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GENETIC ANALYSIS AND MOLECULAR CHARACTERIZATION OF THE NATURALLY-OCCURRING PENICILLINASE-PRODUCING PLASMIDS IN *NEISSERIA GONORRHOEAE*

by

Kwok-Him Yeung, M.Sc.

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Department of Biology
University of Ottawa
Ottawa, Ontario

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Canada

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UNIVERSITY OF OTTAWA

ABSTRACT

Two naturally-occurring beta-lactamase producing plasmids from Neisseria gonorrhoeae, 7.2 kilobases (kB) (Asian-type, pJD4) and 5.1 kb (African-type, pJD5) in size, were analysed and mapped by restriction endonuclease analysis and DNA-DNA heteroduplex analysis. The two plasmids were similar except for a 2.1 kb fragment missing in pJD5, as compared to pJD4, which was located 1.74 kb counter-clockwise from the unique PstI site. In addition, a novel penicillinase-producing plasmid, 4.9 kb (pJD7, 'Toronto-type') in size was discovered during the study and was compared to pJD4 and pJD5. Except for a 2.3 kb fragment, pJD7 was shown to be similar to pJD4 and probably derived from pJD4. Plasmid pJD7 was compared to a plasmid of reportedly smaller size, pGO4717, isolated in the Netherlands and was found to be identical to it. Part of the large BamHI fragment of pJD4 was sequenced and compared to that of Tn3. It was shown that after base 79 from the BamHI site, the sequence starts to differ.

The presence of two replication regions on pJD4 has been identified. Through the construction of mini-plasmids (pJD8, pJD9), the replication region of pJD4 was located on a 1.5 kb fragment, designated region 'a', which included the unique HindIII site. However, fragment 'a' was absent in pJD5. A 1.5 kb fragment (region 'b') was found to be required for the replication of this plasmid. In pJD4, the nucleotide

sequence of region'b' was interrupted by region 'a'. Incompatibility studies showed that, in vitro derived deletion-derivatives from pJD4 and pJD5, containing either region 'a' or region 'b', were compatible. Sequence analysis of region 'a' indicated that this region shares many characteristics of plasmids such as pSC101, P1, F, and R6K.

Ten conjugative plasmids were tested for their ability to mobilize plasmid pJD4 between E. coli strains. Plasmid pJD4 was mobilized by RP4 (IncP), R124 (IncIV), pBG791 (IncIα) and R100 (IncFII), but not by R621a (IncIγ), R199 (IncN), R6K (IncX), R1drd16 (IncFII), TP124 ($IncH^e$) and S-a (IncW). The 2.4 kb BamHI fragment of pJD4, containing the beta-lactamase gene and a 1.0 kb BamHI- HinfI fragment designated region 'M', was cloned into pACYC184, and was shown to be required in cis for the mobilization of the recombinant plasmid by pBG791. The entire DNA sequence of region 'M' was analysed, and contained sequences that were found to be essential for the conjugal transfer of other plasmids. Mobilization of deletion derivatives of the 7.2 kb and the 5.1 kb penicillinase-producing plasmids of N. gonorrhoeae by pBG791 indicated another origin of transfer , located within one of the two replication regions in pJD4.

Résumé

Deux plasmides, d'origine gonocoque, synthétisant la béta-lactamase et de poids moléculaire de 7,2 kilobases (kb) (type asiatique, pJD4) et de 5,1 kb (type africain, pJD5) ont été caractérisés. L'étude de la formation d'hétéroduplexe ADN-ADN et les résultats obtenus avec des endonucléases de restriction, ont permis d'élucider la carte génétique des deux plasmides. L'analyse démontre que les deux plasmides sont similaires à l'exception d'un fragment de 2,1 kb absent chez pJD5 comparativement à pJD4. Ce fragment se situe à 1,74 kb (dans le sens contraire des aiguilles d'une montre) de l'unique site de l'endonuclease PstI. Au cour de cette étude un nouveau plasmide de poids moléculaire de 4,9 kb (type torontois, pJD7) et produisant aussi la béta-lactamase, a été découvert et comparé aux deux plasmides précédants. A l'exception d'un fragment de 2,3 kb, pJD7 était similaire à pJD4 et serait dérivé de celui-ci. pJD7 a été aussi comparé et identifié comme étant le même plasmide que pGO4717, un plus petit plasmide isolé dans les Pays-Bas. Une portion de la séquence nucléique du plus gros fragment de pJD4 généré par l'endonucléase de restriction BamHI, a été déterminé et comparé à Tn3. L'analyse a démontré qu'à partir de la base 79 du site de BamHI, la séquence nucléique de pJD4 diffère de celle du transposon Tn3.

La présence de deux origines de réplications a été identifiée sur le plasmide pJD4. Grace à la construction de

mini-plasmides (pJD8, pJD9) la région de réplication de pJD4 a été localisée sur un fragment de 1,5 kb, désigné `région a', qui inclut l'unique site HindIII. Ce fragment (région `a') était absent chez pJD5. Un autre fragment de 1,5 kb (région `b') a été identifié comme étant nécéssaire aussi pour la réplication adéquate de pJD4. La séquence nucléique de la région `b' est interrompue par la région `a'. Des études d'incompatibilité a demontré que des dérivés in vitro de pJD4 et pJD5 contenant, soit la région `a', soit la région `b', était compatibles. L'analyse de la séquence nucléique de la region `a' a indiqué que cette région partage plusieurs caractéristiques similaires au plasmides pSC101, PI, F et R6K.

Dix plasmides de conjugaison ont été éprouvés pour leur habilité à mobiliser le plasmide pJD4 entre souches de Escherichia coli. pJD4 a été mobilisé par RP4 (IncP), R124 (IncFIV), pBG791 (IncIα) et R100 (IncFII), mais non par R621a (IncIγ), R199 (IncN), R6K (IncX), R1drd16 (Inc FII), TP124 ($IncH^e$), et S-a (IncN). Le fragment de 2,4 kb de pJD4 généré par l'endonucléase BamHI contenant le gène de la beta-lactamase et un fragment de 1.0 kb généré par les endonucleases BamHI et HinfI désigné région `M' ont été cloné dans le vecteur pACYC184. Il a été démontré que ce recombinant était nécéssaire, in cis, pour la mobilisation du plasmide pBG791. L'entière séquence d'ADN de la région `M' a

été analysée et certaines séquences ont été trouves indispensable pour le transfert conjugal efficace d'autres plasmides. La mobilisation des dérivés de délétion des plasmides de 7,2 kb et de 5.1 kb produisant la béta-lactamase chez Neisseria gonorrhoeae, par pBG791, indique la présence d'un autre origine de transfert localisé à l'intérieur d'une des deux origines de réplication de pJD4.

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ATP	adenosine triphosphate
Amp	ampicillin
bp	base pair(s)
C	Celsius
Cm	chloramphenicol
dATP	deoxyadenosine 5'-triphosphate
dCTP	deoxycytidine 5'-triphosphate
dGTP	deoxyguanosine 5'-triphosphate
DNA	deoxyribonucleic acid
dNTP	deoxyribonuclotide triphosphate
ds	double-stranded
DTT	dithiothreitol
dTTP	deoxythimindine 5'-triphosphate
EDTA	ethylenediamine tetracetate
g	grams
Gm	gentamycin
hr	hour
Inc	incompatibility
kb	kilobase pairs
Kdal	kilodalton
Km	kanamycin
λ	bacteriophage lambda
ug	microgram
uL	microlitre
mL	millilitre
min	minute
Nal	nalidixic acid
nm	nanometer
oriT	origin of conjugative transfer
oriV	origin of replication
Pil	pilus
PEG	polyethylene glycol
PPNG	penicillinase-producing Neisseria gonorrhoeae
r	resistance
Rif	rifampicin
RNA	ribonucleic acid
s	sensitivity
ss	single-stranded
SDS	sodium dodeycyl sulfate
Str	streptomycin
Sp	spectinomycin
TEMED	N,N,N',N'-tetramethylethylenediamine
Tet	tetracycline
TE	Tris-HCl, EDTA buffer
Tn	transposon
vol	volume
Xgal	5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside

LITERATURE REVIEW

The disease gonorrhoea has been recognized medically since biblical times (Rosebury, 1971) and the bacteriology of the causative agent, Neisseria gonorrhoeae, has been thoroughly characterized. In recent years, many researchers have begun to apply monoclonal antibody and recombinant DNA methodologies to address questions about the pathogenesis of gonococcal infection and the regulation and function of specific gonococcal components. Despite a very limited genetic map, and few systems for the manipulation of its genome, a great deal has been learned about the genetics of N. gonorrnoeae (Cannon and Sparling, 1984). The following is a summary of what is known about the genetics of this pathogen.

Genetic systems and biology of Neisseria gonorrhoeae

Transformation in the gonococcus - Transformation was the only demonstrated mechanism for the transfer of chromosomal genes between gonococci (Sparling, 1966). There were neither transducing phages (Goldberg et al., 1978) nor conjugal systems for the transfer of chromosomal DNA between N. gonorrhoeae strains (Steinberg and Goldberg, 1980). Therefore, all genetic studies of the gonococcal chromosome must be performed either by mutation or, preferably, by transformation.

The transformation of Neisseria species was first investigated in detail by Catlin and Cunningham (1961) who established that N. meningitidis could be transformed by the DNA from many Neisseria species, such as N. meningitidis, N. perflava, N. flava, N. subflava, N. sicca and N. flavescens. However, it was not until 1966 that the criteria for the transformation of N. gonorrhoeae strains were determined by Sparling. Since then, transformation has proved to be an important tool for genetic studies with gonococci. It has been used extensively in understanding the genetics of antibiotic resistance and the mapping of the corresponding genes (Maier et al.,1975). Two groups, Sparling et al. (1978) and Zubrzycki et al. (1978), identified a cluster of linked antibiotic loci, rif-str-fus-cam-spe(ery)-penB, which comprised about 2% of the chromosome. They also determined that the mechanism of resistance to these antibiotics was due to an alternation of the 30S ribosomal subunit and changes in the molecular weights and quantities of certain outer membrane proteins. With the isolation of temperature-sensitive mutants (Wharton and Zubrzycki, 1976), and the discovery of naturally-occurring auxotrophs (Catlin, 1973), mapping of the N. gonorrhoeae chromosome by searching for double markers using transformation techniques was thought to be possible. However, the search for auxotrophic (nutritional) markers linked to the well-characterized loci of antibiotic resistance has been disappointing (Elwell and

Falkow, 1977). So far, it has been established that the uracil (ura) locus was linked with the rifampin (rif) locus (Sparling et al. 1978).

Bacterial cells were said to be competent when they were able to acquire new genetic information by taking up and incorporating DNA directly from the medium. Competence in Bacillus subtilis and Haemophilus influenzae, was inducible (Tomasz, 1969), while competence in N. gonorrhoeae appeared to be constitutive and did not depend on soluble factors for its induction (Biswas et al.,1977).

The mechanism of DNA uptake in the transformation of gonococci is poorly understood and is still under debate. In B. subtilis, transforming DNA lost its biological activity immdiately after absorption by the competent recipient cell (eclipse), but this activity reappeared after a few minutes of incubation (Venema et al., 1965a; Venema et al., 1965b). The transient loss of donor DNA activity in B. subtilis was due to the conversion of the duplex donor DNA to high-molecular weight but biologically inactive single-stranded DNA (Piechowska and Fox, 1971). Neither the eclipse phenomenon nor free single-stranded DNA was detected in the transformation of H. influenzae and N. gonorrhoeae (Notani and Goodgal, 1966; Biswas and Sparling, 1981). In B. subtilis, the transport mechanism was non-specific - i.e. DNA from any source may be taken up (Soltyk et al., 1975). On the other hand, in H. influenzae and N. gonorrhoeae, DNA uptake

was highly specific for their own DNA (Scocca et al., 1974; Graves et al., 1982). As with H. influenzae, it was suspected that the preferential uptake in gonococci occurred as a result of the specific interaction between membrane receptors and an uptake sequence on the gonococcal DNA. The uptake sequence of Haemophilus DNA has been shown to comprise of eleven base pairs 5'-AAGTGCGGTCA-3' (Danner et al., 1980). Competition studies have shown that the gonococcal uptake sequence was different from that of Haemophilus (Graves et al., 1982). Uptake of gonococcal chromosomal DNA was shown to be more efficient than the uptake of gonococcal plasmid DNA (Graves et al., 1982). This suggested that gonococcal chromosomal DNA contained either a more efficient uptake sequence or more copies of a less efficient sequence. Recently, Goodman and Scocca (1988) claimed to have identified the sequence 5'-GCCGTCTGAA-3' to be the specific uptake sequence in the transformation of N. gonorrhoeae. However, Burnstein et al. (1988) carried out an extensive study on DNA uptake by gonococci, and concluded that although DNA uptake by gonococci was determined by DNA structure, no specific DNA sequence was found to be responsible for specific uptake (Burnstein et al., 1988; Dillon and Gauthier, unpublished data). Therefore, the presence of the specific uptake sequence is still under debate.

Despite their differences, transformation in B. subtilis, H. influenzae and N. gonorrhoeae shared the same phenomenon

of 'marker rescue' (Contente and Dubnau, 1979; Albritton et al., 1981; Biswas et al., 1982). 'Marker rescue' was first demonstrated in the transformation of B. subtilis. Contente and Dubnau (1979) demonstrated that nicked or linearized plamids were much less active than covalently closed circular plasmid in transforming competent B. subtilis. However, the presence of an homologous (totally or partially) plasmid in the recipient markedly increased the efficiency of the transformation of B. subtilis by nicked or linearized plasmid. It was postulated that after the entry of the donor plasmid, recombination took place between the linearized plasmid and the homologous resident plasmid leading to an increase (integration) or decrease (deletion after recombination) in the size of the donor plasmid (Contente and Dubnau, 1979). This postulation was consistent with the findings of Biswas et al. (1982) that isogenic transformations in N. gonorrhoeae by the 42 kb ß-lactamase-producing plasmid (pFA14) always resulted in the deletions of the donor plasmid.

Gonococcal genetics - Using cloning procedures, Lovett and Falkow (1981) reported that they had created a 'gene bank' of N. gonorrhoeae using conventional cloning vectors (i.e. cosmids, plasmids, etc.), and that gonococcal genes were capable of complementating E. coli auxotrophic deficiencies. Indeed, Stein et al. (1984) reported the cloning of certain

genes involved in the biosynthesis of proline using interspecific complementation of E. coli mutants. Koomey et al. (1982) cloned the IgA protease gene from N. gonorrhoeae and found that it was expressed in E. coli.

One of the interesting features of N. gonorrhoeae was that it possessed outer-membrane proteins that undergo phase and antigenic variation (Cannon and Sparling, 1984). This might explain why, as of to-date, there is no effective vaccine against N. gonorrhoeae. Pilus (pil) and opacity (opa or PII) proteins of N. gonorrhoeae were the best examples of variable surface antigens. Both pilus and opacity proteins in N. gonorrhoeae have been shown to promote gonococcal binding to human epithelial cells (Buchanan and Pierce, 1976; Swanson, 1973). The gonococcal pilus is composed of approximately 10,000 identical subunits (pilins) of 18-Kda (Brinton et al., 1978) and undergoes phase variation (from piliated (P^+) to non-piliated (P^-)) at high frequencies (Kellog et al.,1963; Swanson et al.,1971). Many different pilus serotypes have been identified in clinical isolates. Peptide mapping showed that antigenically different pilins shared a common hydrophobic N-terminal sequence and variation occurred in the carboxy-terminal part of the pilin subunits (Rothbard et al.,1984; Hagblom et al.,1985).

The isolation and cloning of a structural pilus gene from N. gonorrhoeae into Escherichia coli was reported by Meyer et al. (1982). Although pilus subunits were produced by

E. coli, they did not assemble into complete structures. Using the cloned pilus gene, which was later identified to be a pilin expression gene (pilE), as a probe, the organization of the pilin gene in N. gonorrhoeae was revealed. The conversion of piliated gonococcal cells to non-piliated gonococcal cells involved the chromosomal rearrangment of the pilus gene. Some of the pil loci were mapped to a chromosomal segment of 50 kb (Meyer, 1987) containing the expression loci pilE1 and pilE2, and the major silent locus pilS1 (Segal et al.,1986). DNA sequencing revealed that unlike the expression locus pilE1 or pilE2, the silent pil loci contained more than one gene copy but did not contain any promoter or the 5' ends of the structural gene that encoded the conserved regions of pilin (Haas and Meyer, 1986). By sequence determination of pilin mRNA transcripts, Hagblom et al. (1985) detected sequence alternations in the expression pilin genes of spontaneous gonococcal phase and antigenic varients. These sequence variations were found to be due to six short gene segments (variable minicassettes) which were flanked by strictly conserved regions (Hagblom et al., 1985; Haas and Meyer, 1986).

Using the same strategy, Stern et al. (1984) reported the isolation and cloning of the opacity gene from N. gonorrhoeae. Their data indicated that the organization of the opacity genes differed from that of the pilus genes. There were at least nine opa genes in N. gonorrhoeae, and

unlike the pilus genes, they were not clustered together in the genome (Stern et al., 1986). However, opa genes of the same cell contained variant sequences which were clustered together and are classified into three major groups: HVa, HVb and HVc (Stern and Meyer, 1987). The most striking finding showed that the 5'-regions of the opa genes were composed of pentameric repeat units (coding repeats, CR), and that the number of CR units was different in most of the opa genes of the same cell (Stern and Meyer, 1987). It was postulated that the number of CR units determined the reading frame and thus the translation of individual opa loci (Meyer, 1987).

In addition to pil and opa genes, genes coding for protein I (Carbonetti and Sparling, 1987; Nicholas et al., 1987), protein III(Gotschlich et al., 1986), H.8 antigen (Black and Cannon, 1985), and the methylase M.NgoPII (Sullivan and Saunders, 1988) were also cloned and studied in E. coli. Because N. gonorrhoeae lacks an efficient cloning vector, the cloning system of E. coli has been used.

Plasmids and antibiotic resistance in N. gonorrhoeae

The search for plasmids in N. gonorrhoeae was stimulated by the desire to have an alternative method for genetic analysis of gonococci. Maness and Sparling (1973), as well as Englekirk and Schoenhard (1973), were the first to demonstrate the presence of extrachromosomal DNA in N. gonorrhoeae. At present, at least six naturally-occurring

plasmids have been described in the gonococcus (Cannon and Sparling, 1984).

Most N. gonorrhoeae strains, except for proline, citrulline and uracil-requiring (PCU^-) auxotrophs and some wild-type strains (Dillon and Pauze, 1981), possessed a small, phenotypically cryptic 4.2 kilobase-pair (kb) plasmid (Mayer et al., 1974; Roberts et al.,1977). The function of this plasmid is not known. There was no evidence that it was involved in the control of piliation, iron utilization, or resistance to serum (Sparling et al., 1980). This plasmid had a mole fraction guanine + cytosine content of 0.50, identical to that of the N. gonorrhoeae chromosome, and had a copy number of 24 to 32 per chromosome equivalent DNA. These data imply that it is an indigenous plasmid (Mayer et al.,1974). Attempts to cure this plasmid from its host have been unsuccessful. Foster and Foster (1976) found the plasmid to be genetically unstable; they observed that it underwent a deletion of about 60 base pairs after continuous subculture of the host strain. This amount of DNA could, upon further cultivation, be regained at or near its original site. Davies and Normark (1980) have constructed a restriction endonuclease map of the 4.2 kb cryptic plasmid, and the entire DNA sequence of one variant pJD1 was known (Korch et al., 1985). Recently, a new 4.4 kb cryptic plasmid was detected and isolated (Roy et al.,1988). This plasmid was found to be the same as the 4.2 kb cryptic plasmid except it

had an insertion of 156 base pairs, which could modify the expression of the cppB gene (Roy et al., 1988).

A large 39.2 kb plasmid that appeared in a small percentage of isolates promotes conjugative transfer of plasmid DNA (Eissenstien et al., 1977; Roberts and Falkow, 1977). Many groups have attempted to demonstrate conjugal transfer of chromosomal genes (Eissenstien et al., 1977; Roberts and Falkow, 1977; Roberts et al., 1978; Steinberg and Goldberg, 1980; Norlander et al., 1979; Sox et al.,1978). Initial results were promising, since recombinants for a variety of chromosomal markers were observed (Roberts et al., 1978). However, at least three groups of investigators have since shown that these initial results were probably due to transformation which occurred despite initial addition of DNase (Steinberg and Goldberg, 1980; Norlander et al., 1979; Sox et al.,1978). A limited restriction endonuclease map of the 39.2 kb plasmid has been constructed by Tenover et al. (1980).

Recently, N. gonorrhoeae strains resistant to high concentrations of tetracycline were isolated and were shown to carry the streptococcal tetM determinant on a 40.3 kb plasmid. This plasmid arose from the insertion of the tetM determinant into the 39.2 kb gonococcal conjugative plasmid (Morse et al., 1986).

In 1976, strains of gonococci resistant to high levels of penicillin were isolated in the United States, England,

and South East Asia (Ashford et al.,1976; Phillips I.,1976; Percival et al., 1976). They have subsequently been described in most countries, including Canada (Dillon et al.,1981). The high level of resistance to penicillin acquired by these isolates was determined by plasmids of two different molecular weights - 7.2 kb and 5.1 kb (Perine et al., 1977; Roberts et al., 1977; Dillon et al., 1981). Both of these plasmids have been shown to produce a TEM-type ß-lactamase in vivo (Eriquez and D'Amato, 1979; Percival et al.,1976) and in vitro (Tenover et al., 1980). Historically, isolates of Asian origin contained the 7.2 kb plasmid, whereas isolates of African origin harboured the 5.1 kb plasmid. Furthermore, the Asian type strains were either prototrophic or proline-requiring, while the African strains were frequently arginine-requiring (Elwell and Falkow, 1977; Dillon et al.,1981). These geographic distinctions have become muted following the spread of these strains around the world. In addition, new ß-lactamase producing plasmids have been detected in clinical isolates around the world (Gouby et al.,1986, Yeung and Dillon, 1985; Yeung et al., 1986, Van Embden et al., 1985).

The gonococcal ß-lactamase-producing plasmids have been shown to contain approximately 40% of the ampicillin-resistance transposon Tn3 (Mayer et al., 1974; Roberts et al.,1977) including one of the two terminal inverted repeats and possibly all or part of the TnpR gene which coded for the

transposition repressor (Fayet et al., 1982).

Using DNA-DNA hybridizations techniques, Roberts et al., (1977) showed that the 7.2 kb and the 5.1 kb plasmids were highly similar in their nucleic acid content and that both were closely related to a penicillin-resistance plasmid (6.4 kb) isolated from H. influenzae. The 7.2 kb and the 5.1 kb gonococcal plasmids have a base content of about 41 mole % G + C which was similar to Haemophilus DNA, but different from the approximately 50 mole % G + C in other gonococcal plasmids and in gonococcal chromosomal DNA (Mayer et al., 1974; Roberts et al., 1977; Sox et al.,1978). These data suggested that the penicillin-resistance plasmids of gonococcae might have originated from Haemophilus species (Sparling et al.,1978). Indeed, the penicillinase-producing plasmids of Haemophilus ducreyi, H. parainfluenzae and H. influenzae have been shown to be highly related to the penicillinase-producing plasmid of N. gonorrhoeae (Brunton et al., 1986). The H. ducreyi plasmids contained the entire penicillin-resistance transposon Tn3 (McNicol et al.,1983), while the gonococcal plasmids contained only the right hand (40%) of Tn3 (Fayet et al.,1982). The 7.2 kb plasmids of H. parainfluenzae and H. influenzae have been shown to be identical to the 7.2 kb plasmid in N. gonorrhoeae. Both H. parainfluenzae and H. ducreyi could conjugally transfer their penicillinase-producing plasmids to gonococci (Sparling et al., 1978; Brunton et al.,1982), and gonococci with the 39.2

kb conjugal plasmid could mobilize the transfer of the Haemophilus penicillin-resistance plasmid. This and other studies have led to the current hypothesis that the penicillinase-producing plasmids may be transferred between Haemophilus and Neisseria in nature (Cannon and Sparling, 1984) and that the penicillinase-producing plasmids of gonococci might have originated from H. parainfluenzae (Brunton et al., 1986). However, it was also possible that the ampicillin transposon Tn3 which was widespread in enteric species, was transposed to a plasmid common to both N. gonorrhoeae. and H. parainfluenzae (Cannon and Sparling, 1984).

Mobilization of the penicillinase-producing plasmids by a 39.2 kb gonococcal conjugative plasmid from N. gonorrhoeae to E. coli and to commensal Neisseria species has also been demonstrated (Flett et al., 1980; Genco et al., 1984) attesting to the wide host range of this plasmid. Furthermore, in 1983, a strain of N. meningitidis, which was isolated from a urogenital sample, was found to contain the 7.2 kb penicillinase-producing plasmid from N. gonorrhoeae (Dillon et al.,1983). This was the first report of plasmid-mediated penicillin resistance in meningococci. A recent study, in which 15% of clinical isolates of Neisseria gonorrhoeae containing both the 7.2 kb plasmid and the 39.2 kb plasmid were able to transfer conjugally the 7.2 kb plasmid to N. meningitidis, has extended these findings

(Ikeda et al., 1986).

We (Yeung and Dillon, unpublished data) and others (Elwell and Falkow; 1977) found that covalently closed circular DNA of any type of ß-lactamase-coding plasmid did not transform N. gonorrhoeae. However, Sox et al.,(1979) succeeded in reintroducing the 7.2 kb plasmid into a N. gonorrhoeae strain cured of the same plasmid at a very low frequency ($\sim 10^{-8}$). They also observed that the introduction of the 7.2 kb plasmid into gonococci by transformation frequently resulted in the formation of deleted plasmids varying in size from 3.7 kb to 5.1 kb. The most common class of transformation-related deletion plasmid was 5.1 kb. This deleted plasmid was identical to the naturally-occurring 5.1 kb plasmid as ascertained by restriction endonuclease mapping (Sox et al.,1979). Hybrid penicillinase-producing plasmids of 11.8 kb and 44.8 kb were also obtained when the 7.2 kb plasmid was transformed into gonococci containing the 39.2 kb conjugative plasmid and the 4.2 kb cryptic plasmid (Stein et al., 1983a; 1983b). These hybrid plasmids gave frequencies of transformation 1000-fold higher than the 7.2 kb plasmid.

Shuttle vectors and restriction-modification systems in N. gonorrhoeae

Two plasmid cloning vectors, pFT180 and pLES2, for N. gonorrhoeae have been described (Stein et al., 1983a; 1983b).

These plasmid cloning vectors could be maintained in both N. gonorrhoeae and E. coli. Plasmid pFT180 was the transformation derived hybrid of the 7.2 kb ß-lactamase producing plasmid and the 4.2 kb cryptic plasmid (Stein et al., 1983a). Despite its high efficiency in transforming N. gonorrhoeae, pFT180 was of limited use due to its size (11.2 kb) and its lack of cloning sites. In addition, this plasmid gave rise to deletion derivatives upon transformation of N. gonorrhoeae (Stein et al.,1983b). This plasmid could mediate site-specific recombination into gonococcal chromosome and other gonococcal plasmids (Hagblom et al.,1986).

The second cloning vector, pLES2 (5.2 kb), was derived from the spontaneous deletion mutant of the first cloning vector (pFT180) (Stein et al.,1983b). It has all the sequences of the cryptic plasmid deleted and insertion of the lacZ gene along with the pUC9 multiple cloning site. This cloning vector, with all the cryptic plasmid sequences deleted, had a very low transformation efficiency. As such, it could only be effectively used to clone DNA fragments from N. gonorrhoeae containing the uptake sequence. Nevertheless, it was used in the cloning and isolation of proline genes from N. gonorrhoeae (Stein et al.,1984).

Another difficulty that must be overcome in studying the genetics of gonococci by cloning was the gonococcal restriction/modification system. In 1979, Clanton et al. reported that N. gonorrhoeae produced restriction enzymes

NgoI and NgoII which were isoschizomers to HaeII and HaeIII. At present, N. gonorrhoeae is known to produce at least five different restriction enzymes and eleven different DNA methylases (Korch et al. 1983; Korch et al., 1985; Norlander et al., 1981; Stein et al., 1988). The gene coding for the methylase, M.NgoPII, in N. gonorrhoeae has recently been sequenced and characterized (Sullivan and Saunders, 1988). It has been postulated that each gonococcal strain contained at least one restriction enzyme (Stein et al., 1988).

Plasmid pFT180, one of the two cloning vectors characterized by Stein et al. (1983a), was unable to transform certain N. gonorrhoeae strains (Stein et al., 1988). Stein et al. (1988) observed that plasmid pFT180 isolated from N. gonorrhoeae strain WR302 was unable to transform N. gonorrhoeae strain PGH3-2 (which produces restriction enzyme NgoSI, an isoschizomer of HaeIII), unless it was first methylated in vitro with methylase M.HaeIII.

On further investigation, Stein et al. (1988) observed that the restriction/modification system of N. gonorrhoeae did not affect acquisition of foreign DNA through conjugation. This observation led to the possibility that gonococcal genes cloned in E. coli could be introduced back into the gonococcus by conjugation provided that the cloning vector could be mobilized, replicated and maintained in the gonococcal recipient.

PLASMID REPLICATION AND INCOMPATIBILITY

Plasmid incompatibility

Plasmid incompatibility is a property which is universally inherited by plasmid and which has generally been used in the study of plasmids relatedness (classification) (Datta, 1975). Two plasmids are considered to be incompatible when they cannot be stably maintained in the same host in the absence of external selection (Novick and Hoppensteadt, 1978). Plasmid incompatibility is caused by the random replication of plasmids sharing common elements involved in plasmid replication control or equipartitioning (Novick and Hoppensteadt, 1978; Novick, 1987). However, incompatibility caused by sharing the same equipartitioning system has been shown to be weak (Nordstrom et al., 1980; Scott, 1984). It has been concluded that incompatibility is mainly caused by the copy number control system and by random selection for replication (Nordstrom et al., 1980).

Incompatibility, seen mainly with co-resident single replicons, was referred to as symmetric when either plasmid (replicon) was lost with equal probability (Novick, 1987). Incompatibility may also be vectoral when one plasmid is lost exclusively or with higher probability than the other. This type of incompatibility usually occurred when the replication of a plasmid was inhibited by cloned plasmid fragments containing elements of the replication control or maintenance

systems (Novick, 1987).

Plasmid replication

Historically, two models have been proposed to relate the rate of plasmid replication with respect to cell growth. Jacob et al. (1963) proposed the 'replicon' model suggesting that replication begais when a plasmid-specific product (i.e. initiator protein) acted at a region of the plasmid, possibly near the origin. This model was also referred to as the positive-control model. Another important feature of this model was that daughter plasmids segregate at cell division by attaching to sites on the cytoplasmic membrane which moved into daughter cells. With this model, incompatibility between plasmids was assumed to arise due to competition for a common membrane attachment site.

The second model, referred to as the 'inhibitor-dilution' (negative-control) model, was proposed by Pritchard et al. (1969). This model suggested that each round of plasmid replication leads to the production of a plasmid-specific repressor (inhibitor) so that further rounds of replication cannot start until this repressor has been diluted by cell growth. In this model, incompatibility was assumed to be due to the interference in replication of one plasmid by the repressor encoded by another plasmid in the same cell. The evidence gathered to date from plasmid replication studies favours the 'inhibitor-dilution'

(negative-control) model (Novick et al., 1987).

It was generally agreed that plasmid replication was controlled at initiation (for reviews, see Pritchard and Grover, 1981; Scott, 1984; Kline, 1985; Novick, 1987; Zverev and Khmel, 1987). Based on current data on the mechanisms of the initiation of plasmid replication, Zverev and Khmel (1987) have recently classified plasmids into three groups.

1) **Plasmids of the ColE1 type (direct-regulation)** - This group included the plasmids ColE1, CloDF13, RSF1010, pBR322, pMB1, and p15A (Scott, 1984). This group of plasmids can replicate in the absence of de novo protein sysnthesis and can be 'amplified' by chloramphenicol. For initiation of replication in vitro, this group of plasmids required host-encoded DNA polymerase I, DNA-dependent RNA polymerase, and RNase H. However, replication of ColE1 in vivo did not require RNase H and can proceed in an alternative manner that requires DNA polymerase III instead of polymerase I (Kogoma, 1984). The first step in the initiation of replication was the synthesis of preprimer RNA (RNA II) by RNA polymerase. The preprimer RNA (RNA II) formed a hybrid with its template DNA near the origin of replication (Itoh and Tomizawa, 1980). This hybrid is cleaved by RNase H, an enzyme spoecific for DNA-RNA hybrids, at the oriV to produce the RNA primer (Itoh and Tomizawa, 1980). Deoxyribonucleotides were added to this primer by the host DNA polymerase I.

The major negative control factor in the regulation of replication initiation in this group of plasmids was a second RNA molecule (RNA I), which was transcribed in the opposite direction from the same region of the DNA that produces the 5'- end region of preprimer RNA (RNA II). Thus, RNA I was complementary to the 5' region of RNA II.

Tomizawa et al. (1981) showed that addition of RNA I to the in vitro replication system of ColE1 DNA inhibits the formation of primer and resulted in the inhibition of the initiation of plasmid replication. It was futher shown that RNA I from ColE1 could also inhibit the initiation of replication of other plasmids incompatible with ColE1 (Tomizawa et al., 1981). It was, therefore, concluded that RNA I was a negative regulator of the copy number of plasmids (ColE1 type) and also determined the incompatibility of these plasmids. The regulation mechanism of replication in this group of plasmids was classified by Novick (1987) as 'inhibitor-target' (direct regulation).

2) **Plasmids of the IncFII type** - In this group of plasmids, the synthesis of the initiator protein (coded by the plasmid) required for the initiation of replication was regulated by the interaction of a small non-translated RNA with the mRNA of the initiator protein. S. aureus plasmid pT181 and also those plasmids of the IncFII group were good representives of this type (Diaz and Ortega, 1984; Khan et al., 1981; Novick

et al., 1984).

Studies with IncFII plasmids R1, R6-5 and R100 showed that they all coded for a RepA1 (RepA) protein (33 kD) (encoded by the rep gene) which was essential for the initiation of plasmid replication (Riise et al.,1982; Rosen et al., 1980; Stougaard et al., 1981a; 1981b). In addition, they also contained two genes, CopA and CopB. The product of CopA was a non-translated RNA (91 nucleotide) termed RNAI which was coded within the DNA region determining the synthesis of RepA protein, except it was transcribed from the opposite strand. Therefore, RNAI and the mRNA (RNAII) for the RepA protein were complementary. As such, RNAI could interrupt the translation of RNAII by simply binding to it. Similar to the ColE1-type plasmids, mutations altering the nucleotide sequence of RNA I resulted in an increase in the number of copies of plasmids, and also caused a decline or loss of the incompatibility with the initial plasmid (Novick, 1987).

The product of CopB gene was a 11 k dalton protein which acted at the promotor of RNAII (Riise et al., 1982) thus preventing the transcription of RNAII. Mutations in the region of the copB gene resulted in an increase in the number of copies of plasmids but did not influence their incompatibility. The basic principles of pT181 replication control are the same as those of IncFII plasmids, except that pT181 coded for a RepC protein, which could be trans-active,

for its own initiation of replication (Kumar et al.,1985).

3) **Plasmids with iteron regulation** - Plasmids of this group were very diverse. They belonged to different incompatibility groups and their sizes varied from 8 kb (RSF1010) to more than 38 kb (R6K) (Scott, 1984; Zverev and Khmel, 1987). However, they all encoded a diffusible plasmid-specific initiator protein which bound to sets of directly repeated oligonucleotides (iterons) in, or near to, their replication origins. The initiator protein has three distinctive features: in addition to being required for replication at low concentration, it inhibited replication initiation directly at high concentration, and was an autorepressor at the level of transcription. The iterons (direct repeats) bearing regions of plasmids belonging to this group have been shown to be responsible for incompatability and plasmid copy-number (Tsutsui et al., 1983). The exact mechanism of iteron-specific incompatibility is still unknown (Novick, 1987). A 'titration' model has been proposed by Tsutsui et al. (1983) suggesting that the concentration of the positively acting initiator protein was lowered by binding to the interons. This resulted in a decline in the suppression of replication initiation by the initiator protein.

The mechanism of action of the initiation protein of this group of plasmids is unknown. Recently, the initiation events at the chromosomal origin of E. coli (oriC) have been

elucidated and a model for initiation at oriC was proposed (Figure L-1; Bramhill and Kornberg, 1988). In this model, the initiator protein (dnaA) bound tightly to the four 9-mer repeats (dnaA boxes) organizing oriC around a protein to form the initial complex. The three adjacent AT-rich 13-mers were then melted by the dnaA protein to form an open complex. Finally, the dnaA protein guided the dnaA-dnaC complex into this melted region to form a prepriming complex which unwound the template for priming and replication.

Comparing the structure of oriC to other prokaryotic replication origins (Figure L-2), Bramhill and Kornberg (1988) discovered that many of them contained AT-rich repeats. In addition, some of them also contain the 9-mer dnaA recognition sequence (Figure L-2). Most of all, from available foot-printing data, there was evidence that an initiator protein bound tightly to the origin of replication via specific AT-rich direct repeated sequences. Based on these similarities, Bramhill and Kornberg (1988) proposed that origins containing AT-rich iterons employed a mechanism of initiation similar to that of oriC in E. coli.

Plasmid mobilization

Bacterial conjugation was recognized as the most efficient mode of gene transfer (Clark and Warren, 1979). In all cases studied so far, the genes for conjugation were carried on self-transmissible plasmids (Willetts and Skurray,

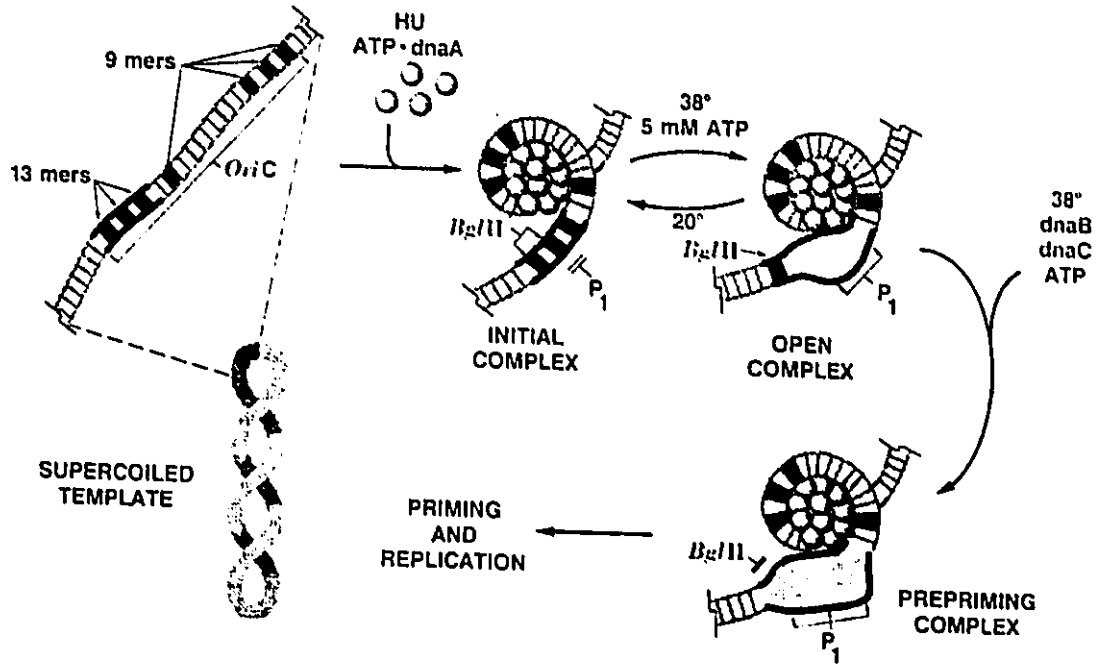


Figure L-1 A model for initiation at oriC. Adopted from Bramhill and Kornberg, 1988.

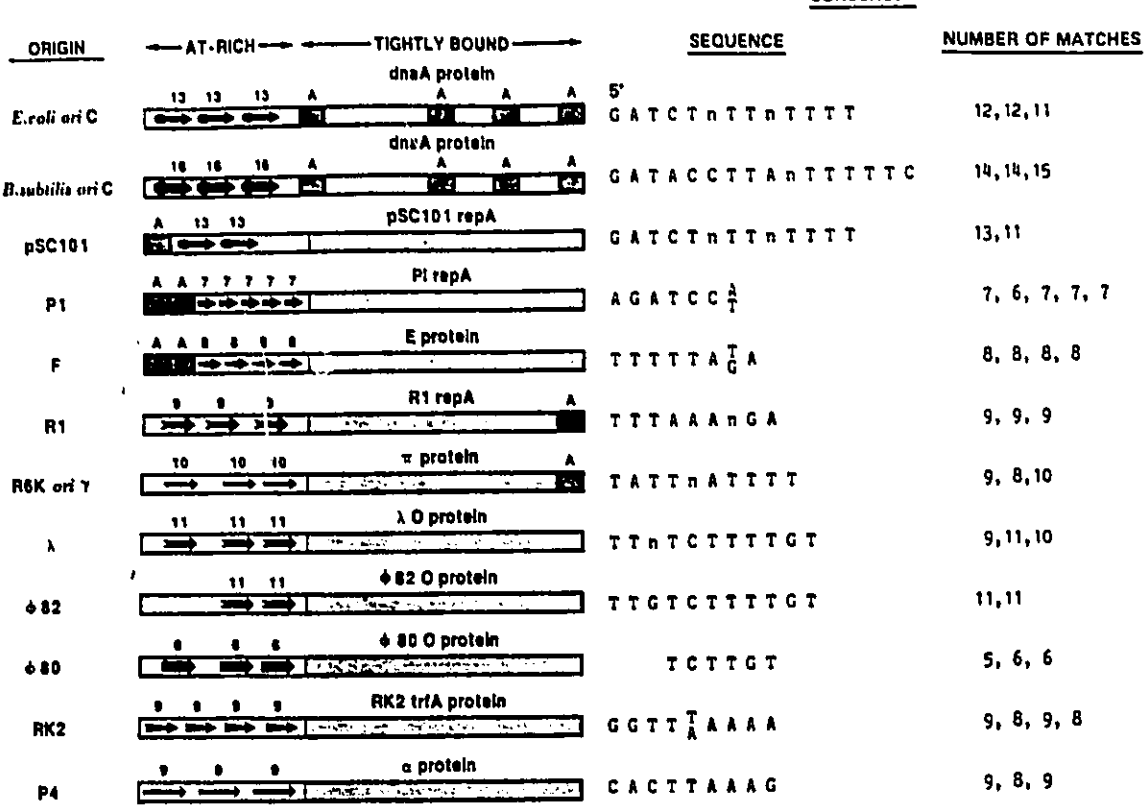


Figure L-2 Comparison of AT-rich repeats in prokaryotic origins. Adopted from Bramhill and Kornberg (1988). The arrows indicate the locations of AT-rich repeats; boxes marked 'A' represent the location of the 9-mer DNA box.

1980). Plasmids that could bring about their own transfer from one cell to another were termed conjugative plasmids, whereas non-conjugative plasmids were those that cannot promote their own transfer. However, non-conjugative plasmids were frequently able to be transmitted into other cells with the help of a conjugative plasmid (Dougan and Sherratt, 1977), and were considered to be mobilizable.

There were two mechanisms by which a non-conjugative plasmid could be transmitted into the recipient cell by a conjugative plasmid (Clark and Warren, 1979). Conduction was defined as the process whereby a conjugative plasmid caused transmission of a non-conjugative plasmid by physical association with it (Clark and Warren, 1979). Donation, in contrast, was defined as the process whereby a non-conjugative plasmid was transferred by a conjugative plasmid without physical association of the two plasmids (Clark and Warren, 1979).

To be mobilized by donation, like conjugative plasmids, the non-conjugative plasmid (such as ColE1, ColDF13, pSC101 and RSF1010) must have a DNA sequence required *in cis* for mobilization. This DNA sequence has been termed *bom* (basis of mobility) (Warren *et al.*, 1979) and was believed to be the origin of transfer (*oriT*). It was at the *oriT* that a plamid DNA-protein relaxation complex, which was associated with mobilization, was formed (Warren *et al.*, 1979). In addition to the origin of transfer, mobilizable plasmids required

plasmid-encoded proteins (*mob*) necessary for their mobilization (Brasch and Meyer, 1987; Derbyshire *et al.*, 1987; Finnegan and Sherratt, 1982; Warren *et al.*, 1979; Willetts and Wilkins, 1984). Some of these have been demonstrated to be components of the relaxation complex. *oriT* sequences of non-conjugative plasmids such as ColE1, ColDF13, pSC101, RSF1010, and RK2 have been shown to be different from each other (Willetts and Wilkins, 1984) and also differed from those conjugative plasmids that were able to mobilize them. Therefore, proteins (encoded by *mob*) that recognize these sites must be different and specific. The ability of some conjugative plasmid to mobilize a non-conjugative plasmid at a high efficiency but others only poorly also suggested that mobilization of non-conjugative plasmids was a specific event.

SCIENTIFIC VALUE AND OBJECTIVES

Although it has been demonstrated that the 7.2 kb and 5.1 kb ß-lactamase-producing gonococcal plasmids were similar, except for a 2.1 kb fragment which was absent in the 5.1 kb plasmid as compared to the 7.2 kb plasmid, no comprehensive restriction maps of the two plasmids had ever been published. In addition, there was controversy concerning the exact location of the 2.1 kb fragment absent in the 5.1 kb plasmid. This controversy definitely hampered the characterization of these plasmids. Thus, an objective of this study was to extend the physical characterization of these plasmids and to resolve the controversies as to the exact location of the 2.1 kb deleted fragment in the 5.1 kb plasmid. A novel 4.9 kb penicillinase-producing plasmid, which was isolated during the course of the study, was also physically characterized and its restriction endonuclease map was compared with the 7.2 and 5.1 kb plasmids. Results from these studies allowed me to expand the current model on the possible evolutionary pathway of the ß-lactamase plasmids from N. gonorrhoeae

The replication and maintenance properties of the 7.2 and 5.1 kb ß-lactamase-producing plasmids which may have been responsible for the establishment of these plasmids in N. gonorrhoeae and other bacterial species were studied. The data presented demonstrated the existence of two replication

regions on the 7.2 kb ß-lactamase plasmid (pJD4). DNA sequence analysis of region 'a', one of the two replication regions suggested that the mechanism of replication initiation was similar to that of oriC in E. coli.

The two penicillinase-producing gonococcal plasmids were non-conjugative, but could be mobilized by the 39.2 kb gonococcal conjugative plasmid to other gonococcal strains and other bacterial species. Data from this thesis suggested that the 7.2 kb plasmid can be mobilized by at least four conjugative plasmids of different incompatibility groups, and that it contained two mobilization regions.

INTRODUCTION

The production of ß-lactamase was mediated by two plasmids in N. gonorrhoeae - 7.2 kb and 5.1 kb in size. Using DNA-DNA hybridization, Roberts et al. (1977) showed that the two penicillinase-producing plasmids in N. gonorrhoeae were closely related to each other. The first physical map of the 7.2 kb plasmid was published in 1979 by Sox et al. mapping restriction sites for four restriction enzymes. It was not until 1983, that the first physical map of the 5.1 kb plasmid was published and compared to that of the 7.2 kb plasmid (Dickgeisser et al., 1982). Supported with heteroduplex analysis, Dickgeisser et al. (1982) showed that the 5.1 kb and the 7.2 kb plasmid were homologous except for a 2.1 kb fragment which was absent in the 5.1 kb plasmid. This deleted fragment was located 1.75 kb clockwise from the unique PstI site of the 7.2 kb plasmid. According to their published maps, this deleted fragment should contain the unique HindIII, AvaI and PvuII sites. In contrast to these observations, McNicol et al.(1983) suggested that the 2.1 kb DNA fragment missing in the 5.1 kb plasmid contained only the unique HindIII site and was located next to the AvaI site of the 7.2 kb plasmid. In order to resolve these major differences as to the location of the deleted fragment, which in turn could confuse further characterization of the two

plasmids, these two plasmids were studied in greater detail.

In April 1984, a novel 4.9 kb (Toronto-type) plasmid, which was involved in an outbreak of penicillinase-producing N. gonorrhoeae that spanned three cities and two provinces in Canada, was isolated and identified in our laboratory. To elucidate the origin of this novel plasmid, it was included in the present study and was physically characterized and compared to the 7.2 kb and 5.1 kb penicillinase-producing plasmids. In the following year, a 4.6 kb (Rio-type) penicillinase-producing plasmid (isolated from two gonococcal strains with different auxotypes) was reported in the Netherlands which appeared to have a similar restriction endonuclease map as the 4.9 kb (Toronto-type) plasmid. To determine whether the 4.9 kb (Toronto-type) and the 4.6kb (Rio-type) were related, they were compared by restriction endonuclease analysis.

MATERIALS AND METHODS

Source of chemicals and reagent kits

Unless specified otherwise, all chemicals were purchased from BDH Chemicals Ltd. (BDH, Toronto, Ontario). Cesium chloride, Tris-base, 5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside (X-gal) and dithiothreitol (DTT) were purchased from Boehringer-Mannheim Canada Ltd. (BMC, Montreal,Quebec). Ethidium bromide, Ficoll, polyvinylpyrolidone and bovine serum albumin were purchased from Sigma (St. Louis, Missouri). 'DNA cloning kit' and 'DNA sequencing kit' were purchased from Bethesda Research Laboratories (BRL, Bethesda, Maryland).

Bacterial strains and plasmids.

Bacterial strains and plasmids used in this study are listed in Table 1-1. The penicillinase-producing N. gonorrhoeae strains GC1-182, GC1-159, and GC1-452, were clinical isolates submitted to the Antimicrobials and Molecular Biology Division, Laboratory Centre for Disease Control, for genetic characterization. E. coli C600 was used as host in all transformation experiments. Plasmid pGO4717 (Rio-type plasmid) was obtained from Dr. J.D.A. van Embden (National Institute of Public Health and Environmental Hygiene, The Netherlands).

Table 1-1 Bacterial strains and plasmids for restriction endonuclease analysis of penicillinase-producing isolates.

Strain	Genotype (plasmid content)	Source
E. coli		
C600	F⁻ *thi*-1 *leu*B6 *thr*-1 *lac*Y1 *ton*A21 *sup*E44	V.N. Iyer
JM103	Δ(*lac pro*) *thi sup*E F'*tra*D36 *end*A *sbc*B15 *hsd*R4 *pro*AB *lac*Z M15	J. Messing collection distributed by BRL
N. gonorrhoeae		
GC1-182	pro[-a] (39.2; 7.2; 4.2)	this lab.
GC1-159	N.R.[b] (39.2; 5.1; 4.2)	this lab.
GC1-452	N.R. (39.2; 4.9; 4.2)	this lab.
Plasmid		
pJD4 (7.2kb)	Amp^R	GC1-182
pJD5 (5.1kb)	Amp^R	GC1-159
pJD7 (4.9kb)	Amp^R	GC1-452
pGO4717	Amp^R	vam Ebden
pMB9	Tet^R	V.N. Iyer
pBR322	Amp^R Tet^R	BMC
M13mp9 (RF)		BRL

[a] pro⁻ = proline-requiring
[b] N.R. = non requiring

Media and growth conditions.

Penicillinase-producing N. gonorrhoeae strains were cultured on GC medium base (GCMB, Difco Laboratories, Detroit, Michigan) supplemented with 1% (final concentration, vol./vol.) defined supplement (glucose 40g; glutamine 1g; 0.5% ferric nitrate solution 10 mL; distilled water 90mL; 20% cocarboxylase 1mL), and 5 mg/L of ampicillin (Ayerst, Montreal, Quebec). Gonococcal strains were incubated at 35^0C, in a CO_2 (5-10%) incubator, in a humid environment, for 18-24 hours (hrs). E. coli transformants were maintained on either MacConkey agar (Difco) or tryptic soy agar (TSA, Difco) supplemented with 100 mg of ampicillin per L. E. coli JM103 (Table 1-1) was maintained on M9 minimal medium (BRL sequencing manual).

Plasmid isolation.

Plasmid DNA was isolated from bacterial cultures using cesium chloride ethidium bromide density gradients as described by Dillon et al. (1985). Bacterial cells harvested from an overnight plate culture (six plates of TSA for E. coli or ten plates of GCMB for N. gonorrhoeae) were suspended in 10 mL of TES buffer (30 mM Tris-Hcl, pH 8.0; 50 mM EDTA; 5 mM NaCl) and pelleted by centrifugation at 10,000 rpm in a Sorvall R5C centrifuge for 10 minutes. The pellet was then resuspended in 3 mL of TES buffer containing 25% sucrose. Ten

mg of lysozyme, followed by 2 mL of 250 mM EDTA, were added to the suspension and kept on ice for 5-10 minutes. Two mL of 2% Triton X-100 were added to the suspension. The mixture was mixed gently. Once the mixture became viscous, it was immediately centrifuged at 16,000 rpm for 20 minutes. The supernatant was transferred to a graduated plastic tube, and 6.5 g of cesium chloride was added. After the cesium chloride was totally dissolved, the volume of the supernatant was topped up to 10mL with TES buffer containing 25% sucrose. 200 uL of ethidium bromide (15 mg/mL) were added and the solution was transferred to a quick-seal Beckman ultracentrifuge tube. Light paraffin oil was used to top up the centrifuge tube before it was sealed. After centrifugation at 55,000 rpm for 20 hours in a Beckman L8-M (type TV65 rotor), the DNA bands were visualized under UV light (Ultraviolet Product Inc., Cal.) (300 nm), and the position of the lower band was marked. The lower band was then collected by puncturing the side of the tube at the mark with a 18 gauge needle. To remove ethidium bromide, 500 uL of water-saturated isobutanol was added (Dillon et al., 1985). After mixing, the mixture was centrifuged at full speed in a microfuge for 3 minutes. The upper organic phase with the extracted ethidium bromide was discarded. This procedure was repeated at least twice to ensure that all ethidium bromide was removed. Plasmid DNA was precipitated twice with 95% ethanol before being suspended in appropriate buffer.

For screening the plasmid content of E. coli transformants, the rapid boiling method of Holmes and Quigley (1981), as modified by Dillon et al. (1983), was used. A loopful of bacterial cells was suspended in 500 uL of STET buffer (50 mM Tris-HCl, pH 8.0; 50 mM EDTA; 8% sucrose; 5% Triton X-100) in a 1.5 mL microfuge tube. After vortex mixing, 100 uL of freshly prepared lysozyme (10 mg/mL in STET buffer) was added. After mixing, the suspension was incubated at room temperature for 5-10 minutes. The mixture was then placed in a boiling water bath for 1 minute, and centrifuged at full speed in an Eppendorff microfuge for 15 minutes. The supernatant was transferred into a new microfuge tube and an equal amount of TES-saturated phenol was added. After mixing, the mixture was separated by centrifugation in a microfuge for 15 minutes. The upper aqueous phase containing the DNA was removed with a plastic pipette into a new microfuge tube. To remove any residual phenol, an equal volume of chloroform was added to the aqueous solution. After mixing and centrifugation, the DNA containing aqueous phase was removed with a plastic pipette into a new microfuge tube. This process was repeated at least twice to ensure total removal of residual phenol. The phenol/chloroform-extracted DNA was then precipitated with 95% ethanol.

Transformation.

Plasmid DNA was transformed into E. coli C600 (JM103 was used as host when the 4.8 kb BamHI fragment was cloned into M13mp9) using a modified procedure of Lederberg and Cohen (1974). An overnight culture of E. coli C600 in YT medium (peptone 8 g/L; yeast extract, 5 g/L; NaCl, 5 g/L) (Dillon et al., 1985) was diluted 20 fold and incubated at 37^0C with constant agitation for 2 hours. The cells were pelletted by centrifugation at 8,000 rpm for 10 minutes. The supernate was discarded and the cell pellet was resuspended in the same volume of cold 0.1M $CaCl_2$ solution and incubated in ice for at least 1 hour. After centrifugation at 8,000 rpm for 10 minutes, the cell pellet was suspended in 1/10 original volume of cold 0.1 M $CaCl_2$. Two hundred uL of the resuspended competent cells were added to 1 ug of DNA (DNA concentration was estimated in a Bausch & Lomb Spectronic 1001 spectrophotometer at 260 nm; 1 A_{260}= 50 ug of DNA/mL) and kept in ice for at least another 40 minutes. The competent cells with DNA were then heat-shocked (42^0C) for 2-5 minutes followed by chilling in ice for 5 minutes. One mL of YT broth (at room temperature) was added and the cells were grown for 1.5 to 2 hours. The transformed cells were then plated out on selective media.

Restriction endonuclease digestion and gel electrophoresis

Restriction endonucleases, AluI, AvaI, AvaII, BamHI, BclI, BglI, BglII, ClaI, EcoRI, HaeII, HaeIII, HincII, HindIII, HinfI, HpaI, HpaII, KpnI, MboI, MluI, PstI, PvuI, PvuII, NciI, TaqI, XbaI and XhoI were obtained from either BRL, Pharmacia (Dorval, Quebec), BMC, or Amersham Canada Ltd. (Toronto, Ontario). Restriction digests were performed using the restriction buffers supplied with the enzymes and the conditions recommended by the suppliers. For multiple digests, if the digestion conditions were the same, the enzymes were added to the DNA simultaneously. However, the total volume of enzymes added was not more than 1/10 volume of the sample. In cases where the enzymes used did not have the same reaction conditions, the enzyme requiring the lower salt buffer was used first. After precipitation with ethanol and resuspension in the second buffer, digestion with the second enzyme was performed.

Restriction enzyme-digested DNA was analysed by electrophoresis either on a 1 to 2% agarose gel or on a 5% polyacrylamide gel (Meyer et al., 1976; Dillon et al.,1985). Bacteriophage lambda DNA (BMC) digested with HindIII or plasmid pBR322 digested with HaeII were used as molecular markers. After electrophoresis, the gels were stained with ethidium bromide (1.0 ug/mL) for 15 minutes and destained with water for another 15 minutes. The destained gel was photographed with a Polaroid MP4 land camera fitted with a

red filter. The types of film used were Polaroid Type 52 or Type 55 (with negative).

Recovery of DNA fragments from agarose gels

DNA fragments were extracted from agarose gels by a modification of the freeze-squeeze method of Tautz and Renz (1983). Appropriate DNA bands were excised from the gel with a razor blade and transferred to a 500 uL microfuge tube with a siliconized glass-wool plug, which was siliconized by soaking in dichlorodimethylsilane followed by rinsing thoroughly with water (Dillon et al.,1985). Two hundred uL of elution buffer (0.3 M sodium acetate; 1 mM EDTA, pH 7.0) was added. The tube was left overnight at -70^{0}C. The tube was then punctured at the bottom with a 18 gauge needle, put in a 1.5 mL microfuge tube and centrifuged at full speed in a microfuge at room temperature for 10 minutes. The filtrate was then extracted with water-saturated isobutanol to eliminate any ethidium bromide as described previously. The DNA in the filtrate was precipitated with the addition of 2 volumes of 95% ethanol.

Ligation

Ligation of DNA fragments was performed as described previously (Dillon et al.,1985; Maniatis et al., 1982). DNA fragments with complementary ends (i.e. ends generated with restriction enzymes recognizing the same or similar sites)

were ligated in 20 uL ligation buffer (20 mM Tris-HCl, pH 7.5; 10 mM $MgCl_2$; 0.6 mM ATP; 10 mM dithiothreitol) with 1 unit of T4 ligase at 16^0C overnight. DNA fragments with non-complementary ends were modified to blunt-ends before ligation. DNA to be blunt-ended was dissolved in 20uL of nick translation buffer containing 2 uL of 0.3 mM dNTPs mix. Two units of the Klenow fragment of DNA polymerase I (BMC) were added. The mixture was incubated at room temperature for 45 minutes before precipitation with 95% ethanol at -70^0C. The blunt-ended DNA fragments were then ligated as described before.

DNA sequencing

DNA was sequenced using the dideoxy method (Sanger et al.,1977). The DNA fragment to be sequenced was first cloned into the M13 cloning vector M13mp9. This was done by ligating the DNA fragment to one of the multiple cloning sites of the M13mp9 RF (replicative form). The recombinant DNA was then transformed into JM103. To select for clones, a mixture of 4 mL YT top agar (YT medium with 0.8% agar) equilibrated to 42^0C; 40 uL of 0.1M IPTG (isopropyl-ß-D-thiogalactopyranoside); 40 uL of 2% X-gal dissolved in dimethylformamide and 0.5 mL of an exponential culture of JM103 was added to the transformed JM103, mixed and poured onto a YT agar plate. The plate was then incubated at 37^0C overnight. Recombinant white plaques were selected.

To prepare single-strand DNA for sequencing, virus obtained from the 'white plaque' was inoculated into 10 mL of YT broth with 0.5 mL of exponential JM103 cells. The culture was grown for 6 hours under vigorous agitation. The bacterial cells were then pelletted by centrifugation at 9,000 rpm for 20 minutes. The supernate was transferred to a clean centrifuge tube. Two mL of 20 % PEG (polyethelene glycol 8000) was added and the tube was left at 4^0C in an upright position for 1 hour. The bacteriophage was then pelletted by centrifugation at 15,000 rpm for 20 minutes. After removal of the supernate, the pellet was resuspended in 0.5 mL of TE buffer (TES buffer without NaCl). The suspension was extracted twice with phenol/chloroform and the single-stranded DNA was precipitated with ethanol at -70^0C.

DNA sequencing was performed as described in the BRL sequencing manual. Single-stranded M13mp9 DNA containing the insert was used as the template. One ug of the single-stranded DNA was annealed with 4ng of the 26 nucleotide universal primer in polymerase buffer (10X buffer: 70 mM Tris-HCl, pH7.5; 70 mM $MgCl_2$; 500 mM NaCl) by boiling the mixture (final volume 12.4uL) for 5 minutes. The mixture (primer/template mix) was then allowed to equilibrate to room temperature slowly for 45 minutes. During this period of time, four microfuge tubes marked A, C, G, T were prepared by adding -

A: 1 ul dideoxy-ATP (0.125 mM), 20 ul deoxy-CTP (0.5 mM),

20 uL deoxy-GTP (0.5 mM), 20 uL deoxy-TTP (0.5 mM).

C: 1 uL dideoxy-CTP (0.25 mM), 1 uL deoxy-CTP (0.5 mM), 20 ul deoxy-GTP (0.5 mM), 20 uL deoxy-TTP (0.5 mM).

G: 1 uL dideoxy-GTP (0.25 mM), 1 uL deoxy-GTP (0.5 mM), 20 uL deoxy CTP (0.5 mM), 20 uL deoxy-TTP (0.5 mM).

T: 1 uL dideoxy-TTP (0.25 mM), 1 uL deoxy-TTP (0.5 mM), 20 uL deoxy-CTP (0.5 mM), 20 uL deoxy-GTP (0.5 mM).

To the primer/template mix, 1 uL of 0.1 M dithiothreitol, 3 uL of [alpha ^{32}P] dATP (>3000 Ci/mmole) and 2 units of the Klenow fragment of polymerase I was added. The mixture was then equally dispensed into the four prepared microfuge tubes marked A, C, G, and T. The microfuge tubes were pulse spinned to make sure the reaction mixtures were at the bottom of the tubes. The reaction mixtures were kept at room temperature for 20 minutes and 1 uL of 0.5 mM deoxy-ATP was added to each tube. The reaction mixtures were again left at room temperature for a further 20 minutes before 10uL of stop buffer, 'blue juice' (0.1% xylene cyanol, 0.1% bromophenol blue, 10 mM EDTA, 95% formamide), was added to each reaction.

The sequencing samples (A, C, G, T) were boiled for 3-5 minutes before loading onto an 8% acrylamide-7 M urea sequencing gel (40 cm X 20 cm X 0.4 mm thick). The sequencing gel was prepared by dissolving 25.2 g of urea in 16 mL of 30% acrylamide (28.6 g acrylamide, 1.4 g Bis-acrylamide), 1.2 mL of 3% freshly prepared 3% ammonium persulphate, and 6 mL

of 10X Tris-borate (1 M Tris-base, 0.8 M boric acid, 10 mM EDTA, pH 8.3). The volume of the mixture was then brought up to 60 mL. The solution was deaerated with a vacuum pump and 22 uL of TEMED (N,N,N',N'-tetramethylethylenediamine) was added. The mixture solution was immediately poured into the gel caster.

Gel electrophoresis was performed at 1600 volts for 4 hours. After electrophoresis, the gel was transferred onto a Whatmann 3MM paper, covered with Saran wrap and autoradiographed. The DNA sequence was determined by recording in which of the four tracks the smallest fragment, which is the first band at the bottom of the gel, appeared. The track in which the next smallest fragment appeared was then recorded and reading was continued up the autoradiograph from track to track. The sequence was recorded in a 5' to 3' direction from the primer.

DNA heteroduplex analysis

DNA heteroduplex analysis was carried out as described by Davis et al (1971). One and one half uL of DNA1, 1.5 uL of DNA2, 1 uL of 1M Tris-HCl (pH 7.0) and 5 uL of ultra-pure formamide were mixed in a microfuge tube and placed in a boiling water bath for 3 minutes (denaturation of DNA). One uL of 2 M $NaClO_4$ was then added to the mixture. After incubation at 40^0C for half an hour (annealing step), 10 uL of ultra-pure formamide and 8 uL of 1 mM Na_2EDTA was added to

the mixture. 0.4 uL of cytochrome C (5 mg/mL) was added to the sample before spreading.

To spread the sample, nitrocellulose-filtered hypophase solution (30% formamide; 0.1 M Tris-HCl, pH 8.5; 10 mM EDTA) was poured into a clean plastic petri dish in such a way that it forms a convex surface. The surface of the hypophase solution was cleaned by passing a throughly cleaned teflon bar across it. A glass ramp was formed by putting one end of a clean glass slide into the hypophase solution and the other end rested on a teflon bar. Three to five uL of the sample was applied onto the ramp about 1 cm from the hypophase surface. The sample flowed down and spread out on the hypophase surface. Part of the spread out sample was picked up on the coated grid by carefully touching the spread area. The spreading procedure was repeated several times.

The DNA adhering to the grid was stained by dipping the grid into 1/100 90% ethanol diluted staining solution (5 X 10^{-3} M uranyl acetate in 0.05 M HCl) for 30 seconds. After air drying on a filter paper, the grid was shadowed at an angle of 6.5 to 7 degrees and a distance of 4.5 cm with a platinum-irridium (80/20) (3.5 cm long) wire in an Edward E-306A shadowing machine.

DNA heteroduplexes were examined with a Zeiss 10 CR transmission electron microscope at 60 kV, and images of interest were recorded on Polaroid Type 52 or 55 (negative) film.

RESULTS

Transformation of E. coli C600 with plasmid DNA from penicillinase-producing N. gonorrhoeae

The gonococcal strain GC1-452 was one of the first strains involved in an outbreak of penicillinase-producing N. gonorrhoeae in Toronto in 1984. This gonococcal strain contained three plasmids, a 4.2 kb cryptic plasmid , a 39.2 kb conjugative plamid and a 4.9 kb plasmid (Figure 1-1). To identify which plasmid was responsible for the production of penicillinase, plasmid DNA extracted from GC1-452 was transformed into ampicillin sensitive E. coli C600. Twenty-four ampicillin resistant transformants were selected and all carried only the 4.9 kb plasmid. None of the transformants analysed carried either the 4.2 kb cryptic plasmid or the 39.2 kb conjugative plasmid. Thus the ampicillin-resistant determinant was located on the novel 4.9 kb plasmid. One of the ampicillin-resistant clones (number three) was chosen and named JD7 and the plasmid was designated pJD7 (Figure 1-1).

Gonococcal strains GC1-182 and GC1-159 were respectively the source of the 7.2 kb (Asian-type) and the 5.1 kb (African-type) penicillinase-producing plasmids. They also contained the 4.2 kb cryptic plasmid and the 39.2 kb conjugative plasmid (Figure 1-1). To facilitate the isolation

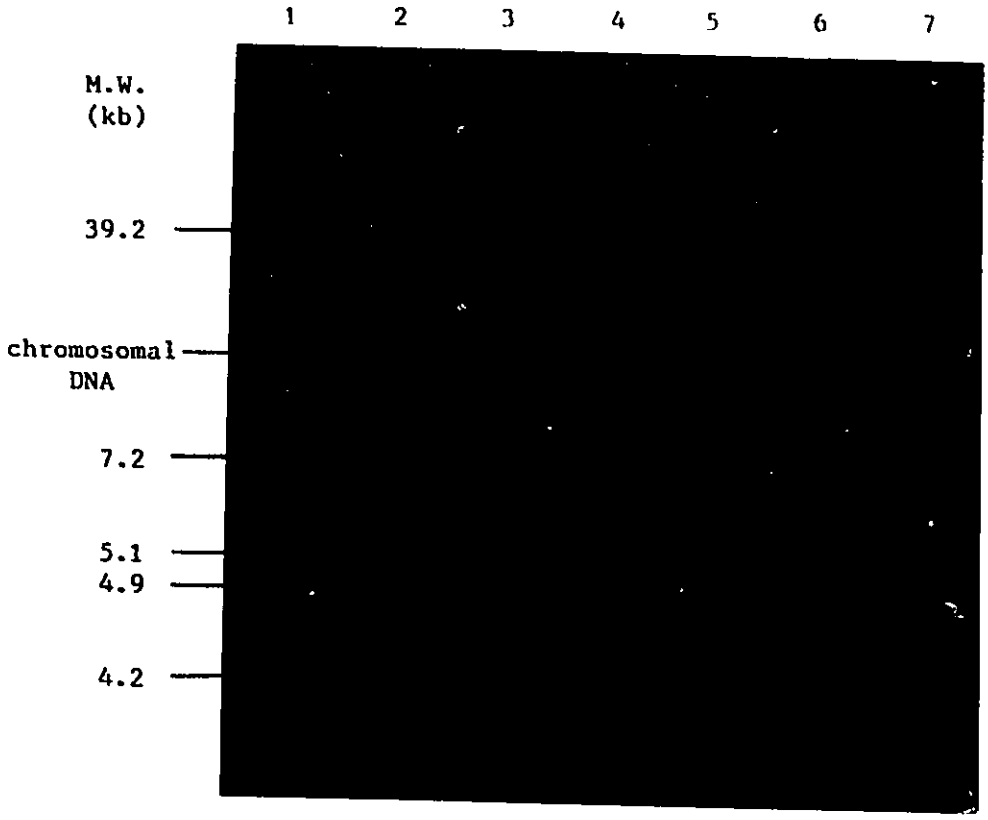


Figure 1-1 Plasmid content of GC1-182, GC1-159, GC1-452, JD4, JD4d, JD5, and JD7. Lane 1 shows that GC1-182 contains three plasmids 39.2 kb, 7.2 kb and 4.2 kb in size; lane 2 shows JD4 contains only the 7.2 kb plasmid; lane 3 shows JD4d contains a plasmid 5.1 kb in size; lane 4 shows GC1-159 contains three plasmids 39.2 kb, 5.1 kb and 4.2 kb; lane 5 shows JD5 contains only the 5.1 kb plasmid; lane 6 shows GC1-452 contains three plasmids 39.2 kb , 4.9 kb and 4.2 kb; lane 7 shows JD7 contains only the 4.9 kb plasmid. Electrophoresis was performed in a 1% agarose gel.

of the 7.2 kb and the 5.1 kb penicillinase-producing plasmids without the contamination by the 39.2 kb conjugative plasmid and the 4.2 kb cryptic plasmid, plasmid DNA extracted from penicillinase producing N. gonorrhoeae (PPNG) strains GC1-182 and GC1-159 was used to transform plasmid-free and ampicillin-sensitive E. coli C600. More than one thousand ampicillin-resistant transformants were obtained when 1 ug of gonococcal plasmid DNA was used in the transformation. Of one hundred and fifty transformants analysed with plasmid DNA isolated from GC1-182, one hundred and forty nine contained the 7.2 kb plasmid (Figure 1-1, lane 2). One transformant contained a smaller 5.1 kb plasmid (Figure 1-1, lane 3). One of the clones (number four) containing only the 7.2 kb plasmid was chosen for further study. This bacterial strain was named JD4 and the plasmid pJD4. The smaller plasmid (5.1 kb) obtained in this transformation was named pJD4d.

Twenty-four transformants obtained from transformation of E. coli C600 with GC1-159 plasmid DNA were analysed and were found to contain only the 5.1 kb plasmid. One of them, (clone number nine) was named JD5 and its plasmid pJD5.

Restriction endonuclease analysis of ß-lactamase-producing plasmids in N. gonorrhoeae

Mapping and comparison of pJD4, pJD5, and pJD7

Restriction endonuclease analysis of pJD4 (7.2 kb), pJD5 (5.1 kb) and pJD7 (4.9 kb) was carried out with a total of twenty-five restriction enzymes. None of the plasmids were digested by the following enzymes: BclI, BglII, ClaI, EcoRI, HaeII, HpaI, KpnI, MboI, MluI and XhoI. The number of restriction sites of the remaining sixteen restriction enzymes is listed in Table 1-2. Except for enzymes that have only one restriction site on the plasmids, restriction patterns and fragment sizes of the three plasmids digested with the enzymes AluI, AvaII, HaeIII and HpaII are shown in Figure 1-2.

The differences between these three plasmids were best demonstrated by analysis with the enzyme BamHI (Figure 1-3 and 1-4). BamHI digestion resulted in the generation of two fragments (Figures 1-3 and 1-4, lanes 2-4) for all three plasmids. The larger BamHI fragments of pJD4, pJD5 and pJD7 were of different sizes (4.8 kb, 2.7 kb, and 2.5 kb respectively). The size of the smaller BamHI fragment was 2.4 kb for all three plasmids. To establish that this fragment was identical in all three plasmids, double digestions were performed using BamHI and either PstI (Figure 1-3, lanes 5-

Table 1-2 Number of restriction endonuclease sites on pJD4, JD5 and pJD7.

Enzymes	Plasmids		
	pJD4	pJD5	pJD7
AluI	>11	>11	>11
AvaI	1	1	0
AvaII	2	2	2
BamHI	2	2	2
BglI	1	1	1
HaeIII	5	5	5
HincII	1	1	1
HindIII	1	0	1
HinfI	4	3	3
HpaII	4	4	4
PstI	1	1	1
PvuI	1	1	1
PvuII	1	1	0
NciI	1	1	1
TaqI	10	7	7
XbaI	1	1	1

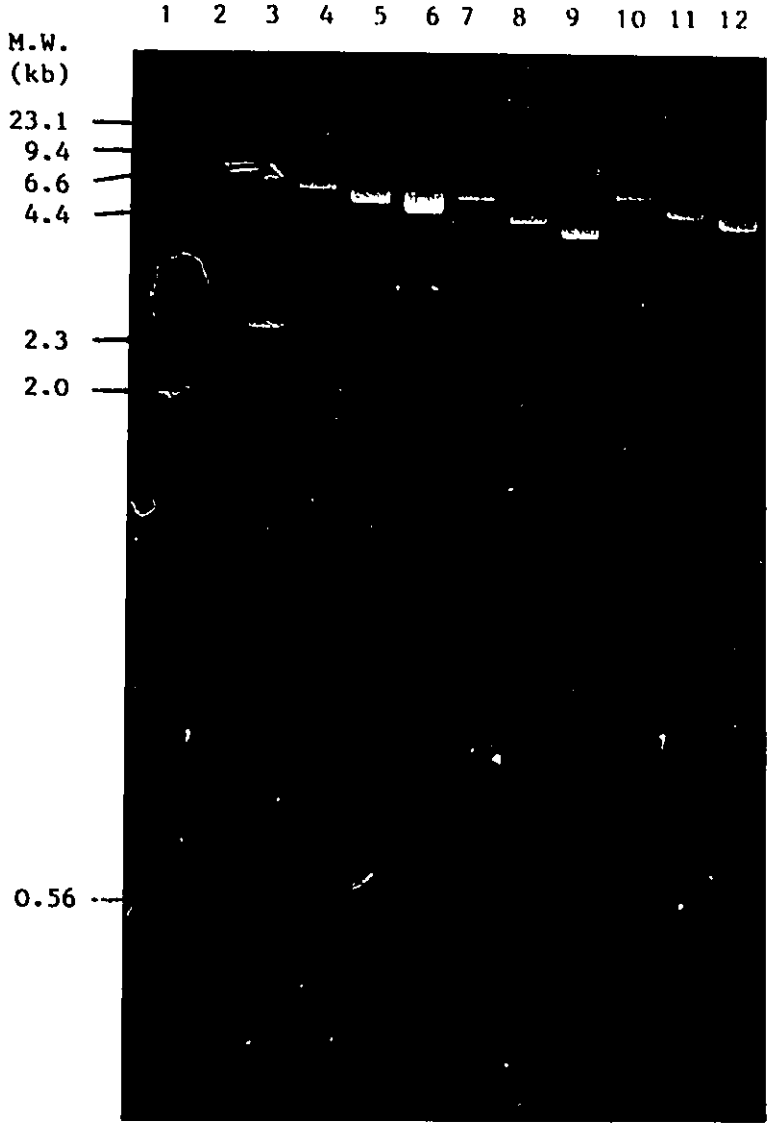


Figure 1-2 Digestion of pJD4, pJD5 and pJD7 with different restriction endonucleases. Digestion patterns of pJD4, pJD5, and pJD7 generated with AluI (lanes 1-3); AvaII (lanes 4-6); HaeIII (lanes 7-9); and HpaII (lanes 10-12).

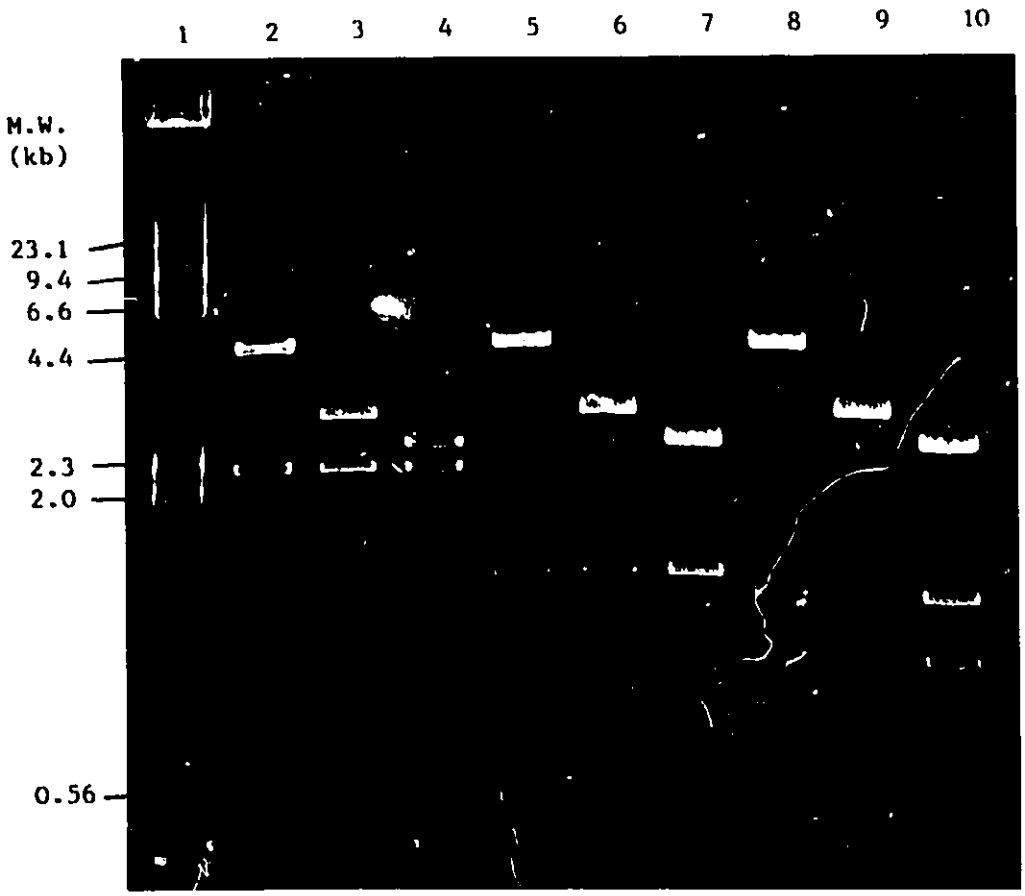


Figure 1-3 Digestion of pJD4, pJD5 and pJD7 with BamHI and either PstI or BglI. Plasmids pJD4, pJD5 and pJD7 digested with BamHI (lanes 2-4); BamHI-PstI (lanes 5-7); BamHI-BglI (lanes 8-10). HindIII digested λ as marker (lane 1). Electrophoresis was performed in a 1% agarose gel.

7), or BglI (Figure 1-3, lanes 8-10). The generation of new fragments of equal size with these digestions indicated that the smaller 2.4 kb fragments were identical (Figure 1-3). Therefore, the differences between plasmids pJD4, pJD5 and pJD7 were located on their larger BamHI fragments. As enzymes HindIII, PvuII, and AvaI each digested the larger BamHI fragment (4.8 kb) of pJD4 once, they were used in double digestions of pJD4, pJD5 and pJD7. The restriction patterns of the three plasmids were compared. Results of double digestions using BamHI and one of HindIII, PvuII and AvaI are shown in Figure 1-4. BamHI-HindIII digestions (lanes 5-7) indicated the presence of a HindIII site on the larger BamHI fragments of pJD4 and pJD7, but not on that of pJD5. PvuII (lanes 8-10) and AvaI (lanes 11-13) sites were found on the larger BamHI fragments of pJD4 and pJD5, but not on that of pJD7. These data show that the larger BamHI fragment (4.8 kb) of pJD4 has a 2.1 kb fragment with a HindIII recognition site which is also present in pJD7 but absent in pJD5. Likewise, this larger BamHI fragment of pJD4 has a 2.3 kb region encoding the recognition sites for PvuII and AvaI which are present in pJD5 but absent in pJD7. Therefore, the larger BamHI fragments of pJD5 and pJD7 are structurally related to pJD4, but not to each other. Detailed restriction maps of pJD4, pJD5 and pJD7 are presented in Figure 1-5.

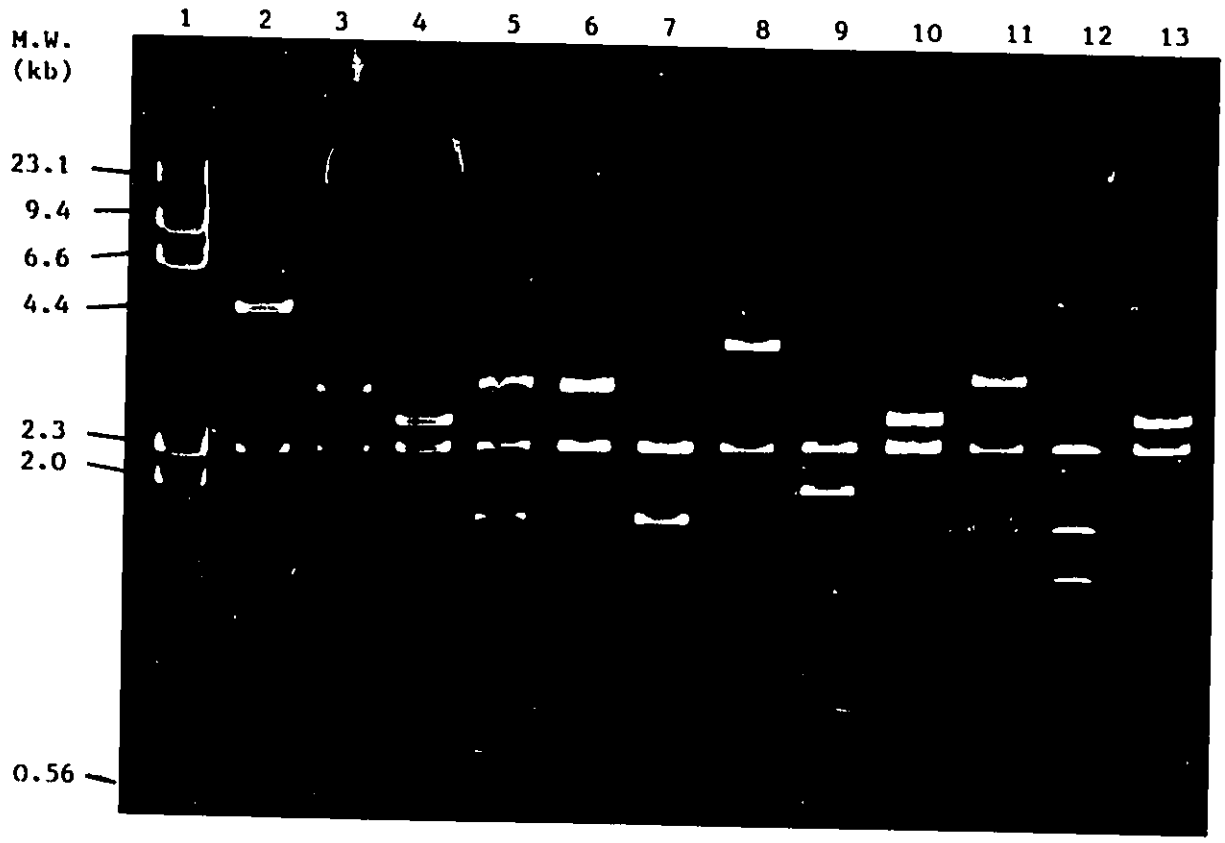


Figure 1-4 Digestion of pJD4, pJD5 and pJD7 with BamHI and either HindIII, AvaI or PvuII. Plasmids pJD4, pJD5 and pJD7 digested with BamHI (lanes 2-4); BamHI-HindIII (lanes 5-7); BamHI-PvuII (lanes 8-10); BamHI-AvaI (lanes 11-13). HindIII digested λ as marker (lane 1). Electrophoresis was performed in a 1% gel.

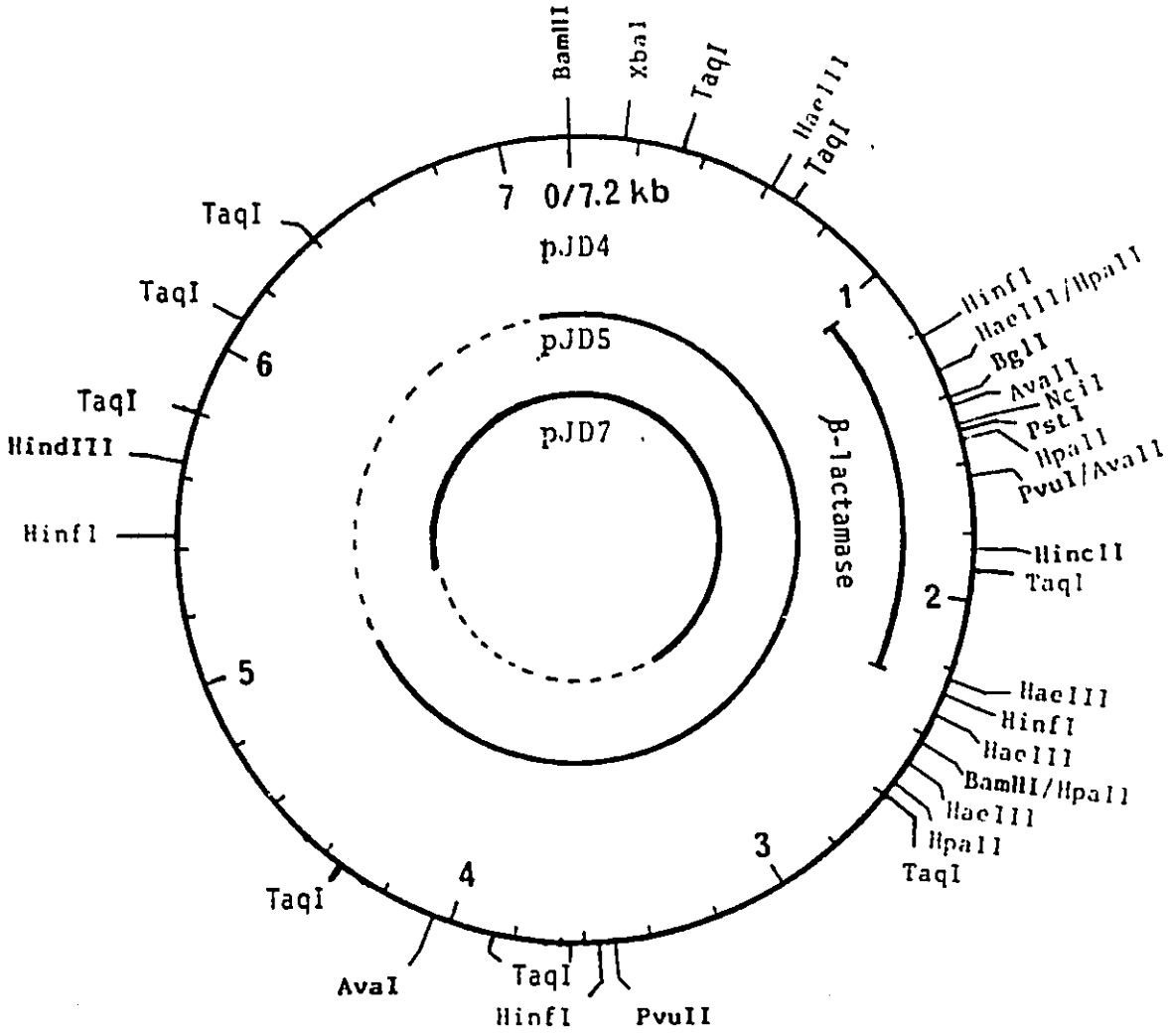


Figure 1-5 Restriction endonuclease maps of pJD4, pJD5 and pJD7. Data used to construct the maps are presented in Appendix A (page 121).

Comparison of plasmid pJD4d and pJD5

Plasmid pJD4d (5.1 kb) was a transformation-derived deletion mutant of pJD4. Figure 1-6 shows that digestions of pJD4d and pJD5 respectively with HinfI (lanes 2-3); BamHI (lanes 4-5); BamHI-HindIII (lanes 6-7); BamHI-PstI (lanes 8-9); BamHI-AvaI (lanes 10-11); and BamHI-PvuII (lanes 12-13) produced the same restriction patterns. Whether pJD4d and pJD5 are completely homologous is not sure. However, it was evident that deletion could occur during transformation of pJD4 and that it gave rise to an altered plasmid similar to pJD5.

Comparison of plasmid pJD7 and the 'Rio-type' (pGO4717) plasmid

The 'Rio-type' plasmid (pGO4717) (4.6 kb), isolated in the Netherlands, was obtained from Dr. van Embden, and was compared to pJD7 (4.9 kb) using restriction endonuclease analysis. HinfI (Figure 1-7, lanes 2-3), BamHI (lanes 4-5); BamHI-HindIII (lanes 6-7), BamHI-PstI (lanes 8-9), BamHI-AvaI (lanes 10-11) and BamHI-PvuII (lanes 12-13) digestions of pGP4717 and pJD7, respectively, showed exactly the same restriction patterns.

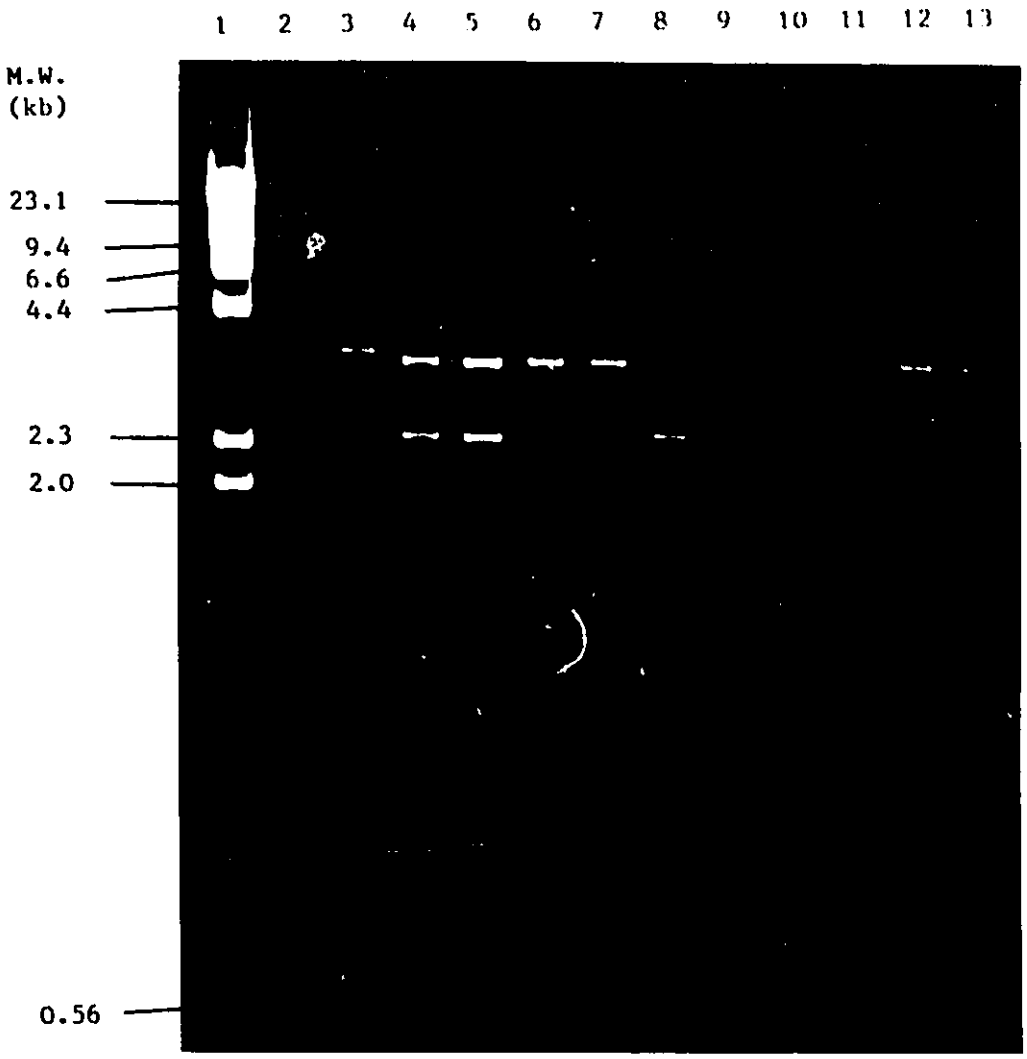


Figure 1-6 Comparison of pJD4d and pJD5 by restriction endonuclease analysis. Plasmids pJD4d and pJD5 digested with HinfI (lanes 2 and 3); BamHI (lanes 4 and 5); BamHI-PstI (lanes 6 and 7); BamHI-AvaI (lanes 8 and 9); BamHI-PvuII (lanes 10 and 11); BamHI-HindII (lanes 12 and 13). HindIII digested λ as markers (lane 1). Electrophoresis was performed in 1% agarose gel.

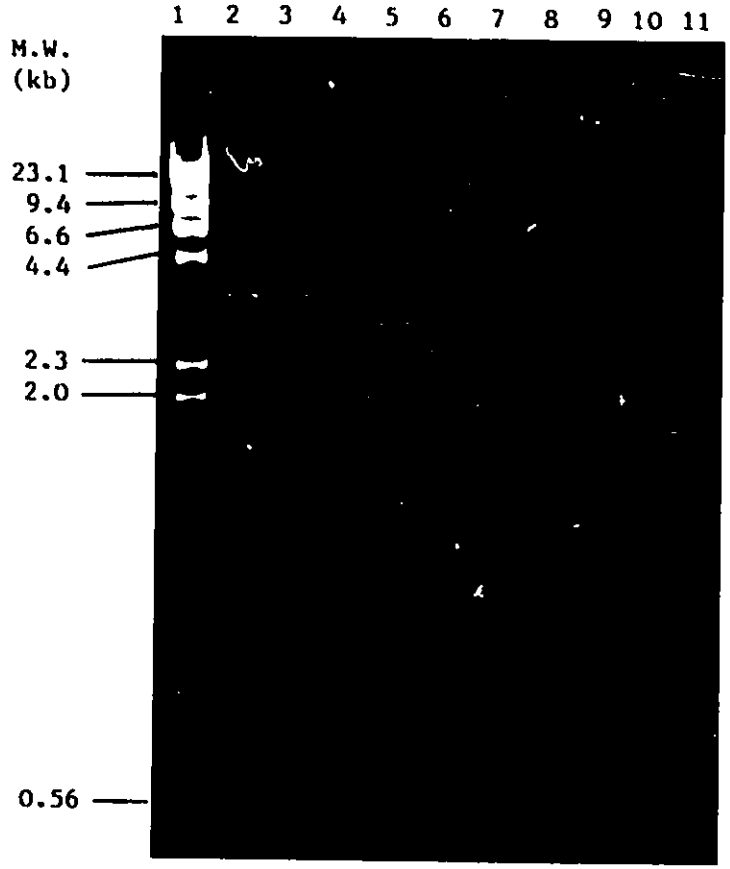


Figure 1-7 Comparison of pJD7 (Toronto-type) and pGO4717 (Rio-type) with different restriction endonucleases. pJD7 and pGO4717 digested with HinfI (lanes 2 and 3); BamHI (lanes 4 and 5); BamHI-HindIII (lanes 6 and 7); BamHI-PstI (lanes 8 and 9); BamHI-AvaI (lanes 10 and 11). HindIII digested λ as marker (lane 1). Electrophoresis was performed in a 1% agarose gel.

Heteroduplex analysis of pJD4, pJD5, and PJD7

To establish the similarity between pJD4 and pJD5, plasmids pJD4 and pJD5 were compared by forming heteroduplexes between them. The results (Figure 1-8) showed that these two plasmids are largely similar. The only apparent difference between them is that pJD4 carries an extra 2.1 kb fragment. Figure 1-8 shows that pJD4 appears to contain an inverted-repeat of 200 base-pairs (at the base of the single-stranded loop), but this phenomenon was not observed on other samples.

To locate this 2.1 kb fragment on pJD4, heteroduplex analysis was repeated with plasmid pJD4 and pJD5 cut with PstI. The result (Figure 1-9) showed that this extra fragment in pJD4 (or the fragment missing in pJD5) is situated 1.74 kb (average of five measurements of five samples, margin of error was $\pm$ 0.1 kb) five from the unique PstI site. Combining with the restriction endonuclease analysis, the fact that both pJD4 and pJD5 contain unique AvaI and PvuII sites, and the absence of the HindIII site in pJD5, the location of the deleted 2.1 fragment was determined to be 1.74 kb counter-clockwise from the PstI site (Figure 1-5).

To establish the extent of similarity between pJD4 and pJD7, and to locate the 2.3 kb fragment missing in pJD7 as compared to pJD4, heteroduplexes were formed and analysed with PstI digested pJD4 and pJD7 DNA (Figure 1-10). It was shown that this 2.3 kb fragment is 1.4 kb from the PstI site

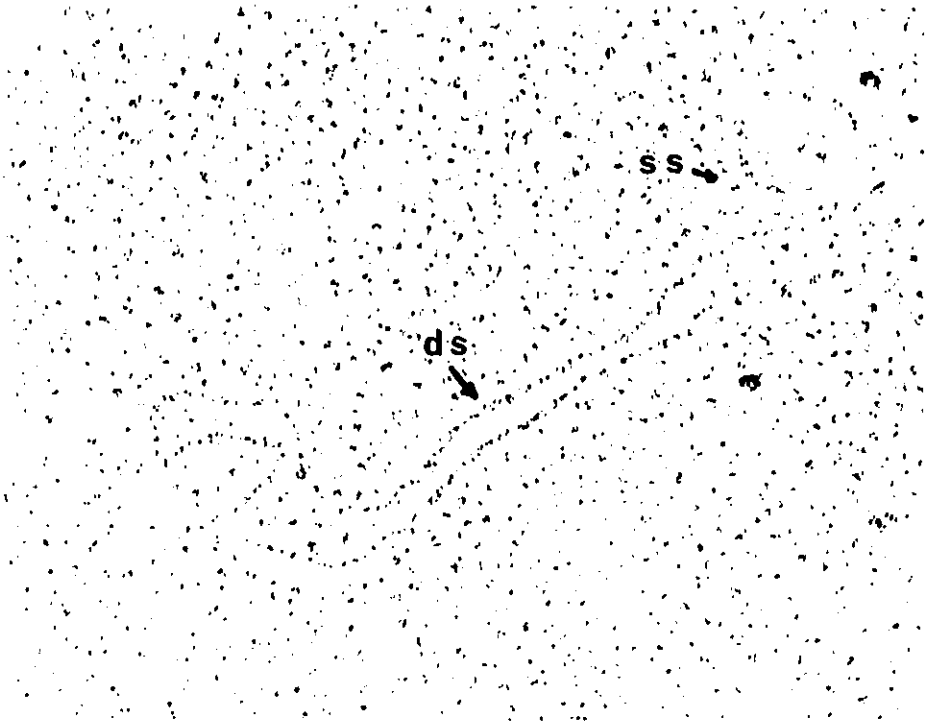


Figure 1-8 Heteroduplex formation between pJD4 and pJD5. The loop is single-stranded (SS) DNA demonstrating the only difference between the two plasmids.

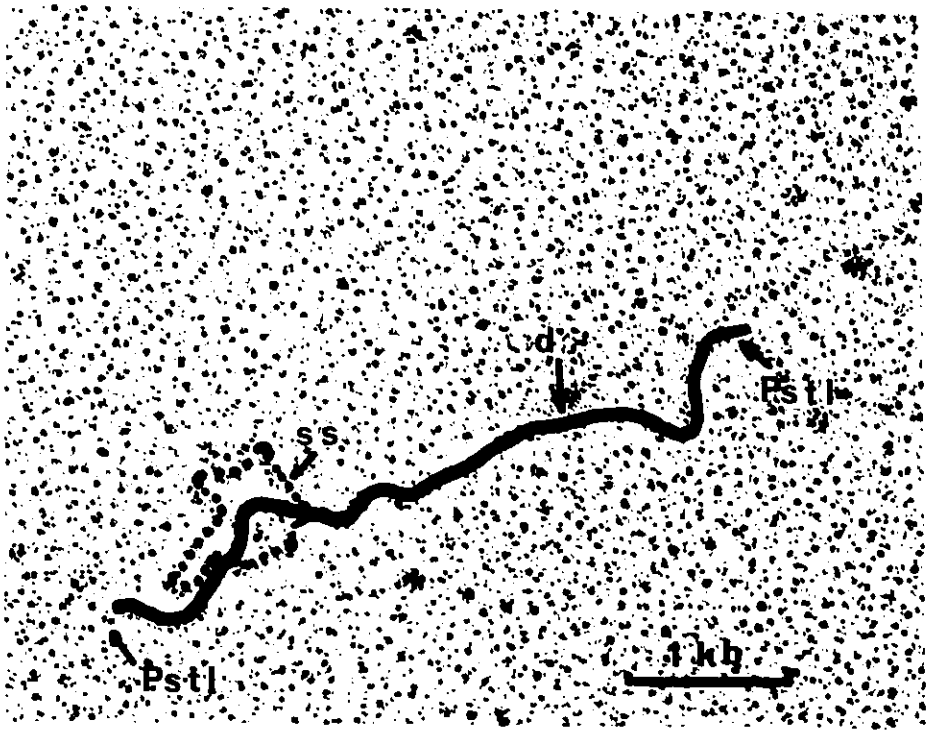


Figure 1-9 Heteroduplex formation between PstI-digested pJD4 and pJD5. The loop is single-stranded (SS) DNA demonstrating the only difference between the two plasmids.

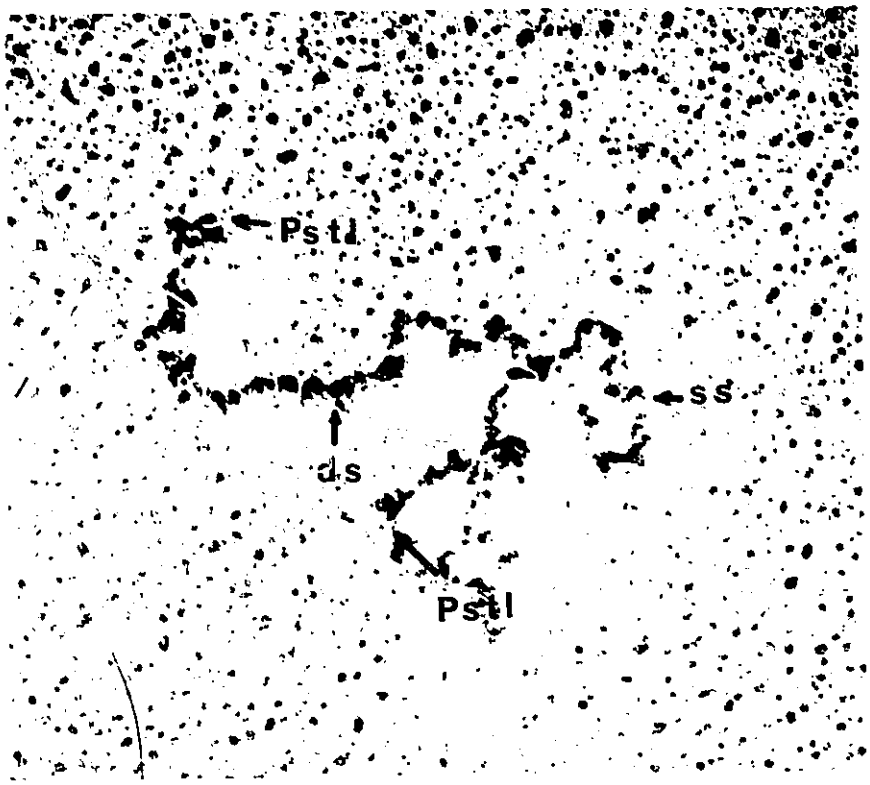


Figure 1-10 Heteroduplex formation between PstI-digested pJD4 and pJD7. The loop is single-stranded (SS) DNA demonstrating the only difference between the two plasmids.

(average of five samples, margin of error was ± 0.1 kb). Since pJD7 contains XbaI and HindIII restriction sites, but not the restriction sites for AvaI and PvuII, the location of this 2.3 kb fragment was determined to be 1.4 kb clockwise from the PstI site (Figure 1-5).

Sequence comparison between pJD4 and transposon Tn3

To determine the extent of homology between pJD4 and Tn3, the sequence clockwise from the BamHI site next to the ß-lactamase gene present on pJD4 was compared to the published sequence of Tn3.

The 4.8 kb BamHI fragment of pJD4 was cloned into the M13mp9 RF (replicative form) at the unique BamHI site. A total of 150 bases were determined. Comparing the sequence starting from the BamHI site to that of Tn3, it was found that there was total homology with the sequence of Tn3 up to base pair 79 (Figure 1-11). After base pair 79 from the BamHI site, the sequence began to differ. Using the published sequence of Tn3, it was calculated that pJD4 contained only 30% of transposon Tn3.

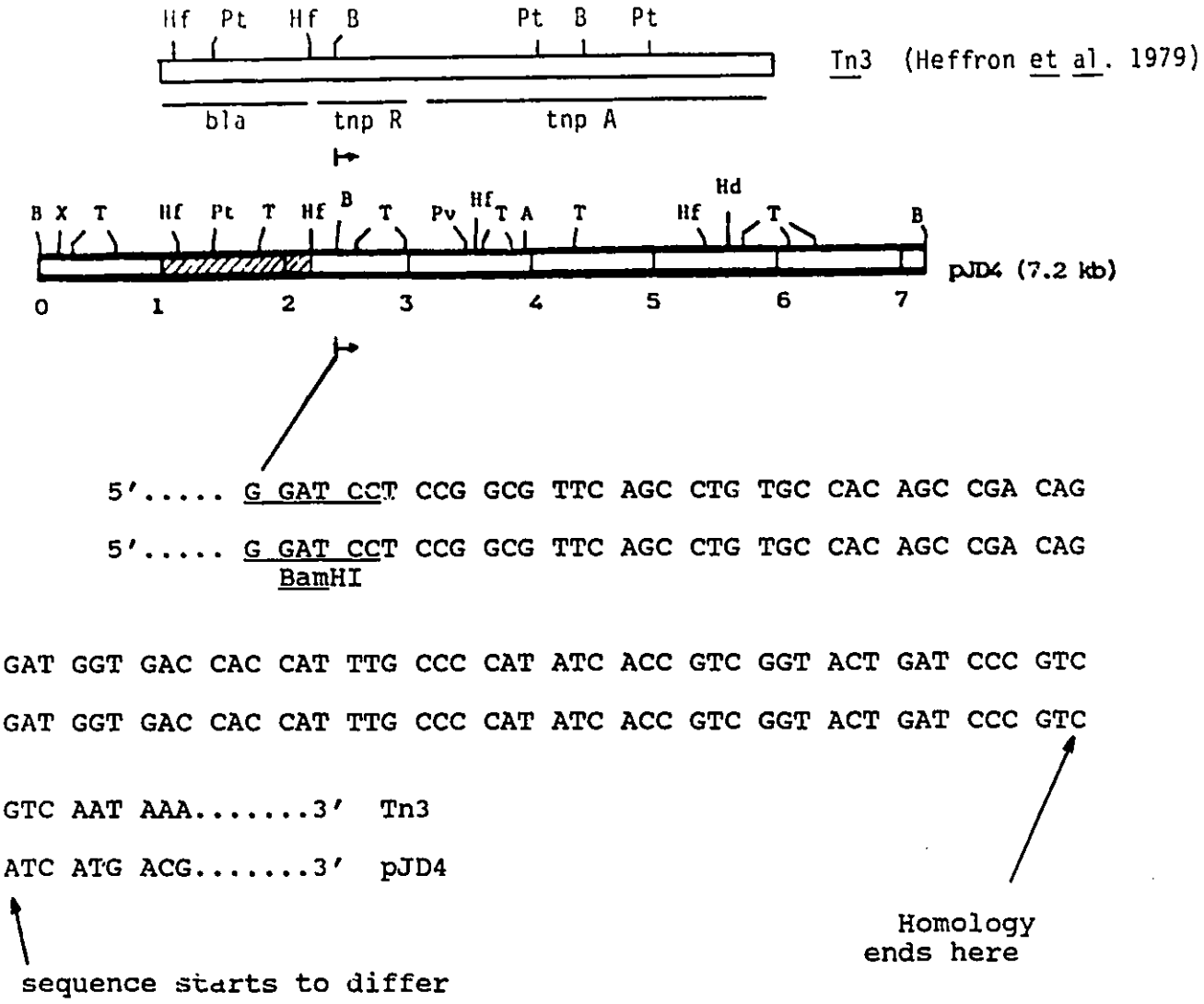


Figure 1-11 Comparison of DNA sequence between pJD4 and Tn3 showing the point of deletion of Tn3 in pJD4. Arrows indicate the direction of sequences from 5' to 3'. ▨ (bla) indicates the region encoding beta-lactamase. Abbreviations: A, AvaI; B, BamHI; Hd, HindIII; Hf, HinfI; Pt, PstI; Pv, PvuII; T, TaqI; X, XbaI.

DISCUSSION

Extensive maps of the three naturally occurring penicillinase-producing gonococcal plasmids, pJD4, pJD5 and pJD7 were constructed and compared (Yeung et al.,1986). The three plasmids were very similar to each other, except pJD5 and pJD7 have deleted DNA fragments, 2.1 kb and 2.3 kb in size respectively, as compared to pJD4. They all contained a common 2.4 kb BamHI fragment with the ß-lactamase gene, and the deletions were located on the 4.8 kb BamHI fragment of pJD4. Although twenty-five restriction enzymes (including those without restriction sites) were used for mapping, the precise locations of these deletions could not be determined by restriction endonuclease analysis alone.

Electron microscopic analysis of the heteroduplexes formed between the PstI digested 7.2 kb and 5.1 kb plasmids showed that the 2.1 kb fragment, which was absent in the 5.1 kb plasmid, was located 1.74 kb from the PstI site, a result previously reported by Dickgiesser et al. (1982; 1983a, b). In conjunction with restriction endonuclease analysis, the deleted 2.1 kb fragment was mapped at 1.74 kb counter-clockwise from the unique PstI site. This result did not agree with that of Dickgiesser et al. (1982; 1983a, b), who placed the deleted 2.1 kb fragment specifically at the other end of the BamHI fragment. If the map of Dickgiesser et al. (1982; 1983a, b) was correct, plasmid pJD5 (the 5.1 kb

penicillinase-producing plasmid) should not contain restriction sites for AvaI and PvuII. However, in the present study and those reported by others (McNicol et al., 1983; Aalen and Gunderson, 1987), the 5.1 kb penicillinase-producing plasmid carries both AvaI and PvuII restriction sites. Therefore, the location of the 2.1 kb deleted fragment as presented by Dickgiesser et al. (1982; 1983a, b) was either incorrect or they may have characterized a different, novel plasmid. Upon examination of their map, however, this later possibility was discounted because of the presence of the single HindIII site, which maps on the 4.9 kb 'Toronto-type' and the 7.2 kb 'Asian-type' plasmids, but not on the 5.1 kb 'African-type' plasmid. The plasmid Dickgiesser (1983) mapped was the 5.1 kb 'African-type' plasmid. Without any data, McNicol et al. (1983) placed the deleted 2.1 kb fragment more than 1.75 kb from the single PstI site, a result contradictory to studies using heteroduplex analysis. Most likely, they just simply placed the deleted 2.1 kb fragment next to the unique AvaI site of the 7.2 kb plasmid.

When the 4.6 kb (Rio-type) plasmid was isolated and characterized by van Embden et al.(1985), its restriction map was compared to that of the 7.2 kb and 5.1 kb plasmid. The restriction endonuclease map of the 5.1 kb plasmid used in the comparison was reported to be derived from that of Brunton et al. (1982). However, Brunton et al. (1982) did not

attempt to locate the coordinates of the deleted 2.1 kb fragment, and the map appeared to be similar to that published by McNicol et al. (1983). By extrapolating from the map published by McNicol et al.(1983), the deleted fragment mapped ~100 base pairs from the single AvaI site. In this study, the deletion was placed 900 base pairs from the same site. Recently, the location of the deletion as presented in this study was confirmed (Aalen and Gunderson, 1987).

Sox et al. (1979) suggested that the 5.1 kb plasmid might have been derived through a transformation-associated deletion of the 7.2 kb plasmid, while Dickgiesser et al. (1982) suggested that the 7.2 kb plasmid might have been generated from the 5.1 kb plasmid by the transpositional acquisition of an insertion element. The identification and isolation of a transformation-related deletion mutant, pJD4d, of the 7.2 kb plasmid which was similar in size and restriction endonuclease digestion pattern to the 5.1 kb plasmid supported Sox et al.'s hypothesis. Whether this event happens in nature is unknown, as the transformation frequency of N. gonorrrhoeae with the 7.2 kb plasmid was very low ($<10^{-8}$). Exhaustive characterization of the novel, naturally-occurring 4.9 kb plasmid reported in the present study reveals that it is related directly to the 7.2 kb plasmid but not to the 5.1 kb plasmid. This finding thereby further favored the hypothesis proposed by Sox et al. (1979).

The 4.9 kb 'Toronto-type' plasmid which was involved in an outbreak of penicillinase-producing N. gonorrhoeae (PPNG) in Toronto was compared to the 4.6 kb 'Rio-type' plasmid, pGO4717, which was isolated from a sailor who acquired the disease in Durban, South Africa. This plasmid, pGO4717 (strain GO4717), was not involved in any reported outbreak of PPNG in the Netherlands. Van Embden et al. (1985) observed that in addition to the absence of a 2.3 kb fragment in the 3.1 kb large BamHI-HindIII fragment, there was an additional 100 bp absent on the small 1.7 kb BamHI-HindIII fragment of the 7.2 kb plasmid. However, in the present study, the restriction endonuclease digestion patterns of these two plasmids pJD7 and pGO4717, were found to be identical. The difference in the estimation of their molecular weights between this study and that of van Embden et al.(1985) remains unexplained, as the figure of BamHI-HindIII digestion of pGO4717 was not published by the Dutch group. Since the 'Rio' plasmid was not involved in any outbreak of the disease and was reported in 1985, later than the report of the 'Toronto' plasmid at Asilomar, 1984, it is proposed that this novel plasmid be named 'Toronto'.

Part of the DNA sequence of the 2.4 kb BamHI fragment of pJD4 was determined and compared to that of the published Tn3 sequence (Heffron et al., 1979). Results obtained further supported the fact that the ß-lactamase producing plasmids in

that the ß-lactamase producing plasmids in N. gonorrhoeae probably obtained their ß-lactamase gene from transposon Tn3 (Fayet et al., 1982). The point of deletion was determined to be 79 base pairs from the BamHI site. By using the published Tn3 sequence (Heffron et al.1979), it was calculated that pJD4 contained only 30% of transposon Tn3 (including the right inverted repeat). This would include all of the ß-lactamase gene and only 46% of the TnpR gene. In a more recent publication, Chen and Clowes (1987) who have compared over 1,195 base-pairs of Tn1, Tn2, and Tn3, showed that there were twenty base-pair differences, scattered along the 1,195 base-pair segment compared, between Tn2 and Tn3 , and that the ß-lactamase encoding region of a 7.2 kb plasmid (pFA3) is more related (by 18 base-pairs) to Tn2 than Tn3. They also determined that pFA3 contained 40% of Tn2. The minor discrepancy on the location of deletion suggested that there could be minor variants within the 7.2 kb ß-lactamase-producing plasmids in N. gonorrhoeae as has been suspected.

N. gonorrhoeae was known to produce five restriction endonucleases NgoI, NgoII, NgoIII, NgoIV and NgoV which were isoschizomers of HaeII, HaeIII, SstII, NaeI and NiaIV respectively (Clanton et al., 1978; Roberts, 1980; Ritchot and Roy, 1988). Therefore, to survive in such an environment, DNA containing recognition sites for these enzymes must be modified in gonococci. Indeed, methylases M.NgoI, M.NgoII, M.NgoIII, M.NgoIV and M.NgoV have been detected in N.

gonorrhoeae (Ritchot and Roy, 1988). The 7.2 kb penicillinase-producing plasmid, when extracted from N. gonorrhoeae, was found to be resistant to both restriction enzymes HaeII and HaeIII (Norlander et al., 1981). In addition, SacII and BamHI were unable to restrict the 7.2 kb plasmid extracted from certain N. gonorrhoeae strains (Norlander et. al., 1981). Thus, the restriction endonuclease maps constructed from penicillinase-producing plamids extracted from E. coli would be different from those constructed from plasmids extracted directly from N. gonorrhoeae.

More recently, a 6.6 kb penicillinase-producing (Nimes) plasmid in N. gonorrhoeae was isolated (Gouby et al., 1986). In contrast to the 5.1 kb (African), the 4.9 kb (Toronto) and the 4.6 kb (Rio) plasmids, which appear to have evolved by independent deletions of DNA fragments from the 7.2 kb (Asian) plasmid, the 6.6 kb (Nimes) plasmid was shown to have been formed by the insertion of a fragment with two HinfI sites on the 2.4 kb BamHI fragment of the 5.1 kb plasmid (Gouby et al., 1986). All these observations indicate that the penicillinase-producing plasmids are still evolving.

Data presented in this chapter have shown that previous restriction endonuclease maps of the 7.2 kb and 5.1 kb plamids were inaccurate particularly in describing the exact site of the 2.1 kb fragment missing in pJD5, which could have confused the characterization of the two plasmids. The

isolation of pJD4d and the discovery of the 4.9 kb (Toronto) plasmids, particularly the isolation of the 'Rio' plasmid from two gonococcal strains with different auxotypes, supported the hypothesis of Sox et al. (1979) that the 5.1 kb plasmid could have been derived through a tranformation-associated deletion of the 7.2 kb plasmid. It also attests to the evolutionary fluidity of gonococcal ß-lactamase-producing plasmids.

CHAPTER TWO

THE 7.2 KB PENICILLINASE-PRODUCING PLASMID IN *N. GONORRHOEAE* HAS TWO INDEPENDENT REPLICATION REGIONS

INTRODUCTION

The 7.2 kb (pJD4) plasmid of N. gonorrhoeae had a broad host-range and could be mobilized to and could replicate in a variety of genera: other Neisseria species, E. coli, Salmonella minnesota, H. influenzae, H. parainfluenzae, H. ducreyi and N. meningitidis (Sparling et al., 1978; Brunton et al. 1982; Guiney and Ito, 1982; Dillon et al., 1983; Genco et al., 1984; Ikeda et al., 1986). The host-range of the 5.1 kb plasmid has not been extensively studied, as it has been assumed that the 5.1 kb plasmid has the same replication system as the 7.2 kb plasmid. Because small plasmids do not generally possess such a broad host-range, the replication system of both the 7.2 kb and the 5.1 kb plasmids was studied. In addition, the understanding of the replication system(s) of these plasmids could give some insight as to the establishment of these plasmids in N. gonorrhoeae.

Before the replication mechanism of the 7.2 kb plasmid could be determined, the essential replication region of the plasmid must be identified. McNicol et al. (1984) and Johnson (1985) cloned the DNA fragment responsible for the maintenance and replication of the 7.2 kb plasmid of N. gonorrhoeae. Their results were contradictory. McNicol et al. (1984) found that the essential replication region was located on a 2.4 kb BamHI fragment excluding the ß-lactamase (bla) gene . Conversely, Johnson (1985) reported that the replication region was located on the 3.2 kb BamHI-PvuII

fragment, which was part of a second BamHI fragment (4.8 kb) of the 7.2 kb plasmid. In both published reports, DNA fragments from the 7.2 kb plasmid were cloned into a cloning vector which could not replicate in a polA⁻ (deficient in DNA polymerase I) host. Since the replication origin and control system of the cloning vectors were still intact, it was therefore not conclusive whether the DNA fragment cloned from the 7.2 kb plasmid contained a polA⁻ independent origin of replication or a gene that could provide a product that could help the vector to survive in a polA⁻ host. In view of this controversy, the exact location of the essential replication region of the 7.2 kb plasmid was studied. Deletion has never been used to construct mini-plasmids from the 7.2 kb ß-lactamase plasmid in N. gonorrhoeae, although it has been widely used in the construction of mini-plasmids (mini-replicons) to locate the essential replication region of other plasmids (Nordstrom, 1983; Scott, 1984). Experiments were designed to construct mini-plasmids by in vitro deletions of the 7.2 kb and the 5.1 kb plasmids, in order to verify or discount previous observations. By comparing naturally-occurring and in vitro derived variants of the 7.2 kb plasmid, two independent replication systems have been identified. Incompatibility studies further confirmed that these two replication systems were different.

MATERIALS AND METHODS

Bacterial strains and plasmids

All plasmids were maintained in E. coli C600 (F⁻ thi-1 thr-1 leuB6 lac Y1 tonA2 supE44), which was supplied by Dr. V.N. Iyer (Carleton University, Ottawa) (Bachmann, 1972). Penicillinase-producing N. gonorrhoeae plasmids used in this study, pJD4 (7.2 kb) and pJD5 (5.1 kb), were described previously (Chapter one; Yeung et al., 1986). In addition, plasmid pACYC184 was utilized in some experiments (Chang and Cohen, 1978). All plasmid-containing derivatives of E. coli C600 were cultured on either Mackonkey agar (Difco, Detroit, Michigan, USA), or Tryptic Soy Agar (TSA, Difco). Antibiotics, when required, were used at the following concentrations : ampicillin (Ayerst, Montreal, Canada), 100 mg/L; chloramphenicol (Sigma, St. Louis, Mo., USA), 30 mg/L.

DNA manipulations

DNA digestions with restriction endonucleases were performed in the same manner as described in Chapter one (materials and methods). Other manipulations, such as transformation, ligation, and extraction from agarose gels, were also carried out as described in materials and methods in Chapter one.

Digestion with exonuclease Bal31 was performed as

described in Maniatis (1982). One ug linearized DNA was precipitated with ethanol and resuspended in 100 uL of Bal31 buffer (20 mM Tris-HCl, pH 8.0; 12 mM $CaCl_2$; 12 mM $MgCl_2$; 0.2 M NaCl; 1 mM EDTA). 0.5 unit of Bal31 was then added. To stop the activity of Bal31, the reaction mixture was extracted with phenol/chloroform as described in Chapter one.

Transfer of DNA to solid matrices

DNA fragments were transferred from agarose gels to nitrocellulose sheets (Amersham) using the method of Southern (1975). After electrophoresis, the agarose gel was placed in a denaturing solution (0.4 N NaOH; 0.8 M NaCl) for 30 minutes with gentle agitation to denature the DNA in the gel. The gel was then rinsed once with water, and neutralized in a neutralization solution (0.5 M Tris-Hcl, pH 8.0; 1.5 M NaCl) for another 30 minutes.

A piece of nitrocellulose sheet cut to the same size as the gel was completely wetted with water before soaking in 10X SSC solution (3 M NaCl; 0.3 M sodium citrate). Whatmann 3MM chromatography paper (26 cm X 15 cm) to act as a wick was soaked with 10X SSC before it was placed onto a raised plexiglass platform. The gel was then placed on the 3MM paper ensuring that no air was trapped between the gel and the 3MM paper. To prevent a 'short-circuit' (i.e. the nitrocellulose sheet or absorbant papers touching the 3MM paper), spacers were placed around the gel before the nitrocellulose paper

was placed on top of the gel. Two pieces of Whatmann No. 1 paper (a bit larger than the gel) were placed on the nitrocellulose paper. Extra care was used to make sure no air was trapped between the Whatmann No. 1 paper and the nitrocellulose paper. A stack of absorbent paper was then positioned on top of the Whatmann No. 1 paper. A piece of plexiglass and a weight were then placed at the very top of the assembly. The whole set-up was left overnight at room temperature.

The assembly was taken apart the next morning. With the gel still stuck to the nitrocellulose paper the loading slots were traced with a pencil onto the nitrocellulose paper. After the gel was removed, the nitrocellulose paper was gently washed with 2X SSC to remove any residual agarose. After air drying, the nitrocellulose paper was exposed to UV illumination for 5 minutes to fix the DNA fragment transferred, as suggested by the manufacturer (Amersham).

Nick translation

DNA to be used as a probe was labelled with [alpha-^{32}P] dATP using the procedure of 'nick translation' first developed by Rigby et al. (1977). Purified DNA (1 ug) was suspended in 5 uL of 10X nick translation buffer (0.5 M Tris-HCl, pH 7.5; 0.1 M $MgSO_4$; 0.01 M dithiothreitol). One uL each of unlabelled 1 mM dCTP, dGTP and dTTP stock solution were added along with 100 pmole [alpha-^{32}P] (> 3000 Ci/mmol)

(ICN U.S.A.). The volume of the mixture was brought up to 48 uL with sterile double distilled water. To the mixture, 1 uL of DNase I (0.05 ug/mL) and 1 uL of DNA polymerase I (5 units/uL) were added. The reaction mixture was then incubated at 16^{0}C for 2 hours. The reaction was stopped by the addition of 2 uL of EDTA (0.5 M). Unincorporated nucleotides were removed by precipitation of the labelled DNA with 95% ethanol.

DNA-DNA hybridization and autoradiography

A modified procedure of Dillon et al.(1985) was followed. The nitrocellulose paper with bound DNA was prehybridized in a sealed plastic bag with 10 mL of prehybridization solution (6X SSC; 0.5% SDS; 100 ug/mL herring sperm DNA; and 5X Denhardt solution [0.1% Ficoll; 0.1% Polyvinylpyrolidone ; 0.1% bovine serum albumin]). After incubation in a 70^{0}C water bath for at least 2 hours, the prehybridization solution was removed. Ten mL of radioactive DNA probe in hybridization solution (prehybridization solution (see above) without SDS) was denatured by boiling for 5 minutes before being added to the nitrocellulose sheet. With most of the air in the bag removed, the plastic bag was resealed and incubated in a 70^{0}C rotating water bath overnight. After the probe was removed, the nitrocellulose paper was washed three times (15 minutes each) with 300 mL of washing solution (0.1X SSC; 0.5% SDS) in a plastic container

at 70°C. The nitrocellulose sheet was then air dried, wrapped in Saran wrap and exposed overnight at -70°C to an X-ray film (Dupont Cronex film, Dupont, Canada) in a cassette with Hi-speed intensifier (Dupont, Canada). The exposed X-ray film was developed with a Dupont QC1-R/T processor.

Incompatibility studies

Incompatibility studies were carried out as described by Bergquist (1987). Incoming plasmid (i.e. pJD6 or pJD9) was transformed into E. coli C600 already harbouring a resident plasmid (i.e. pJD4Cm or pJD5Cm) through transformation. Transformants were selected for resistance to ampicillin and chloramphenicol, and were then checked for plasmid content. To get a better representation, ten single colonies with the same plasmid profile were inoculated into 5 mLs of Tryptic Soya Broth (TSB) without any antibiotics. The culture was incubated with shaking at 37°C for 15 - 20 generations (10 hrs). At time zero and 10 hrs., samples (0.1 mL) were taken, serially diluted and plated onto TSA without any antibiotics, and allowed to incubate overnight at 37°C. At least 100 colonies were picked and reinoculated onto TSA containing either ampicillin or chloramphenicol, or no antibiotics to observe plasmid segregation. Incompatibility was expressed as percent of colonies resistant to both ampicillin and chloramphenicol.

RESULTS

Location of the first replication region

Digestion of plasmid pJD4 of N. gonorrhoeae with BamHI produced two DNA fragments, 2.4 kb and 4.8 kb in size (Figure 1-3). Attempts to obtain a mini-ß-lactamase producing plasmid consisting of only the 2.4 kb BamHI fragment, by religation of the BamHI-generated ends followed by transformation of E. coli C600 and selection on ampicillin, were unsuccessful. Since the 4.8 kb BamHI fragment lacked an antibiotic resistance marker for selection, it was not certain whether the 4.8 kb BamHI fragment could form a mini-plasmid. The following experiments were performed to investigate this possibility.

A. Construction of pJD4C from pJD4

In order to ascertain whether the replication region was located on the 4.8 kb BamHI fragment, a plasmid containing the entire 4.8 kb BamHI fragment and the chloramphenicol resistance determinant from pACYC184 was constructed following the strategy outlined in Figure 2-1. The chloramphenicol resistance determinant of pACYC184 is located on a 1.2 kb HaeII fragment (Chang and Cohen, 1978). Following HaeII digestion of pACYC184, this fragment was extracted from agarose gels. The 3′ protruding ends generated by HaeII

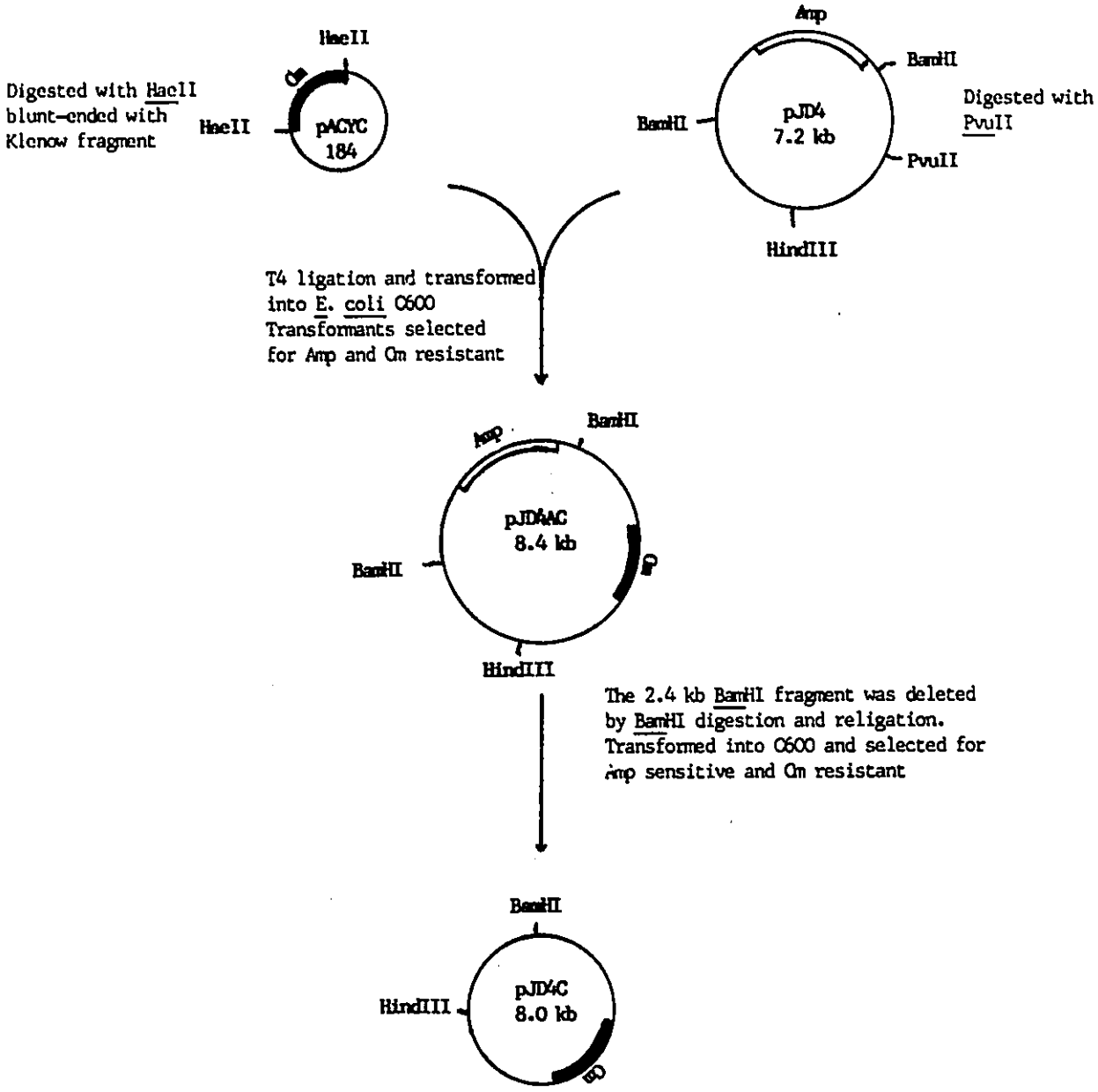


Figure 2-1 Strategy for the construction of pJD4AC and pJD4C from pJD4.

digestion were blunt-ended with Klenow fragment. Plasmid pJD4 was linearized with PvuII. Following ligation of the 1.2 kb HaeII fragment and the linearized 7.2 kb pJD4, the recombinant DNA molecules were transformed into E. coli C600. Bacterial colonies resistant to ampicillin and chloramphenicol were selected. Plasmid DNA isolated from ampicillin and chloramphenicol-resistant clones were analysed by digestion with BamHI and HindIII and compared with a similar digestion of pJD4 (Figure 2-2A). Digestion of pJD4 with BamHI and HindIII produced three fragments of 3.1 kb, 2.4 kb and 1.7 kb, respectively (Figure 2-2A, lane 2). Digestion of the recombinant plasmid with the same enzymes produced fragments 4.1 kb, 2.4 kb and 1.7 kb in size. Thus, the 1.2 kb HaeII fragment had been inserted within the 3.1 kb BamHI-HindIII fragment of JD4 at its unique PvuII site. The plasmid with this construct was named pJD4AC.

Plasmid pJD4C was derived from plasmid pJD4AC by the deletion of the 2.4 kb BamHI fragment containing the B-lactamase gene. To minimize religation of the 2.4 kb BamHI fragment, plasmid pJD4AC was digested with BamHI and PstI before ligation with T4 ligase. The ligation mixture was then transformed into C600. Transformed cells, which were ampicillin sensitive but chloramphenicol resistant, were lysed and their plasmid content analysed. One of the transformants with the smallest recombinant plasmid was selected, and named pJD4C. BamHI and HindIII digestion of

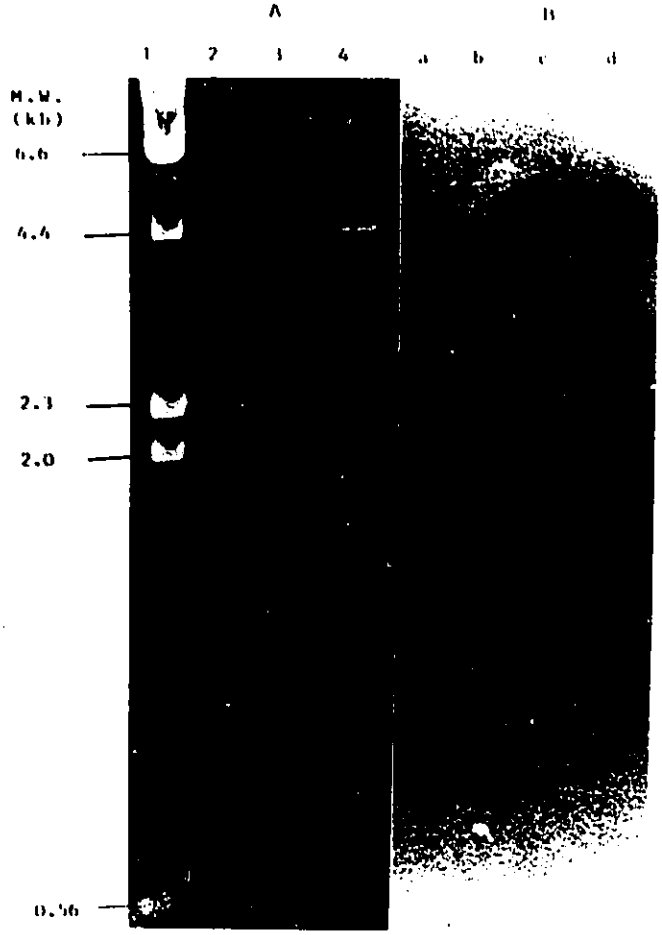


Figure 2-2A. Restriction endonuclease analysis of pJD4, pJD4AC and pJD4C. Plasmid pJD4 (lane 2), pJD4AC (lane 3), and pJD4C (lane 4) digested with BamHI-HindIII and run on a 1.5% agarose gel. Lane 1 - λ HindIII marker.

Figure 2-2B. Hybridization of (A) with pJD4C. Hybridization of DNA fragments generated by digestion with BamHI-HindIII of pJD4 (lane b), pJD4AC (lane c) and pJD4C (lane d) with pJD4C.

pJD4C (Figure 2-2A, lane 4) showed the absence of the 2.4 kb BamHI fragment in pJD4C as compared to pJD4 and pJD4AC.

To ascertain that pJD4C did not contain the 2.4 kb BamHI fragment of pJD4, plasmid pJD4C was labelled with ^{32}P and was used as a probe to detect homology with the 2.4 kb BamHI fragment. Plasmid pJD4C did not hybridize the 2.4 kb BamHI of either pJD4 (Figure 2-2B, lane 2) or pJD4AC (Figure 2-2B, lane 3). Thus the essential replication region of pJD4 is located on the 4.8 kb BamHI fragment.

B. Construction of mini-plasmids pJD8 and pJD9 from pJD4

To further delineate the location of the essential replication region on pJD4, plasmids pJD8 and pJD9 were constructed by *in vitro* deletion of pJD4. Figure 2-3 outlines the strategy used. Plasmid pJD4 was linearized with XbaI and then digested with Bal31. At different times, the activity of Bal31 was stopped, the digested DNA extracted, modified to blunt-ends, ligated and transformed into C600. A series of deletion derivatives were obtained and the smallest one was chosen for further analysis. Digestion of pJD4 with HinfI, produced four DNA fragments of 2.9 kb, 2.0 kb, 1.2 and 1.1 kb, respectively (Figure 2-4, lane 2). HinfI digestion of the smallest deletion derivative of pJD4, designated pJD8 (Figure 2-4, lane 3), indicated that the 2.0 kb, 1.2 kb and the 1.1 kb fragments remained intact, while the 2.9 kb fragment was reduced in size to 1.7 kb, with an overall reduction of 1.2

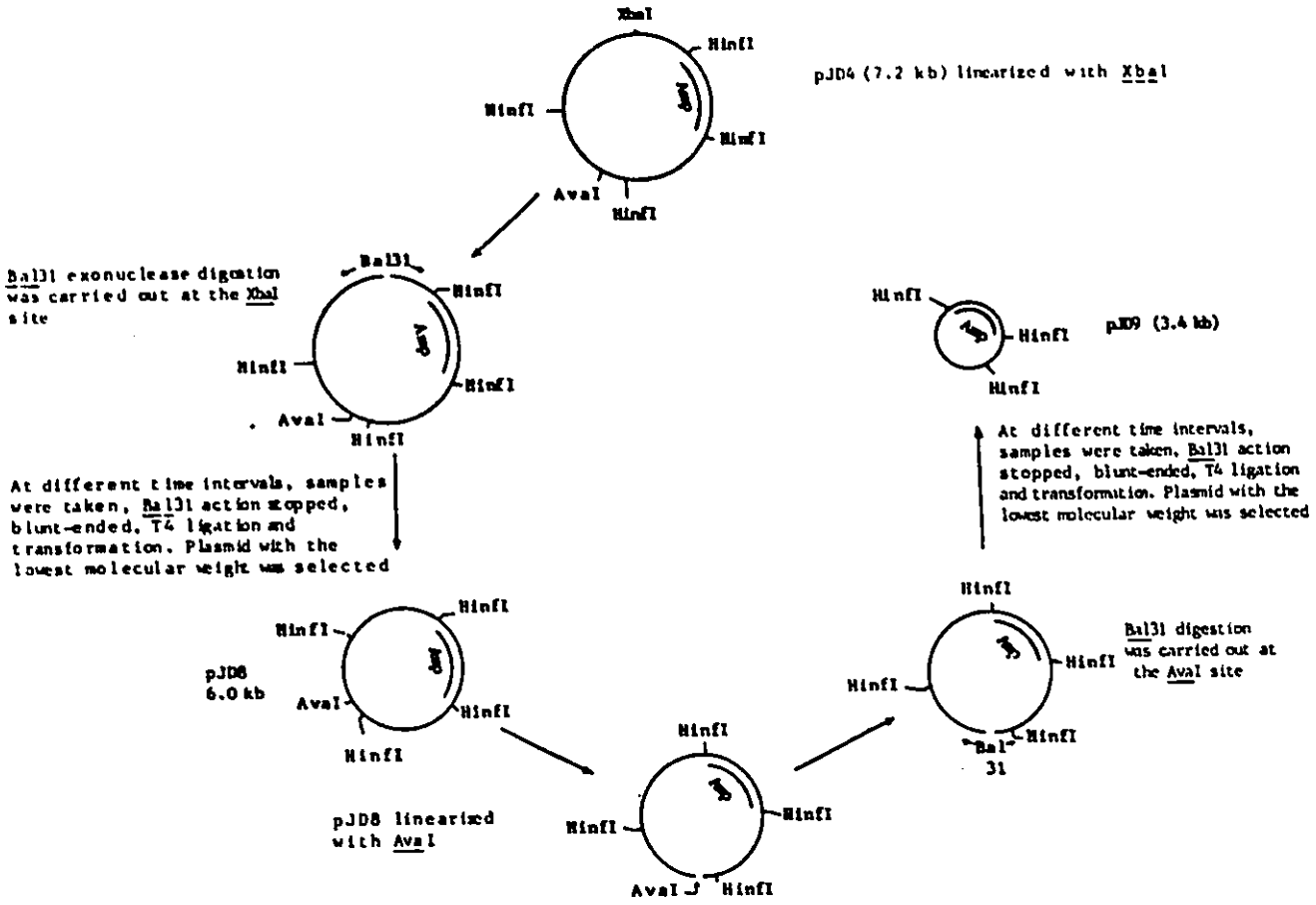


Figure 2-3 Strategy for the construction of mini-plasmids pJD8 and pJD9 from pJD4

kb as compared to pJD4. A further 2.6 kb was deleted from pJD8 by first linearizing pJD8 at its unique AvaI site followed by digestion with Bal31. The resultant plasmid was named pJD9. Digestion of this plasmid with HinfI (Figure 2-4, lane 4) showed that while the 1.7 kb and 1.1 kb fragments remained the same as those of pJD8, the 2.0 kb and 1.2 kb fragments were absent, and a new 0.6 kb fragment was present. Further analysis of pJD8 and pJD9 with TaqI (Figure 2-5) delineated the regions deleted (Figure 2-6). The 2.4 kb BamHI fragment was previously shown not to be required for the replication of pJD4. The essential region of replication for pJD9 is therefore attributed to a 1.5 kb region (region 'a') as shown in Figure 2-6.

Second replication region: construction of plasmids pJD6 and pJD5C from pJD5

In comparing the restriction map of pJD9 to that of pJD5 (Figure 2-6), a 5.1 kb naturally-occurring deletion derivative of pJD4, it was observed that region 'a' was absent in pJD5. This strongly suggested that there might be another region responsible for the replication of pJD5.

Digestion of pJD5 with BamHI produced two fragments of 2.7 kb and 2.4 kb. As with pJD4, attempts to obtain a mini-plasmid comprising only the 2.4 BamHI fragment of pJD5 were unsuccessful. It was possible to reduce the size of pJD5 to produce a construct, plasmid pJD6, by digestion and

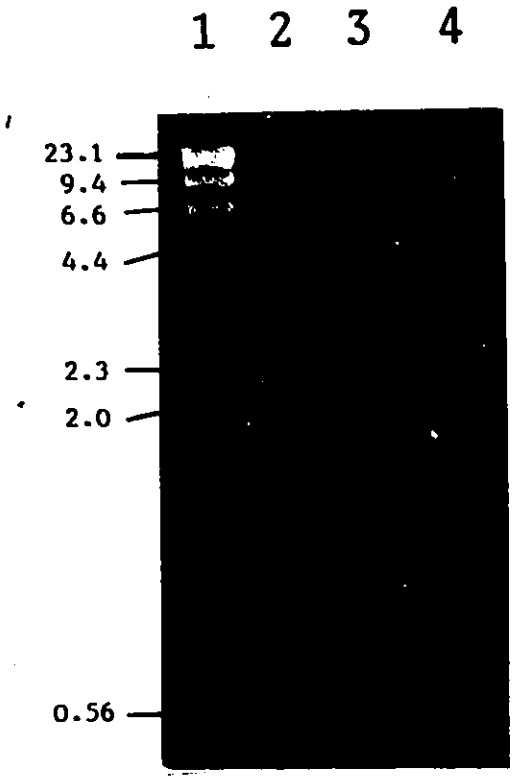


Figure 2-4 Analysis of pJD4, pJD8 and pJD9 with HinfI. Plasmid pJD4 (lane 2); pJD8 (lane 3); and pJD9 (lane 4). Samples were run on a 1.5% agarose gel. HindIII digested λ as markers (lane 1).

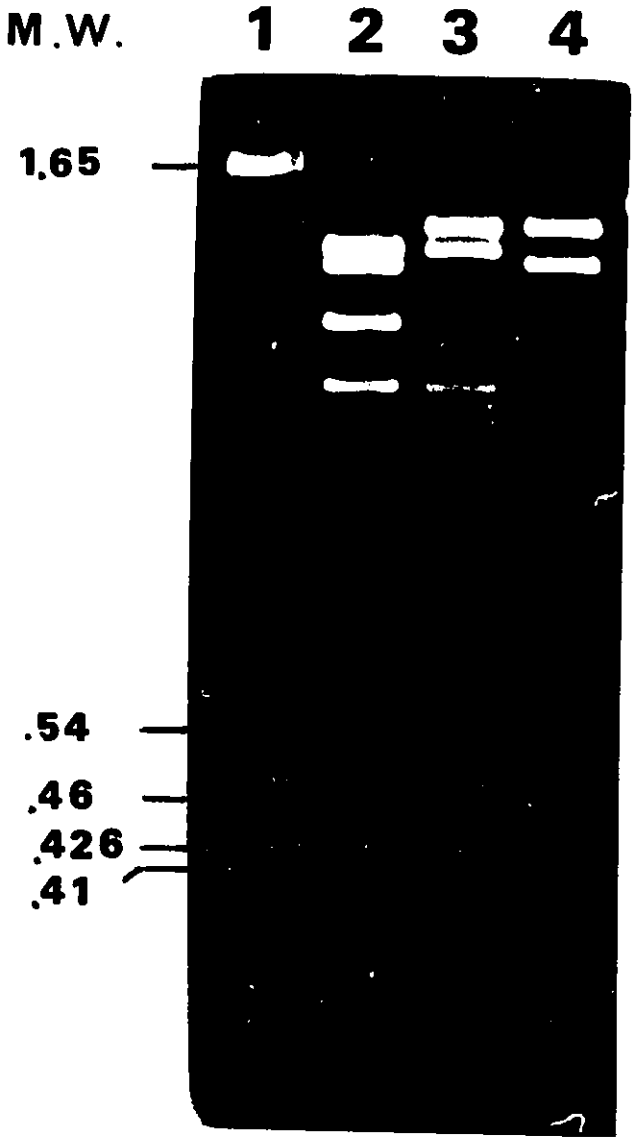
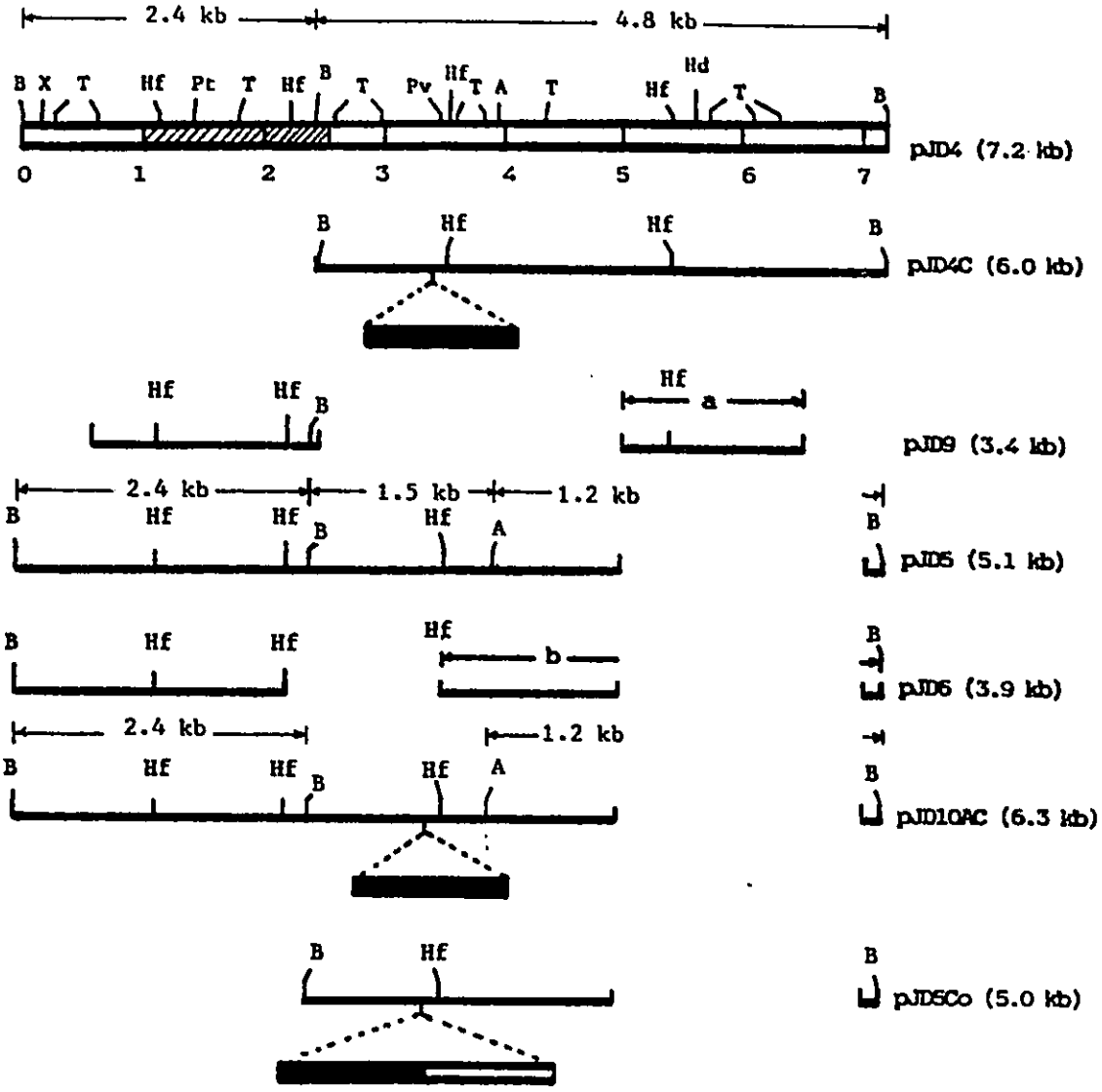


Figure 2-5 TaqI digestion of pJD4, pJD8 and pJD9. Plasmid pJD4 (lane 2); pJD8 (lane 3) and pJD9 (lane 4). DdeI digested pBR322 was used as markers (lane 1). Samples were run on a 1.5% agarose gel.

Figure 2-6. Restriction endonuclease maps of pJD4, pJD5 and their derivatives.  indicates the region encoding beta-lactamase.  represents chloramphenicol-resistance gene from pACYC184, while  represents the region containing oriV of pACYC184. Abbreviations: A, AvaI; B BamHI; Hd, HindIII; Hf, HinfI; Pt, PstI; Pv, PvuII; T, TaqI; X, XbaI. - a - replication region 'a'; - b - replication region 'b'.


2.4 kb
4.8 kb
B X T Hf Pt T Hf B T Pv Hf T A T Hf Hd T B
0 1 2 3 4 5 6 7
pJD4 (7.2 kb)
B Hf Hf B
pJD4C (6.0 kb)
Hf Hf B
Hf a
pJD9 (3.4 kb)
2.4 kb
1.5 kb
1.2 kb
B Hf Hf B Hf A
B
pJD5 (5.1 kb)
B Hf Hf
Hf b
B
pJD6 (3.9 kb)
2.4 kb
1.2 kb
B Hf Hf B Hf A
B
pJD10AC (6.3 kb)
B Hf
B
pJD5Co (5.0 kb)

religation of pJD5 with HinfI. HinfI digestion of pJD5 produced three fragments, 2.8 kb, 1.2kb and 1.1 kb in size (Figure 2-7, lane 2), while HinfI digestion of pJD6 produced two fragments 2.8 kb and 1.1 kb in size (Figure 2-7, lane 3). This plasmid pJD6, could not be reduced in size by the exonuclease activity of Bal31 at either the XbaI or the AvaI sites. Since previous experiments had shown that the 2.4 kb BamHI fragment was not required for the replication of pJD4, it was tentatively concluded that the region designated 'b' on pJD6 (Figure 2-6) was required for replication.

To further specify the location of the replication region of pJD5, the chloramphenicol resistance gene of pACYC184 (1.2 kb) was cloned into pJD5 (5.1 kb) by blunt-end ligation at its unique PvuII site. More than 150 (out of approximately 400) ampicillin and chloramphenicol resistant transformants were analysed. Only one harboured a plasmid, designated pJD10AC, of appropriate size (6.3 kb). BamHI-AvaI digestion of pJD5 produced three fragments 2.4 kb, 1.5 kb and 1.2 kb in size (Figure 2-8, lane 2). BamHI-AvaI digestion of the 6.3 kb plasmid, pJD10AC, indicated that the 2.4 kb and the 1.2 kb fragments were common to both plasmids (pJD5 and pJD10AC) but that a new 2.7 kb fragment (Figure 2-8, lane 3) was produced as a result of the insertion of a 1.2 kb fragment from pACYC184. In order to delete the 2.4 kb BamHI fragment, plasmid pJD10AC was digested with PstI and BamHI, ligated with T4 ligase, and then transformed into E. coli

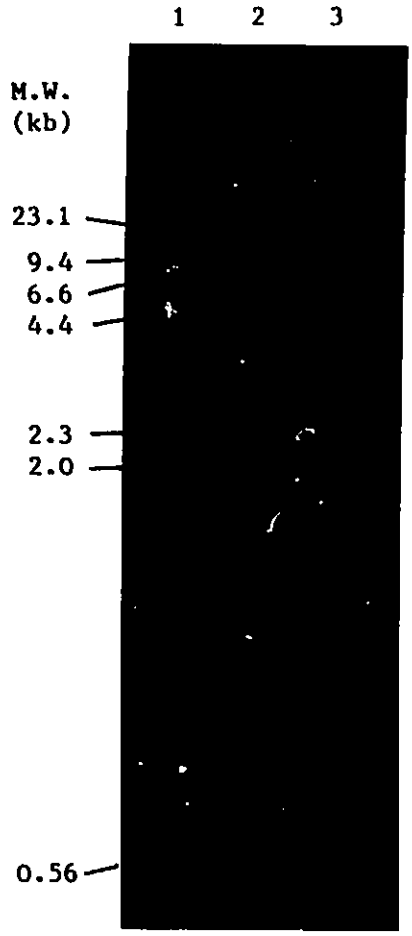


Figure 2-7. Restriction endonuclease analysis of pJD5 and pJD6 with HinfI. Plasmid pJD5 (lane 2) and pJD6 (lane 3). Samples were run on a 1.5% agarose gel. λ HindIII digested DNA as marker.

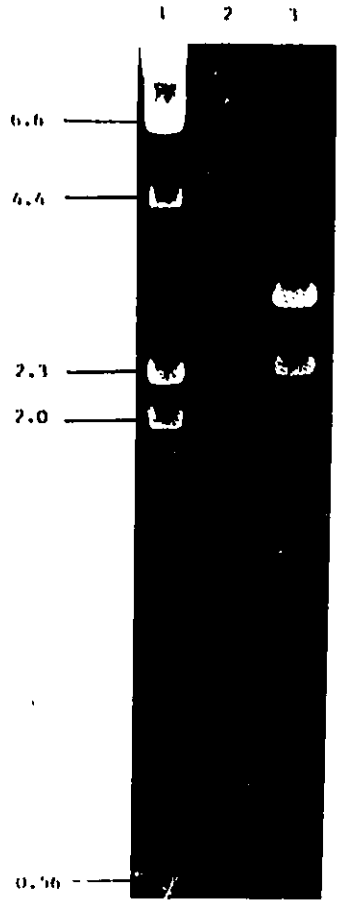


Figure 2-8 Restriction endonuclease analysis of pJD5 and pJD10AC with BamHI-AvaI. Plasmid pJD5 (lane 2) and pJD10AC (lane 3). Samples were run on a 1.5% agarose gel. HindIII digested λ DNA as marker.

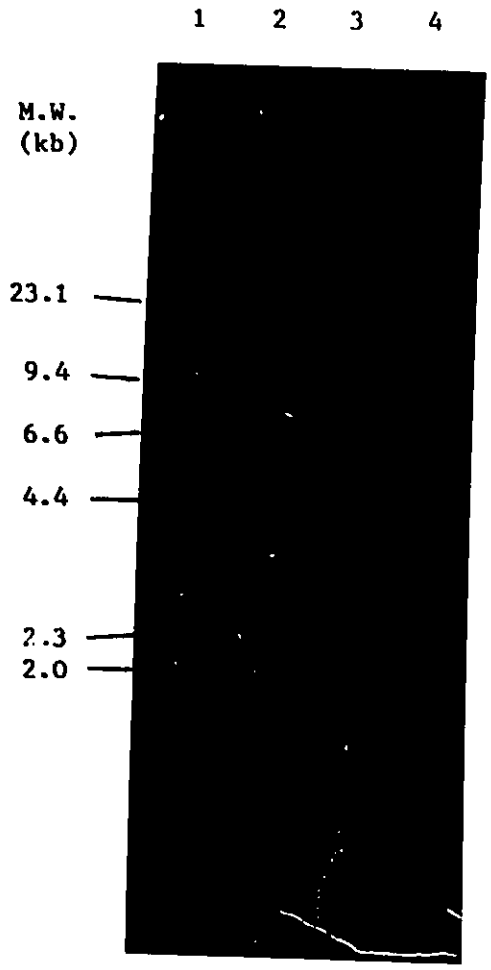


Figure 2-9 Restriction endonuclease analysis of pJD5, pJD5Co and pJD5ACo with BamHI-AvaI. Samples of pJD5 (lane 2), pJD5Co (lane 3) and pJD5ACo (lane 4) were run on a 1.5% agarose gel. HindIII digested λ DNA as markers.

C600. About 400 chloramphenicol resistant transformants were obtained and about 10% of them were ampicillin sensitive, suggesting the deletion of the ß-lactamase gene. However, plasmids with the expected size of 3.9 kb could not be obtained, suggesting that the 2.4 BamHI fragment is required for stable maintenance (most likely the partition system) of pJD5.

A 2.3 kb HaeII partial-digested fragment from pACYC184, containing the pACYC184 origin of replication and the chloramphenicol-resistance gene was cloned into the PvuII site of pJD5 to produce pJD5ACo (Figure 2-9). In contrast to pJD10AC, the 2.4 kb BamHI fragment could be deleted from pJD5ACo. Chloramphenicol-resistant, ampicillin-sensitive transformants were lysed and found to contain a 5.0 kb plasmid (pJD5Co). Analysis with restriction enzyme BamHI showed that the 2.4 kb BamHI fragment present in pJD5 and pJD5ACo was absent in pJD5Co (Figure 2-9).

Incompatibility relationship between pJD4, pJD5 and their derivatives

Since replication and maintenance properties of plasmids are associated with incompatibility functions (Pritchard et al.,1981; Timmis et al., 1975), the relationships between pJD4, pJD5 and their derivatives were studied. Their relationship to pACYC184 was also studied, since some of the

recombinant plasmids (pJD5AC, pJD5C) contained the origin of replication of this plasmid.

Plasmids pJD4, pJD5, pJD6 and pJD9 were separately introduced by transformation into C600 already harboring plasmid pACYC184. The incompatibility of the plasmids was tested as outlined in Materials and Methods. Plasmid pACYC184 was found to be compatible with pJD4, pJD5, pJD6 and pJD9 (Table 2-1). Thus any incompatibility shown among pJD4, pJD5 and their derivatives would not be due to the presence of the cloned origin of replication from pACYC184, which is being used for replication by pJD5Co.

When plasmids pJD4, pJD6 and pJD9 were introduced individually into C600 containing pJD4C, incompatibility between the incoming plasmid and the resident plasmid was observed. After 10 hours of incubation in antibiotic-free medium, all bacterial cells, containing pJD4C and pJD6 or JD9 initially, had lost the incoming plasmid. However, 11% of the cells harboured both pJD4C and pJD4, while 52% contained only pJD4C and 37% harbored only pJD4 (Table 2-1). When either pJD5, pJD6 or pJD9 were tested for incompatibility with pJD5Co, pJD5 and pJD6 were 100% incompatible with pJD5C, while pJD9 could maintain itself with pJD5C (Table 2-1). These data confirmed that the replication system in pJD9 is different from that of pJD5 and pJD6. Since both pJD9 and pJD6 are incompatible with pJD4C, it appears that pJD4 contained both the replication systems of pJD9 and pJD6.

TABLE 2-1. INCOMPATIBILITY RELATIONSHIPS OF pJD4, pJD5 AND THEIR DERIVATIVES

Incoming plasmid	Resident plasmid	% of Cells containing			Incompatible
		Incoming only	Resident only	Both	
pJD4	pACYC184	0	0	100	-
pJD5	pACYC184	0	0	100	-
pJD6	pACYC184	0	0	100	-
pJD9	pACYC184	0	0	100	-
pJD4	pJD4C	37	52	11	+
pJD6	pJD4C	0	100	0	+
pJD9	pJD4C	0	100	0	+
pJD5	pJD5Co	0	100	0	+
pJD6	pJD5Co	0	100	0	+
pJD9	pJD5Co	0	0	100	-

Data presented are the average of at least three separate experiments.

DISCUSSION

Plasmids that contain more than one origin of replication are usually large, conjugative plasmids (Robinson et al., 1985). This is the first report of a small (7.2 kb) naturally-occurring, non-conjugative plasmid containing two replication regions. The presence of multiple replication regions broadens the host range of plasmids (Bergquist, 1987). This appears to be the case with the 7.2 kb penicillinase-producing plasmid in N. gonorrhoeae.

Through the construction of in vitro deletion derivatives of the 7.2 kb penicillinase-producing plasmid of N. gonorrhoeae, it was found that one of the replication regions (region 'a') was located on a 1.5 kb fragment containing the unique HindIII site (Figure 3). This finding extends the preliminary results reported by Johnson (1985), who described the replication region as being on a 3.7 kb BamHI-PvuII fragment of this plasmid. Both of the above results are contrary to the findings of McNicol et al. (1984) who located the replication region for a 7.2 kb plasmid on an approximately 1.0 kb BamHI-HinfI fragment adjacent to the beta-lactamase gene. It has been suggested that the results of McNicol et al. (1984) could be due to the transformation of wild-type pol revertants of the polA1 mutant SF800 that was used (Johnson, 1985).

The replication system of the naturally-occurring 5.1 kb

penicillinase-producing plasmid (pJD5) of N. gonorrhoeae, which lacks replication region 'a', has not been studied. Although there is clear evidence that the 2.4 kb BamHI fragment is not required for replication of the 7.2 kb penicillinase-producing plasmid, this conclusion cannot be categorically made regarding the 5.1 kb plasmid. The minimal in vitro deletion derivative that could be constructed from the 5.1 kb plasmid (pJD5) was a 3.9 kb plasmid (pJD6). Thus, the replication and maintenance of this plasmid is attributed to the 2.5 kb region which excludes the bla gene.

McNicol et al. (1983) mapped the oriV of the 7.2 kb plasmid between the XbaI and BamHI sites of the 1.0 kb BamHI-HinfI fragment. They indicated that only this fragment was required for the plasmid to survive in a polA$^-$ host. However, we found that the 1.0 kb BamHI-HinfI fragment was non-essential for the replication of the 7.2 kb penicillinase-producing plasmid, but appeared to be required for the stability of the 3.9 kb (pJD6)and the 5.1 kb (pJD5) plasmids. Based on the present study, it appears that the entire 1.0 kb BamHI-HinfI fragment does not contain all the necessary information required for replication; a miniplasmid with this region alone plus the bla gene cannot be constructed. Furthermore, region 'b' (Figure 3), is required for the replication of the 5.1 kb plasmid and its derivatives. Incompatibility studies indicated that region 'b', which is distinct from region 'a', is required for replication and

functions independently from the 1.0 kb BamHI-HinfI fragment.

The essential replication region on the 5.1 kb penicillinase-producing plasmid is also present on the 7.2 kb penicillinase-producing plasmid. In the 7.2 kb plasmid the coding sequence of region 'b', which was essential for the replication of the 5.1 kb plasmid, was interrupted by the apparent insertion of replication region 'a'. Plasmids with replication region 'a' were more stable. Therefore, in both N. gonorrhoeae and E. coli replication region 'a' is dominant. The contribution of replication region 'b' when both regions are present is unknown, but the insertion of the replication region 'a' may render the 'b' replicon non-functional. This hypothesis is consistent with previous observations in which the 7.2 kb plasmid was found to be more stable than the 5.1 kb plasmid (Dillon et al.,1981; Roberts et al., 1977; Sox et al., 1979). In addition, as stated previously, the presence of multiple replication regions would serve to broaden the host-range of a given plasmid. Thus, the host-range of the 7.2 kb plasmid should be broader than that of the 5.1 kb plasmid. These experiments have not as yet been undertaken, since it has been widely assumed that the two plasmids have the same replication system.

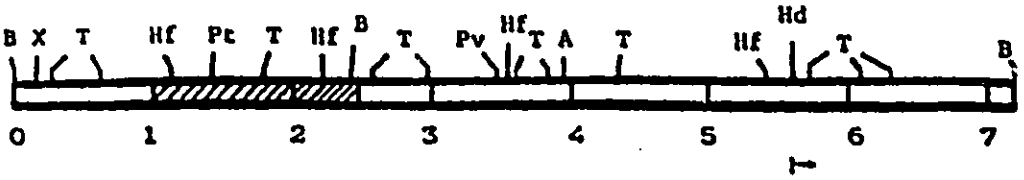
The mechanisms of plasmid replication have been categorized into three major groups (Zverev and Khmel, 1987). Unlike plasmids of the first group (e.g. ColEI, ColDF13, pBR322, pMB1, p15A), plasmids in the second and third group

require plasmid-specific, positively-acting proteins , but not DNA-polymerase I, encoded by polA, provided by the host (Nordstorm, 1983; Nordstorm et al., 1984; Scott, 1984) for initiation of replication. The 7.2 kb penicillinase-producing plasmid can replicate and maintain itself in polA$^-$ hosts (McNicol et al., 1984; Johnson, 1985). Thus, by deduction, it seemed unlikely that the replication mechanism of region 'a' of the 7.2 kb plasmid belongs to the first group.

Plasmids of the second and third group code for and require a plasmid specific positively acting protein (initiator protein) for their initiation of replication. The synthesis of this protein by plasmids of the third group is autoregulated and these plasmids share one similar feature: they all have repeating sequences in or near the origin of replication. It had been shown that these repeating sequences are the recognition/binding site of the initiator protein and that they are required for the expression of incompatibility of the plasmid (Tolum and Helinski, 1981; Tsutsui et al., 1983).

To further elucidate the mechanism by which pJD4 replicated, 752 bases have been sequenced (Figure 2-10; Hasnain et al., 1988). Computer sequence analysis shows that this region is A-T rich and there are many direct repeats and inverted repeats. There were 7 sets of more than three direct repeats (Figure 2-10) within this region, a characteristic of plasmids that code for plasmid-specific initiator proteins

Figure 2-10 Nucleotide sequence of 752 bases of the replication region 'a' of pJD9. Analysis of this sequence shows there are seven sets (underlined) of perfect direct repeats. Sequences with a line above and marked dnaA Box are the putative recognition sequence of dnaA protein. Underlined sequence marked IHF is the recognition sequence of IHF. ▨ indicates the region homologous to Tn2. Abbrevations: A, AvaI; B, BamHI; Hd, HindIII; Hf, HinfI; Pt, PstI; Pv, PvuII; R, RsaI; T, TaqI; X, XbaI. ⟶ indicates direction of sequencing.



pJD4 (7.2 kb)

pJD9 (3.4 kb)

HindIII dnaA Box

5'...TTCGAACCCGATGTAAATCTGATATAGCTTACACACAAGTTAGAAATGCGTTTTAAAAAT 60

TAAAGCGTTGGGTTACATGGATAACGAACAGAATTAACAGAAGTAGCTTTAATTCATAAG 120

CGTTCTATTTTAAAAGAAGGGCATTTATATCGAGTTCCACGATGAGCTAATTCCTTACAT 180

TTCTGCCTTACACGATAACTACACAAAATATAAATTGATCAATCTTGGGGCTTTGGGTAG 240

TACGCTATCGCCTTTATGAATTGTGTTCTCAATATCGTGATACAGTTGGCGACAAACAAG 300

IHF

CGTAGAAGATATTAAAAGTTGGTTAAGTATCTCTGATAAGTACCACTGTTTAAGGATTTT 360

AAAAAGCGAGTATTAACTCCATCCATAAATGAAATTAATGCTAAATCCGATCTGCTTGTT 420

ATGTTGAACCTATCAAGCGTGGGCGAACAATCGTAGCATTAAAATTCACGATCAAGAGTA 480

AGAAAAGTGCGGTAAAAATCGAACAGAAACGAAATGTCATTTCGCATAAACACTACGGGC 540

CGTTTGTGATTGATACTCAAAATCCCAAAATGAGTAATGCTGAATATGGAAACTATGCGA 600

dnaA Box

GAGATTGCCTAAAAATCCTTGAAGATTTTTATTCTGATCTTGCCGATGTTACACAGAAGA 660

ATTTACGCATTATTGGGTTTTTTTGACTAGCAATGCTAGTTTTAGATCAAAACTTGGTAA 720

TaqI

GCGGTCAGTTTTTAAACGAGTTGCAAAATCGA....3' 752

such as pSC101, F, P1 and R6K (Chattoraj et al.,1984; Germino et al.,1983). In addition, two sequences, TTACACACA and TTACACAGA, which were similar to the consensus dnaA box sequence TTATACACA found in pSC101 (Churchward et al., 1983; Vocke and Bastia, 1983) were identified. The presence of these sequences may indicate that the 7.2 kb plasmid requires dnaA function from the host for its replication. Recently, it has been reported that the integration host factor (IHF), encoded by the chromosome gene himA and hip, was required for the replication of pSC101 (Gamas et al., 1986). IHF recognizes the consensus sequence C/TAANNNNTTGATA/T (Gamas et al., 1986; Leong et al., 1985). Such a sequence CAATATCGTGATA (Figure 2-10), with only one mismatch, was also found in region 'a'. Based on these data and the replication initiation model of Bramhill and Kornberg (1988), the mechanism of replication of region 'a' should be very similar to that of oriC in E. coli.

Open reading frames coding for hypothetical proteins, 2.0 kd, 3.1 kd, and 6 kd were also found. It is not known whether these proteins are actually produced and involved in the replication of pJD9. The mechanism of replication for the second replication region designated 'b' has yet to be determined.

Restriction endonuclease maps in Figure 1-6 showed that region 'a' and region 'b' did not contain any overlap. However, when the 1.5 kb BamHI fragment of pJD6 was used as a probe, it did show some homology with region 'a' (data not shown) suggesting there might be some overlap between the two regions.

The extent of the overlap was estiminated to be 50-100 base-pairs. This was one of the reasons for performing the incompatibility studies. Plasmids pJD5 and pJD6, without replication region 'a', were found to be compatible with plasmid pACYC184. However, these two plasmids showed vectoral incompatibility with plasmid pJD5Co, which utilized the replication system of pACYC184. Vectoral incompatibility usually occurrs when the replication of a plasmid is inhibited by cloned plasmid fragments containing elements of the same replication control or partition system (Novick, 1987). Since it has been suggested that the 2.4 kb BamHI fragment of pJD5 was responsible for stability and pJD5Co contained only region 'b' from pJD5, it was concluded that region 'b' was responsible for replication control in pJD5 and therefore exhibited vectoral incompatibility with pJD5Co.. Plasmid pJD9 containing only replication region 'a', was shown to be compatible with pJD5Co; therefore the mechanisms of replication control of regions 'a' and 'b' were different.

Obviously, complete DNA sequence analysis of region 'a' and region 'b', and construction of more deletion mutants would allow a better understanding of the replication of these plasmids. However, the identification of two replication regions in pJD4 is the first step in understanding the mechanism by which the ß-lactamase-producing plasmids established themselves in N. gonorrhoeae.

CHAPTER THREE

DETECTION OF THE REGION REQUIRED *IN CIS* FOR THE MOBILIZATION OF THE 7.2 KB PENICILLINASE-PRODUCING PLASMID IN *N. GONORRHOEAE*

INTRODUCTION

The 7.2 kb and 5.1 kb penicillinase-producing plasmids are non-conjugative and the spread of these plasmids has been attributed to a 39.2 kb conjugative plasmid which could mobilize the 7.2 kb and the 5.1 kb penicillinase-producing plasmids between gonococcal isolates (Eisenstein *et al.*, 1977; Roberts and Falkow, 1977). The 39.2 kb conjugative plasmid could also mobilize the 7.2 kb and 5.1 kb plasmids from *N. gonorrhoeae* to *E. coli*, *Haemophilus* species, *N. meningitidis*, and *N. cinerea* (Flett *et al.*, 1980; Genco *et al.*, 1984; Ikeda *et al.*, 1986). In addition, a conjugative plasmid from *H. ducreyi*, pHD147, could mobilize these two ß-lactamase-producing plasmids between *Haemophilus* species (McNicol *et al.*, 1983). Guiney and Ito (1982) demonstrated that the 7.2 kb plasmid could be mobilized by the broad-host range *Inc*P plasmids, pRK231, pRK2013 and R751, from *E. coli* to *Salmonella minnesota* and *H. influenza*. A protein-DNA relaxation complex similar to those found with *Col*E1 was also detected in their study (Guiney and Ito, 1982).

The presence of the 39.2 kb gonococcal conjugative plasmid has never been detected in *E. coli* and this observation has been indirectly confirmed in the present study. To study the mobilization mechanism of the ß-lactamase-producing plasmids and to identify the region required for their mobilization, a number of conjugative plasmids were tested for their abilities to mobilize a 7.2 kb

plasmid (pJD4) from E. coli DH1 to E. coli C600 (rif^r). In addition, the 2.4 kb BamHI fragment from the 7.2 kb plasmid, pJD4, was cloned into pACYC184 and was found to be responsible for the mobilization of the 7.2 kb plasmid by conjugative plasmid pBG791 (IncIκ).

MATERIALS AND METHODS

Bacterial strains and plasmids

Bacterial strains and plasmids used in this study are listed in Table 3-1. All the conjugal plasmids, originally obtained from the collection of Dr. V.N. Iyer (Carleton University, Ottawa, Ontario), were conjugally transferred into and maintained in E. coli CSH56 (Bezanson et al., 1981). Plasmid pBG791 was isolated from an unknown Salmonella species and characterized by Bezanson et al. (1981)

All bacterial strains, with or without plasmids, were maintained on either MacConkey agar (Difco, Detroit, Mi.) or Tryptic Soy Agar (TSA, Difco). Antibiotics, when required, were added at the following concentrations: ampicillin (Amp; Sigma, St. Louis, Mo.) 100 g/mL; rifampin (Rif; Sigma) 200 ug/mL; chloramphenicol (Cm; Sigma) 30 ug/mL; tetracycline (Tet; Sigma) 15 ug/mL; streptomycin (Str; Sigma) 30 ug/mL; kanamycin (Km; Sigma) 30 ug/mL.

DNA manipulations

Plasmid DNA was isolated using a cleared lysate procedure followed by cesium chloride-ethidium bromide density gradient ultracentrifugation as described in Chapter one and by Dillon et al. (1985).

DNA digestions with restriction endonucleases were erformed in the manner described in Chapter One.

Table 3-1 Bacterial strains and plasmids for mobilization studies

STRAIN	Revelent characteristics	Source or Reference
E. coli DH1	recA1 endA1 gyrA96 thi-1 hsdR17 (r_K^- m_K^-) SupE44	Maniatis et al. (1982)
E. coli CSH56	ara Δ(lacpro) supD nalA thi	Miller (1972)
E. coli C600(rif)	Rif^r; F^- thi-1 leuB6 lacY1 tonA21 supE44	spontaneous mutant of C600 (this lab.)
PLASMID		
pJD4	Amp^r	this lab. (chapter 1)
pJD5	Amp^r	this lab. (chapter 1)
pJD6	Amp^r	this lab. (chapter 2)
pJD7	Amp^r	this lab. (chapter 1)
pJD8	Amp^r	this lab. (chapter 2)
pJD9	Amp^r	this lab. (chapter 2)
pACYC184	Cm^r Tet^r	Chang and Cohen (1978)
CONJUGATIVE PLASMID (Inc)		
R124 (FIV)	Tet^r	Benzanson et al.(1981)
R621a (Iγ)	Tet^r	Benzanson et al.(1981)
R100 (FII)	Tet^r Sm^r Sp^r Cm^r	Benzanson et al.(1981)
R199 (N)	Tet^r	Benzanson et al.(1981)
R6K (X)	Amp^r Sm^r	Benzanson et al.(1981)
RP4 (P)	Amp^r Km^r Nm^r Tet^r	Benzanson et al.(1981)
R1drd16 (FII)	Km^r	Benzanson et al.(1981)
Tp124 (H^e)	Cm^r Sm^r Tet^r Su^r	Benzanson et al.(1981)
S-a (W)	Cm^r Gm^r Km^r Sm^r	Benzanson et al.(1981)
pBG791 (Iκ)	Sm^r	Benzanson et al.(1981)

Digestions with the exonuclease Bal31 and DNA ligation with T4 ligase were performed as described in Chapter Two.

Conjugal transfer of plasmids to E. coli recipients

Conjugations were performed using a filter-mating technique (Guiney and Ito, 1982). Bacterial strains were grown overnight at 37^0C with shaking in tryptic soy broth (TSB). One mL of the overnight culture was used to inoculate 10 mL of fresh TSB which was subsequently incubated for another 2 hours at 37^0C in a shaking water bath. An equal volume of donor and recipient cells (0.5 mL of each) was mixed and filtered onto a sterile 0.45 um membrane filter (type HA) (Millipore Canada Ltd.).The membrane filter was placed, cells side up, on tryptic soy agar, and incubated at 37^0C for 1 to 2 hours. The cells on the membrane were then resuspended in 1 mL of TSB. From the suspension, ten-fold serial dilutions were performed. One hundred uL of each dilution were plated on appropiate selective media and incubated overnight at 37^0C. Transconjugants were subcultured at least twice on appropiate selective medium to ensure phenotypic consistency.

Mobilization of non-conjugative plasmids

Non-conjugative plasmids were transformed into E. coli DH1 using the procedure of Lederberg and Cohen (1974). Conjugative plasmids belonging to different incompatibility

groups (Table 3-1) were introduced to the same cell by conjugation as described above. The bacterial strain now harbouring both the conjugative and non-conjugative plasmids was used as donor in the mobilization experiments. The same filter-mating technique as described above was used in the mobilization of non-conjugative plasmid by the presence of the conjugal plasmid. The frequency of mobilization was expressed as the number of transconjugants per donor.

RESULTS

Mobilization of pJD4 in E. coli by conjugal plasmids of different incompatibility groups

Ten conjugative plasmids belonging to different incompatibility groups were tested for their ability to mobilize pJD4 from E. coli DH1 to E. coli C600(rif^r). These plasmids were R124 (Inc FIV), R621a (Inc IY), R100 (Inc FII), R199 (Inc N), R6K (Inc X), RP4 (Inc P), R1drd16 (Inc FII), TP124 (IncH^e), S-a (Inc W), and pBG791 (Inc Iκ), (Table 3-1). When ampicillin and rifampicin were used as selective markers, three of the plasmids -- R124, R100 and pBG791 -- were able to mobilize pJD4 from E. coli DHI to E. coli C600 (rif^r) (Table 3-2). Plasmids R124 and R100 mobilized pJD4 at a frequency of 1.25 X 10^{-2} and 1.72 X 10^{-2} respectively, while pBG791 mobilized pJD4 at a frequency of 4.15 X 10^{-4} (Table 3-2). Conjugative plasmids R199, R621a, R1drd16, Tp124 and S-a were unable to mobilize pJD4 from E. coli DH1 to E. coli C600 (rif^r).

Since plasmids R6K and RP4 were resistant to ampicillin, as was pJD4, ampicillin was not used as a selective marker to detect the ability of R6K and RP4 to mobilize pJD4. Plasmid pJD4AC , a derivative of pJD4 having the 1.2 HaeII fragment containing the chloramphenicol resistance determinant of pACYC184 cloned at the unique PvuII site of pJD4 (46), was resistant to ampicillin and chloramphenicol. Since R6K and

Table 3-2 Mobilization frequencies of pJD4 by conjugative lasmids belonging to different Inc groups.

Plasmids in Donor strain (Inc)	Selective marker	Frequency[a] per donor	Ratio[b]
pJD4/R124 (FIV)	Rif Amp lac	1.25×10^{-2}	7.4×10^{-3}
pJD4/R100 (FII)	Rif Amp lac	1.72×10^{-2}	1.6×10^{-2}
pJD4/pBG791 (Iα)	Rif Amp lac	4.15×10^{-4}	3.3×10^{-4}
pJD4/R621a (Iγ)	Rif Amp lac	$< 10^{-8}$	--
pJD4/R199 (N)	Rif Amp lac	$< 10^{-8}$	--
pJD4/R1drd16 (FII)	Rif Amp lac	$< 10^{-8}$	--
pJD4/TP124 (H^e)	Rif Amp lac	$< 10^{-8}$	--
pJD4/S-a (W)	Rif Amp lac	$< 10^{-8}$	--
pJD4AC/RP4 (P)	Rif Cm lac	1.5×10^{-2}	6.8×10^{-3}
pJD4AC/R6K (X)	Rif Cm lac	$< 10^{-8}$	--

[a]Data presented were the average of at least three independent experiments.

[b]Ratio is the transfer frequency of the plasmid being mobilized divided by the transfer frequency of the conjugative plasmid in the same experiment.

RP4 do not code for chloramphenicol resistance, the mobilization of pJD4AC by these plasmids would indirectly indicate their ability to mobilize pJD4. When chloramphenicol and rifampicin were used as selective markers, only RP4 mobilized pJD4AC from DH1 to C600 at a frequency of 3.2×10^{-3}.

Location of the region required for the mobilization of pJD4

In order to identify the region required for the mobilization of pJD4, different DNA fragments of pJD4 were cloned into pACYC184 using the strategy outlined in Figure 3-1. Digestion of plasmid pJD4 with restriction endonucleases BamHI and HindIII, generated three fragments - 3.1 kb (fragment I), 2.4 kb (fragment II) and 1.7 kb (fragment III). The recombinant plasmid obtained by cloning the 2.4 kb fragment into pACYC184 at the unique BamHI site was named pACJDII. Those obtained by cloning the 3.1kb and the 1.7 kb fragments, at the BamHI-HindIII sites, were named pACJDI and pACJDIII, respectively. Confirmation of the clones is shown in Figure 3-2.

Plasmid pACYC184 was mobilized at very low frequency (10^{-6} to 10^{-8}) by pBG791 (Table 3-3). Mating experiments were performed to detect which of the plasmids pACJDI, pACJDII and pACJDIII could be mobilized by pBG791 at a frequency characteristic of pJD4 (10^{-4}). Plasmid pBG791 was first introduced by conjugation into DHI which already harboured

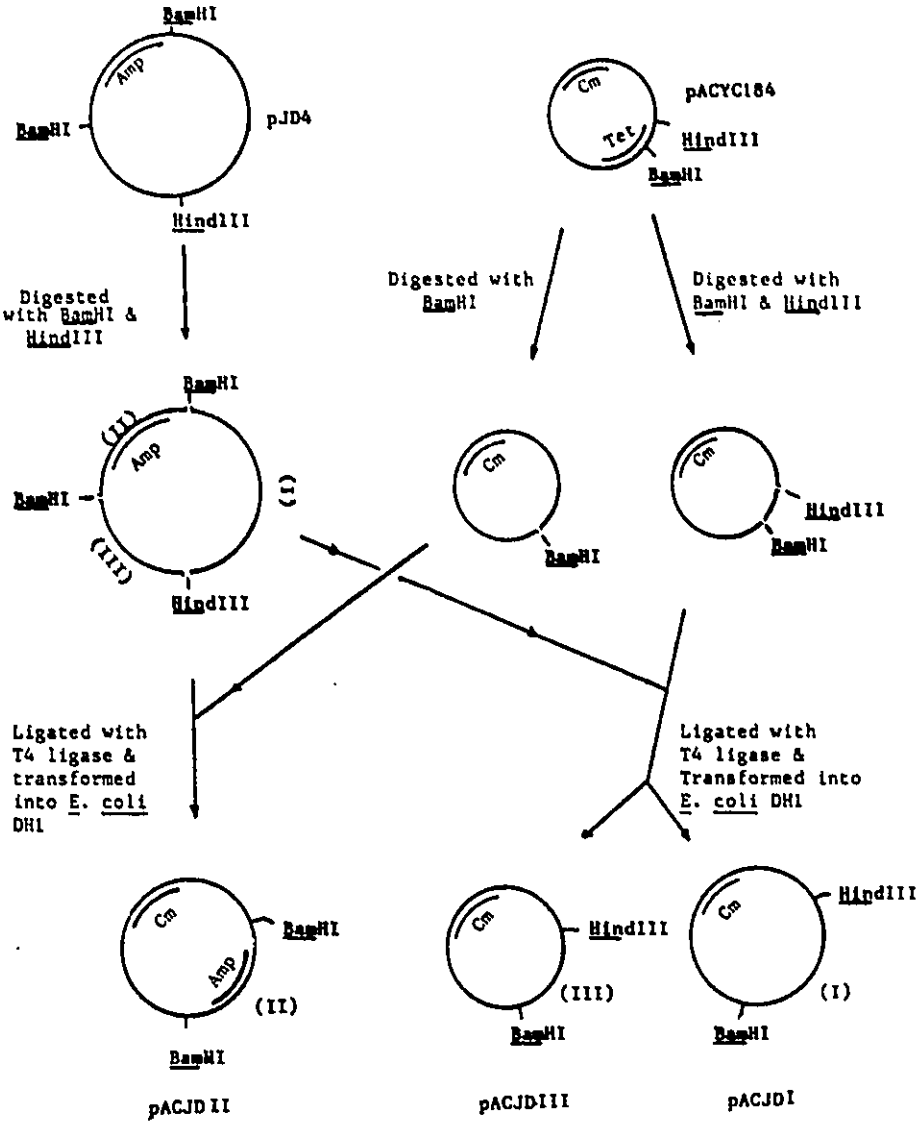


Figure 3-1 Strategy for the cloning of different fragments from pJD4 into pACYC184.

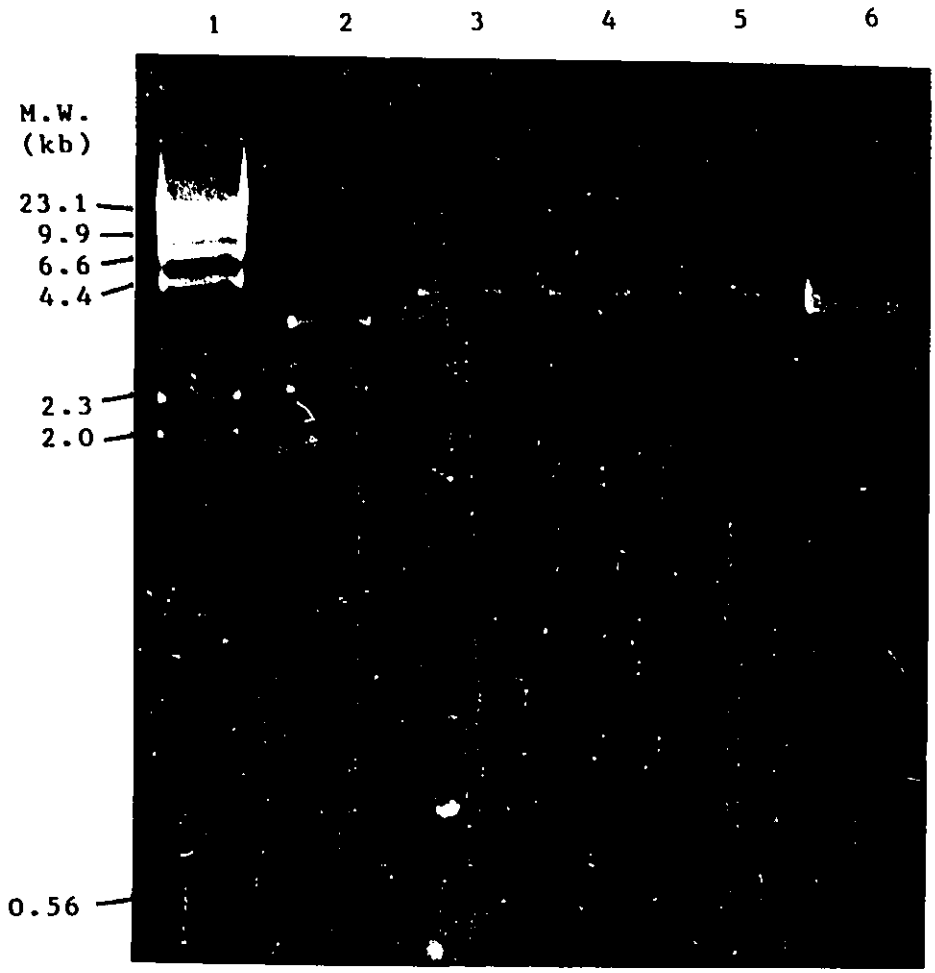


Figure 3-2 Restriction endonuclease analysis of pJD4, pACJDI, pACJDII, pACJDIII and pACYC184. Plasmid pJD4 (lane 2), pACJDI (lane 3), pACJDII (lane 4) and pACJDIII (lane 5) digested with BamHI-HindIII. HindIII digested λ was used as molecular weight markers (lane 1). Samples were run on a 1.5 % agarose gel.

either pACJDI, pACJDII or pACJDIII. Mobilization of these plasmids by pBG791 was then tested by mating with C600 (rif^r). Plasmid pACJDII, containing the 2.4 BamHI fragment (II) of pJD4, was mobilized by pBG791 at a frequency (10^{-4}) similar to that of pJD4, whereas pACJDI and pACJDIII were mobilized at frequencies of 10^{-7} and 10^{-6} respectively, a frequency shared with pACYC184 (Table 3-3). Therefore, the 2.4 kb BamHI fragment of pJD4 carried functions required for the mobilization of this plasmid. This fragment contained a 1.4 kb portion of TnA which has never been shown to be responsible for mobilization. Therefore, it was concluded that the region required for the mobilization of pJD4 was located within the remaining 1 kb fragment, designated region 'M' (Figure 3-3).

To ascertain whether the cloned 2.4 kb BamHI fragment, and thus region 'M', was required in cis or in trans in the mobilization of plasmid pACJDII, the following assumptions were made. If the cloned 2.4 kb BamHI fragment was required in trans, the presence of pJD4 would increase the frequency of mobilization of pACYC184 by pGB791. On the other hand, if this fragment was required in cis, the frequency of mobilization of pACYC184 would remain the same. Plasmid pACYC184 was transformed into DHI already containing pJD4 and pBG791. After mating with C600 (rif^r), the frequencies of mobilization of both pACYC184 and pJD4 were unchanged (Table 3-3). Thus, fragment 'II' (the 2.4 kb BamHI fragment) with

Table 3-3 Mobilization of pACYC184 with cloned pJD4 fragments by pBG791 (IncIα) from DH1 to C600(Rif^r)

Donor	Selective markers	Frequency[a] per donor	Ratio[b]
pJD4	Amp Rif lac	4.1×10^{-4}	3.3×10^{-4}
pACYC184	Cm Rif lac	1.2×10^{-6}	6.6×10^{-7}
pJD4/pACYC184	Cm Rif lac	4.8×10^{-7}	3.0×10^{-7}
	Amp Rif lac	11.7×10^{-4}	7.3×10^{-4}
pACJDI	Cm Rif lac	1.4×10^{-7}	1.0×10^{-7}
pACJDII	Cm Rif lac	2.0×10^{-4}	1.5×10^{-4}
pACJDIII	Cm Rif lac	3.2×10^{-6}	2.2×10^{-6}

[a]Data presented were the average of at least three individual experiments.

[b]Ratio is the transfer frequency of the plasmid being mobilized divided by the transfer frequency of the conjugative plasmid.

the region 'M' was required in cis and therefore contained the origin of transfer of pJD4.

Mobilization of deletion derivatives of pJD4 by pBG791

To determine whether region 'M' was the only region responsible for the mobilization of pJD4, plasmid pBG791 was used to mobilize deletion derivatives of pJD4 (Figure 3-3; chapter two; Yeung and Dillon, 1988). Conjugative plasmid pBG791 was able to mobilize both naturally-occurring (pJD5 and pJD7) (Chapter One; Yeung et al., 1986) and in vitro-derived deletion mutants (pJD6, pJD8 and pJD9) (Chapter Two; Yeung and Dillon, 1988) of pJD4 from E. coli DH1 to E. coli C600 (rif^r). All derivatives of pJD4 were mobilized at frequencies of 10^{-4} - 10^{-5} (Table 3-4), which were comparable to those of pJD4.

Figure 3-3 Restriction endonuclease maps of pJD4, pJD5, pJD6, pJD7 and pJD9, showing deleted regions as compared to pJD4. ▨ indicates the region encoding beta-lactamase. ⊢---⊣ indicates the region sequenced by Sanchez-Pescador *et al.* (1988). ↓ indicates the site where the sequence TGGCTTA, containing the *nic* site of *Col*E1, was located. Abbreviations: A, *Ava*I; B, *Bam*HI; Hd, *Hin*dIII; Hf, *Hin*fI; Pt, *Pst*I; Pv, *Pvu*I; T, *Taq*I; X, *Xba*I.


M
B X T Hf Pt T Hf B T Pv Hf T A T Hf Hd T B
0 1 2 3 4 5 6 7
pJD4 (7.2 kb)
pJD7 (4.9 kb)
a
pJD9 (3.4 kb)
pJD5 (5.1 kb)
pJD6 (3.9 kb)

Table 3-4 Mobilization of pJD4 deletion derivatives by pBG791 (IncIα) from DH1 to C600(Rif^r)

Plasmid mobilized	Selective markers	Frequency[a] per donor	Ratio[b]
pJD5	Rif Amp lac	4.0×10^{-4}	5.7×10^{-4}
pJD6	Rif Amp lac	1.8×10^{-4}	2.5×10^{-4}
pJD7	Rif Amp lac	5.0×10^{-5}	1.0×10^{-4}
pJD8	Rif Amp lac	5.0×10^{-5}	8.3×10^{-5}
pJD9	Rif Amp lac	4.7×10^{-5}	9.4×10^{-5}

[a]Data presented was the average of at least three individual experiments.

[b]Ratio is the transfer frequency of the plasmid being mobilized divided by the transfer frequency of pBG791 in the same experiment.

DISCUSSION

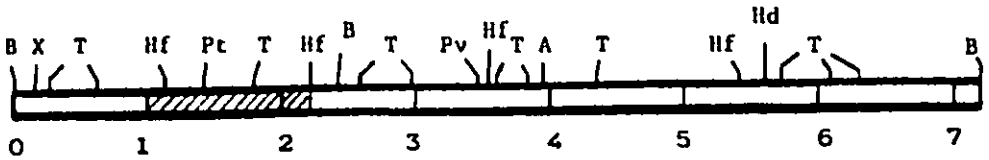
It has previously been reported that IncP plasmids, pRK231, pRK2013 and R751 could mobilize the 7.2 kb plasmid from E. coli to S. minnesota and H. influenzae (Guiney and Ito, 1982). In the present study, in addition to IncP plasmid RP4, three other conjugative plasmids, R124 (IncFIV), R100 (IncFII) and pBG791 (IncIα), were able to mobilize pJD4, a 7.2 kb penicillinase-producing plasmid from N. gonorrhoeae, between E. coli strains. Despite a frequency of 3×10^{-5}, Guiney and Ito (1982) reported that R64drd11 (IncIα) did not mobilize the 7.2 kb plasmid. Evidently, they were using 10^{-4} as the lowest acceptable frequency of mobilization. In the present study, plasmid pBG791 (IncIα) was able to mobilize pJD4 at a frequency of 4.2×10^{-4}.

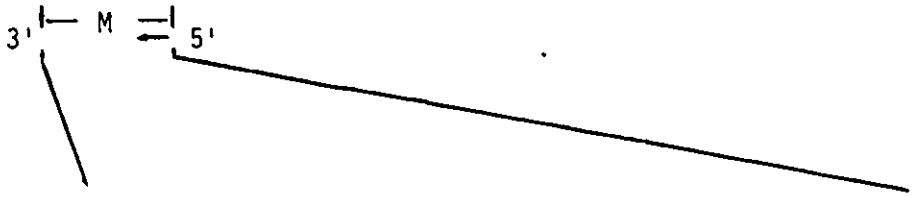
In this study, R100 was able to mobilize pJD4 efficiently at a frequency of 10^{-2}. Guiney and Ito (1982) found that R100drd1, a derepressed mutant of R100, was unable to mobilize the 7.2 kb plasmid (1982). Plasmid R100 is known to dissociate and reassociate with the mediation of either IS1 or IS2 (Rownd et al., 1978; Timmis et al, 1978). Since the plasmid R100 used in the present study has been circulated in different research laboratories, it is possible that the R100 used in the present study is different from the R100drd1 used by Guiney and Ito (1982). Conversely, the possibility that the drd mutation of R100 might have affected its ability to mobilize pJD4 cannot be discounted.

To be mobilized, ColE1 and other non-conjugative plasmids, such as RSF1010 and R1162, require both a specific region of DNA (the 'bom' site or OriT) and a plasmid-encoded protein (Brasch and Meyer, 1987; Derbyshire et al., 1987; Finnegan and Sherratt, 1982; Warren et al., 1979; Willetts and Wilkins, 1984). It has been reported that the 1.0 kb 'M' region fragment of a 7.2 kb penicillinase-producing gonococcal plasmid encoded a 41 kilodalton (kDa) protein (Tenover et al., 1985), which might be the protein required for the mobilization of pJD4. The 1.86 kb HincII-BamHI fragment, containing the 'M' region, of the 7.2 kb plasmid has recently been sequenced by Sanchez-Pescador et al. (1988). The sequence was kindly supplied to us in prepublication (Figure 3-4). Our analysis of this 'M' region sequence indicated that it does not contain an open reading frame (ORF) for a 41 kDa protein. Instead, it contains three ORFs encoding hypothetical proteins of 2.2 kda, 5.4 kda and 4.8 kda. The possibility that these hypothetical proteins were produced and involved in the mobilization of pJD4 is under further investigation.

In comparing the DNA sequence of the 'M' region to OriT sequences of pBR322 (the same as ColE1), R1010 and RK2, several regions of sequence similarity were found (Figure 3-4). The 'M' region contained two sequences, ACCCAGT and TGGCTTA, which were found within the 56 bp 'bom' region of pBR322 and ColE1 (Covarrubias et al., 1981; Willetts and

Figure 3-4 Nucleotide sequence of the 'M' region. This sequence was kindly provided by Dr. R. Sanchez-Pescador. Sequence analysis was performed using the 'Basic Program for sequence analysis' developed by W. Schwindinger. Sequence underlined and designated: n' = the recongnition sequence for the n'-protein; nic = the sequence containing the nic site in ColE1; 3 = sequence found in the OriT of ColE1; 1 = the sequences, with only one mismatch, similar to the CCTACTT sequences found in RK2 and RSF1010; 2 = sequence having one mismatch as compared to sequence GGTTTCTC which was required for the mobilization of RSF1010; IR = the sequences very similar to the inverted repeats found in R100; IHF = recognition sequences for the integration host factor (IHF).






```
5'...TATCTATAAACTCTTGGCTTGGTTCTAATCCCTCTAAACGATTATTATCAATAGCCGCTC  60
     ATAGATATTTGAGAACCGAACCAAGATTAGGGAGATTTGCTAATAATAGTTATCGGCGAG

     TAACCGCTTTTTCTCGGCTTAATTTTTCTGTCTCTGTTATAAAATTGCTTATTCATTCTT 120
     ATTGGCGAAAAAGAGCCGAATTAAAAAGACAGAGACAATATTTTAACGAATAAGTAAGAA
        n'                    1
     GTTCTTCTTTCAAAAAAAAGTTAAGTAAAATACCTACCTAAATTTTTACTAGTTGCAAT 180
     CAAGAAGAAAGTTTTTTTTCAATTCATTTTATGGATGGATTTAAAAATGATCAAGCGTTA
                                                        1
     CTACGAGCTTATAACCTCGTTTTTTCAATTCATTTAAAAAAATCAGATTTTGAGCCTAATT 240
     GATGCTCGAATATTGGAGCAAAAAAGTTAAGTAAATTTTTTAGTCTAAAACTCGGATTAA
                          3
     TGATCTATTGCTATCGTTACCCGCTAGAAATACCCAGTAATTACGCAAATCTTCATTGGT 300
     ACTAGATAACGATAGCAATGGGCGATGTTTATGGGTCATTAATGCGTTTAGAAGTAACCA
                                          IR
     AACTTTCGTAATATCTGTGTAATGATCTTCGAGTATTTTTAAGCAATCTCTAGCCCATAA 360
     TTGAAAGCATTATAGACACATTACTAGAAGCTCATAAAAATTCGTTAGAGATCGGGTATT
          IR                                                   1
     ACCGTACTCGTGATTGCTCATCTTAGGGTTTTGCTTATCGAGTTTGACGAACTTCCCATA 420
     TGGCATGAGCACTAACGAGTAGAATCCCAAAACGAATAGCTCAAACTGCTTGAAGGGTAT

     CTTGTTTTTATGTGGAAATACTGGCCGTTTGCAACTTCTTCAATTTTTTGAGCTGTTCGT 480
     GAACAAAAATACACCTTTATGACCGGCAAACGTTGAAGAAGTTAAAAAACTCGACAAGCA

     TTTTTACTACCAATCACAAAATTTAAAGAGTGAATAGTACGCCCACGCTTGATTTGTTCA 540
     AAAAATGTTGGTTAGTGTTTTAAATTTCTCACTTATCATGCGGGTGCGAACTAAACAAGT
                2                                          IHF
     ACCTCAACGACTAAATCAGATTTCTCGTTAATCTCAGTTATTGCAGGTTCCAAAACACGT 600
     TGGAGTTGCTGATTTAGTCTAAAGAGCAATTAGAGTCAATAACGTCCAAGGTTTTGTGCA

     TGATTTAATGAATTAAATCTAGGTATTATTCAACCTGAAGCCATTCTTTAGTTTTCTACT 660
     ACTAAATTACTTAATTTAGATCCATAATAAGTTGGACTTCGGTAAGAAATCAAAAGATGA
                    IHF
     GTAATTTCACGACTACCAACAGAGCGATATTGTGTAATTAGCTCATAAATTCGAATTGAA 720
     CATTAAAGTGCTGATGGTTGTCTGCTATAACACATTAATCGAGTATTTAAGCTTAACTT

     TGTACACTGTTGAAATAAGCGATATGTTTGAGTTGATATTGCGTGAATTGCCCTTTAAGT 780
     ACATGTGACAACTTTATTCGCTATACAAACTCAACTATAACGCACTTAACGGGAAATTCA

     TGCGTTAGGTATGGCATAACTTCATCAGTCATTGCAATTCTAAAACGCCCCTCTTTCTGA 840
     ACGCAATCCATACCGTATTGAAGTAGTCAGTAACGTTAAGATTTTGCGGGGAGAAAGACT

     AATATGTTCTAGAGGAAACCCAACGAAATTCAGTTACACGGTCTTTATCTTCAGTTTTAA 900
     TTATACAAGATCTCCTTTGGGTTGCTTTAAGTCAATGTGCCAGAAATAGAAGTCAAAATT
                                                        nic
     CACTTCGGTCATAAATCCGTTTTATAGCCGCCTGAATTTGCTTATAGGCGTTATCTTGGC 960
     GTGAAGCCAGTATTTAGGCAAAATCTGGCGGACTTAAACGAATATCCGCAATAGAACCG
                                                IHF
     TTATTTCTGGAAACTCACGGACAAAATCAGCCACCGTAAAATCAAAAATTTTTTGATTAG 1020
     AATAAAGACCTTTGAGTGCCTGTTTTAGTCGGTGGCATTTTAGTTTTTAAAAAACTAATC
                                                IHF
     ATTTCGGATCC...3'                                              1031
     TAAAGCCTAGG
```

Wilkins, 1984). The sequence TGGCTTA has been determined to be the nic site of ColE1. In addition, it contained the recognition site AAGCGG for the n' protein, which was found in the OriTs of F and ColE1 (Willetts and Wilkins, 1984). These sequence similarities provided some evidence that the mechanism of mobilization of pJD4 could be similar to that of ColE1. In addition, it contained three 7 bp sequences CCTACCT, CCTAATT, and CATACTT, each having one base pair mismatch as compared to the CCTACTT sequence reported in RK2 and RSF1010 and which was suspected as being the recognition site of a mobilization protein (Derbyshire and Willetts, 1987). The sequence GATTTCTC which has one base pair mismatch as compared to the GGTTTCTC sequence required for the mobilization of RSF1010 (Derbyshire and Willetts, 1987) was also found in the region 'M'.

Further analysis of the DNA sequence of the 'M' region (Figure 3-4) revealed that it also contained a pair of inverted repeats AGCAATCTCTAG and CTCGTGATTGCT very similar to sequences AGCAATCGGC and GCAGATTGCT, which were found in R100 (McIntire and Dempsey, 1987). It has recently been reported that the integration host factor (IHF) encoded by the chromosomal genes himA and hip, was required for the transfer of R100 and the replication of pSC101 (Gamas et al., 1986; McIntire et al., 1987). IHF recognizes the consensus sequence C/TAANNNNTTGATA/T (Gamas et al., 1986; Leong et al., 1985; McIntire and Dempsey, 1987). Four such consensus

sequences, AAACACGTTGATT, TAATACCTAGATT, AAATTTTTTGATT and AAAATTTTTGATT, with only one mismatch were found in the region 'M'. Therefore, plasmid R100 should be able to mobilize pJD4 as indicated in the present study.

The significance of these sequence similarities between the region 'M' and the OriT regions of R100, ColE1, pBR322, REF1010 and RK2 remains to be established. They suggested that the 7.2 kb plasmid has, through evolution, acquired the potential of being mobilized by a wide-range of conjugative plasmids.

Plasmid pJD4 contained two replication regions and thus two origins of replication (OriV) (Chapter two; Yeung and Dillon, 1988). Our finding that there were also two OriT on pJD4 was consistent with other observations that OriV and OriT are usually located physically close to one another (Norheim et al., 1980), and that plasmids such as R6K, which has two OriV, contained a nic site (OriT) close to each of the OriV. In addition to region 'M', it was observed that region 'a', which was essential for the replication and maintenance of pJD9 (a deletion derivative of pJD4) (see chapter two), was also responsible for the mobilization of pJD4.

The ability of pBG791 to mobilize pJD5 and pJD6, both of which contained the 1.0 kb M region but not the replication region 'a' (Figure 3-3), was in good agreement with the present finding that region 'M' was required for the

mobilization of pJD4. The mobilization of pJD8 and pJD9, both lacking the region 'M' (Figure 3-3), strongly suggested that there was another mobilization region. This region was most likely located within region 'a', which also contained a replication region (OriV) (Chapter two; Yeung and Dillon, 1988). The second mobilization region was not detected in the cloning experiment. This could be due to either the truncation of the plasmid specific mobilization protein or the separation of the second OriT with the DNA sequence encoding for the mobilization protein. These findings supported a previous observation that OriT was located on the 1.7 kb BamHI-HindIII fragment of the 7.2 kb plasmid (McNicol et al., 1983). Since plasmid pJD5, a 5.1 kb plasmid of N. gonorrhoeae, did not contain region 'a', it should not contain the second OriT as indicated by previous researchers (McNicol et al., 1983).

The mobilization of the 7.2 kb plasmid from N. gonorrhoeae to other bacterial species, such as E. coli, has been demonstrated previously (Flett et al., 1980). However, it was not until the discovery and isolation of a penicillinase-producing N. meningitidis (Dillon et al., 1983) that the possibility of N. gonorrhoeae itself serving as a reservoir for the dissemination of the 7.2 kb plasmid was seriously investigated. Genco et al. (1984) observed that the 39.2 kb conjugal plasmid of N. gonorrhoeae was unable to transfer the 7.2 kb plasmid to N. meningitidis, and concluded

that such a possibility was very unlikely. However, in a subsequent study using clinical isolates of N. gonorrhoeae containing both 39.2 kb and 7.2 kb plasmids, Ikeda et al. (1986) were able to demonstrate that in 15% of the isolates, the 7.2 kb plasmid could be transfered to N. meningitidis through conjugation. In the same study, those 39.2 kb conjugal plasmids which were unable to mobilize the 7.2 kb plasmid into N. meningitidis, were able to mobilize the 7.2 kb plasmid to E. coli. These observations could only be explained if there were two mechanisms by which the gonococcal conjugative plasmids could mobilize the 7.2 kb penicillinase-producing plasmid in N. gonorrhoeae as implicated in this study, and that one of the mechanisms was non-functional in those cases where the 7.2 kb plasmid was not mobilized into N. meningitidis. In view of this, the possibility that N. gonorrhoeae acted as a potential reservoir for the dissemination of this plasmid to other Neisseria species should be reevaluated.

CONCLUSIONS

Since the discovery of the 7.2 kb and 5.1 kb ß-lactamase-producing plasmids in N. gonorrhoeae, researchers have been attempting to identify the origin of these two plasmids and their relatedness. These two plasmids have been shown to be homologous, except for a 2.1 kb fragment which was absent in the 5.1 kb plasmid (this thesis; Brunton et al., 1986). There has never been agreement on the exact location of this 2.1 kb fragment on the 7.2 kb plasmid, until the data presented in chapter one of this study was published (Yeung et al., 1986) and confirmed (Aalan et al., 1987).

The 'Toronto' plasmid and the 'Rio' plasmid were shown to have identifical restriction patterns. Since the 'Toronto' plasmid was involved in an outbreak of PPNG, it was suggested that the novel 4.9 kb (or 4.6 kb) plasmid be named 'Toronto' plasmid.

Plasmid pJD4 was the smallest plasmid shown to contain two replication regions and the presence of these two replication regions may explain the wide host-range of the gonococcal 7.2 kb ß-lactamase plasmids. The location of replication region 'a' (Figure C-1), one of the two replication regions, confirmed the findings of Johnson (1985). DNA sequence analysis showed that region 'a' contained seven sets of more than three direct repeats, a characteristic of plasmids, such as pSC101, F, P1 and R6K,

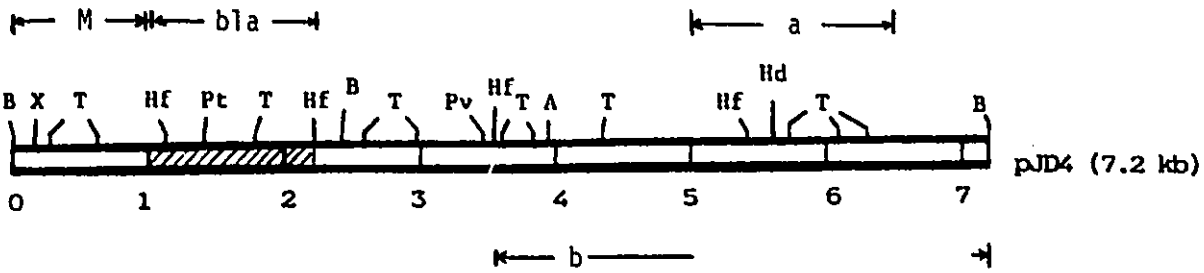


Figure C-1. Genetic map of the 7.2 kb B-lactamase-producing plasmid pJD4. -bla- indicates the region encoding the ß-lactamase gene; -a- indicates replication region 'a'; -b- indicates the replication region 'b'; -M- indicates the region required for mobilization <u>in cis</u> by conjugative plsmid pBG791.

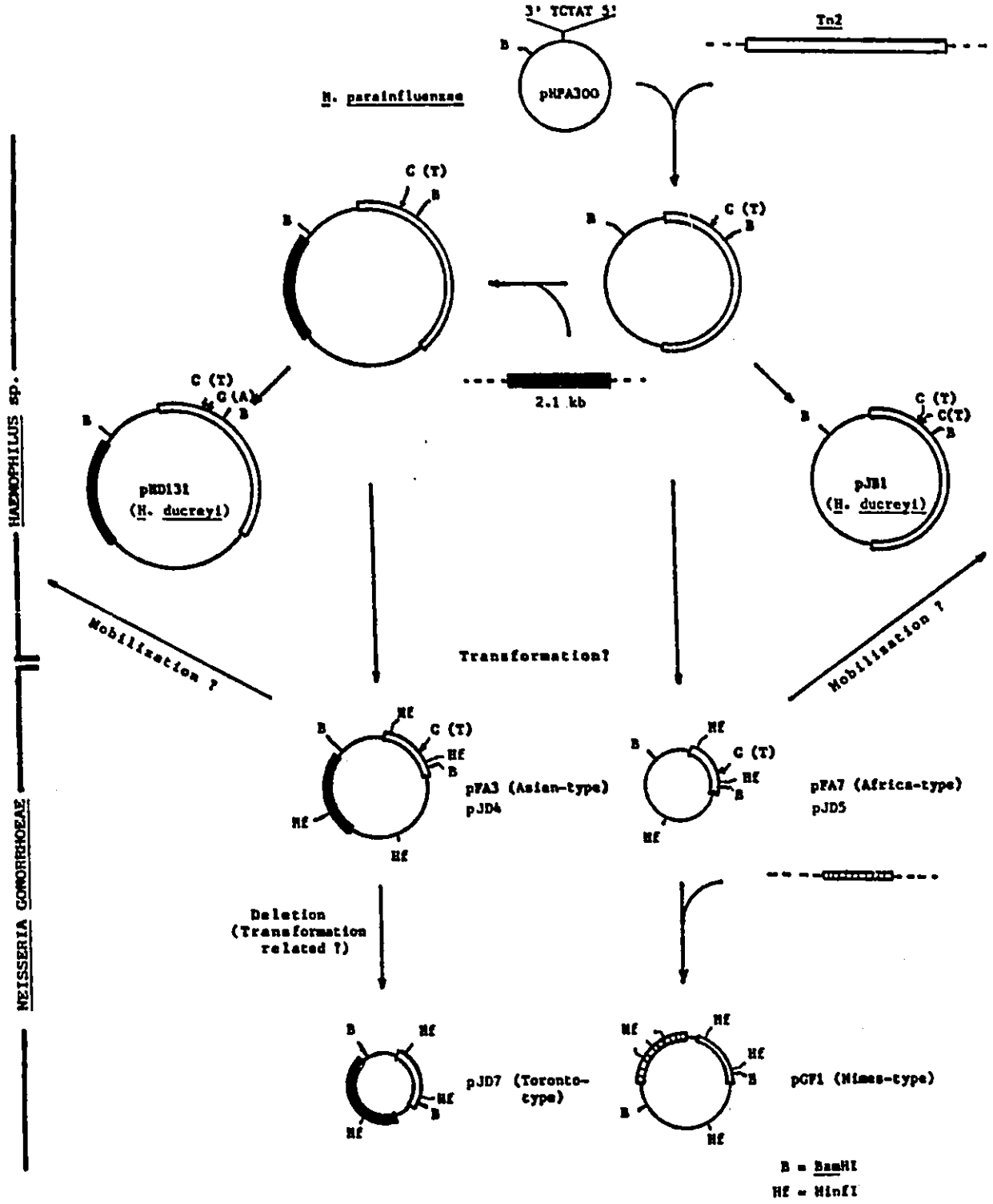
that code for plasmid-specific initiator proteins. In addition, sequences similar to dnaA box and the IHF recognition sequence which were required for the replication of pSC101 were also detected. In view of these similarities, the replication mechanism of region 'a' could be very similar to that of pSC101. Recently, Bramhill and Kornberg (1988) proposed that replicons with oriV containing AT-rich repeats employed a mechanism of initiation similar to that of oriC. Therfore, it was suggested that region 'a' probably employed a similar mechanism of initiation.

A 1.0 kb region ('M', Figure C-1) of pJD4 was shown to be required in cis for its mobilization by conjugative plasmid pBG791 (IncIα). The entire previously published DNA sequence of this region was analysed. It contained sequences that are homologous or similar (with one or two base-pair difference) to those found in the oriT and shown to be essential in the mobilization or transfer of other plasmids, such as RK2, ColE1, and R100. The significance of these sequences in pJD4 is not known. However, it appeared that pJD4, through evolution, has acquired the potential of being mobilized by a wide-range of conjugative plasmids. Such a result has not been previously noted. In addition, the location of a second mobilization region (within replication region 'a') on pJD4 was also identified, which was consistent with the results obtained by McNicol et al. (1983).

Sox et al. (1979) speculated that the 5.1 kb plasmid could have been derived from the 7.1 kb plasmid through a transformation-related deletion. The isolation and characterization of a novel 4.9 kb (Toronto) plasmid described in this study, which was directly related to the 7.2 kb plamsid but not to the 5.1 kb plasmid, appeared to support their hypothesis. However, the discovery that there was a replication region ('a') located within another replication region ('b', Figure C-1) in pJD4 strongly suggested that the 7.2 kb plasmid could have been derived from the 5.1 kb plasmid with a 2.1 kb miniplasmid inserted within its replication region. This observation is further supported by the isolation of the 6.6 kb 'Nimes' plasmid, which was shown to be derived from the 5.1 kb plasmid by an insertion of a DNA fragment containg two HinfI sites..

The hypothesis that the ß-lactamase-producing plasmids in N. gonorrhoeae originated in Haemophilus (Brunton et al., 1982; Brunton et al., 1986; Cannon and Sparling, 1984; Sparling et al., 1980; Sparling et al., 1978) received strong support with the isolation of a small cryptic plasmid pHPA300 from H. parainfluenzae which was homologous to the non-TnA portion of the 5.1 kb penicillinase-producing plasmid (pFA7) in N. gonorrhoeae (Brunton et al., 1986). Recently, a model was proposed suggesting that the 7.2 kb and the 5.1 kb plasmids were derived separately from the progenitor plasmid pHPA300 (Chen and Clowes, 1987). In this model, transposon

Figure C-2. Evolutionary pathways of naturally occurring ß-lactmase-producing gonococcal plamsids. See text for detailed explanation.


HAEMOPHILUS sp.
NEISSERIA GONORRHOEAE
3' TCTAT 5'
Tn2
H. parainfluenzae
pHPA3OO
C (T)
B
2.1 kb
C (T)
G (A)
pHD131
(H. ducreyi)
C(T)
pJB1
(H. ducreyi)
Mobilization ?
Transformation?
Mobilization ?
Hf
pFA3 (Asian-type)
pJD4
pFA7 (Africa-type)
pJD5
Deletion
(Transformation
related ?)
pJD7 (Toronto-
type)
pGP1 (Nimes-type)
B = BamHI
Hf = HinfI

Tn2 transposed onto pHPA300 at the target site 5'-TATCT-3' (Figure C-2). At some early stage, cytosine was replaced by thymidine at 3773 bp from the left inverted repeat terminus of Tn2. Divergence in the evolution of this plasmid then occurred, as a 2.1 kb fragment, which has been shown to contain replication region 'a' (Chapter 2), was inserted resulting in the formation of two distinctive plasmids. These two plasmids probably evolved independently in Haemophilus and Neisseria species. Analysis of two such plasmids from H. ducreyi indicated that each underwent unique point mutations (C to T on pJB1; G to A on pHD131) (Figure C-2). These mutations did not appear on gonococcal ß-lactamase-producing plasmids, thereby inferring a separate evolutionary pathway. It seemed likely that gonococcal plasmids arose through the transformation of plasmid DNA from H. parainfluenzae or some unknown intermediate followed by the subsequently deletion of the left hand end of Tn2. Although one report noted the transfer of a 7.2 kb plasmid from H. parainfluenzae to N. gonorrhoeae, other groups have been unable to conjugally transfer plasmids from Haemophilus spp. to N. gonorrhoeae (Sparling et al., 1978). The gonococcus itself then acted as a reservoir for ß-lactamase-producing plasmids, transferring them to other N. gonorrhoeae and to Haemophilus spp., possibly through conjugation.

Although ß-lactamase-producing plasmids were also present in Haemophilus species, they appeared to be evolving

only in N. gonorrhoeae as novel, naturally-occurring derivatives of the 7.2 kb and the 5.1 kb plasmids have been detected only in N. gonorrhoeae. In fact, all the plasmids found in N. gonorrhoeae appeared to be evolving. Recently the 39.2 kb conjugal plasmid in N. gonorrhoeae was shown to have acquired the tetM determinant, and the 4.2 kb cryptic plasmid has obtained an insertion of ~200 basepairs within its cppB gene (Roy et al., 1988).

APPENDIX

Appendix A

Data used for the construction of restriction maps of pJD4, pJD5 and pJD7.

RESTRICTION ENDONUCLEASE	PLASMID	FRAGMENTS IN KILOBASE PAIRS						
		A	B	C	D	E	F	G
HindII (HincII)	pJD4	7.2						
	pJD5	5.2						
	pJD7	4.9						
PstI	pJD4	7.2						
	pJD5	5.2						
	pJD7	4.9						
BamHI	pJD4	4.8	2.4					
	pJD5	2.7	2.4					
	pJD7	2.5	2.4					
HpaII	pJD4	6.16	0.64	0.2	0.2			
	pJD5	3.9	0.64	0.2	0.2			
	pJD7	3.6	0.64	0.2				
HaeIII	pJD4	5.3	0.9	0.72	0.142	0.1		
	pJD5	3.1	0.9	0.72	0.142	0.1		
	pJD7	2.8	0.9	0.72	0.142			
HinfI	pJD4	3.08	1.9	1.2	1.1			
	pJD5	2.8		1.2	1.1			
	pJD7	3.08			1.1	0.56		
HindIII	pJD4	7.2						
	pJD7	4.9						
BamHI/HincII	pJD4	4.8	1.79	0.64				
	pJD5	2.7	1.79	0.64				
	pJD7	2.5	1.79	0.64				
HaeIII/BamHI	pJD4	4.7	0.89	0.72	0.62	0.125	0.1	
	pJD5	2.6	0.89	0.72	0.62	0.125	0.1	
	pJD7	2.5	0.89	0.72	0.62	0.125		
HaeIII/HincII	pJD4	5.3	0.72	0.46	0.4	0.142	0.1	
	pJD5	3.1	0.72	0.46	0.4	0.142	0.1	
	pJD7	2.8	0.72	0.46	0.4	0.142		
HpaII/BamHI	pJD4	4.8	1.36	0.64	0.2	0.2		
	pJD5	2.7	1.36	0.64	0.2	0.2		
	pJD7	2.5	1.36	0.64	0.2			
HpaII/HincII	pJD4	6.16	0.64	0.2	0.2			
	pJD5	3.9	0.64	0.2	0.2			
	pJD7	3.6	0.64	0.2				
HinfI/BamHI	pJD4	1.9	1.84	1.25	1.17	1.1		
	pJD5	1.6		1.25	1.17	1.1		
	pJD7	1.9			1.17	1.1	0.5	
HinfI/HincII	pJD4	2.98	1.888	1.2	0.88		0.19	
	pJD5	3.1		1.2	0.88		0.19	
	pJD7	2.98			0.88	0.56	0.19	
PstI/BamHI	pJD4	4.8	1.5	0.9				
	pJD5	2.7	1.5	0.9				
	pJD7	2.5	1.5	0.9				
PstI/HincII	pJD4	6.8	0.32					
	pJD5	4.7	0.32					
	pJD7	4.5	0.32					
HindIII/BamHI	pJD4	3.04	2.4	1.76				
	pJD7	1.8	2.4		0.7			
HindIII/HincII	pJD4	3.84	3.36					
	pJD7	3.84		1.1				
HindIII/HinfI	pJD4	2.9	1.9	1.2	1.1		0.208	
	pJD7	2.9			1.1	0.56		

APPENDIX B

Other observations: In vitro transcription/translation of plasmid pJD4

To better understand the role of plasmids in the biology of the gonococcus, several groups of researchers (Tenover et al., 1983; 1985; Biswas et al., 1986) have attemped to identify and examine proteins produced by gonococcal plasmids, particularly the gonococcal R plasmid. Using 'In vitro transcription/translation kit' from Amersham Canada Ltd., and pJD4 as template, in vitro DNA-directed protein synthesis experiments were performed to detect proteins encoded by pJD4. The results were compared to those published (Table A-1).

The total molecular weight of proteins detected with pJD4 was 427 kda. The theoretical maximum coding capacity of pJD4 was only 240 kda. This discrepancy could be due to some of the proteins bands were precursors of the others. When compared to proteins detected and published by other researchers (Table A-1) using the 7.2 kb ß-lactamase-producing gonococcal plasmid as template, all proteins (except for the low molecular weight proteins) detected in the present study were detected by one or the other research group. Therefore, the proteins detected using the in vitro

Table B-1 Comparison of pJD4 transcription/translation products to those observed by other research groups

This study	Tenover et al. (1983)	Tenover et al. (1985)	Biswas et al. (1986)
56.5*			55
43	43	43	
41	41	41	41
			40
36			36.5
31	30	30	
28		28	29
26		26	
24		24	
22		20	
			18
17.5			17
16.5	16	16	
		15	
	14	14	14
12.8			12
10.8			
8.8			
7.8			
6.5			
5.5			

* molecular weight in kilodalton

transcription/translation system were consistent with those detected by other researchers using mini-cell (Tenover et al., 1985) or maxi-cell systems (Biswas et al., 1986).

In order to locate the genes encoding for the proteins detected, in vitro DNA-directed protein synthesis experiments were performed using deletion mutants (pJD5, pJD6, pJD7 and pJD8, see chapter one and two) of pJD4 as templates. As indicated in Table A-1, some proteins varied in their appearance from one experiment to another, possibly indicating that they may be incomplete proteins or precursors of other proteins. No new protein bands were observed relative to the pJD4 control in any of the in vitro protein synthesis experiments, therefore it appeared that no fused protein was produced by any of the other plasmids.

Using the proteins produced by pJD4 as control, protein patterns produced by the other four plasmids were analysed on 12, 15 and 20% polyacrylamide-sodium dodecyl sulfate gels. The molecular weight of 12 proteins (of a total 18) produced in vitro by pJD4 could be determined on 12% gels (Figure A-1, lane 2). In this particular gel, three proteins (26 kda, 17.5 kda and 16.5 kda) produced by pJD4 were present in very small amount. They were only detectable after three days exposure. Although no obvious differences were noted between proteins produced by pJD4 and pJD5 on a 12% gel (Figure A-1, lanes 2 and 3), a 56.5 kda protein produced by pJD4 was found to be absent in pJD7 (Figure A-1, lane 4) and pJD6

Table B-2 Molecular weights of transcription/translation products of pJD4, pJD5, pJD7, pJD6 and pJD8.

pJD4	pJD5	pJD7	pJD6	pJD8
56.5	56.5	-- [1]	-- [1]	56.5
43	43	43 [2]	43 [2]	43
41	41	41	41 [2]	41
36	36	36	36	-- [1]
33	33	33	33	33
31 [3]	31 [3]	31 [3]	31 [3]	31 [3]
28	28	28	28	28
26 [2]	26	26	26	26
24.5	24.5	24.5	24.5	-- [1]
22	22	22 [2]	22 [2]	22
17.5 [2]	17.5 [2]	17.5 [2]	17.5 [2]	17.5 [2]
16.5 [2]	16.5 [2]	17.5 [2]	17.5 [2]	17.5 [2]
12.8	12.8	12.8	12.8	12.8
10.8	10.8	-- [1]	10.8	10.8
8.8	8.8	8.8	8.8	8.8
7.8	-- [1]	7.8	-- [1]	7.8
6.5	6.5	-- [1]	6.5	6.5
5.5	5.5	5.5	5.5	5.5
Total 427.2	419.4	353.4	362.9	402.7

[1] Not detected.

[2] Intensity and appearance of the protein band on autoradiogram vary from experiment to experiment.

[3] Using pAt153 as control and from observations of other researchers, this band was identified as B-lactamase.

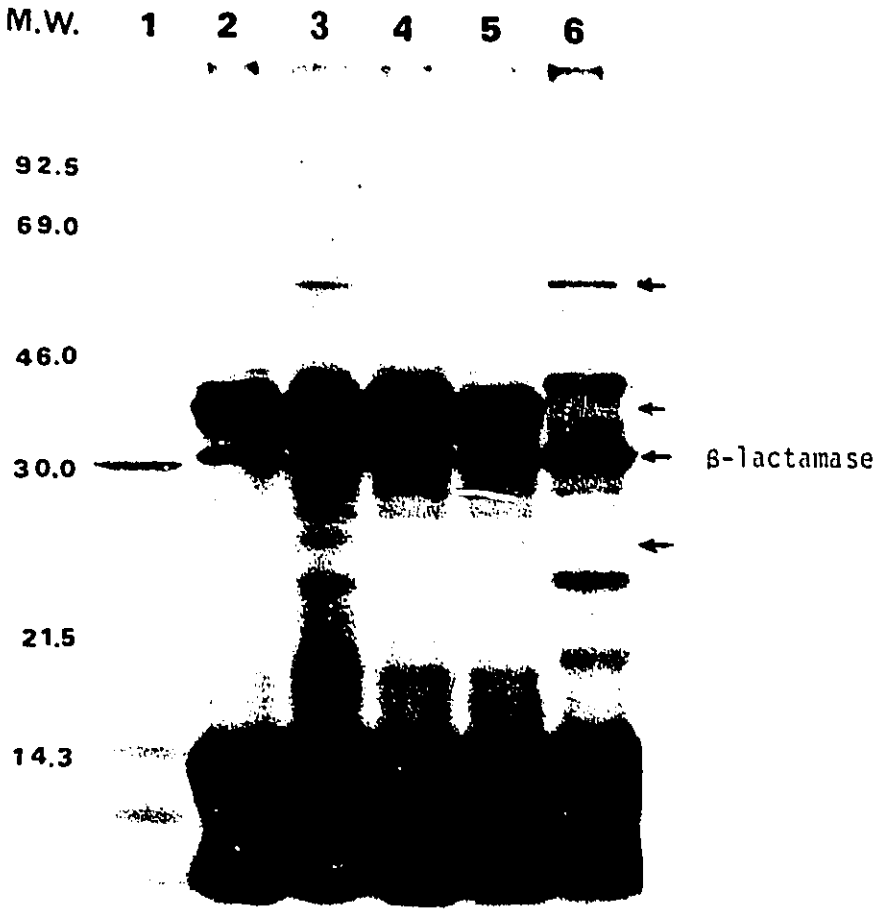


Figure B-1 In vitro translation products of plasmids pJD4, pJD5, pJD7, pJD6 and pJD8. ^{35}S-labeled proteins produced were separated on 12% polyacrylamide gels and then autoradiographed. Lane 1, ^{14}C-labeled molecular weight markers in thousands (Amersham Canada Ltd.); lane 2, protein specific to pJD4; lane 3, proteins specific to pJD5; lane 4, proteins specific to pJD7; lane 5, proteins specific to pJD6; lane 6, proteins specific to pJD8. Arrow indicates protein band missing from one or the other samples.

(Figure A-1, lane 5). Likewise, proteins of 36K and 24.5K were not produced in vitro by pJD8 (Figure A-1, lane 6). The difference between pJD4 and pJD5 became obvious after the same samples were analysed on a 20% polyacrylamide gel (not shown). Six additional protein bands of low molecular weight were detected in pJD4. One of them, a 7.8 kda protein, was not produced by pJD5 (Table A-1). Analysis on the 20% gel also indicated that proteins of 10 kda and 6.5 kda were not coded by pJD7, nor was a 7.8 kda protein produced by pJD6 (Table A-1). In all of the in vitro protein synthesis experiments performed, two proteins (31 kda and 12.8 kda) were found to be very prominent. From previous publications (Tenover et al., 1983; 1985; Biswas et al., 1986), the 31 kda protein was determined to be the ß-lactamase protein.

In vivo protein synthesis with pJD4 and its derivatives using mini-cell and maxi-cell systems were very unsatisfactory. Either there was a very high background or no protein bands were detected al all.

Only two other groups of investigators (Dickgiesser, 1983; Tenover et al., 1983) performed in vitro DNA-directed transcription/translation studies with the 7.2 kb and the 5.1 kb plasmids. Tenover et al. (1983) observed that the 7.2 kb plasmid produced 10 proteins, two of them, 43 kda and 42.5 kda were absent in the in vitro system directed by the 5.1 kb plasmid. Dickgiesser (1983) reported that the 7.2 kb plasmid, not the 5.1 kb plasmid produced a translation product of

10.8K. However, in the present study, pJD5 (5.1 kb) did not code for a 7.8 kda protein which was produced by pJD4. In addition, five other proteins were found among the in vitro transcription/translation products directed by pJD4 that were not detected in one or the other three remaining deleted plasmids (pJD6, pJD7, & pJD8). As their absence did not interfere with the survival of the plasmid, these six proteins were probably non-essential.

Appendix C

Papers, articles and abstracts pretaining to this thesis published or in preparation.

Refereed articles:

Yeung, K-H, J.R. Dillon, M. Pauze, and E. Wallace. 1986. A novel 4.9 kilobase plasmid associated with an outbreak of penicillinase-producing Neisseria gonorrhoeae. J. Infect. Dis. 153: 1162-1165.

Dillon, J.R., M. Pauze, and K.-H. Yeung. 1983. Spread of penicillinase-producing and transfer plasmids from the gonococcus to Neisseria meningitidis. Lancet i:779-781.

Dillon, J.R., M. Pauze, and K.-H. Yeung. 1986. Molecular and epidemiological analysis of PPNG isolated in Canada 1976-1984. Evolution of novel auxotypes and ß-lactamase producing plasmids. Genitourin. Med. 62:151-157.

Yeung, K.-H., and J.R. Dillon. 1989. Construction of miniplasmids from the 7.2 and 5.1 kb penicillinase-producing plasmids of N. gonorrhoeae reveals two replication regions. Plasmid 20:In press.

Yeung, K.-H., and J.R. Dillon. 1989. Mobilization of the penicillinase-producing plasmids of Neisseria gonorrhoeae by the conjugative plasmid pBG791 and other plasmids. In preparation.

Refereed conference proceedings:

Yeung, K.-H., and J.R. Dillon. 1985. In vitro transcription/translation products and molecular characterization of naturally occurring and in vitro deletion derivatives of the 7.2 kb plasmid of Neisseria gonorrhoeae. p. 209-215. In The Pathogenic Neisseriae. Proceedings of the fourth international symposium, Asilomer, California,21-25 October 1984. American Society for Microbiology,Washington, D.C..

Conference presentation and abstracts:

Yeung, K.-H., and J.R. Dillon. 1988. The 7.2 kb penicillinase-producing plasmid in Neisseria gonorrhoeae has two replication regions. Abstracts of American Society for Microbiology Annual Meeting (88th), 8-13 May, Miami Beach, Florida.

Hasnain, S., B. Gauthier, K.-H. Yeung, and J. R. Dillon. 1988 DNA sequence and putative origin of replication of pJD9 a mini-plasmid derived from a penicillinase-producing plasmid in N. gonorrhoeae. Abstracts of the 38th Canadian Society of Microbiologists annual meeting, 19-22 June, Windsor, Ont..

Dillon, J.R., S.H. Hasnain, B.R. Gauthier and K.-H. Yeung. 1988. Sequence analysis of one of the two replication regions in the 7.2-kb penicillionase-producing plasmid of N. gonorrhoeae. Sixth International Pathogenic Neisseria Conference. 16-21 October, Pine Mountain. Georgia.

Dillon, J.R., and K.-H. Yeung. 1988. Invited paper. Molecular biology and evolution of gonococcal R-factor. Sixth International Pathogenic Neisseria Conference. 16-21 October, Pine Mountain, Georgia.

Yeung, K.-H., and J.R. Dillon. 1987. Origin of replication and mobilization of a ß-lactamase-producing plasmid in N. gonorrhoeae. Abstracts of 37th annual meeting of the Canadian Society of Microbiologists, 14-18 June, Saskatoon, Saskatchewan.

Dillon, J.R., M. Pauze, and K.-H. Yeung. 1986. Evolution of novel auxotypes serotypes and ß-lactamase-producing plasmids amongst PPNG isolated in Canada. Second World Congress on Sexually transmitted diseases, 25-28 June, Paris, France.

Yeung, K.-H., and J.R. Dillon. 1985. Identification of proteins encoded by the penicillinase-producing plasmids of Neisseria gonorrhoeae. Abstracts of the 35th annual meeting of the Canadian Society of Microbiologists, 9-13 June, Halifax, Nova Scotia.

Yeung, K.-H. and J.R. Dillon. 1984. In vitro transcription/translation products and molecular characterization of naturallu-occuring and in vitro deletion derivatives of the 7.2 kb plasmid of Neisseria gonorrhoeae. 21-25 Oct., Asilomar, California.

Yeung, K.-H., G.S. Bezanson and J.R. Dillon. 1982. Molecular and functional characterization of penicillin-resistance plasmids on N. gonorrhoeae. Annual Meetings of the Canadian Society of Microbiologists. 12-16 June, Quebec City, Quebec.

Yeung, K.-H., G.S. Bezanson and J.R. Dillon. 1982. Mobility and other genetic studies with gonococcal plasmids. Presented at the International conference on pathogenic Neisseria, 11-14 Aug., Montreal, Quebec.

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