominant in Figure 21 (Lane E) as in other partial digestion preparations (data not shown). Thus, *Hin*dII restriction sites were detected 3.7 kb, 1.4 kb and 0.8 kb from the *Hin*dIII terminus of the 10.7 kb end-labelled *Hin*dIII/*Sac*I restriction fragment (Figure 23c).

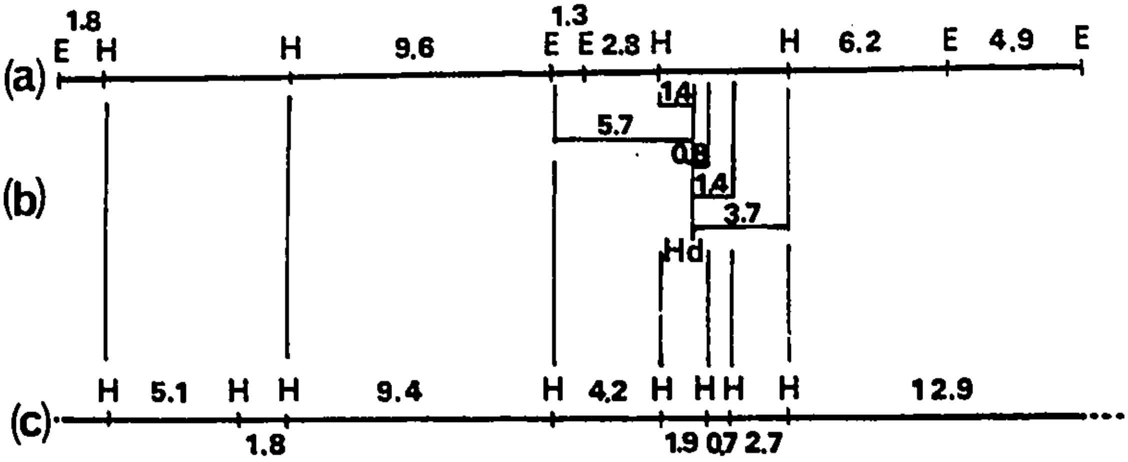


FIG. 23. HindII restriction endonuclease mapping of pJD38. Restriction fragment sizes are expressed in kilobases. (a) HindII sites mapped by digestion of end-labelled EcoRI restriction fragments. Distance of HindII (H) restriction sites from EcoRI (E) restriction sites as determined from HindII digestion of end-labelled EcoRI restriction fragments of pJD38. (b) HindII sites mapped by partial digestion mapping. HindII (H) restriction sites mapped relative to the HindIII (Hd) restriction site by partial digestion mapping of pJD38. (c) HindII restriction map of pJD38. HindII restriction fragment sizes have been adjusted to correspond to the average restriction fragment sizes observed following HindII digestion of pJD38 (see Table 11). ---Indicates the 12.9 kb HindII restriction fragment includes the 1.8 kb EcoRI/HindII restriction fragment.

Partial digestion mapping and *Hin*dII digestion of end-labelled *Eco*RI restriction fragments of pJD38 mapped all the *Hin*dII restriction sites except for those within a 6.2 kb segment of pJD38. The average restriction fragment sizes observed following digestion of pJD38 with *Hin*dII+*Eco*RI (Figure 20, Lane D, Table 13) correlated with the expected sizes predicted by the restriction maps of *Hin*dII (Figure 23c) and *Eco*RI (see Results Section IIIc, Figure 8) except for two observed *Hin*dII restriction fragments of 1.8 kb and 5.1 kb which were not predicted by the incomplete *Hin*dII restriction map. Since the sum of these two restriction fragments (1.8 kb and 5.1 kb) corresponded approximately to the size of the unmapped 6.2 kb region in pJD38, it was concluded that these fragments were adjacent in this region. The orientation of the 1.8 kb and 5.1 kb restriction fragments was ascertained by comparing the observed and expected restriction fragment sizes of pJD38 cleaved with *Pvu*I+*Hin*dII (Table 13, Figure 20, Lane C).

d) *Bcl*I

Mapping of *Bcl*I restriction sites was initiated by complete *Bcl*I digestion of end-labelled *Eco*RI restriction fragments of pJD38 (Figure 18a, Lanes H, I and J). Figure 24a shows the preliminary map of *Bcl*I restriction sites relative to *Eco*RI restriction sites. *Bcl*I digestion of the end-labelled 18.7 kb EcoRI restriction fragment generated 4.5 kb and 3.4 kb restriction fragments (Figure 18a, Lane H). 'Ghost bands' were observed and they were considered to reflect restriction fragments observed following BclI digestion of the 14.0 kb end-labelled EcoRI restriction fragment (Figure 18a, Lane I). Moreover, additional restriction fragments which did not represent 'ghost bands' were also observed (Figure 18a, Lane H). These restriction fragments likely represented partially cleaved end-labelled EcoRI restriction fragments.

BclI cleaved the end-labelled 14.0 kb EcoRI restriction fragment to generate 1.9 kb and 1.0 kb restriction fragments (Figure 18a, Lane I). BclI digestion of the end-labelled 4.9 kb EcoRI restriction fragment did not generate any restriction fragments larger than 0.075 kb as determined by the absence of restriction fragments following electrophoresis on a 5.0% polyacrylamide gel (data not shown) or a 1.0% agarose gel (Figure 18a, Lane J). However, it was concluded that BclI must cleave within 0.075 kb of the EcoRI restriction site in the 4.9 kb EcoRI restriction fragment in order to generate the observed 6.6 kb BclI restriction fragment (Figure 24b). If BclI did not cleave at this position, then a 10.2 kb BclI restriction fragment of pJD38 would be expected and this was not observed. BclI did not appear to cleave the end-labelled 1.3 kb EcoRI restriction fragment as determined by the absence of restriction fragments smaller than 1.3 kb (or larger than 0.075 kb) following electrophoresis on a 5.0% polyacrylamide gel (data not shown).

Partial *Bcl*I digestion mapping of the 28.2 kb and 10.7 kb end-labelled *Hin*dIII/*Sac*I restriction fragments of pJD38 confirmed the position of *Bcl*I restriction sites (Table 14, Figure 21). The location of *Bcl*I restriction sites determined by partial digestion mapping is shown in Figure 24b. *Bcl*I digestion of the 28.2 kb end-labelled *Hin*dIII/*Sac*I restriction fragment located *Bcl*I restriction sites 3.4 kb and 10.0 kb from the *Hin*dIII terminus (Figure 21, Lane I). *Bcl*I digestion of the 10.7 kb end-labelled *Hin*dIII/*Sac*I restriction fragment indicated a *Bcl*I restriction site 2.8 kb and 6.8 kb from the *Hin*dIII terminus (Figure 21, Lane J).

Such mapping approaches left one major area of approximately 12.6 kb unmapped on pJD38. *Bcl*I restriction fragments which were predicted by the incomplete restriction map and which corresponded to observed *Bcl*I restriction fragments included the 6.6 kb, 6.6 kb, 5.8 kb, 4.0 kb and 3.3 kb restriction fragments. This left the 5.3 kb, 2.8 kb, 1.1 kb and 0.86 kb *Bcl*I restriction fragments unaccounted for by the predictions of the incomplete map. Since the sum of these restriction fragments was only 10.06 kb it could not be ruled out that additional *Bcl*I restriction fragments were present in the 12.6 kb region of pJD38. The location and exact orientation of these restriction fragments was not examined further. The tentative *BclI* restriction map of pJD38 is shown in Figure 24c.

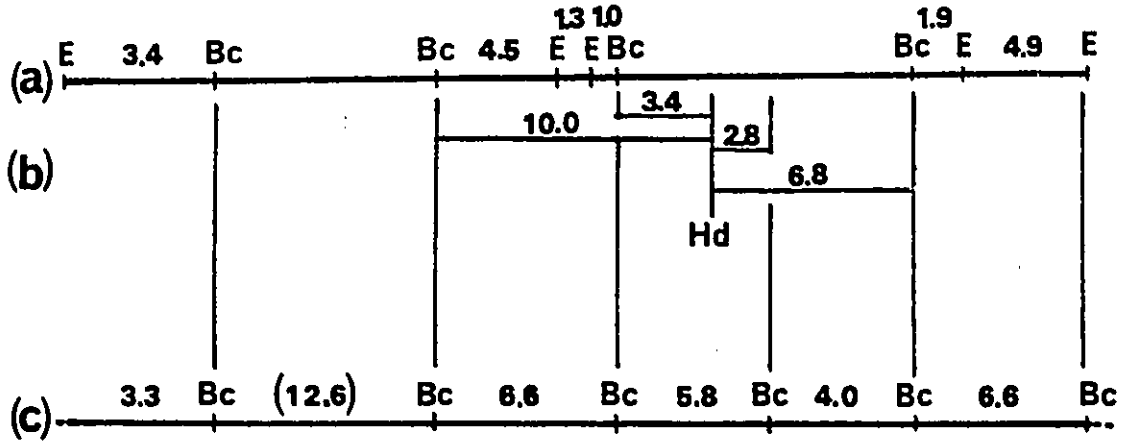


FIG. 24. *BclI* restriction endonuclease mapping of pJD38. Restriction fragment sizes are expressed in kilobases. (a) *BclI* sites mapped by digestion of end-labelled *EcoRI* restriction fragments. Distance of *BclI* (Bc) restriction sites from *EcoRI* (E) restriction sites as determined from *BclI* digestion of end-labelled *EcoRI* restriction fragments of pJD38. (b) *BclI* sites mapped by partial digestion mapping. *BclI* (Bc) restriction sites mapped relative to the *HindIII* (Hd) restriction site by partial digestion mapping of pJD38. (c) Tentative *BclI* restriction map of pJD38. *BclI* (Bc) restriction fragment sizes have been adjusted to correspond to the average restriction fragment sizes observed following *BclI* digestion of pJD38 (see Table 11). The 12.6 kb region indicated in parenthesis represents an unmapped region of pJD38 (see text for details). ---Indicates the 3.3 kb *BclI* restriction fragment contains a portion of the adjacent 4.9 kb *EcoRI* restriction fragment.

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