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STRUCTURAL STUDIES OF THE $\beta$-LACTAMASE-PRODUCING PLASMIDS OF

NEISSERIA GONORRHOEAE.

By

ABU THOLIB AMAN

Thesis submitted to the School of
Graduates Studies and Research
University of Ottawa
in partial fulfilment of
the requirements for the degree of
Masters of Science
Ottawa-Carleton Institute of Biology

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ABSTRACT

The β-lactamase producing plasmids of *Neisseria gonorrhoeae* pJD4 (Asia-type plasmid), pJD5 (Africa-type plasmid), pJD7 (Toronto-type plasmid), pGC4717 (Rio-type plasmid) and pGF1 (Nimes plasmid) are structurally related. Previous studies have shown that plasmids pJD5, pJD7 and pGO4717 are deletion derivatives of pJD4 and that plasmid pGF1 is an insertion derivative of pJD5. However, the exact location of these deletion has not been determined. The primary sequence of pJD4, the primary sequence around the deletion of pJD5, pJD7 and the Rio plasmid, and the insertion of pGF1 were determined using the dideoxy termination method. This is the first report which identified the exact size and location of deletion of the β-lactamase plamids of *N. gonorrhoeae* based on DNA sequence analyses. The primary sequence of pJD4 was used as reference for all other plasmids. Coordinates were numbered from the *PstI* site. In addition, regions around the deletion of four other Toronto-type plasmids pGC1213, pGC4538, pGC5221, pGC5228, were also sequenced.

Based on the DNA sequence of pJD4, a comprehensive restriction endonuclease map of pJD4 was constructed and compared to previously published maps. Analysis of the primary sequence of pJD4 including the region homologous to Tn4 shows at least 62 direct repeats (DR) and 24 inverted repeats greater or equal to 10 base pairs (bp) in length. The longest direct repeat, DR-30, comprised 507-bp (DR-30A) and 509-bp (DR-30B).
The deletion of pJD5 was 1827-bp in size corresponding to coordinates 1881 to 3707 of pJD4. This deletion corresponded to the sequence between the longest direct repeat and DR-30A up to the end of the sequence DR-30B. The deletion of pJD7 was 2272 bp in size, corresponding to coordinates 3803 to 6074 of pJD4. The size and location of the deletion of other Toronto-type plasmids pGC1213, pGC4538, pGC5221, pGC5228, and pGC5230 all were identical, and were also identical to that of pJD7. The deletion of pGO4717 (Rio plasmid) also found to be identical to that of pJD7. The deletion characterizing all of the Toronto type plasmids and the Rio plasmid was identical.

Structural analysis of pJD5, pJD7, Rio plasmid and five other Toronto-type plasmids suggested that the deletion occurred through a similar process. This fact support the hypothesis that the Africa-type, Toronto-type and Rio plasmids may have been deletion derivatives of the Asia-type plasmid.

The insertion of pGF1 was found to be insertion sequence IS5, 1119-bp in length, and it is located 604-bp downstream of the single PstI site. The insertion is flanked by direct repeats 5'-TTAA-3', and including one of the repeats. The target sequence 5'-TTAA-3' is different from the consensus target sequence, C.T/A.A.G/A, reported previously for IS5.

The region homologous to TnA in pJD4 is similar to that of pJD7 and pFA3. The region homologous to TnA in pJD5 is identical to that of pFA7. The Africa-type, Toronto-type and Rio plasmids are deletion derivatives of pJD4, and Nimes plasmid (pGF1) is a deletion derivative of pJD5.
iii

Analysis of the primary sequence of pJD4 showed an AT-rich region with several direct and inverted repeats which resemble replication regions found in many plasmids. A sequence homologous to the consensus sequence of integration host factor which could involved in the mobilization of this plasmid was also found in several regions.
iv

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I am very grateful to Dr. Jo-Anne R. Dillon, my supervisor. Her vast knowledge and her great personality has greatly influenced me in finishing this thesis. Throughout my study, she has been very helpful, patient and deeply understand. This work would be impossible without her help and encouragement.

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DEDICATION

To the most gracious and the most merciful

To my mom and dad.
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>bla</td>
<td>β-lactamase gene</td>
</tr>
<tr>
<td>bp</td>
<td>base pair(s)</td>
</tr>
<tr>
<td>C</td>
<td>Celsius</td>
</tr>
<tr>
<td>CMRNG</td>
<td>chromosomally resistant <em>Neisseria gonorrhoeae</em> (isolates)</td>
</tr>
<tr>
<td>dATP</td>
<td>deoxyadenosine 5′triphosphate</td>
</tr>
<tr>
<td>dCTP</td>
<td>deoxycytidine 5′triphosphate</td>
</tr>
<tr>
<td>dGTP</td>
<td>deoxyguanosine 5′triphosphate</td>
</tr>
<tr>
<td>dITP</td>
<td>deoxyinosine 5′triphosphate</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>DR</td>
<td>direct repeat</td>
</tr>
<tr>
<td>ds</td>
<td>double stranded</td>
</tr>
<tr>
<td>dTTP</td>
<td>deoxothymidine 5′triphosphate</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediamine tetracete</td>
</tr>
<tr>
<td>g</td>
<td>gram</td>
</tr>
<tr>
<td>GCMB</td>
<td>GC medium base</td>
</tr>
<tr>
<td>IHF</td>
<td>integration host factor</td>
</tr>
<tr>
<td>Inc</td>
<td>incompatibility group</td>
</tr>
<tr>
<td>IR</td>
<td>inverted repeat</td>
</tr>
<tr>
<td>IS</td>
<td>insertion sequence</td>
</tr>
</tbody>
</table>
kb    kilobase pairs
kdal  kilodalton
L     liter
LB    Luria Bertani (broth)
Mdal  megadalton
mm    millimeter
mob   mobilization (gene)
MIC   minimal inhibitory concentration
µg    microgram
µL    microliter
nm    nanometer
OD    optical density
ORF   open reading frame
oriT  origin of conjugative transfer
oriV  origin of replication
PPNG  penicillinase-producing *Neisseria gonorrhoeae* (isolates)
ss    single stranded
TE    Tris-HCl EDTA
Tn    transposable element
TRNG  tetracycline resistant (plasmid mediated) *Neisseria gonorrhoeae* (isolates)
TSA   tryptic soy agar
TSB   tryptic soy broth
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>US</td>
<td>uptake sequence</td>
</tr>
<tr>
<td>uv</td>
<td>ultraviolet</td>
</tr>
<tr>
<td>X-gal</td>
<td>5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside</td>
</tr>
</tbody>
</table>
CHAPTER I: INTRODUCTION

1.1. Epidemiology of antibiotic resistance in Neisseria gonorrhoeae

Infections caused by Neisseria gonorrhoeae have been documented in Biblical times and in ancient prescriptions (Rosebury, 1971; Fekete, 1993). For many decades, gonorrhoea has been one of the most prevalent diseases in Canada (Health and Welfare Canada, 1991). Gonorrhoea is currently the fourth most prevalent (1461 cases) notifiable disease in Canada after chlamydia (43,939 cases), chicken pox (13,687 cases) and campylobacter (12,741 cases) infections (Health and Welfare Canada, 1991). The number of reported cases peaked in 1981 at 56,336 cases with a rate of 233 cases per 100,000 population (Health and Welfare Canada, 1988), and declined to 9,307 in 1992 with a rate of 35 (Health Canada, unpublished data).

The incidence of gonorrhoea in most industrialized countries as well as some developing countries also declined during the 1980’s and early 1990’s (Health and Welfare Canada, 1991; Hook, 1993; Ison et al., 1993). For example, in the United States, the incidence of gonorrhoea fell from 395 cases to 245 per 100,000 population between 1981 and 1991 (Hook, 1993). In England, the total number of gonococcal isolates at St. Mary’s Hospital, London, declined from 3670 isolates in 1980 to 485 isolates in 1992 (Ison et al., 1993). Several reports indicate that the
incidence of gonorrhoea in the developing countries was very high. The highest incidence is in Africa with 3-10% of the population), Latin American countries - (2-8% of the population), and Asian countries with the lowest (1% of the population) (Wasserheit, 1989; Schryver and Meheus, 1990). From 1987 to 1991 the annual incidence rate has also decreased in 8 Caribbean countries and in 18 Latin American countries (Pan American Health Organization, 1991).

Historically, a number of treatments were proposed for this infection, including catheterization and the insertion of a louse into the urethral meatus (Rosebury, 1971; Waugh, 1990). Mercury, the first antimicrobial agent, was used to combat venereal infections, such as syphilis and gonorrhoea, at the end of the fifteenth century (Waugh, 1990). It was not until the eighteenth century that syphilis and gonorrhoea were considered to be different diseases, as a result of the Hunter experiment (Sparling, 1990). The real understanding of *N. gonorrhoeae* was achieved after cultivation of the organism by Leistikow and Loffler in 1882 (Sparling, 1990). Silver nitrate was introduced to prevent gonococcal blindness in the 1880s (Waugh, 1990). Effective antimicrobial agents against *N. gonorrhoeae* were not available until the introduction of protonsil, the first sulfonamide, in 1937 (Dunlop, 1949; Kampmeier, 1983). However, resistance to sulfonamide developed very rapidly: five to six years after its introduction, most gonococcal isolates were resistant to sulfonamides resulting in a high proportion of treatment failures (Campbell, 1944; Dunlop, 1949). In 1943, penicillin was introduced as an antimicrobial agent against *N. gonorrhoeae* (Sparling, 1990; Lind, 1990). During the first 10 years of penicillin therapy, 50,000
units given intramuscularly in a single dose, the cure rate exceeded more than 95% in treated patients (Lind, 1990). *In vitro* testing indicated that all gonococcal isolates were susceptible to 0.1 μg/mL penicillin or less (Reyn *et al.*, 1958).

In 1957, *N. gonorrhoeae* isolates that were not susceptible to 0.1 μg/mL penicillin were reported in several European countries (Reyn *et al.*, 1958). Nevertheless, most of the less susceptible isolates could be treated effectively by increasing the dose of penicillin (Lind, 1986; Nicol *et al.*, 1968) coupled with the use of probenecid (Lesinski *et al.*, 1973), a drug which enhances and prolongs the serum level of penicillin (Willcox, 1970; Willcox and Woodcock, 1970). By the early 1970s, a significant percentage of gonococci isolated in the Far East were not susceptible to 1 mg/L penicillin (Watko *et al.*, 1975; Hart, 1973). In Canada during 1973/74, 35.5% of all gonococcal isolates were resistant to 0.5 mg/L or more of penicillin (Dillon *et al.*, 1978). By 1982, gonococcal isolates with a minimum inhibitory concentration (MIC) > 2 mg/L were noted (Health and Welfare Canada, 1982). In the mid 1970s penicillin resistance had become a significant problem in parts of Africa and the Far East (Jaffe *et al.*, 1976; Piot *et al.*, 1979). As the MIC of less susceptible isolates increased, the dose of penicillin was increased to achieve successful treatment, until a level (5 X 10⁶ units plus 1g probenecid) was reach that could no longer be physiologically tolerated by patients (Olsen, 1973; Fluker and Hewitt, 1969; Norton-Brandao, 1971). Thus, other treatment strategies, such as dual antibiotic therapies, for example as ampicillin plus probenecid with tetracycline (Lesinki *et al.*, 1973; Nicol *et al.*, 1968), or single therapies with newer, more efficacious, and
ultimately more expensive drugs, such as spectinomycin, quinolone, and third
generation of cephalosporins such as ceftriaxone and cefixime were used (Fekete,
1993; Health and Welfare Canada, 1991; Bryan et al., 1990; Centres for Disease
Control, 1987; Wilcox and Woodcock, 1970). Resistance to penicillin was followed
subsequently by resistance to other antimicrobial agents used in therapy, such as
tetracycline, thiamphenicol, spectinomycin and streptomycin (Reyn et al., 1973;
Zenilman et al., 1987; Easmon and Ison, 1991).

1.1.1. Chromosomally mediated resistance of N. gonorrhoeae

Resistance to antimicrobial agents in N. gonorrhoeae can be mediated by
mutation of the chromosomal DNA or by plasmids. Chromosomal mutation at various
loci can mediate resistance to a single antimicrobial agent or variable degrees of
resistance to a wide spectrum of antimicrobial agents (Easmon, 1985; Sarubi et al.,
1974; Sarubi et al., 1975; Sparling et al., 1975; Reyn, 1976). For most antibiotics,
resistance due to chromosomal resistance is relatively low-level (Cannon and Sparling,
1984). Three genetic loci causing low-level resistance to penicillin have been
identified: penA, mtr and penB (Cannon and Sparling, 1984). Mutations at these loci
also increase resistance to cephalosporins (Rice et al., 1986; Ison et al., 1987).
Mutations at these genes collectively increase the MIC for penicillin from 0.01 μg/mL
to about 0.5-1 μg/mL (Sparling et al., 1975; Maier et al., 1975; ). Mutation at penA
causes an alteration of penicillin-binding protein 2 (PBP2) resulting in 4 to 8-fold
increases in resistance to β-lactam antibiotics (Sparling et al., 1975; Maier et al.,
1975; Barbour, 1981). Mutations at penB and mtr also cause an increase in resistance to many antibiotics, including penicillin, tetracycline, erythromycin, and chloramphenicol (Sparling et al., 1975; Maier et al., 1975). The mtr mutation results in changes in the outer membrane protein (Guymon et al., 1978) that reduce cellular uptake of dyes and other compounds, including antibiotics, resulting in multiple-drug resistance (Guymon and Sparling, 1975). Mutations at penB results in low-level increases in resistance to penicillin and tetracycline (Sparling et al., 1975). Mutations at two other loci, tet and chl mediate resistance to tetracycline and chloramphenicol, respectively (Sarubi et al., 1975; Sparling et al., 1975). Isolates with high level chromosomal resistance to one or more antibiotics have been called chromosomally resistant N. gonorrhoeae (CMRNG; Centres for Diseases Control, 1984). The first report of an outbreak caused by CMRNG was in 1983, in Durham, North Carolina, subsequent outbreaks occurred in Tennessee, New Mexico and Oregon (Kimberly et al., 1984; Faruki et al., 1985). Retrospective analysis of Canadian data identified CMRNG outbreaks in Manitoba in 1988 and in Ontario in 1990 (Dillon et al., unpublished data).

I.1.2. Plasmid-mediated penicillinase-producing Neisseria gonorrhoeae (PPNG)

The first gonococcal isolates to produce penicillinase (i.e., plasmid-mediated penicillinase producing N. gonorrhoeae, PPNG) were isolated simultaneously in the United Kingdom and in North America in 1976 (Phillips, 1976; Percival et al., 1976; Ashford et al., 1976; Bowmer, 1976), and those in the United Kingdom were
epidemiologically linked to Africa (Phillips, 1976; Percival et al., 1976). Strains isolated in North America were epidemiologically linked to East Asia (Ashford et al., 1976; Bowmer, 1976). Both strains contained plasmids encoding a TEM1-type β-lactamase (Roberts et al., 1977) and both plasmids carried DNA sequences homologous to about 40% of Tn2, a transposable element encoding β-lactamase (Fayet et al., 1982; Norlander et al., 1981; Roberts et al., 1977; Chen and Clowes, 1987a).

Since the report of the first isolates in 1976, PPNG strains have been isolated throughout the world (Centres for Diseases Control, 1979; Phillips, 1976; Percival et al., 1976; Ashford et al., 1976; Bowmer, 1976; Dickgiesser et al., 1982; Jephcott, 1986). In Canada, the first PPNG was isolated in 1976, by 1981 the number of cases had increased to 58 and peaked at 1,282 in 1991 (Dillon et al., unpublished data). As the number of cases of gonorrhoea decreased, the percentage of PPNG isolates increased (Figure 1). In Canada in 1985, PPNG represented 0.5% of all gonococcal isolates, this number increased to 10.3% in 1991 (Dillon et al., unpublished data). However the incidence of PPNG decreased to 6.3% of all gonococcal isolates in 1992 (Dillon et al., unpublished data). Similarly, in the United States of America, the prevalence of PPNG infections increased steadily since 1976, and from 1983 to 1989, the percentage of PPNG isolates increased from 0.41% (Centre for Disease Control, 1984) to 7.4% of total gonorrhoea cases (Centres for Disease Control, 1990). In England and Wales, the total number reported cases of PPNG were 1223
cases in 1982 and 1227 in 1983, then declined to 235 cases in 1987 (Ison and Easmon, 1989). In Japan, the incidence of PPNG peaked in the mid 80’s (5-10% of gonococcal isolates), and in 1993 PPNG strains accounted for about 5% of the total gonococcal isolates (Nishimura et al., 1993).

Several reports from other countries show that the percentage of PPNG strains was generally much higher than in the developed countries (Kam et al., 1992; Poh et al., 1991; Clendennen et al., 1992). In Singapore in 1984, PPNG accounted for 41.6% of all gonococcal isolates from the Middle Road Hospital (Poh et al., 1991). In the Philippines, PPNG accounted for 55% of isolates collected in September 1989 (Clendennen et al., 1992). In Malaysia, in 1992, PPNG accounted for 40.3% of all gonococcal isolates (Cheong et al., 1993). In African countries from 1982 to 1986, the percentage of PPNG among gonococcal isolates varied greatly from 31% to 81% (Lind et al., 1991; Mason et al., 1990). Reports from several large cities in Africa, i.e., Tripoli, Libya, Harare, Zimbabwe and Nairobi, Kenya, showed that PPNG accounted for 35% to 70% of all gonococcal isolates (Elghouli and Joshi, 1990; Mason et al., 1990; Obette et al., 1993)

I.1.3. Tetracycline resistant *Neisseria gonorrhoeae* (TRNG)

In 1985, plasmid mediated resistance to tetracycline was reported in the United states (Centre for Disease Control, 1985; Carson et al., 1985). The isolates carrying the plasmids were called tetracycline-resistant *Neisseria gonorrhoeae* (TRNG)
for Disease Control, 1985; Carson et al., 1985). In 1986, TRNG was reported in Canada (Shaw et al., 1986) followed by another report in the Netherlands (Morse et al., 1986; Roberts et al., 1988). Now TRNG has been reported in many other countries including Zaire, the United Kingdom, France and Spain (Heritage and Hawkey, 1988; Ison et al., 1988; Gascoyne-Binzi et al., 1992; Waugh et al., 1988). Since 1985, the incidence of TRNG strains has significantly increased (Dillon and Carballo, 1990; Carballo et al., 1990; Klinger et al., 1989; Dyck et al., 1992). From October 1987 to May 1989, 84 isolates were confirmed as TRNG in Canada (Dillon and Carballo, 1990), and in 1992, there were 773 confirmed isolates (Dillon et al., 1993). In Malaysia in 1992, 28.1% of all gonococcal isolates were TRNG (Cheong et al., 1993), and in Zaire in 1988, 10% were TRNG (Dyck et al., 1992).

These TRNG isolates carry a 40.5-kb (25.2-MDa) plasmid, which probably arose from the insertion of the _tetM_ determinant, a transposon-borne determinant initially found in the genus _Streptococcus_ into the 39.2-kb transfer plasmid found in _N. gonorrhoeae_ isolates (Morse et al., 1986). Subsequently, Gascoyne et al. (1991), showed that there were two different TRNG plasmid types based on the restriction endonuclease analysis of plasmids of TRNG strains isolated in the United Kingdom, the United States and the Netherlands (Gascoyne et al. 1991). Some TRNG isolates also carry plasmids that mediate resistance to penicillin (PP/TRNG). In Canada from October 1987 to May 1989, 84 isolates were confirmed as TRNG and 20.2% of them were also PPNG (Dillon and Carballo, 1990). In 1989, the percentage of PP/TRNG was 66.7% of TRNG isolates (Carballo et al., 1990).
1.2. Characterization of penicillinase-producing plasmids in *N. gonorrhoeae*

PPNG isolates, first reported from the United Kingdom, carried a 5.1-kb β-lactamase-producing plasmid as well as a 4.2-kb cryptic plasmid, which are common to most gonococcal isolates (Roberts et al., 1977; Dillon et al., 1981; Ison and Easmon, 1989); their auxotypes (specific growth requirement on a chemically defined medium) were arginine-requiring (Perine et al., 1977). The first PPNG isolates in North America were auxotyped as either proline-requiring or non-requiring and carried a β-lactamase-producing plasmid estimated to be 7.4 kb in size as well as a 4.2-kb cryptic plasmid; some also carried a 39.2-kb conjugative plasmid (Dillon et al., 1981; Perine et al., 1977). These two β-lactamase-producing plasmid types, which were called Africa-type and Asia-type, respectively, now have been reported throughout the world (Dillon et al., 1987; Dickgiesser et al., 1982; Jephcott, 1986). Today, the host range of these two plasmid types has expanded to include other gonococcal auxotypes (Dillon et al., 1987; Poh et al., 1991; Ison and Easmon, 1989), and these isolates, irrespective of plasmid types, may harbour a 39.5 conjugative plasmid (Dillon et al., 1987; Poh et al., 1991; Ison and Easmon, 1989).

In 1982, a third β-lactamase-producing plasmid, which was 6.6-kb in size, designated the Nimes plasmid (pGF1), was isolated in Nimes, France (Gouby et al., 1986). This isolate also carried a 4.2-kb cryptic plasmid but not a 39.2-kb
conjugal plasmid (Gouby et al., 1986). Isolates with Nimes plasmid have not been reported in other parts of the world.

Van Embden et al. (1985) isolated two PPNG isolates carrying 4.6-kb β-lactamase-producing plasmids. One strain was epidemiologically linked to Durban, South Africa, and the other to Rio de Janeiro (Rio plasmid) (Van Embden et al., 1985). These isolates also carried 4.2-kb cryptic plasmids and 39.2-kb conjugative plasmids and their auxotypes classes were either methionine-requiring or non-requiring (van Embden et al., 1985). A PPNG outbreak in Toronto in 1984, was found to be caused by isolates containing a 4.9-kb β-lactamase plasmid, designated "Toronto" (Yeung and Dillon, 1985). These strains also carried a 4.2-kb cryptic plasmid and a 39.2-kb conjugative plasmid, and their auxotype was non-requiring (Yeung and Dillon, 1985; Yeung et al., 1986). Isolates containing this plasmid have now been isolated in Europe (Reimann et al., 1992), Japan, Taiwan and the Philippines (Sarafian et al., 1991).

Recent β-lactamase-producing plasmid variants isolated in New Zealand (Brett, 1989) were 9.0-kb in size, and one was epidemiologically linked to New Zealand, and designated the New Zealand plasmid (Brett, 1989). This strain also carried a 4.2-kb cryptic plasmid but not the 39.2-kb conjugative plasmid (Brett et al., 1989). The auxotype of the strain was proline-leucine-requiring (Dillon et al., unpublished data). So far, plasmids of this type have not been described elsewhere.
1.3. Molecular characterization of \( \beta \)-lactamase-producing plasmids of \( N. \) gonorrhoeae

The \( \beta \)-lactamase plasmids of \( N. \) gonorrhoeae are structurally related (Yeung and Dillon, 1985; Yeung et al., 1986; Aalen and Gundersen, 1987; Dickgiesser et al., 1982; McNicol et al., 1983; van Embden et al., 1985; and Gouby et al., 1986). At least 9 different Asia-type plasmids have been studied using restriction endonuclease analysis (Table 1). Using restriction endonuclease and electron microscope analysis, groups of researchers reported that the size of the Asia-type plasmid varies between 7.1 and 7.5-kb (4.4 and 4.7 M\( \text{d} \)). The problem of sizing may have occurred because researchers used different different restriction endonucleases and running conditions for their analysis. Yeung and Dillon (1985) reported that \( \text{BamHI} \) cut pJD4 into two fragments: 2.4-kb and 4.8-kb. Aalen and Gundersen (1987) used the \( \text{HindIII} \) site as the zero reference position and reported the size of pRosB as 7.3-kb in size. Dickgiesser et al. (1982) studied pNG10 and reported its size as 7.4-kb. Fayet et al. (1982) who used \( \text{BamHI} \) as the zero reference position, reported the size of pPJ102 as 7.4-kb, while Chen and Clowes (1987b), reported pFA3 as 7.5-kb in size. Seven other groups, using a variety of restriction endonuclease enzymes, reported the size
Table 1. Size of Asia-type of $\beta$-lactamase-producing plasmids of *N. gonorrhoeae* as reported in the literature.

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Reported plasmid size</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pJD4</td>
<td>7.2-kb</td>
<td>Yeung and Dillon, 1985</td>
</tr>
<tr>
<td>pRosB</td>
<td>7.3-kb</td>
<td>Aalen and Gundersen, 1987</td>
</tr>
<tr>
<td>pFA3</td>
<td>7.5-kb</td>
<td>Chen and Clowes, 1987</td>
</tr>
<tr>
<td>pFA3</td>
<td>7.1-kb</td>
<td>Sox <em>et al.</em>, 1979</td>
</tr>
<tr>
<td>pFA3</td>
<td>7.1-kb</td>
<td>Brunton <em>et al.</em>, 1982</td>
</tr>
<tr>
<td>p22209</td>
<td>7.1-kb</td>
<td>McNicol <em>et al.</em>, 1983</td>
</tr>
<tr>
<td>pFT1</td>
<td>7.1-kb</td>
<td>Tenover <em>et al.</em>, 1985</td>
</tr>
<tr>
<td>pNG10</td>
<td>7.4-kb</td>
<td>Dickgiesser, 1982</td>
</tr>
<tr>
<td>pMR0360</td>
<td>7.1-kb</td>
<td>Roberts <em>et al.</em>, 1977</td>
</tr>
<tr>
<td>pMR0360</td>
<td>7.1-kb</td>
<td>Mayer and Robbins, 1983</td>
</tr>
<tr>
<td>pCDC66</td>
<td>7.1-kb</td>
<td>Elwell <em>et al.</em>, 1977</td>
</tr>
<tr>
<td>pPJ102</td>
<td>7.4-kb</td>
<td>Fayet <em>et al.</em>, 1982</td>
</tr>
</tbody>
</table>
of the Asia-type plasmid as 7.1-kb (Elwell et al., 1977; Roberts et al., 1977; Sox et al. 1979; Brunton et al., 1982; McNicol et al., 1983; Mayer and Robbins, 1983; Tenover et al., 1985).

The other gonococcal plasmids (Africa, Toronto, Nimes, Rio and New Zealand) are structurally related to each other as well as to the Asia-type (Yeung and Dillon, 1985; Yeung et al., 1986; Aalen and Gundersen, 1987; Dickgiesser et al., 1982; McNicol et al., 1983; van Embden et al., 1985; and Gouby et al., 1986). Because a number of different plasmids of the same type have been investigated by various group of researchers, some have reported differences in the structure of the plasmids, notably the location of deletion relative to the Asia-type plasmid as well as the size of the Africa-type plasmid. Using restriction endonuclease digestion and electron microscope analysis, groups of researchers have reported that the size of the Africa-type plasmid varies between 5.1 and 5.5-kb (3.2 and 3.4 Md). Aalen and Gundersen (1987) using the Hincll site as the zero reference position, reported pTorB as 5.5-kb in size. Dickgiesser et al. (1982) studied pNG18 by restriction endonuclease and electron microscope analysis, and reported its size to be 5.3 kb. Chen and Clowes (1987b) who used BamHI as the zero reference position reported pFA7 as 5.4 kb in size. Tenover et al. (1985) used Hincll as the zero reference position reported the size of pFT300 as 5.2 kb. Six other groups, using a variety of restriction endonuclease enzymes, reported the size of the Africa-type plasmid as 5.1-kb (Dillon and Yeung, 1985; Elwell et al., 1977; Roberts et al., 1977; Sox et al. 1979; Brunton et al., 1982; McNicol et al., 1983).
Table 2. Size of Africa-type plasmids of *N. gonorrhoeae* as reported in the literature.

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Reported plasmid size</th>
<th>Reported deletion size</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>pJD5</td>
<td>5.1-kb</td>
<td>2.1-kb</td>
<td>Dillon and Yeung, 1985</td>
</tr>
<tr>
<td>pTorB</td>
<td>5.5-kb</td>
<td>1.8-kb</td>
<td>Aalen and Gundersen, 1987</td>
</tr>
<tr>
<td>pFA7</td>
<td>5.4-kb</td>
<td>2.1-kb</td>
<td>Chen and Clowes, 1987</td>
</tr>
<tr>
<td>pFA7</td>
<td>5.1-kb</td>
<td>2.0-kb</td>
<td>Brunton <em>et al.</em>, 1981</td>
</tr>
<tr>
<td>pFA7</td>
<td>5.1-kb</td>
<td>2.0-kb</td>
<td>Sox <em>et al.</em>, 1979</td>
</tr>
<tr>
<td>pFT300</td>
<td>5.2-kb</td>
<td>1.9-kb</td>
<td>Tenover <em>et al.</em>, 1985</td>
</tr>
<tr>
<td>p88557</td>
<td>5.1-kb</td>
<td>2.0-kb</td>
<td>McNicol <em>et al.</em>, 1983</td>
</tr>
<tr>
<td>pNG18</td>
<td>5.3-kb</td>
<td>2.1-kb</td>
<td>Dickgiesser, 1984</td>
</tr>
<tr>
<td>pMR0200</td>
<td>5.1-kb</td>
<td>2.0-kb</td>
<td>Roberts <em>et al.</em>, 1977</td>
</tr>
<tr>
<td>pLPL</td>
<td>5.1-kb</td>
<td>2.0-kb</td>
<td>Elwell <em>et al.</em>, 1977</td>
</tr>
</tbody>
</table>
Several groups have reported the size and location deletion of the Africa-type plasmid relative to the Asia-type plasmid (Figure 2). Dickgiesser et al. (1982) located the 2.1-kb deletion of pNG18, at 1.75 kb upstream of the PstI site (Dickgiesser et al., 1982). Yeung and Dillon (1985) and Yeung et al. (1986) located the 2.1-kb deletion of pJD5 at 1.75 kb downstream of the PstI site (opposite to the direction of reported by Dickgiesser et al. (1982). McNicol et al. (1983) located a 2.0 deletion on p88557 approximately 2.5-kb downstream of the PstI site. Brunton et al. (1981) located a 1.87-kb (1.2 Mdal) deletion of pFA7 at 1.87-kb downstream of the PstI site. Sox et al. (1979) located a 2.0-kb deletion of pFA7 at 2.77-kb downstream of the PstI site.

Two other studies (Roberts et al., 1977; Elwell et al., 1977) reported a 2.0-kb deletion but the exact location of the deletion was not reported. The latest studies, by Chen and Clowes (1987b), reported a 1.9-kb deletion of pFA7, however, the exact location was not identified.

Several other β-lactamase plasmids (Table 3) have been structurally characterized. Restriction endonuclease analysis of pJD7, a Toronto-type plasmid, was found to be structurally related to the Asia-type plasmid, with a 2.3-kb fragment deleted from the Asia-type plasmid (Yeung and Dillon 1985; Yeung et al., 1986). Heteroduplex studies located the 2.3-kb deletion starting at 3.7 kb downstream of the PstI site (Figure 3; Yeung and Dillon 1985).

The Rio (4.6-kb) plasmid (pGO4717) is identical to pGO181, an Asia-type plasmid except for the 2.6-kb deletion (Figure 3; van Embden et al., 1985). Based on restriction endonuclease analysis of the Rio and the Asia-type plasmids using BamHI
Figure 2. Comparison of restriction endonuclease maps of Africa-type plasmid as compared to plasmids pJD4 (Asia) of *N. gonorrhoeae*. The map coordinates and the restriction sites in pJD4 are those generated in this study. Plasmids are: pJD5 (Yeung and Dillon, 1985); pNG18 (Dickgiesser, 1982); p88557 (McNicol et al., 1983); pTorB (Aalen and Gundersen, 1987); pFA7a (Sox et al., 1979); pFA7b (Brunton et al., 1981).
Table 3. Size of the Toronto, Rio, Nimes and New Zealand plasmids of *N. gonorrhoeae* as reported in the literature.

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Reported size*</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pGF1 (Nimes)</td>
<td>6.6-kb</td>
<td>Gouby <em>et al.</em>, 1986</td>
</tr>
<tr>
<td>pGF1 (Nimes)</td>
<td>6.6-kb</td>
<td>Dillon and Yeung, 1989</td>
</tr>
<tr>
<td>pJD7 (Toronto)</td>
<td>4.9-kb</td>
<td>Yeung <em>et al.</em>, 1986</td>
</tr>
<tr>
<td>pGO4717 (Rio)</td>
<td>4.6-kb</td>
<td>vanEmbden, 1985</td>
</tr>
<tr>
<td>pGO4717 (Rio)</td>
<td>9.0-kb</td>
<td>Yeung <em>et al.</em>, 1991</td>
</tr>
<tr>
<td>pAS84/417 (New Zealand)</td>
<td>9.0-kb</td>
<td>Brett, 1989</td>
</tr>
</tbody>
</table>

a. Some sizes were originally reported in Megadaltons (Mdal).
Figure 3. Comparison of the restriction endonuclease map "Toronto" (pJD7), "Rio" (pGO4717), "Nimes"/pGF1 and New Zealand, pAS84/417 plasmids with pJD4 (an Asia-type plasmid). The map of pJD4 was generated in this study.
and \textit{Hind}III,\ van\ Embden \textit{et al} (1985) reported that the region homologous to the small (1.8-kb) \textit{Bam}HI-\textit{Hind}III fragment of the Asia-type plasmid was 0.1-kb smaller in the Rio, but the exact location of the putative deletion was not established. The remaining 2.5-kb of the differences was located within the 3.2-kb \textit{Bam}HI-\textit{Hind}III fragment of pGO181. The claim that the small \textit{Bam}HI-\textit{Hind}III fragment of Rio was 0.1-kb smaller than that of the Asia plasmid was not supported by double digestion of the Rio plasmid with \textit{Bam}HI and \textit{Hind}III. Based on restriction endonuclease analysis of the Rio plasmid, pJD7 (Toronto) and pJD4 (Asia), Dillon and Yeung (1989) did not observe the 0.1-kb deleted fragment. Analysis of double digestions of pJD7 and pGO4717 with \textit{Bam}HI-\textit{Hind}III showed that pJD7 (Toronto) was identical to pGO4717 (Dillon and Yeung, 1989). Only DNA sequencing will clarify whether the Toronto plasmid are different from the Rio plasmid.

The Nimes (6.6-kb, pGF1) plasmid was found to be similar to the Africa-type plasmid except for a 1.2-kb insertion located inside the 2.4-kb \textit{Bam}HI fragment (Gouby \textit{et al}., 1986; Dillon and Yeung, 1989). However, the exact location or the nature of the insertion was not established (Gouby \textit{et al}., 1986; Dillon and Yeung, 1989).

Restriction endonuclease analysis indicated that the New Zealand plasmid was identical to the Asia plasmid except for the addition of 1.8-kb (Yeung \textit{et al}., 1991a), which is located at approximately 2.4-kb downstream of the \textit{Pst}I site (Bigelow and
Dillon, personal communication). The deletions in the Africa and Toronto plasmids and the insertion in the Nimes plasmid were not present in the New Zealand plasmid (Yeung et al., 1991a).

The ampicillin resistance transposon (Tn4) is widely distributed in prokaryotic genera (Heffron et al., 1977). It comprises closely related transposons that were designated as Tn1, Tn2, and Tn3 (Heffron et al., 1977; Calos and Miller, 1980). Tn3 is the first transposable element encoding antibiotic resistance to be reported (Hedges and Jacob, 1974). Tn3 is derived from IncF plasmid R1dps19, while its very similar relatives, Tn1 and Tn2, are derived from broad host range IncP1 plasmid RP4 and non-conjugative plasmid RSF1030, respectively (Heffron, 1983). They differ only a few base pairs (Heffron, 1983; Chen and Clowes, 1987a).

Tn3 has been completely sequenced; it is a 4,957-bp DNA segment flanked by two (38-bp) inverted repeat sequences (IR-L and IR-R) (Figure 4; Heffron et al., 1979). It contains three genes: a transposase (tnpA gene), which encodes transposase consists of 1015 amino acids; a regulatory (specific repressor) gene, tnpR, which encodes 185 amino acids; and a β-lactamase (bla) gene, which encodes 185 amino acids (Heffron et al., 1979). Between the tnpA and tnpR genes, and between the tnpR and bla genes are the 118-bp and 182-bp non-coding (intervening) regions, respectively. Between bla gene and IR-R is a 109-bp non-coding region (Heffron et al., 1979).
Figure 4. Genetic map of ampicillin transposon Tn3 (Heffron et al., 1979). Arrows indicate the length of the genes and direction of transcription. □, terminal inverted repeats (IR-L and IR-R). ■, region present in the β-lactamase-producing plasmids of *N. gonorrhoeae*. 
The β-lactamase (bla) gene of the Asia-type and Africa-type plasmids are located in a DNA segment homologous to about 40% of the ampicillin transposon, Tn2 (Roberts et al., 1977). By heteroduplex analysis, Fayet et al. (1982) found that the Asia-type plasmid contains a 1.6-kb segment homologous to Tn3-like transposon Tn2301. The 1.6-kb segment is not transposable, however, it became a functional transposon when linked to the left part of Tn2301. Chen and Clowes (1987b) proposed that the TnA sequence in plasmid pFA3, an Asia-type plasmid, and pFA7, an Africa-type plasmid of N. gonorrhoeae are derived from Tn2, since they are more similar to Tn2 than to Tn1 or Tn3 (Chen and Clowes, 1987b). Nucleotide sequence analysis of pFA3, an Asia-type plasmid, and pFA7, an Africa-type plasmid, showed that the DNA segment homologous to TnA on both plasmids contained IR-R, bla gene and part (469 bp of pFA3 and 468 bp of pFA7) of the tnpR gene (Roberts et al., 1977; Chen and Clowes, 1987b).

1.4. Structurally related penicillinase-producing plasmids found in species other than N. gonorrhoeae

β-lactamase production in Haemophilus influenzae, H. parainfluenzae, H. ducreyi, and N. gonorrhoeae is mediated by plasmids (Table 4) which are structurally similar to gonococcal plasmids (Table 4)(Brunton et al., 1981; Brunton et al., 1982; McNicol et al., 1983; Chen and Clowes, 1987b).
Table 4.  Size of several structurally related plasmids found in other species than *N. gonorrhoeae* plasmids, as reported in the literature.

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Species origin</th>
<th>Reported size*</th>
<th>Related gonococcal plasmid</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pRSF0885</td>
<td><em>H. influenzae</em></td>
<td>6.6-kb</td>
<td>Africa/5.3-kb</td>
<td>Albritton, personal</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>communication</td>
</tr>
<tr>
<td>pRSF0885</td>
<td><em>H. influenzae</em></td>
<td>6.6-kb</td>
<td>Africa/5.3-kb</td>
<td>Roberts <em>et al.</em>, 1986</td>
</tr>
<tr>
<td>pKC83</td>
<td><em>H. ducreyi</em></td>
<td>5.1-kb</td>
<td>Africa/5.3-kb</td>
<td>Anderson <em>et al.</em>, 1984</td>
</tr>
<tr>
<td>pVe445</td>
<td><em>H. influenzae</em></td>
<td>7.1-kb</td>
<td>Asia/7.4-kb</td>
<td>Lauf <em>et al.</em>, 1979</td>
</tr>
<tr>
<td>pVe445</td>
<td><em>H. influenzae</em></td>
<td>7.4-kb</td>
<td>Asia/7.4-kb</td>
<td>Dickgiesser, 1984</td>
</tr>
<tr>
<td>pHD747</td>
<td><em>H. ducreyi</em></td>
<td>10.5-kb</td>
<td>Asia/7.4-kb</td>
<td>McNicol <em>et al.</em>, 1983</td>
</tr>
<tr>
<td>pHD131</td>
<td><em>H. ducreyi</em></td>
<td>10.5-kb</td>
<td>Asia/7.4-kb</td>
<td>Chen and Clowes, 1987</td>
</tr>
<tr>
<td>pHD131</td>
<td><em>H. ducreyi</em></td>
<td>10.5-kb</td>
<td>Asia/7.4-kb</td>
<td>Brunton <em>et al.</em>, 1982</td>
</tr>
<tr>
<td>pJB1</td>
<td><em>H. ducreyi</em></td>
<td>8.6-kb</td>
<td>Africa/5.3-kb</td>
<td>McNicol <em>et al.</em>, 1983</td>
</tr>
<tr>
<td>pJB1</td>
<td><em>H. ducreyi</em></td>
<td>8.6-kb</td>
<td>Africa/5.3-kb</td>
<td>Brunton <em>et al.</em>, 1982</td>
</tr>
<tr>
<td>pJB1</td>
<td><em>H. ducreyi</em></td>
<td>8.6-kb</td>
<td>Africa/5.3-kb</td>
<td>Chen and Clowes, 1987</td>
</tr>
</tbody>
</table>

a. Some sizes were originally reported in Megadaltons (Mdal).
Based on restriction endonuclease analysis and Southern blot hybridization, two plasmids from *H. ducreyi*, 8.6-kb (5.7-MDa; pJB1) and 10.5-kb (7.0-MDa; pHD131) in size, were identical to the Africa-type and the Asia-type plasmids of *N. gonorrhoeae*, except that the *Haemophilus* plasmids carried a complete Tn2 sequence, while the gonococcal plasmids carry about 40% of Tn2 (Brunton *et al.*, 1982; McNicol *et al.*, 1983; Chen and Clowes, 1987b).

Laufs *et al* (1979) reported a 7.1-kb (4.4-MDa) β-lactamase plasmid (pVe445) from *H. influenzae* which shared a high degree of homology with the Asia-type gonococcal plasmid, which carried about 40% of Tn2. Roberts *et al.* (1977) reported that pRSF0885, a 6.6-kb (4.1-Mdal) β-lactamase producing plasmid of *H. influenzae* (pRSF0885) also carried about 40% of Tn2 and showed a high degree of homology (95%) to pMR360, an Asia-type plasmid. Recently, Albritton (personal communication) found that pRSF0885, a 6.6-kb plasmid of *H. influenzae*, was identical to the Africa plasmid with insertion of an insertion sequence IS1. In 1984, Anderson *et al.* reported that pKC83, a 3.2-MDa (5.1-Kb) β-lactamase plasmid isolated from *H. ducreyi*, had restriction endonuclease patterns identical to the 5.3-Kb (Africa) plasmid of *N. gonorrhoeae*. Furthermore, a 4.2-kb cryptic plasmid from *H. parainfluenzae*, which did not carry the Tn2 sequence, was found to be homologous to the β-lactamase plasmids from *H. ducreyi* and *N. gonorrhoeae* (Brunton *et al.*, 1986).
Roberts et al. (1977) proposed that the $\beta$-lactamase plasmids in *N. gonorrhoeae* were acquired from a foreign origin since the 7.4-kb and the 5.3-kb plasmids had a G+C content (40%) which was different from that of the chromosomal DNA (50%) of *N. gonorrhoeae*. The 7.4-kb and the 5.3-kb plasmids have G+C contents similar to pRSF0885, a 4.1-MDa $\beta$-lactamase plasmid in *H. influenzae* (Roberts et al., 1977). The 7.4-kb and 5.3-kb $\beta$-lactamase-producing plasmids of *N. gonorrhoeae* were also homologous to a variety of $\beta$-lactamase-producing plasmids isolated from *H. ducreyi*, *H. influenzae* and *H. parainfluenzae* (Brunton et al., 1986; Chen and Clowes, 1987b; McNicol et al., 1983) which were structurally similar to gonococcal plasmids (Brunton et al., 1982; McNicol et al., 1983; Chen and Clowes, 1987b).

Based on the above observations, it was suggested that gonococcal plasmids might have arisen through the transformation of plasmid DNA from *H. ducreyi* or other *Haemophilus* spp. to *N. gonorrhoeae* (Brunton et al., 1982; Dillon and Yeung, 1989). The deletion of the left-hand inverted repeat (IR-L), *tnpA* and part of *tnpR* of Tn2 could have occurred during the transfer of the plasmid to *N. gonorrhoeae* (Aalen and Gundersen, 1987). *N. gonorrhoeae* itself then acted as a reservoir for $\beta$-lactamase plasmids, transferring them to other *N. gonorrhoeae*, *N. meningitidis* and *Haemophilus* spp. (Flett et al., 1981; Dillon et al., 1983; Genco et al., 1984). Roberts et al. (1977) and Sox et al. (1979) suggested that the Africa plasmid was a deletion derivative of the Asia plasmid. Sox et al. (1979) observed a 5.3-kb plasmid deletion
derivative of pFA3 after transformation of pFA3 into *N. gonorrhoeae* or *E. coli*. Similar observations were reported by Yeung (1990). Restriction endonuclease analysis of the 5.3-kb deleted plasmid was found to be identical to pFA7, an Asia-type plasmid (Sox *et al.*, 1979). An alternate hypothesis was proposed by Dickgiesser *et al.* (1982) who suggested that pNG10, an Asia-type plasmid, was an insertion derivative of pNG18, an Africa-type plasmid. Electron microscopic analysis of heteroduplex between pNG10 and pNG15 indicated that pNG10 carried a 2.1-kb fragment which was hypothesized as an insertion sequence bounded by inverted repeats of 300-bp (Dickgiesser *et al.* 1982).

The 4.9-kb (Toronto) plasmid (Yeung *et al.*, 1986) and the 4.6-kb (Rio) plasmid (van Embden *et al.*, 1985), structurally related to the Asia-type but not to the Africa-type plasmid, favour the hypothesis proposed by Sox *et al.* (1979), and may indicate that transformation-associated deletion may have occurred in nature (Aalen and Gundersen, 1987).

Recently, insertion derivatives of the Asia and the Africa plasmid have also been demonstrated. The New Zealand-type plasmid (9.0-kb) is structurally related to the Asia-type plasmid but not to the Africa plasmid. In contrast, the Nimes plasmid (a 6.6-kb plasmid) which was reported in Nimes, France, is related to the Africa plasmid with a 1.2-kb insertion.
1.5. DNA sequence of the $\beta$-lactamase producing plasmids of *N. gonorrhoeae*

None of the $\beta$-lactamase producing plasmids of *N. gonorrhoeae* has been sequenced entirely. Sanchez-Pescador *et al.* (1988) sequenced 1,811 bp of pFA7 (an Africa-type plasmid). The sequence corresponds to 780 bp of the 3'end of the $\beta$-lactamase (*bla*) gene and 1031 bp of pFA7 from one of the *Bam*HI sites outside Tn4 to the right hand of inverted repeat (IR-R) of Tn4 (including the entire IR-R) (Figure 5). Chen and Clowes (1987b) sequenced 1447-bp of the region homologous to Tn4 in pFA3, an Asia-type plasmid, corresponding to 469-bp of the 3'terminus of the *tnpR* gene, the entire intervening (non-coding) region (182-bp), 491-bp 5' terminus of *bla* ($\beta$-lactamase) gene, and 305-bp from the end of IR-R of Tn4 (Chen and Clowes, 1987b). Chen and Clowes (1987b) had also sequenced a 896-bp of pFA7, an Africa-type plasmid, corresponding to 468-bp of the 3' terminus of the *tnpR* gene, the entire intervening (non-coding) region (182-bp) and 246-bp of the 5' terminus of the *bla* gene of Tn2 (Chen and Clowes, 1987b). In pFA7 Chen and Clowes (1987b) did not sequence the IR-R region of Tn4. Gilbride and Brunton (1990) sequenced approximately 1.6-kb of the 3.4-kb *Hind*III-*PstI* fragment of pFA3, an Asia-type plasmid, starting at the 480-bp proximal of a *Bam*HI site toward the *PstI* site, including the IR-R of Tn4, and ending at 48 bp 3'terminus of *bla* gene. Therefore, part of this latter sequence overlapped sequences obtained by Sanchez-Pescador *et al.* (1988). Yeung and Dillon (1988) sequenced 752-bp of the 1.8-kb *Hind*III-*Bam*HI fragment of pJD4, the 7.4-kb gonococcal plasmid, starting from the *Hind*III site.
Figure 5. Regions of the β-lactamase producing plasmids of *N. gonorrhoeae* that have been previously sequenced. Plasmid pJD4, which was sequenced in this study, was used for comparison.
I.6. Studies to identify phenotype and function on the $\beta$-lactamase plasmids of *N. gonorrhoeae*

Regions required for the replication and maintenance (McNicol et al., 1984; Johnson, 1985; Yeung and Dillon, 1988; Gilbride and Brunton, 1990) and for the mobilization (McNicol et al., 1983; Tenover et al., 1985; Dillon and Yeung, 1989; Gauthier, 1990) of the 7.4-kb and 5.3-kb $\beta$-lactamase plasmids of *N. gonorrhoeae* have been identified.

I.6.1. Regions required for the replication

Two approaches were used to study regions required for the replication of the $\beta$-lactamase plasmids of *N. gonorrhoeae*: 1) cloning fragments and checking for the ability of the clones to replicate in a polA host, and 2) creating deletion derivatives of plasmids. McNicol et al., (1984) cloned the 2.4-kb *BamH*I fragment and the large *BamH*I fragment of p22209, an Asia-type plasmid and p88557, an Africa-type plasmid of *N. gonorrhoeae*, into pAT2 or pAT153, both derivatives of plasmid pBR322, and proposed that the essential region for the replication of the Asia plasmid (p22209) and the Africa plasmid (p88557) was located on the 0.9-kb non-Tn2 portion of the 2.4-kb *BamH*I fragment, 0.5-kb downstream of the *PstI* site (Figure 6). Studies by Johnson (1985) did not support this conclusion. Johnson (1985) cloned the 2.4-kb *BamH*I fragment, the 1.1-kb and the 3.8-kb *BamH*I-*PvuII* fragments of an Asia-type plasmid
Figure 6. Reported replication regions of the Asia-type and the Africa-type plasmids of *N. gonorrhoeae*.
pGR9091 into pBR322 or pMB8, and found that recombinant plasmids containing either the 2.4-kb BamHI or the 1.1-kb BamHI-Pvull fragments of pGR9091 could not replicate in *Escherichia coli* W3110, a polA1 mutant. Johnson (1985) proposed that the region required for replication was on a 3.8-kb BamHI-Pvull fragment. Both McNicol *et al.* (1984) and Johnson (1985) cloned the DNA fragments into pBR322 or its derivates, vectors which can not replicate in a host deficient in DNA polymerase I (polA'). Later, it was found that the results of McNicol *et al.* (1984) were most likely due to the reversion of the polA mutant host that was used (Gilbride and Brunton, 1990).

By creating mini plasmids from pJD4 and pJD5, Yeung and Dillon (1988) proposed two replication regions. First, a 1.5-kb region, designated as "a" which was not present in pJD5, is located approximately 2.0-kb downstream of the PstI site. Second, a 1.5-kb region, designated "b", was located in the 3.1-kb BamHI fragment of pJD5, 1.5-kb downstream of the PstI site. Since the replication region "b" was interrupted by replication region "a" in pJD4, Yeung and Dillon (1988) hypothesized that the replication region "b" was not functional in pJD4. By creating mini plasmids, Gilbride and Brunton (1990) proposed that the essential region for the replication of pFA3 (the Asia plasmid) was within the 3.3-kb PstI-HindIII fragment, which starts from approximately 460-bp proximal to the BamHI site and includes all non-Tn2 portions of the 2.4-kb BamHI fragment. Gilbride and Brunton (1990) also confirmed the previous report by Yeung and Dillon (1988) and Johnson (1985) that the 2.4-kb BamHI fragment did not direct replication.
The replication region proposed by Johnson (1985) is in agreement with replication region "a" proposed by Yeung and Dillon (1988). A 0.4-kb fragment, 1.5-kb to 1.9-kb downstream of the PstI site, is common to the replication regions proposed by Johnson (1985), Gilbride and Brunton (1990) and replication region "b" proposed by Yeung and Dillon (1988).

1.6.2. Regions required for the mobilization

Conjugation is the process of genetic exchange that requires intimate cell to cell contact (Guiney and Lanka, 1989). In most cases, the DNA transfer from the donor to the recipient is a plasmid (Dale, 1989). Plasmids capable of promoting their own transfer are called conjugative, while those that can not promote their own transfer are called non-conjugative (Dale, 1989). Transfer of non-conjugative plasmids requires a DNA sequence in cis, which is called "basis of mobility" (bom), and is believed to be the origin of transfer (oriT) (Waren et al., 1979).

The β-lactamase-producing plasmids of N. gonorrhoeae are non-conjugative. The Asia- and Africa-type plasmids can be mobilized among N. gonorrhoeae isolates and from N. gonorrhoeae isolates to other species, such as N. meningitidis (Ikeda et al., 1986; Genco et al., 1984), N. cinerea, N. flava, N. perflava, N. sicca, N. subflava, N. flavascens, and N. mucosa (Genco et al., 1984; Roberts and Falkow, 1977; Eisenstein et al., 1977; Sarafian et al., 1990) by the 39.2-kb conjugative plasmid of N. gonorrhoeae. The Asia-type plasmid can also be mobilized and established in a variety of N. gonorrhoeae strains with different auxotypes as well as other genera, such as
E. coli, Salmonella minesota, H. influenzae, H. parainfluenzae and H. ducreyi (Sparling et al., 1978; McNicol et al., 1984; Brunton et al., 1982). The 39.2 kb gonococcal conjugative plasmid can not mobilize the Toronto plasmid. IncP plasmids such as pRK231, pRK2013, and pR751), and Inclα (R64drd-11) could also mobilize the Asia-type plasmid from E. coli to E. coli, from E. coli to S. minnesota, and from E. coli to H. influenzae (Guiney and Ito, 1982). Piffaretti et al. (1988) and Dillon et al. (1990) found that conjugative plasmid pUB307 (IncP) could mobilize the Asia-type plasmid (pPJ121, pPJ122 and pJD4) and the Africa-type plasmid (pJD5) but not the Toronto plasmid from E. coli to N. gonorrhoeae. Conjugative plasmid pR100-1 (IncFIII), pR124 (IncFIV), and pBG791 (Incl) mobilized the Asia plasmid (pJD4) between E. coli strains (Dillon et al., 1990). Conjugative plasmid pBG791 (Inc791) was also able to mobilize pJD4, pJD5 (Africa) and pJD7 (Toronto) from E. coli to N. gonorrhoeae (Dillon et al., 1990).

It appears that mobilization of the Asia- and Africa-type plasmids of N. gonorrhoeae by various conjugative plasmids involves different mechanisms of transfer. Several regions (Figure 7) required for the mobilization of the Asia-type and the Africa-type plasmids have been proposed (McNicol et al., 1983; Tenover et al., 1985; Dillon and Yeung, 1989; Gauthier, 1990). Using a H. ducreyi conjugative plasmid pHD147, and a nick labelling procedure, McNicol et al., (1983) located the origin of transfer (oriT) of p22209, an Asia-type plasmid, on the 1.8-kb BamHI-HindIII fragment, and the oriT of the Africa-type plasmid (p88557) in the 1.4-kb BamHI-AvaI fragment (McNicol et al., 1983).
Figure 7. Location of the putative mobilization regions of the $\beta$-lactamase plasmids of *N. gonorrhoeae*. 
Tenover et al. (1985) found that a recombinant plasmid pFT2, which comprised pFT1, an Asia-type plasmid lacking its 1.9-kb HindII fragment (3.7-kb downstream of the PstI site) and the entire 4.2-kb cryptic plasmid, could not be mobilized by the 39.5-kb gonococcal conjugative plasmid from E. coli to N. gonorrhoeae or E. coli (Tenover et al., 1985). Transcription and translation studies found that compared to pFT1, pFT2 did not produce one protein which was 16-kd in size. The mobilization of ColE1 requires that a specific 16-kd plasmid-encoded protein interact with two other proteins at oriT to initiate the formation of relaxation complex (Waren et al., 1978). Therefore, Tenover et al., (1985) proposed a mobilization region in the 1.9-kb HindII fragment, 3.7-kb downstream of the PstI site.

Dillon and Yeung (1989) cloned three fragments of pJD4 (an Asia-type plasmid): the 2.4-kb BamHI, the 1.8-kb BamHI-HindIII and the 3.2-kb BamHI-HindIII fragment, into pACYC184. Only the recombinant plasmid containing the 2.4-kb BamHI fragment could be mobilized by pBG791 (IncIa) (Dillon and Yeung, 1989). This plasmid was mobilized at a frequency similar to that of the 7.4-kb plasmid. Because the 2.4-kb BamHI fragment contained the β-lactamase gene, it was concluded that the mobilization region was located in the 0.8-kb non-TnA BamHI fragment, which was designated region "M" (Dillon and Yeung, 1989).

By creating mini-plasmid derivatives of pJD4 and using the conjugative plasmid pUB307 (IncP), Gauthier (1990) located the oriT in the 1.8-kb BamHI-HindIII fragment of pJD4. Gauthier (1990) also proposed the 0.6-kb Pvull-Aval fragment of pJD4 as
the essential part of the mob region, since a deletion of the derivative plasmid lacking the 0.6-Kb *PvuII*-Aval fragment could not be mobilized by pUB307. It is not conclusive whether or not the mob region was located entirely within the 0.6-kb *PvuII*-Aval fragment.

1.7. Scientific value and objectives of the present research

The physical structure of the β-lactamase plasmids of *N. gonorrhoeae* as determined by restriction endonuclease analysis has been extensively studied. Partial DNA sequences of several plasmids (the Asia- and the Africa-type plasmids) have also been obtained. Disagreement exists concerning the exact location and the size of the deletion in the Africa plasmid and in the 4.9-kb (Toronto) plasmid. In order to clarify some of these issues, the present studies were undertaken with the following objectives:

1. To obtain the complete DNA sequence of the Asia-type plasmid (pJD4), except for the region homologous to Tn4 (the 0.9-kb *BamHI*-PstI fragment), which has been published previously (Heffron *et al.*, 1979)

2. To specify the exact location of the region deleted from pJD4 to create pJD5 (Africa plasmid) and pJD7 (Toronto plasmid), by obtaining the primary sequence around the deletion and by comparing the sequence to the sequence in pJD4.

3. To analyze the sequences flanking the deletion of the Toronto type plasmids from
various strains and Rio-type plasmid to determine whether the deletion occurred at identical sites and were of similar size.

4. To locate the region homologous to the ampicillin transposable element, TnA, in pJD4, pJD5 and pJD7 and to compare the regions flaking TnA in the various $\beta$-lactamase-producing plasmids in *N. gonorrhoeae*.

5. To specify the location of the insertion of Nimes plasmid (pGF1) and to obtain the sequence of the insertion. Sequencing of the insertion will clarify the nature and relationship of the insertion which, in turn, will shed light on the nature of the $\beta$-lactamase-producing plasmids of *N. gonorrhoeae* in general.

6. To analyze the structure of the $\beta$-lactamase-producing plasmids of *N. gonorrhoeae* and compare their structure to reports in the literature.
Chapter II: MATERIALS AND METHODS

II.1. Bacterial strains and plasmids

*E. coli* strain C600-JD4, C600-JD5 and C600-JD7 containing plasmids pJD4 (Asia), pJD5 (Africa) and pJD7 (Toronto) (Table 5), respectively, were obtained from the National Laboratory for Sexually Transmitted Diseases (NLSTD), Laboratory Centre for Disease Control (LCDC). Plasmids pJD4, pJD5, and pJD7 had been transformed previously into *E. coli* C600 (F' thi-1 leuB6 thr-1 lacY1 tonA21 supE44) (Young and Davis, 1983; Yeung and Dillon, 1985). PPNG strains GC1213, GC4538, and GC5221 containing Toronto-type plasmids pGC1213, pGC4538 and pGC5221, respectively, were also obtained from the National Laboratory for Sexually Transmitted Diseases (NLSTD), Laboratory Centre for Disease Control (LCDC; Table 5). PPNG stains GC5228, GC5230 and GO4717 containing Toronto-type plasmids pGC5228, pGC5230 and "Rio" plasmid (pGO4717), respectively, were obtained from Dr. J. van Embden (National Institute of Public Health and Environmental Hygiene, Bilthoven, The Netherlands). The Nimes plasmid (pGF1) was originally obtained from Dr A. Gouby (Faculté de Medicine, Institute National de la Santé et de la Recherche Medicalé, Nimes, France). Plasmids pGF1 had been transformed (Table 5) previously into *E. coli* HB101 (supE44 supF58 hsdS3 (r6 m b) recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1) (Gouby *et al.*, 1986; Bolivar and Backman, 1979).
Table 5. Bacterial strains and plasmids

<table>
<thead>
<tr>
<th>E. coli strain</th>
<th>PPNG Strains</th>
<th>Plasmid Content</th>
<th>Description/Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>C600-JD4</td>
<td>pJD4</td>
<td>pJD4 transformed into E. coli C600 (Yeung and Dillon, 1985)</td>
<td></td>
</tr>
<tr>
<td>C600-JD5</td>
<td>pJD5</td>
<td>pJD5 transformed into E. coli C600 (Yeung and Dillon, 1985)</td>
<td></td>
</tr>
<tr>
<td>C600-JD7</td>
<td>pJD7</td>
<td>pJD7 transformed into E. coli C600 (Yeung and Dillon, 1985)</td>
<td></td>
</tr>
<tr>
<td>GC1213</td>
<td>Toronto-type</td>
<td>PPNG strain containing Toronto-type plasmid from NLSTD, LCDC</td>
<td></td>
</tr>
<tr>
<td>GC4538</td>
<td>Toronto-type</td>
<td>PPNG strain containing Toronto-type plasmid from NLSTD, LCDC</td>
<td></td>
</tr>
<tr>
<td>GC5221</td>
<td>Toronto-type</td>
<td>PPNG strain containing Toronto-type plasmid from NLSTD, LCDC</td>
<td></td>
</tr>
<tr>
<td>GC5228</td>
<td>Toronto-type</td>
<td>PPNG strain containing Toronto-type plasmid from Dr. J. van Embden</td>
<td></td>
</tr>
<tr>
<td>GC5230</td>
<td>Toronto-type</td>
<td>PPNG strain containing Toronto-type plasmid from Dr. J. van Embden</td>
<td></td>
</tr>
<tr>
<td>HB101-GF1</td>
<td>pGF1</td>
<td>Gouby et al., 1986</td>
<td></td>
</tr>
<tr>
<td>JM83-STD32</td>
<td>pSTD32</td>
<td>1.5-kb BamHI-PstI fragment of pJD4 cloned into pBluescript II KS (+) (Hutnik and Dillon, unpublished data).</td>
<td></td>
</tr>
<tr>
<td>JM83-STD41</td>
<td>pSTD41</td>
<td>3.2-kb BamHI fragment of pJD4 cloned into pBluescript II KS (+) (Hutnik and Dillon, unpublished data).</td>
<td></td>
</tr>
</tbody>
</table>
A recombinant plasmid, pSTD32, which contained a 1.5 kb BamHI/PstI fragment and pSTD41, which contained a 3.2 kb BamHI/HindIII fragment of pJD4 in the phagemid pBluscript II KS (+) (Stratagene Cloning System, La Jolla, California, USA), was also constructed previously (Hutnik and Dillon, unpublished data). *E. coli* JM83 (ara, Δlac pro, thi, strA, p80dlacZΔM15) (Messing, 1979) was used as a host for all cloning experiments and as host in the generation of nested deletions.

II.2. Sources of chemicals and reagent kits

Unless specified otherwise, all chemicals were purchased from British Drug House Inc. (B.D.H. Inc., Toronto, Ontario). Ampicillin was purchased from Sigma Chemical Company, (Sigma, St Louis, Missouri). Luria Bertani (LB) Medium Broth, Tryptic Soy Agar (TSA), Tryptic Soy Broth (TSB) and GC Medium Base (GCMB) were Difco products (Difco Laboratories, Detroit, Michigan). 5-bromo-4-choro-4-indolyl-β-D-galactoside (X-gal) was purchased from Boehringer Mannheim Canada Ltd. (BMC, Laval, Quebec). Restriction buffers, restriction endonuclease enzymes (*Aval, BamHI, HindIII, HinfI, KpnI, PstI, PvuII, SacI, SpeI, TaqI and XbaI*), and bovine serum albumin (BSA) were purchased from Promega (Promega Corporation, Madison, Wisconsin).
The DNA sequencing kit, Sequenase® version 2.0 was purchased from United States Biochemical Corporation (USB, Cleveland, Ohio, USA). The TaqTrack™ Sequencing Kit, Klenow fragment, T4 DNA ligase, ligation buffer, Erase-a-Base® System kit, Magic™ Minipreps and Magic™ Maxipreps DNA purification system kit were purchased from Fisher Scientific. The non-radioactive labelling kit (Dig-kit) was purchased from BMC. ³⁵[S]-dATP was purchased from NEN® Research Products (Dupont Canada Incorporation, Mississauga, Canada).

II.3. Growth of bacteria

*E. coli* strains C600, JM83 and HB101, harbouring β-lactamase-producing plasmids, were cultured on either TSA supplemented with 100 mg ampicillin per litre (L) or TSB supplemented with 60 mg of ampicillin per L. Cultures were incubated at 37°C for 18-24 hours. *E. coli* JM83 was cultured on either TSA or LB medium broth at 37°C for 18-24 hours.

PPNG strains were cultured on GCMB supplemented with 1% Kellogg’s defined supplement (glucose 40 g, glutamine 1 g, 0.5% ferric nitrate solution 10 mL, 20% carboxylase 1 mL) (Dillon, 1983) and 5 mg of ampicillin per L (Sigma). Cultures were incubated at 35°C, in a CO₂ (5-10%) incubator, in a humid environment, for 18-24 hours.
II.4. Transformation of plasmid DNA

II.4.1. Preparation of competent cells

Competent *E. coli* JM83 was prepared as described by Sambrook *et al.* (1989). Either a single colony from a culture grown for 16-20 hours at 37°C or 1 mL of an overnight culture grown in LB medium broth at 37°C was transferred to 100 mL of LB broth in a 1 L flask. After incubation for approximately 3 hours at 37°C, in a shaking water bath (New Brunswick Scientific, Edison, New York, USA), the culture was aseptically transferred to sterile, ice-cold 50 mL centrifuge tubes and cooled to 0°C on ice for 10 minutes. Cells were recovered by centrifugation in a 50 mL sterile centrifuge tube at 4000 rpm, using a type SS-34 rotor (Sorvall Instrument, RC5C, Dupont) for 10 minutes at 4°C. After discarding the media and inverting the tube to let the last traces of media drain away, the cell pellet was resuspended in 10 mL of ice-cold 0.1M CaCl₂ and placed on ice for 10 minutes. Cells were pelleted by centrifugation as described above, and the pellet was resuspended in 4 mL of 0.1M CaCl₂.

II.4.2. Transformation

Transformation was carried out by adding 50 ng of DNA to 200 μL of competent cells in a centrifuge tube, and the contents were mixed by swirling gently. After placing the tube on ice for 30 minutes, cells were heat shocked at 42°C for 90
seconds, followed by chilling on ice for 1-2 minutes. Following the addition of 0.8 mL of LB broth or SOC medium (950 ml deionized water, Bacto-tryptone 20 g, Bacto yeast extract 5 g, NaCl 0.5 g and 20 mM glucose), the culture was incubated in a 37°C waterbath for 45 minutes. Transformed competent cells were plated on selective medium (TSA supplemented with 100 mg/L ampicillin).

II.5. Plasmid DNA isolation

II.5.1. Mini-scale recombinant plasmid DNA preparation

A. A modification of the procedure of Birnboim and Doly (Ng et al., 1987) was used to prepare a high copy number of plasmid DNA, such as pJD4, pJD7 and all recombinant plasmid DNA. One-third loopful of bacterial cells was suspended in 100 μL of ice-cold Solution I (50 mM glucose; 25 mM Tris-Cl, pH 8.0; 10 mM EDTA, pH 8.0) in a microcentrifuge tube, and placed on ice for 10 minutes. Two hundred microlitres (μL) of Solution II (0.2 N NaOH; 1% SDS) were added and mixed by inversion several times to homogeneity. After 5 minutes on ice, 150 μL of Solution III (3 M sodium acetate, pH 4.8) were added and mixed by inversion. The tube was placed on ice for 3-5 minutes. After centrifugation at 12,000 x g (Brinkman Centrifuge type 5414) for 5 minutes at 4°C, the supernatant was transferred to a new tube. The DNA was precipitated with two volumes of 95% ethanol, at 70°C, for at least 10 minutes. Finally, the DNA was centrifuged at 12,000 x g (full speed) for 5
minutes at 4°C. The supernatant was removed and the pellet was air dried. The DNA was suspended either in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) or in sterile double distilled water (ddH₂O).

B. To prepare plasmid DNA for sequencing, the Magic Minipreps™ DNA purification system was used, as described in the manufacture’s protocol (Promega Technical Bulletin, TB 117: 6/91). This method is a modification of the method of Birnboim and Doly (1979).

II.5.2. Large-scale recombinant plasmid DNA preparation

A. To prepare plasmid DNA for restriction endonuclease digestion and subsequent ligation, for the generation of nested deletions as well as for DNA sequencing, DNA was isolated using the method of Birnboim and Doly (1979) followed by purification by cesium chloride (CsCl)-ethidium bromide (EtBr) ultracentrifugation (Sambrook et al. 1989). The bacterial pellet from a 500 mL culture or from 5 to 10 plates was suspended in 10 mL of Solution I in a bottle. Twenty miligrams (mg) of lysozyme were added and mixed thoroughly, and 20 mL of Solution II were added and mixed gently by inverting the tube several times. The tube was placed on ice for 5-10 minutes. Fifteen millilitres of Solution III were added and mixed and the bottle was then placed on ice for at least 10 minutes until a flocculent white precipitate formed. The bacterial lysate was centrifuged at 4000 rpm for 15 minutes at 4°C. The supernatant was transferred to a new bottle and the DNA was
precipitated by adding 2 volumes of 95 % ethanol and stored in -70°C for 2-3 hours. The DNA was recovered by centrifugation at 5000 rpm for 15 minutes. The supernatant was removed and the bottle was inverted to allow remaining supernatant to drain away. The DNA pellet was suspended in 9 mL TE (pH 8.0), and the suspension was transferred to a disposable graduated tube (15 mL). Eight grams of CsCl were added and the contents were mixed gently until the CsCl was completely dissolved. While keeping the tube in the dark, 150 μL ethidium bromide (10 μg/mL) were added and mixed. The mixture was then transferred to a Quick-seal ultracentrifuge tube (Beckman, Palo Alto, California, USA), which was filled to the top with liquid paraffin. After balancing tubes in pairs, they were sealed with a heat sealer and centrifuged at 55,000 rpm for 20-24 hours in a Type 70 Ti rotor (Beckman). The lower band (the plasmid band) was collected by puncturing the side of the tube with a sterile 5 mL gauge needle or by opening the top of the tube then aspirating the plasmid band with a sterile pasture pipet. To remove ethidium bromide, two volumes of water-saturated isobutanol were added (Dillon et al., 1985). After mixing, the mixture was centrifuged in a microcentrifuge tube at full speed for 3-5 minutes. The upper phase (containing ethidium bromide) was discarded. This procedure was repeated several times to minimize the ethidium bromide remaining in the DNA (to ensure that all ethidium bromide was removed). Plasmid DNA then was precipitated with 90% ethanol before being suspended in water or appropriate buffer.
B. A modification of the procedure of Birnboim and Doly (1979) using the Magic Maxiprep™ DNA purification system (Promega) was used to isolate plasmid DNA for sequencing as described in the manufacturer’s protocols (Promega Technical Bulletin, TB 139: 3/92). This method was used especially to obtain plasmid DNA such as pJD5, since using Magic Miniprep™ DNA purification system (Promega) or the Birnboim and Doly method, followed by cesium chloride (CsCl)-ethidium bromide (EtBr) ultracentrifugation (Sambrook et al. 1989) did not yield enough plasmid DNA.

II.6. Calculation of DNA concentration

DNA concentration was measured using the Spectronic® 1001 Spectrophotometer (Bausch & Lomb, Rochester, New York). The optical density (OD) of the solution containing the DNA was taken at wavelengths of 260 nm and 280 nm. The DNA concentration was calculated according to the following formula: a solution containing 50 μg/mL of double-stranded DNA has an absorbance of 1 at 260 nm (Sambrook et al., 1989). The purity of DNA was estimated by comparing the values between the readings at 260 nm and 280 nm (OD_{260/OD_{280}}). Pure DNA preparations have an OD_{260/OD_{280}} value of 1.8 (Sambrook et al., 1989).
II.7. Restriction endonuclease digestion and agarose gel electrophoresis

Restriction endonuclease digests were performed using enzymes and appropriate restriction buffers provided by the manufacturer and under conditions recommended by the manufacturer (Promega). One to 5 μg DNA were dissolved in 25 to 50 μl appropriate restriction buffer. Five to 10 units of restriction enzyme were added and the reaction mixture was incubated at 37°C (except if specified otherwise) for 2-3 hours. To stop the reaction, the tube was incubated at 65°C for 5 minutes. For double and multiple digestions, if conditions for enzyme activity were the same, enzymes were added simultaneously. However, if conditions were different, after digestion with the first enzyme, the DNA was precipitated with ethanol and was subsequently digested with the second enzyme in its appropriate buffer. DNA was analyzed by electrophoresis through 1% agarose gels or 5% polyacrylamide gels to ensure complete digestion (Dillon et al., 1985).

The molecular weight markers used were either the 1 kb DNA ladder (Bethesda Research laboratories Life Technologies, Inc., BRL, Burlington, Canada) or bacteriophage lambda DNA digested with HindIII (BRL). Electrophoresis was performed in tris acetate buffer (40mM Tris base, 20mM sodium acetate, 1.8 mM EDTA, pH 7.8) at 50-60 volts for 3-5 hours. After electrophoresis, the gel was stained with ethidium bromide (1μg of ethidium bromide per mL of water) for 5 to 10 minutes followed by destaining in water for 15 minutes.
II.8. DNA visualization and photography

DNA was visualized using an ultraviolet (UV) transilluminator (Fotodyne, Fotodyne Inc. New Berlin, Wisconsin, USA). An ethidium bromide-stained gel was placed above a UV (300nm) light source with a UV pass-visible block filter. The gel was photographed with a Polaroid MP4 Land camera, using type 52 or type 55 Polaroid film.

II.9. Cloning

DNA fragments were cloned by the shot gun method as described by Dillon et al. (1985). Various DNA fragments were cloned into the corresponding sites of pBluescript II KS (+) to produce the following recombinant plasmids: pSTD32, pSTD41, pATA1, pATA2, pATA3 and pATA4.

II.9.1. Ligation

Ligation was performed as described by Dillon et al. (1985) and Sambrook et al. (1989). Plasmid DNA (0.5 μg) was digested with restriction endonuclease enzymes to generate fragments with cohesive ends and the vector (0.25 μg) was digested with similar enzymes. The vector and DNA fragment were co-precipitated with 70% ethanol followed by suspension in 20 μL of Ligation Buffer (30 mM Tris-
HCl, pH 7.8; 10 mM MgCl₂; 1.0 mM ATP; 10 mM dithiothreitol) containing 1 unit of
T4 DNA ligase. The ligation mixture was incubated overnight in a 16°C water bath.
Ligation reactions were monitored by running 2 μL of the ligation reaction on a 1 %
arose gel. The ligation reaction was considered successful if the DNA produced
ladder bands in the gel.

Recombinant DNAs were then used to transform E. coli JM83 using the method
described in section II.4. Cells then were plated on TSA supplemented with 100 mg/L
ampicillin, covered with 100 μl of 10 mg/L X-gal for each plate. Bacterial cells
forming white colonies, indicative of recombinant plasmids, were isolated. A number
of independently transformed bacterial colonies were subcultured and their plasmid
DNAs were isolated (Birnboim and Doly, 1979; small scale preparation). Plasmid
DNAs were digested with restriction endonuclease enzymes similar to those used for
cloning to release the DNA insert, and were analyzed by electrophoresis in 1 %
arose gels. The fragment sizes were determined to find out whether the insert was
similar to the DNA fragment, which had been cloned.

Recombinant plasmid DNAs could be verified further by limited DNA
sequencing. Approximately 200-250-bp of both ends of the DNA insert of some
recombinant plasmids were sequenced. The nucleotide sequenced obtained was
compared to the primary sequence of pJD4 in the corresponding area. Sequence
similarity indicated that the proper fragment had been cloned.
II.10. Generation of nested deletions

Nested deletions were constructed according to the protocol provided by the supplier (Erase-a-base Kit, Promega). Recombinant plasmid DNAs, prepared using CsCl density gradient ultracentrifugation, were digested with an appropriate restriction endonuclease to generate a 3' overhang, which protects DNA from activity of exonuclease III. The DNA was checked for complete digestion (i.e., linearity) in a 1% agarose gel for linearity, and was then digested with the second restriction enzyme to generate a 5' overhang. Exonuclease III digestion, which acts on 5' overhangs, was performed at 30°C at 60 second intervals. Ligation and transformation procedures were completed as described by the supplier (Promega). Several (3 to 5) single colonies from each time point were subcultured in TSA supplemented with 100 mg/L of ampicillin. Their plasmid DNAs were isolated (see section III.4.1) and analyzed in a 1% agarose gel, in comparison to the size of the undeleted parent plasmid. These plasmids were used for DNA sequencing.

II.11. DNA sequencing

Double-stranded plasmid DNAs were sequenced on both strands by the dideoxy chain termination method (Sanger et al., 1977) using Sequenase® Version 2.0 (USB) and Taq DNA polymerase (Promega). Nucleotides were labelled with [α-35S]-dATP
(Dupont). To prepare a single-stranded DNA template, approximately a 4-5 µg double-stranded DNA was denatured using 0.2M NaOH and 2mM EDTA. Sequencing reactions were carried out according to the instruction manual provided by the supplier (Step-By-Step Protocols for DNA Sequencing With Sequenase® Version 2.0 T7 DNA Polymerase, 1990).

Sequencing reactions were analyzed in 5 % polyacrylamide (sequencing) gels containing 7 M urea (Sequi-Gene® Nucleic Acid Sequencing Cell Instruction Manual, Bio-Rad laboratories). Gel electrophoresis was performed at 1700-1800 volts using the Sequi-Gene® Nucleic Acid Sequencing Cell (Bio-Rad Laboratories, Canada). After electrophoresis, the gel was transferred to Whatman® 3MM Chromatography paper (Fisher). The gel was air dried or dried in a slab gel drier (Model 583, Bio-Rad), as described in the supplier’s protocol (Bio Rad). The gel was autoradiographed using Chronex film (Dupont) in a film cassette for 1-3 days. The film was developed in a Cordell developer (Cordell Engineering Inc., Peabody, Massachusetts, The USA). To resolve gel anomalies caused by high G-C content and secondary structure, a dITP labelling mix was used to substitute the dGTP labelling mix (Mizusawa et al., 1983). Taq DNA polymerase (Taq-Track® Sequencing Kit, Fisher) was used to resolve gel anomalies that could not be resolved when Sequenase® 2.0 was used (Sambrook et al., 1989). Reactions were performed as described in the supplier’s protocol (Fisher).

The primary sequence of the 3.2 kb BamHI-HindIII insert of pSTD41 was obtained previously (Dillon and Ng, unpublished data) but had not been analyzed. To obtain the primary sequence of DNA inserts in pSTD32, pATA1 and pATA2, a
combination of nested deletions as described above (Erase-a-base kit, Fisher) and primer extension (Sambrook et al., 1989) was used. The primary sequences surrounding the deletion in pJD5 and pJD7, and around the area homologous to TnA in pJD5 and pJD7, were obtained by primer extension (Sambrook et al., 1989).

Primers for DNA sequencing were selected based on the primary sequence of pJD4, using "Primer Designer" software (Scientific and Educational Software, State Line, USA). The sequences of the primers and the position of the primers as compared to pJD4 are listed in Table 6. Primers were purchased from General Synthesis and Diagnostics (GSD, Toronto, Canada).

II.12. DNA sequence analysis

The primary DNA sequences generated in this study and the primary sequence of the insert of pSTD41 were analyzed for homology with DNA sequences in the Data Bank Microgenie®, restriction endonuclease sites, direct and inverted repeats, open reading frames (ORFs) and other structural features by Microgenie® Sequence Analysis Program (Beckman Instrument Inc., Palo Alto, California) and PC gene (Stratagene, Canada).
Table 6. Primers used for DNA sequencing

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* The coordinates of primers are calculated according to the primary sequence of pJD4 with the *PstI* as coordinate 1.

b. Coordinate of the Nimes.1 primer is based on the primary sequence of pGF1, with the *PstI* site as coordinate 1.
Chapter III: RESULTS

III.1. Construction of recombinant plasmids for sequencing

For sequencing purposes, the following recombinant plasmids were constructed: pSTD32, pSTD41, pATA1, pATA2, and pATA3. DNA fragments cloned were from plasmids pJD4, pGF1, pJD5, and pJD7 (Figure 8). To construct pSTD32, pSTD41 and pATA1, the 1.5-kb BamHl-PstI, the 3.2-kb BamHl-HindIII and the 2.0-kb XbaI-HindIII fragments of pJD4 were cloned into the appropriate sites of pBluescript II KS (+) as described in the Material and Methods (section II.8). Plasmids pSTD32 and pSTD41 were constructed previously (Hutnik and Dillon, unpublished data). To construct pATA2, pATA3 and pATA4, the 1.8 kb SpeI/PstI fragment of pGF1, the 3.1 kb BamHl fragment of pJD5 and the 2.7 kb BamHl fragment of pJD7 were also cloned into the appropriate sites of pBluescript II KS (+) as described in the Materials and Methods.
Figure 8. Various DNA fragments cloned from pJD4, pGF1, pJD5 and pJD7 into pBluescript II KS (+) to produce recombinant plasmids pSTD32, pSTD41, pATA1, pATA2, pATA3, and pATA4.
Nested deletions of pSTD32 were constructed in both directions. Restriction endonucleases Kpnl (3' overhang) and HindIII (5' overhang) were used to produce nested deletions from the PstI site toward the BamHI site (Figure 9a), and restriction endonucleases SacI (3' overhang) and BamHI (5' overhang) were used to produce nested deletions from the BamHI site toward the PstI site.

Nested deletions of pATA1 were constructed in both directions, from the Xbal site and from the HindIII site. Restriction endonucleases SacI (3' overhang) and Xbal (5' overhang) were used to produce nested deletions from the Xbal site toward the HindIII site (Figure 9b). Restriction endonucleases SacI (3' overhang) and Xbal (5' overhang) were used to produce nested deletions from the HindIII site toward the Xbal site.

Nested deletions of pATA2 were constructed in both directions, from the PstI site and from the SpeI site. Restriction endonucleases Kpnl (3' overhang) and HindIII (5' overhang) were used to produce nested deletions from the PstI site toward the SpeI site (Figure 9c). Restriction endonucleases SacI (3' overhang) and SpeI (5' overhang) were used to produce nested deletions from the SpeI site toward the PstI site.
Figure 9. Restriction endonucleases used to generated nested deletions of pSTD32, pATA1, and pATA2.
III.3. DNA sequence of pJD4

The primary sequence of pJD4 excluding a 0.9-kb *BamH*I-*Pst*I fragment, which is homologous to Tn4, was obtained by sequencing the DNA inserts from a series of recombinant plasmids: pSTD32, pSTD41, and pATA1. The DNA insert of pSTD41 was sequenced previously (Dillon, unpublished data), but the sequence had not been analysed.

To sequence the insert of pSTD32, nested deletions were constructed to produce a number of subclones (Figure 10) from the *BamH*I site (pSTD32B1, pSTD32B13, pSTD32B18, pSTD32B27, pSTD32B32, pSTD32B30, pSTD32B37, pSTD32B36, pSTD32B43, pSTD32B41, pSTD32B59, pSTD32B51, pSTD32B29, pSTD32B54, pSTD32B47, and pSTD32B24), and from the *Pst*I (pSTD32P2, pSTD32c, pSTD32i, pSTD32P5, pSTD32P6, pSTD32P7, pSTD32P9, pSTD32P11, pSTD32P12, pSTD32P13, pSTD32P14 and pSTD32P15). Clones were numbered sequentially and then ordered by size on a 1 % agarose gel (Figure 10).

The sequence of the 1.5-kb *BamH*I-*Pst*I insert of pSTD32 from the *BamH*I site (Figure 11) was generated from overlapping sequences obtained from nested deletion and were numbered 32B0, 32B1, 32B13, 32B18, 32B27, 32B32, 32B30, 32B37, 32B36, 32B43, 32B41, 32B59, 32B51, 32B29, 32B54, 32B47, and 32B24. Sequence number 32B0 was generated directly from pSTD32. The sequence of the
Figure 10.  Sizing deletions from the *BamHI* site of pSTD32 on an 1% agarose gel. Subclones were digested with *PvuII*. Lane 1, pBluscript II KS (+); lane 2, pSTD32; lane 3, pSTD32B1; lane 4, pSTD32B9; lane 5, pSTD32B13; lane 6, pSTD32B18; lane 7, pSTD32B14; lane 8, pSTD32B27; lane 9, pSTD32B28; lane 10, pSTD32B32; lane 11, pSTD32B30; lane 12, pSTD32B37; lane 13, pSTD32B36; lane 14, pSTD32B43; lane 15, pSTD32B41; lane 16, pSTD32B48; lane 17, pSTD32B20; lane 18, pSTD32B51; lane 19, pSTD32B59; lane 20, pSTD32B29; lane 21, pSTD32B54; lane 22, pSTD32B50; lane 23, pSTD32B47; lane 24, pSTD32B15; lane 25, pSTD32B24; lane 26, pSTD32B35; lane 27, 1 Kb DNA ladder.
Figure 11. Strategy used to determine the primary sequence of the 1.5-kb $PstI$-$BamHI$ insert of $pSTD32$. The length of the arrows corresponds to the number of nucleotides actually sequenced from each subclone.
1.5-kb *BamHI-PstI* insert of pSTD32 from the *PstI* site (Figure 11) was generated from overlapping sequences numbered 32P0, 32P2, 32Pc, 32Pi, 32P5, 32P6, 32P7, 32P9, 32P11, 32P12, 32P13, 32P14, and 32P15. Sequence number 32P0 was generated directly from pSTD32.

The primary sequence of the 2.0-kb *XbaI-HindIII* fragment of pJD4 was obtained by a combination of nested deletions and primer extensions of the insert of pATA1 (Figure 12). Nested deletions from the *HindIII* site produced subclones pATA1H57, pATA1H62, pATA1H68, pATA1H83, pATA1H76, and pATA1H67. Nested deletions from the *XbaI* site produced subclones pATA1X3.1, pATA1X2.1, and pATA1X9.5. The sequence from the *HindIII* site was generated from overlapping sequences ATA1H0 (which was generated directly from pATA1), ATA1H57, ATA1H62, ATA1H68, ATA1H83, ATA1H76, ATA1HJD4.1, ATA1HJD4.2, and ATA1H67. Sequences ATA1HJD4.1 and ATA1HJD4.2 were generated from pATA1 using primers JD4.1 and JD4.2 (Materials and Methods). The sequence from *XbaI* was generated from overlapping sequences of ATA1X0 (which was generated directly from pATA1), ATA1X3.1, ATA1X2.1, ATA1X9.5 and ATA1XJD4.11, ATA1XJD4.9, ATA1XJD4.7, ATA1XJD4.6, ATA1XJD4.5, ATA1XJD4.4, and ATA1XJD4.3. Sequences ATA1XJD4.11, ATA1XJD4.9, ATA1XJD4.7, ATA1XJD4.6, ATA1XJD4.5, ATA1XJD4.4, and ATA1XJD4.3 were generated from pATA1 by primer extension with primers JD4.11, JD4.9, JD4.7, JD4.6, JD4.5, JD4.4, and JD4.3. Primers were generated from the complementary sequence which was determined first.
Figure 12. Strategy used to determine the primary sequence of the 2.0-kb \( XbaI \)-
\( HindIII \) insert of pATA1. The length of the arrows corresponds to the
number of nucleotides actually sequenced from each subclone. *,
sequence derived by primer extension. The bottom line indicates the
scale in base pairs (bp) with \( XbaI \) as zero reference position.
One problem affecting sequence interpretation was anomalous patterns of migration in which adjacent bands of DNA became compressed, usually due to secondary structures or GC-rich areas. This problem was resolved by either substituting 2'-deoxyguanosine 5'-triphosphate (dGTP) with 2'-deoxyinosine 5'-triphosphate (dITP) in sequencing reactions with Sequenase 2.0 or by performing the sequencing reaction with Taq-Track® DNA polymerase. For example in pSTD32 at coordinates 1 to 26, using dGTP, the sequence obtained was 5'-CTGCA GCAAT C?G?A?G?A? CAACG TTGCG C-3' (Figure 13), and the bands were not clear at coordinates 11 to 15. The sequence obtained using dITP was 5'-CTGCA GCAAT GGCAA C?AACG TTGCG C-3', with only the band coordinate 16 giving an interpretation problem. Since the C at coordinate 16 in the sequencing reaction using dGTP was clear, the final deduced sequence was interpreted as 5'-C'iGCAGCAAT GGCAACAAACG TTGCG C-3'.

DNA sequences were determined in both directions except for nucleotide sequences from coordinates 1321 to 1504 (a 185-bp XbaI-BamHI fragment of pJD4). This fragment was sequenced four times, since this region is an overlapping sequence between the insert of pSTD32 and pATA1.
Figure 13. Autoradiogram of sequencing reactions of pSTD32 from the PstI site using dGTP and dITP at coordinates 1 to 26.
III.4. Primary sequence of pJD4

The complete primary sequence of pJD4, written in the 5' to 3' direction, is presented in Figure 14, using the PstI site as coordinate 1. The nucleotides from coordinates 6514 to 7426 were taken from the Tn3 sequence as described by Heffron et al. (1979). The total length of pJD4 is 7,426 bp.

The G-C contents of pJD4, including the 0.9-kb BamHI-PstI fragment comprising Tn3, is 38.4 %, and the GC content of pJD4 excluding the region homologous to TnA, is 35.7 %. Two regions, at coordinates 1535 to 1964 and 3115 to 3792, have G-C content of 29.0 % and 34.5 %, respectively. Two other regions, at coordinates 1696 to 1744 and 1771 to 1860 have especially low G-C content (26.5 % and 21 %, respectively).

III.5. Analysis of the primary sequence of pJD4

III.5.1. Restriction endonuclease sites

Restriction endonuclease sites deduced from the primary sequence of pJD4, including the 0.9-kb BamHI-PstI fragment of Tn3 (Heffron et al., 1979), as identified using the Micro Genie® program, are listed in Table 7. Twenty-three restriction endonucleases (AatII, AffIII, ApaLI, AsuII, Aval, BanI, BglII, BspMI, BstEII, DrdI,
Figure 14. The primary sequence of pJD4. It was written in 5' to 3' direction. Coordinate 1 is the *PstI* site. Coordinates 6514 to 7426 were taken from the Tn3 sequence (Heffron *et al.*, 1979). The sequence homologous to Tn4 is written in bold. A 507/509-bp direct repeat (DR-30) is underlined. IHF, sequence homologous to the consensus sequence of integration host factor. US, sequence homologous to gonococcal uptake sequence.
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Table 7 cont’d. Location of restriction endonucleases sites on pJD4.

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Table 7 cont’d. Location of restriction endonucleases sites on pJD4.

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Table 7 cont'd. Location of restriction endonucleases sites on the primary sequence of pJD4.

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<th>Number</th>
<th>Restriction endonuclease</th>
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<th>Coordinate(s) of restriction sites</th>
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<tr>
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<td><em>PstI</em></td>
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<tr>
<td>55</td>
<td><em>PvuII</em></td>
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<td>56</td>
<td><em>PvuII</em></td>
<td>1</td>
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<td>57</td>
<td><em>Rrul</em></td>
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<td><em>RsaI</em></td>
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<td><em>ScrFI</em></td>
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<td><em>Spal</em></td>
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<td>71</td>
<td><em>XmnI</em></td>
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</table>

A restriction endonuclease map of pJD4 comprising restriction endonucleases that have 11 sites or less is shown in Figure 15. Most of the recognition sites were located from coordinates 6251 to 460, where the GC content is relatively high, 47%. Two regions have only a few restriction endonuclease recognition sites: from coordinates 1501 to 2500 and from 3400 to 4500. These two region have low GC contents, 31.4% and 33.4%, respectively.
Figure 15. Restriction endonuclease map of pJD4. Coordinate 1 is the PstI site.

Figure 15a, coordinates 1-500; Figure 15b, coordinates 500-2300;
Figure 15c, coordinates 2200-4400; Figure 15d, coordinates 4400-6000; Figure 15e, coordinates 6000-6900; Figure 15f, coordinates 6900-7426; Figure 15g, restriction endonuclease map of pJD4 which has $\geq 12$ recognition sites (Ahal, Alul, Ddel, Fnu4HI, MboI, MnlI, Msel, RseI, Rsal, and Sau3AI). $\rightarrow$, region homologous to TnA.
Figure 15d
III.5.2. Structural features of pJD4 based on the primary sequence

The primary sequence of pJD4 has 62 direct repeats (DRs) and 24 inverted repeats (IRs) which are \( \geq 10 \)-bp in length. The direct repeats (Table 8; Appendix 1) are ordered chronologically by coordinates, and called DR-1 to DR-62. Forty-seven of these are perfect direct repeats, and the remaining 15 are imperfect repeats. The longest of these 62 different direct repeats, DR-30, is present in two copies, 507 and 509-bp in length. Fifty-four other DRs are also present in two copies, while sequences DR-3, DR-5, DR-18, DR-38, and DR-48, are present in three copies, and sequences DR-26 and DR-37 are present in four copies. One direct repeat, DR-27, is present in five copies (Figure 16).

The two copies of the longest direct repeat, DR-30, are designated DR-30A and DR-30B; with DR-30A (507-bp) located from coordinates 1881 to 2387, and DR-30B (509-bp) from coordinates 3708 to 4216. The repeats is not perfect; two nucleotides in DR-30B, corresponding to coordinates 3738 and 3828, are not present in DR-30A (Figure 17). Six other nucleotide mismatches between these two sequences, including coordinates 1917 (A), 1983 (A), 1991 (T), 2037 (T), 2042 (G), and 2214 (C) of DR-30A, corresponding to coordinates 3746 (T), 3811 (T), 3818 (C), 3866 (C), 3871 (T), and 4043 (T) of DR-30B, respectively.
Figure 16. Map of direct and inverted repeats (≥10-bp) found in pJD4. -->, direct repeats (DR); <-->, inverted repeats (IR). Figure 16a, coordinates 1-500; Figure 16b, coordinates 500-2300; Figure 16c, coordinates 2200-4400; Figure 16d, coordinates 4400-6000; Figure 16e, coordinates 6000-6900; Figure 16f, coordinates 6900-7426.
Figure 16b
Figure 16c
Figure 16d
Figure 16f
Table 8. Location of direct repeats on the primary sequence of pJD4\(^a\).

<table>
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<th>Direct repeats</th>
<th>Coordinate of the first sequences</th>
<th>Coordinate of the other sequences</th>
<th>Number of nucleotides</th>
<th>Number of mismatched nucleotides</th>
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<td>DR-1</td>
<td>312 to 325</td>
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<tr>
<td>DR-2</td>
<td>502 to 593</td>
<td>2386 to 2477</td>
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<td>DR-3</td>
<td>527 to 539</td>
<td>2411 to 2423</td>
<td>23</td>
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<td>7321 to 7333</td>
<td>23</td>
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<td>DR-4</td>
<td>608 to 617</td>
<td>5428 to 5437</td>
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<tr>
<td>DR-5</td>
<td>a. 665 to 674</td>
<td>b. 2279 to 2288</td>
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<td>c. 4108 to 4117</td>
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<td>DR-6</td>
<td>677 to 690</td>
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<td>DR-7</td>
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\(^a\) DNA sequence of direct repeats found in Appendix 1.
Table 8 cont’d. Location of direct repeats on the primary sequence of pJD4a).

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<th>DR</th>
<th>Coordinate of the first sequence</th>
<th>Coordinate of the other sequences</th>
<th>Number of nucleotide</th>
<th>Number of mismatched nucleotides</th>
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<td>1665 to 1676</td>
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<td>c. 1745 to 1766</td>
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<tr>
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<td>d. 1767 to 1788</td>
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<td>b. 1723 to 1744</td>
<td>c. 1745 to 1766</td>
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<td>d. 1767 to 1788</td>
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<td>d. 1767 to 1779</td>
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<td>1871 to 1881</td>
<td>6918 to 6928</td>
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<td>1881 to 2387</td>
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<td>507-509</td>
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<td>1911 to 1921</td>
<td>3352 to 3362</td>
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a) DNA sequence of direct repeats found in Appendix 1.
Table 8 cont’d.  Location of direct repeats on the primary sequence of pJD4<sup>a</sup>.

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<td>2207 to 2216</td>
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<td>2279 to 2292</td>
<td>4108 to 4121</td>
<td>14</td>
<td>0</td>
</tr>
<tr>
<td>DR-37</td>
<td>a. 2292 to 2303</td>
<td>b. 2331 to 2342</td>
<td>12</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>c. 4121 to 4232</td>
<td>12</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>d. 4160 to 4171</td>
<td>12</td>
<td>0</td>
</tr>
<tr>
<td>DR-38</td>
<td>2296 to 2305</td>
<td>4125 to 4134</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6850 to 6859</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>DR-39</td>
<td>2603 to 2614</td>
<td>6165 to 6176</td>
<td>12</td>
<td>1</td>
</tr>
<tr>
<td>DR-40</td>
<td>2655 to 2665</td>
<td>3344 to 3354</td>
<td>11</td>
<td>0</td>
</tr>
<tr>
<td>DR-41</td>
<td>2885 to 2894</td>
<td>4223 to 4232</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>DR-42</td>
<td>2915 to 2924</td>
<td>3155 to 3164</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>DR-43</td>
<td>3278 to 3287</td>
<td>3737 to 3746</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>DR-44</td>
<td>3280 to 3292</td>
<td>5597 to 5609</td>
<td>13</td>
<td>1</td>
</tr>
<tr>
<td>DR-45</td>
<td>3514 to 3525</td>
<td>6459 to 6470</td>
<td>12</td>
<td>0</td>
</tr>
<tr>
<td>DR-46</td>
<td>3542 to 3551</td>
<td>5621 to 5630</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>DR-47</td>
<td>3543 to 3570</td>
<td>3587 to 3614</td>
<td>28</td>
<td>0</td>
</tr>
<tr>
<td>DR-48</td>
<td>a. 3543 to 3564</td>
<td>b. 3565 to 3586</td>
<td>22</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>c. 3587 to 3608</td>
<td>22</td>
<td>0</td>
</tr>
</tbody>
</table>

<sup>a</sup> DNA sequence of direct repeats found in Appendix 1.
Table 8 cont’d. Location of direct repeats on the primary sequence of pJD4*

<table>
<thead>
<tr>
<th>Name of DR</th>
<th>Coordinate of the first sequence</th>
<th>Coordinate of the other sequences</th>
<th>Number of nucleotides</th>
<th>Number of mismatched nucleotides</th>
</tr>
</thead>
<tbody>
<tr>
<td>DR-49</td>
<td>3652 to 3663</td>
<td>3664 to 3675</td>
<td>12</td>
<td>0</td>
</tr>
<tr>
<td>DR-50</td>
<td>3783 to 3792</td>
<td>4285 to 4294</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>DR-51</td>
<td>3915 to 3924</td>
<td>7328 to 7337</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>DR-52</td>
<td>4121 to 4132</td>
<td>4160 to 4171</td>
<td>12</td>
<td>0</td>
</tr>
<tr>
<td>DR-53</td>
<td>4231 to 4240</td>
<td>5766 to 5775</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>DR-54</td>
<td>4249 to 4258</td>
<td>4283 to 4292</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>DR-55</td>
<td>4306 to 4315</td>
<td>5133 to 5142</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>DR-56</td>
<td>4597 to 4606</td>
<td>5682 to 5691</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>DR-57</td>
<td>4950 to 4959</td>
<td>5238 to 5247</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>DR-58</td>
<td>5185 to 5194</td>
<td>5229 to 5238</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>DR-59</td>
<td>5188 to 5198</td>
<td>5211 to 5221</td>
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<td>0</td>
</tr>
<tr>
<td>DR-60</td>
<td>5286 to 5295</td>
<td>5687 to 5696</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>DR-61</td>
<td>5316 to 5325</td>
<td>5931 to 5940</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>DR-62</td>
<td>5324 to 5333</td>
<td>5888 to 5897</td>
<td>10</td>
<td>0</td>
</tr>
</tbody>
</table>

a) DNA sequence of direct repeats found in Appendix 1.
Figure 17. Comparison of the primary sequences of DR-30A (coordinates 1881 to 2387) and DR-30B (coordinates 3708 to 4216). Sequences are written in 5' to 3' direction. Nucleotide differences were written in bold. * indicates that there is no nucleotide at a given coordinate. n, indicates locations of nucleotide differences.
Most of the direct repeats are clustered around two regions of pJD4 (Figure 16). The first region, from coordinates 1535 to 1964, contains DR-20, DR-21, DR-22, DR-23, DR-24, DR-25, DR-27, DR-28, DR-29, DR-30, DR-31, and DR-32. This region is located upstream of the DR-30A and has a G-C content of 28.9%. In particular, at coordinates 1696 to 1744 where DR-25, DR-26 and DR-27 are located, the G-C content is low (26.5 %). DR-26 consist of a 22-bp sequence which is repeated four times, all in this region. DR-27 consist of a 13-bp sequence which is repeated five times, four of these sequences are located in this region. The second region spans coordinates 3115 to 3792, and includes DR-16, DR-17, DR-20, DR-22, DR-28, DR-30, DR-31, DR-42, DR-43, DR-44, DR-45, DR-46, DR-47, DR-48, DR-49, and DR-50. DR-48 consist of a 22-bp sequence, which is repeated three times.

The primary sequence of pJD4, including the TnA coding region, carries 24 inverted repeats which are \( \geq 10 \)bp, and were numbered sequentially (Table 9; Appendix 2). All except 2 of these inverted repeats are imperfect having 1-3 mismatches. Most of these inverted repeats (IRs) are located at 2 regions which are A-T rich (\( > 66\% \))(Figure 16). The first region, which located within the first region of direct repeats, spans coordinates 1607 to 1973, and contains IR-4, IR-5, IR-6, IR-7, IR-8, IR-9, IR-10, IR-11 and IR-12. The second region spans coordinate 3556 to 4358, is located within the second direct repeat region, and contains IR-13, IR-14, IR-15, IR-16 and IR-17.
Table 9. Location of inverted repeats (IRs)* of the primary sequence of pJD4.

<table>
<thead>
<tr>
<th>IR</th>
<th>Coordinate of the first sequence</th>
<th>Coordinate of the second sequence</th>
<th>Number of nucleotides</th>
<th>Number of mismatched nucleotides</th>
</tr>
</thead>
<tbody>
<tr>
<td>IR-1</td>
<td>397 to 406</td>
<td>390 to 381</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>IR-2</td>
<td>1201 to 1210</td>
<td>1104 to 1094</td>
<td>10/11</td>
<td>1</td>
</tr>
<tr>
<td>IR-3</td>
<td>1282 to 1295</td>
<td>1194 to 1182</td>
<td>14/13</td>
<td>2</td>
</tr>
<tr>
<td>IR-4</td>
<td>1652 to 1661</td>
<td>1617 to 1607</td>
<td>10/11</td>
<td>1</td>
</tr>
<tr>
<td>IR-5</td>
<td>1663 to 1682</td>
<td>1662 to 1644</td>
<td>20/19</td>
<td>3</td>
</tr>
<tr>
<td>IR-6</td>
<td>1696 to 1720</td>
<td>1630 to 1607</td>
<td>25/24</td>
<td>1</td>
</tr>
<tr>
<td>IR-7</td>
<td>1711 to 1742</td>
<td>1637 to 1607</td>
<td>32/31</td>
<td>3</td>
</tr>
<tr>
<td>IR-8</td>
<td>1709 to 1721</td>
<td>1675 to 1663</td>
<td>13</td>
<td>1</td>
</tr>
<tr>
<td>IR-9</td>
<td>1743 to 1764</td>
<td>1628 to 1607</td>
<td>22</td>
<td>1</td>
</tr>
<tr>
<td>IR-10</td>
<td>1828 to 1840</td>
<td>1817 to 1805</td>
<td>13</td>
<td>1</td>
</tr>
<tr>
<td>IR-11</td>
<td>1898 to 1909</td>
<td>1848 to 1837</td>
<td>12</td>
<td>1</td>
</tr>
<tr>
<td>IR-12</td>
<td>1962 to 1973</td>
<td>1939 to 1928</td>
<td>12</td>
<td>1</td>
</tr>
<tr>
<td>IR-13</td>
<td>3556 to 3566</td>
<td>3481 to 3470</td>
<td>11</td>
<td>1</td>
</tr>
<tr>
<td>IR-14</td>
<td>3578 to 3588</td>
<td>3481 to 3470</td>
<td>11/12</td>
<td>1</td>
</tr>
<tr>
<td>IR-15</td>
<td>3696 to 3706</td>
<td>3693 to 3683</td>
<td>11</td>
<td>1</td>
</tr>
<tr>
<td>IR-16</td>
<td>3790 to 3801</td>
<td>3767 to 3756</td>
<td>12</td>
<td>1</td>
</tr>
<tr>
<td>IR-17</td>
<td>3848 to 3857</td>
<td>3755 to 3745</td>
<td>10/11</td>
<td>1</td>
</tr>
<tr>
<td>IR-18</td>
<td>4473 to 4486</td>
<td>4431 to 4417</td>
<td>14/15</td>
<td>1</td>
</tr>
<tr>
<td>IR-19</td>
<td>4478 to 4491</td>
<td>4474 to 4461</td>
<td>14</td>
<td>2</td>
</tr>
<tr>
<td>IR-20</td>
<td>4662 to 4672</td>
<td>4644 to 4635</td>
<td>11/10</td>
<td>1</td>
</tr>
<tr>
<td>IR-21</td>
<td>5488 to 5498</td>
<td>5469 to 5460</td>
<td>11/10</td>
<td>1</td>
</tr>
<tr>
<td>IR-22</td>
<td>6053 to 6073</td>
<td>6050 to 6030</td>
<td>21</td>
<td>2</td>
</tr>
<tr>
<td>IR-23</td>
<td>6157 to 6167</td>
<td>6084 to 6075</td>
<td>11</td>
<td>1</td>
</tr>
<tr>
<td>IR-24</td>
<td>6531 to 6540</td>
<td>6483 to 6474</td>
<td>10</td>
<td>0</td>
</tr>
</tbody>
</table>

a) DNA sequence of IRs found in Appendix 2.
III.5.3. Open reading frames (ORFs) analysis

The primary sequence of pJD4, including the region homologous to TnA, was analyzed for potential open reading frames (ORFs) using the software Micro Genie® and PC gene. In this study, only ORFs with the potential to encode 20 amino acids or more are presented. The sizes were used here because in *E. coli* nearly every gene has an ORF of at least 50 codons (Stormo, 1987), and the average protein is 250 amino acids (Creighton, 1993). The method used was a one to detect the protein coding regions in procaryotic cells (Kolaskar and Reddy, 1985), using PC gene software package. This method detects protein coding regions by analyzing nucleotides from -18 to +18 of the initiation codon (ATG and GTG), and the nucleotides within the ORFs. A scoring system was applied to the nucleotides between -18 and +18 of the initiation codon and to the nucleotides between initiation and stop codons. If the value is $\geq 26$ for the initiation and $\geq 6$ for the contents, the region is defined as protein coding sequence. ORFs as small as 20 codons (60 bp) were detected.

Thirty protein coding regions (ORFs) in all possible frames were identified throughout the sequence of pJD4 (Table 10). Twenty of the ORFs have ATG as the initiation codon, and the remaining 10 ORFs have GTG as the initiation codon. Ten ORFs- ORF3, ORF4, ORF5, ORF6, ORF8, ORF9, ORF15, ORF20, ORF23 and ORF24-
Table 10. Summary of the most probable ORFs in pJD4 identified by the method of Kolaskar and Reddy.

<table>
<thead>
<tr>
<th>ORF</th>
<th>Coordinates</th>
<th>Start/Stop codon</th>
<th>Size of amino acids</th>
<th>Molecular weight</th>
<th>Initiation score</th>
<th>Coding score</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1192-1260</td>
<td>ATG/TAG</td>
<td>22</td>
<td>2.745</td>
<td>26</td>
<td>7</td>
</tr>
<tr>
<td>2</td>
<td>2894-3013</td>
<td>ATG/TGA</td>
<td>39</td>
<td>4.418</td>
<td>26</td>
<td>8</td>
</tr>
<tr>
<td>3</td>
<td>4561-5997</td>
<td>ATG/TAG</td>
<td>478</td>
<td>56.212</td>
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<td>8</td>
</tr>
<tr>
<td>4</td>
<td>4699-5997</td>
<td>ATG/ATG</td>
<td>432</td>
<td>50.867</td>
<td>26</td>
<td>10</td>
</tr>
<tr>
<td>5</td>
<td>5035-5997</td>
<td>ATG/TAG</td>
<td>320</td>
<td>37.943</td>
<td>31</td>
<td>10</td>
</tr>
<tr>
<td>6</td>
<td>5335-5997</td>
<td>ATG/TAG</td>
<td>220</td>
<td>25.917</td>
<td>27</td>
<td>9</td>
</tr>
<tr>
<td>7</td>
<td>5569-5997</td>
<td>ATG/TAG</td>
<td>142</td>
<td>16.876</td>
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<tr>
<td>8</td>
<td>6886-318</td>
<td>ATG/TAA</td>
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<tr>
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<td>ATG/TAA</td>
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<td>4575-4429</td>
<td>ATG/TAA</td>
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<td>ATG/TGA</td>
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<td>6.199</td>
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<td>3498-2479</td>
<td>ATG/TGA</td>
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<td>16</td>
<td>2466-2374</td>
<td>ATG/TAG</td>
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<td>6</td>
</tr>
<tr>
<td>17</td>
<td>2295-2173</td>
<td>ATG/TAA</td>
<td>40</td>
<td>4.801</td>
<td>28</td>
<td>7</td>
</tr>
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<td>ATG/TGA</td>
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<tr>
<td>19</td>
<td>2019-1933</td>
<td>ATG/TAA</td>
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<td>3.106</td>
<td>24</td>
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<tr>
<td>20</td>
<td>1269-622</td>
<td>ATG/TAG</td>
<td>215</td>
<td>25.400</td>
<td>26</td>
<td>10</td>
</tr>
</tbody>
</table>
Table 10 cont’d. Summary of the most probable ORFs in pJD4 identified by the method of Kolaskar and Reddy.

<table>
<thead>
<tr>
<th>ORF</th>
<th>Coordinates</th>
<th>Start/Stop codon</th>
<th>Size of amino acids</th>
<th>Molecular weight</th>
<th>Initiation score</th>
<th>Coding score</th>
</tr>
</thead>
<tbody>
<tr>
<td>21</td>
<td>3886-4038</td>
<td>GTG/TAG</td>
<td>50</td>
<td>6.010</td>
<td>27</td>
<td>8</td>
</tr>
<tr>
<td>22</td>
<td>5208-5270</td>
<td>GTG/TAA</td>
<td>20</td>
<td>2.308</td>
<td>27</td>
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</tr>
<tr>
<td>23</td>
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<tr>
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<td>180</td>
<td>21.363</td>
<td>26</td>
<td>10</td>
</tr>
<tr>
<td>25</td>
<td>4559-4467</td>
<td>GTG/TAA</td>
<td>30</td>
<td>3.312</td>
<td>29</td>
<td>6</td>
</tr>
<tr>
<td>26</td>
<td>4310-4245</td>
<td>GTG/TAG</td>
<td>21</td>
<td>2.649</td>
<td>31</td>
<td>8</td>
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<tr>
<td>27</td>
<td>4093-3836</td>
<td>GTG/TAG</td>
<td>85</td>
<td>10.491</td>
<td>32</td>
<td>10</td>
</tr>
<tr>
<td>28</td>
<td>3608-3543</td>
<td>GTG/TAG</td>
<td>21</td>
<td>2.515</td>
<td>30</td>
<td>8</td>
</tr>
<tr>
<td>29</td>
<td>3586-3512</td>
<td>GTG/TAA</td>
<td>24</td>
<td>2.668</td>
<td>27</td>
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<tr>
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<td>2264-2007</td>
<td>GTG/TAG</td>
<td>85</td>
<td>10.525</td>
<td>32</td>
<td>9</td>
</tr>
</tbody>
</table>
have the potential to encode a polypeptide with a molecular weight higher than 20-Kdal. Five ORFs- ORF7, ORF10, ORF12, ORF27 and ORF30- have the potential to encode polypeptides with a molecular weight between 10.0 to 20.0-Kdal. Four ORFs (ORF11, ORF14, ORF18 and ORF21) have the potential to encode polypeptides with a molecular weight between 5.0 to 10.0-Kd and the remaining ORFs have the potential to encode polypeptides with a molecular weight less than 5.0-Kdal. A map of the 30 putative ORFs can be found in Figure 18.

ORFs identified in pJD4 were compared to protein sequences in the Data Bank. ORF8 has the potential to encode a 31.5-kd protein (286 amino acids), β-lactamase (Heffron et al., 1979). This protein was found to be 100 % homologous to the β-lactamase precursor of E. coli plasmid pBR322 and showed significant homology to a variety of β-lactamase found in a variety of bacteria: 228 of 286 amino acids were 43.9 % homologous to a β-lactamase precursor of Bacillus cereus; 262 amino acids were 68.7 % homologous to a β-lactamase precursor of E. coli plasmid p453; 273 amino acids were 64.5 % homologous to a β-lactamase precursor of Klebsiella pneumoniae.

The ORFs found in the primary sequence of pJD4 were compared to the proteins reported in the transcription/translation studies by several research groups (Yeung and Dillon, 1985; Tenover et al., 1985; Biswas et al., 1986). Comparison of the potential ORFs found in pJD4 and the proteins reported from in vitro transcription and translation studies (Yeung and Dillon, 1985; Tenover et al., 1985; Biswas et al., 1986) shows that almost all proteins detected in the transcription translation studies
Figure 18. Map of the potential Open Reading Frames (ORFs) found in the primary sequence of pJD4. Figure 18a, coordinates 1-500; Figure 18b, coordinates 500-2275; Figure 15c, coordinates 2200-4400; Figure 15d, coordinates 4400-6000; Figure 15e, coordinates 6000-6900; Figure 15f, coordinates 6900-7426.
Figure 18c
were found in these ORFs. Some proteins reported in the transcription and translation studies, such as the 33 and 15 Kd proteins were not found in these ORFs (Table 11).

III.5.4. Homology comparison between the primary sequence of pJD4 and DNA sequences in the Data Bank

The primary sequence of pJD4 excluding Tn4 sequences was compared to the sequences in the Data Bank of MicroGenie® and PCgene. It was found that the primary sequence of pJD4 from coordinates 300 to 1504 showed a high degree homology to the primary sequence of pFA7 (an Africa-type plasmid) (Sanchez-Pescador et al., 1988). Seventeen nucleotide differences were found between the primary sequence of pJD4 and pFA7 corresponding to coordinates 369, 577, 583, 595, 596, 604, 706, 781, 916, 1090, 1095, 1099, 1100, 1123, 1130, 1311 and 1486 of pJD4 (Figure 19). The nucleotide sequence of pJD4 at coordinates 1077 to 1107 is 5'-AATTA AATCT AGGGT ATTTA TTTTC AACCT G-3' (Figure 20A), while the reported nucleotide sequence of pFA7 at the corresponding area is 5'-AATTA AATCT AGG* T ATT*A TT** C AACCT G-3' (*) indicates that there is no nucleotides) (Sanchez-Pescador et al., 1988). At coordinates 1114 to 1134 (20B) the nucleotide sequence of pJD4 is 5'-CCATT CTTTT AGTTT TTCTA C-3', while the nucleotide sequence of pFA7 is 5'-CCATT CTTT* AGTTT T*CTA C-3' (Sanchez-Pescador et al., 1988). Nucleotides at coordinates 1090, 1095, 1099, 1100, 1123 and 1130 of pJD4 do not exist in pFA7.
Table 11. Comparison of open reading frames found in the primary sequence of pJD4 and reports of transcription/translation products observed by other research groups.

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\textsuperscript{a} ORFs that have the potential to encode protein with molecular weight 4.5 Kdal or less are not indicated since no product were detected in other studies.
Figure 19. Comparison of the primary sequence of pJD4 (top), the primary sequence of pFA3 (middle; Gilbride and Brunton, 1990) and pFA7 (bottom; Sanchez-Pescador et al., 1988). The nucleotide differences among pJD4, pFA3 and pFA7 sequences are indicated by n. Asterisks (*) indicate that there is no nucleotide at a given coordinate. Coordinates are based on the primary sequence of pJD4. End, end of sequence determination.
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Figure 20. Autoradiogram of the primary sequence of pJD4 at coordinates 1077 to 1107 (20A) and 1114 to 1134 (20B), the region where the nucleotide sequences are different from that of pFA7 (Sanchez-Pescador et al., 1988). Coordinates are based on the coordinates of the primary sequence of pJD4.
The primary sequence of pJD4 also showed homology to pFA3 (an Asia-type plasmid) (Gilbride and Brunton, 1990), from coordinates 386 to 1987. Six nucleotide differences were found between the sequence of pJD4 and pFA3 (Figure 19) corresponding to coordinates 388, 1873, 1874, 1888, 1892, and 1893 of pJD4.

III.5.5. Distinctive sequence in the primary sequence of pJD4

Goodman and Scocca (1988) showed the 10-bp sequence 5'-'GCCGTCTGAA-3' or 5'-'TTCAGACGGC-3' is required for the gonococcal transformation. In 1991, Elkins et al. reported that uptake of DNA by N. gonorrhoeae is increased by the presence of the 10-bp sequence, and a single copy of the uptake sequence was sufficient for efficient uptake. No sequences 100% homologous to the uptake sequence were detected in pJD4. One sequence was found to be 90% homologous to the uptake sequence (from coordinates 1400 to 1409, 5'-'GCCGCCTGAA-3'). Five sequences were 80% homologous to the uptake sequence: coordinates 3243 to 3252 (5'-'TTAAACGGC-3'), 3383 to 3345 (5'-'TT*AGACGG-3'), 4947 to 4956 (5'-'TTCAGAACGC-3'), 4961 to 4968 (5'-'TT**GACGGC-3'), and 6108 to 6116 (5'-'GCGGT*TGAA-3').

Mcintire and Dempsey (1987) reported that integration host factor (IHF) is required for the transformation of plasmid R100. IHF was first detected as a host factor integrative recombination in bacteriophage λ (Nash and Robertson, 1981). The
IHF in the transformation of plasmid R100 recognizes the consensus sequence 5'-C/TAANNNTTGATA/T-3' or the complementary strand 5'-A/TATCAANNNTTA/G-3'. In the primary sequence of pJD4, this consensus sequence is present in three copies, from coordinates 1794 to 18006 (5’-TAACTTATTGATT-3’), 2223 to 2235 (5’-TATCAATACATTG-3’), and 4052 to 4064 (5’-TATCAATACATTG-3’). Six other sequences located at coordinates 1059-1071 (5’-AAACACGTTGATT-3’), 1479-1491 (5’-AAATCTTTTGATA-3’), 2171-2159 (5’-AAACGGATTGATA-3’), 2247 to 2236 (5’-AATCATTTTTA-3’), 4000 to 3988 (5’-AAACGGATTGATA-3’), and 4065 to 4076 (5’-TAAAAAAATGATT-3’) were found with one base pair mismatched to the consensus sequence recognized by the IHF.

III.6. Location of the deletion of pJD5 as compared to pJD4

It has been shown by restriction endonuclease analysis and by limited DNA sequence analysis that the Asia-type plasmid of *N. gonorrhoeae* is homologous to the Africa-type plasmid except for a deletion reported from 1.8 to 2.1-kb by various groups (Yeung and Dillon, 1985; Yeung et al., 1986; Dickgiesser, 1982; Aalen and Gundersen, 1987). In addition, reports differ regarding the exact location of the deletion in comparison to the map of the Asia-type plasmid (Figure 2). To determine the exact location and the size of the deletion of pJD5, the region around the deletion of pJD5, an Africa-type plasmid was sequenced.
To obtain the primary sequence around the deletion of pJD5, a recombinant plasmid pATA3 which was constructed from pJD5. To construct pATA3, the 3.1-kb BamHI fragment of pJD5 was cloned into the appropriate site of pBluescript II KS (+), as described previously (section II. Materials and Methods). Based on the map of pJD5 generated by Yeung and Dillon (1985), the putative deletion of pJD5 is located within the 3.1-kb BamHI fragment.

The primary sequence around the deletion of pJD5 was obtained by sequencing pATA3 by a primer extension using the universal primer T3, primers JD5.1, JD5.2 and JD5.5 (Figure 21). Primers JD5.1, JD5.2 and JD5.5 were generated using the primary sequence of pJD4. The primary sequence was generated from overlapping sequences ATA3.1, ATA3.2, ATA3.5 and ATA3.T3 which were obtained by sequencing pATA3 using primers JD5.1, JD5.2 and JD5.5 and T3, respectively. Since the region sequenced in pJD5 was homologous to pJD4, the DNA was sequenced in only one direction.

Eight hundred and eighty-four base pairs of the primary sequence around the deletion of pJD5 is shown in Figure 22. This sequence is compared to the analogous region of pJD4 and the coordinates used are those of pJD4. Analysis of the sequence from coordinates 3707 to 4216 indicated similarities to DR-30, the 507/509 bp direct repeat of pJD4. This pJD5 sequence was compared to both DR-30A and DR-30B. The sequence of pJD5 and DR-30A were different in 6 nucleotides at coordinates 1983, 1990, 2000, 2037, 2042, and 2214 (data not shown). Only two nucleotide
Figure 21. Strategy used to determine the primary sequence around the deletion of pJD5. The region sequenced, which was located inside 1.1-kb BamHI-\textit{Earl} fragment was scaled up. Sequencing reactions from each of the primer sites are indicated in the bottom of the figure. The length of the arrows corresponds to the number of nucleotides actually sequenced from each primer start site. The direction of the arrows indicate the direction sequenced from 5' to 3'.
Figure 22. Primary sequence around the deletion of pJD5 (bottom) and the primary sequence of pJD4 (top). Coordinate 1 being the PstI site of pJD4. The end of the nucleotide determined from pJD5. DR-30B is underlined. Nucleotide differences between pJD4 and pJD5 were written in bold. \( n \), indicates location of nucleotide differences. \( * \), indicates that there is nucleotide at a given coordinate.
1524      1583
pJD4  CTAATTTTCTGATTTTCATCAATATCTCAGTTAATTTGCTTCAATAAGGCTATTAGCCT
pJD5  CTAATTTTCTGATTTTCATCAATATCTCAGTTAATTTGCTTCAATAAGGCTATTAGCCT

1643
pJD4  TTAACACAACCTAAATCATATTGTCACAGAAGACACAAATTTTCTGTTTAAAAACAAACAGCAA
pJD5  TTAACACAACCTAAATCATATTGTCACAGAAGACACAAATTTTCTGTTTAAAAACAAACAGCAA

1703
pJD4  AATATACCTGTGTTTTATATAATAAAAACAAACAAAGTATTTTTCTAAAGGTTGCTATATAACAG
pJD5  AATATACCTGTGTTTTATATAATAAAAACAAACAAAGTATTTTTCTAAAGGTTGCTATATAACAG

1717      1739
pJD4  GAAATTGGTGTCCCTATATACAGAAATTTTGTGTCGATATAACAGAAATTTTGTGTCGTA
pJD5  GAAATTGGTGTCCCTATATACAGAAATTTTGTGTCGATATAACAGAAATTTTGTGTCGTA

1763
pJD4  TAAACAGAAAATTTGTGTCGATAAGTTTTGTAACATTATTGTATTTTACGTGTTTTAATAAAAAC
pJD5  TAAACAGAAAATTTGTGTCGATAAGTTTTGTAACATTATTGTATTTTACGTGTTTTAATAAAAAC

1823
pJD4  GCCGAAAACACAGTAAGAAACACAAATATAAAATATAGGGAACCGCTGCTCCCGTTTTTTGGG
pJD5  GCCGAAAACACAGTAAGAAACACAAATATAAAATATAGGGAACCGCTGCTCCCGTTTTTTGGG

1883
pJD4  CTTCAGCCCTAATTTTTTTTTCTCTCTCTATCGGATTTAAATTACAAAAACCTTACAGA
pJD5  CTTCAGCCCTAATTTTTTTTTCTCTCTCTATCGGATTTAAATTACAAAAACCTTACAGA

1943
------------ sequence does not exist in pJD5 ---------------

3707      3737      3745      3758
pJD4  ..CTTTTTGGGGCTTCCAGCTCTATTTTTTTTTTTCAGGATTAAATTACAAAAAC
pJD5  -----------GGGGCTTTCAGCCCTAATTTTTTTTTTTT*CAAGGATTAAATTACAAAAAC

3818
pJD4  CTTACAGGGACAGTAAAGTTTTGCTTCTTTGCAAGGTTTCACAGCAACGTAGGCGTC
pJD5  CTTACAGGGACAGTAAAGTTTTGCTTCTTTGCAAGGTTTCACAGCAACGTAGGCGTC

3878
pJD4  AGGCCTAGGGCCTGACCTATAAAGGGCAATTATAATTTTATCTTAAACCTTCCTTTAAAA
pJD5  AGGCCTAGGGCCTGACCTATAAAGGGCAATTATAATTTTATCTTAAACCTTCCTTTAAAA

3938
pJD4  GCTTTGAGGTGGTCCTCTTTTATCGACTCATCATTCTCTCTTTTGACTTTCTCTTTTTGGATCT
pJD5  GCTTTGAGGTGGTCCTCTTTTATCGACTCATCATTCTCTCTTTTTTGCATTTTCTCTTTTTGGATCT

3998
pJD4  TTGTGATCGGGCAATTTTTGGGAATAGTTTTTCTCATTCTCTCATCTAAGTTCTTTTTTTTTTTGCT
pJD5  TTGTGATCGGGCAATTTTTGGGAATAGTTTTTCTCATTCTCTCATCTAAGTTCTTTTTTTTTTTGCT
differences were noted (at coordinates 3737 and 3745), when pJD5 was compared to DR-30B (Figure 22). Therefore, the 508-bp of the primary sequence of pJD5 from coordinates 3708 to 4216 corresponded to DR-30B in pJD4. The region of pJD4, which is deleted to produced pJD5, was 1827 bp and corresponds to coordinates 1881 to 3707 of pJD4 (Figure 22). The deletion of pJD5 is adjacent to coordinates 1500 to 1800, a region with many direct and inverted repeats. Thus, the 884 bp of the primary sequence of pJD5 corresponds to coordinates 1524 to 1880 and 3708 to 4234 of pJD4. The size of pJD5 was estimated to be 5.6 kb (5599 bp). Comparison of the 884-bp of the primary sequence of pJD5 to pJD4 showed four nucleotide differences corresponding to coordinates 1717, 1739, 3737 and 3745 of pJD4.

III.7. Primary sequence around the deletion of pJD7

In order to determine the exact location and the size of the deletion of pJD7, the map of pJD7 reported previously (Yeung and Dillon, 1985) was used to estimate the approximate location of the deleted fragment. The deletion was located approximately 3.4 kb downstream of the PstI site (Yeung and Dillon, 1985). Then the nucleotide sequence around the deletion were determined.
The primary sequence around the deletion of pJD7 was obtained by primer extension using primers JD7.4, JD7.5 and JD7.7 (Figure 23), which flanked the deletion. These primers were generated based on the primary sequence of pJD4. The primary sequence around the deletion of pJD7 was generated from overlapping sequences of ATA4.4, ATA4.5 and ATA7.

Four hundred and thirty-three bp of pJD7 were sequenced corresponding to coordinates 3506-3802 and 6075-6209 of pJD4 (Figure 24). By comparing the primary sequence of pJD7 with that of pJD4, the deletion of pJD7 was found to be 2273 bp corresponding to coordinates 3803-6074 of pJD4. Upstream of the deleted fragment (coordinates 3117 to 3800) is a region with a cluster of direct and inverted repeats. The 433 bp nucleotide sequenced in pJD7 were 100 % homologous to pJD4. Therefore the estimate size of pJD7 is 5.15 kb (5153 bp). This 433-bp nucleotide sequence of pJD7 contains IR-15, IR-16, IR-23 and DR-46, DR-47 and DR 49.
Figure 23. Strategy used to determine the primary sequence around the deletion of pJD7. The length of the arrows corresponds to the number of nucleotides actually sequenced from each primer start site. The arrows indicate the direction of the sequence from 5' to 3'.
Figure 24. Comparison of the primary sequence flanking the deletion of pJD7 and the primary sequence of pJD4 in the corresponding area. The coordinates of the deleted sequences are 3803 to 6074. The primary sequence of pJD7 presented is the sequence determined in this study.

..., sequence of pJD4 (not written) ; ---, sequence is not present in pJD7. End, end sequence determination.
pJD4  3515 3525 3535 3545 3555 3565
   ATACAGTTAAATGGTGTCA ATAAAAACAAAGACCATTA TAACAATATAATTTGTCACCC
   ATACAGTTAAATGGTGTCA ATAAAAACAAAGACCATTA TAACAATATAATTTGTCACCC
   DR-48
pJD7

pJD4  3575 3585 3595 3605 3615 3625
   TATAACAATAAATTTTGTCAC CCTATAACAATAAAATTGTGTC CACCTATAAATCTCGCAAGC
   TATAACAATAAATTTTGTCAC CCTATAACAATAAAATTGTGTC CACCTATAAATCTCGCAAGC
   DR-48  DR-48
pJD7

pJD4  3635 3645 3655 3665 3675 3685
   CTTGTGTAACAAGGAGGAGCC AGAGCCCTACAACAAAGAGATA CAABAACAAGAATACAAAAAAA
   CTTGTGTAACAAGGAGGAGCC AGAGCCCTACAACAAAGAGATA CAABAACAAGAATACAAAAAAA
   DR-49  DR-49
pJD7

pJD4  3695 3705 3715 3725 3735 3745
   TAGAGCCCTAAAGGCTCTTTTT TGGGGCTTTTGCACCACTAATT TTTTTTTTTTTCAAGGATTT
   TAGAGCCCTAAAGGCTCTTTTT TGGGGCTTTTGCACCACTAATT TTTTTTTTTTTCAAGGATTT
pJD7

pJD4  3755 3765 3775 3785 3795 3805
   AAAATTACAAAAAACCCCTACA GAGCAAGTTAATTGGTGTGTC TGTTCGCAAGGGTTGAC
   AAAATTACAAAAAACCCCTACA GAGCAAGTTAATTGGTGTGTC TGTTCGCAAGGGTTGAC
pJD7

pJD4  3815 3825 3835 3845 3855 3865
   AACCCTAGCCGCCTACGGGTCA GGGCGGTAGCCTATAAAAGC CATTATAATTTTATTTCTTT...
   --------------------------------- sequence not present in pJD7 ---------------------------------

pJD4  6070 6080 6090 6100 6110 6120
   ...TTTAAAGGCTTGCAATA AAAACAACCCTAAACATTT TGAGGGCCGCCTAGTAAAATT
   ------------------- GCRATA AAAACAACCCTAAACATTT TGAGGGCCGCCTAGTAAAATT
pJD7

pJD4  6130 6140 6150 6160 6170 6180
   TACCTATTCCCACCATTCAAT GATCAGCGAGAACATTTTTG TGATTGCCGTAATAATGTCCGT
   TACCTATTCCCACCATTCAAT GATCAGCGAGAACATTTTTG TGATTGCCGTAATAATGTCCGT
pJD7

pJD4  6190 6200 6209
   ATATCTAGTGGAGGCAACAC CGCCCAAG
   ATATCTAGTGGAGGCAACAC CGCCCAAG
pJD7  6nd

pJD4  6200 6209
   ATATCTAGTGGAGGCAACAC CGCCCAAG
   ATATCTAGTGGAGGCAACAC CGCCCAAG
III.8. Comparison of the primary sequence around the deletion of Toronto-type plasmid pGC1213, pGC4538, pGC5221, pGC5228, pGC5230, and "Rio" plasmid (pGO4717).

A number of Toronto-type plasmids have been isolated (Yeung et al., 1986). I predicted that the deletions of all the Toronto-type plasmids are identical. To ascertain that, the regions around the deletion in various other Toronto-type plasmids - pGC1213, pGC4538, pGC5221- were also sequenced. Since those plasmids were all Toronto-type plasmids, it was predicted that the nucleotide sequence should be identical to pJD7, the "Toronto" plasmid. In addition, the DNA sequence surrounding "Rio" plasmid (pGO4717) was initially reported to be smaller (4.6 kb) than the Toronto plasmid (van Embden et al., 1985); however, based on restriction endonuclease analysis it was subsequently reported to be identical to pJD7 (Dillon and Yeung, 1989). The strategy and the primers used to obtain the nucleotide sequence around the deletion of these plasmids were the same as described above for pJD7 (Figure 23).

The primary sequence around the deletions of pGC1213, pGC4538, pGC5221, pGC5228, pGC5230, and pGO4717 were compared to the primary sequence of pJD7 (Figure 25). The primary sequence determined for each of the plasmids using coordinates of pJD4 is as follows: pGC1213, coordinates 3712-3802 and 6075-6202; pGC4538, coordinates 3676-3802 and 6075-6202; pGC5221, coordinates
Figure 25. Comparison of the primary sequences of pJD7, pGC1213, pGC4538, pGC5221, pGC5228, pGC5230 and pGO4117. Coordinates used are coordinates of the primary sequence pJD4. ----, indicates areas not sequenced. ..., indicates region deleted in comparison to pJD4. *End,* sequence determination.
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3755-3802 and 6075-6167; pGC5228, coordinates 3694-3802 and 6075-6203; pGC5230, coordinates 3740-3802 and 6075-6179; and pGO4717, coordinates 3724-3802 and 6075-62023. All these sequences were identical to pJD7, including the Rio plasmid, and the deleted sequence relative to the sequence of pJD4 was identical to that of pJD7. The deletion in each of the Toronto/Rio-type plasmids was 2273 bp corresponding to coordinates 3803 to 6074 in pJD4. The direct and inverted repeats present in each of the Toronto/Rio-type plasmids were also identical to that of pJD7.

III.9. Analysis of sequences homologous to TnA in pJD4, pJD5 and pJD7

The ampicillin resistance transposon (TnA) comprises closely related transposons that were designated Tn1, Tn2, and Tn3 (Heffron et al., 1977). Tn3 has been completely sequenced (Heffron et al., 1979). Comparison of the primary sequence of pFA3 and pFA7 to that of Tn1 and Tn3 in the bla region, intervening non-coding region and tnpR region, Chen and Clowes (1987b) found 26 bp differences, while only one bp difference was found when the primary sequence of pFA3 and pFA7 was compared to that of Tn2 at the same region. Therefore, the TnA present in pFA3 and pFA7 is Tn2 (Chen and Clowes, 1987b).
The TnA sequence present in pFA3 and in pFA7 is different (Chen and Clowes, 1987b). I would like to know whether the TnA/Tn2 sequence is present in pJD4, pJD5 and pJD7. To find out the exact location of the region homologous to TnA/Tn2 in pJD4, pJD5 and pJD7, the primary sequences of pJD4, pJD5 and pJD7 were compared to the primary sequences of TnA/Tn2 (Heffron et al., 1979; Chen and Clowes, 1987a).

III.9.1. Region homologous to IR-R of TnA in pJD4, pJD5 and pJD7

Members of TnA were similar on their inverted repeat (IR) termini and their mode of transmission (Sherratt, 1989). Since Tn3 is the only ampicillin transposon that has been sequenced in the IR-R region, the primary sequence of pJD4 was compared to that of IR-R of TnA (Tn3). The primary sequence of pJD4 in the IR-R region of TnA is located within 1.5-kb PstI-BamHI insert of pSTD32. The primary sequence of pJD4 in that region was obtained by sequencing the DNA insert of pSTD 32 (Results, section III.5; Figure 11).

The primary sequence of pJD4 from coordinates 1 to 467 was 100% homologous to TnA from coordinates 4991 (within bla gene) to 4957 (the end of IR-R sequence)(Figure 26). The 38-bp IR-R sequence of TnA (Heffron et al., 1979) corresponded to coordinates 430 to 467 of pJD4. Thus, in the IR-R region, the primary sequence of pJD4 is identical to that of TnA.
Figure 26. Primary sequences of pJD4, pJD5, and pJD7 as compared to the primary sequence of TnA (Tn3). The coordinates correspond to position in pJD4, with the *PstI* site as coordinate 1. *, end of IR-R of TnA. IR-R sequence were underlined. ____, not sequenced.
571
pJD4  TCGGCTTAATTTTTCTGTCATCGTTAAAATTGCTATTTCATCTTCTCTTTCCTCCTTA 600
TnA  ---------------------------------------
pJD5  TCGGCTTAATTTTTCTGTCATCGTTAAAATTGCTATTTCATCTTCTCTTTCCTCCTCAAAA 636
pJD7  TCGGCTTAATTTTTCTGTCATCGTTAAA^end  -------------------------------------

636
pJD4  AAAGTTAAGTAAATACCTACCTAAATTTTTACACTAGTTCGCAATCTACGAGCTTATAACC 660
TnA  ---------------------------------------
pJD5  AAAGTTAAGTAAATACCTACCTAAATTTTTACACTAG^end  
pJD7  ---------------------------------------
The primary sequences around the region homologous to IR-R of TnA in pJD5 and pJD7 were obtained by primer extension, using primers Tn3.1 and Tn3.3 (Materials and Methods, Table 6), which flanks the IR-R region. Two hundred and eighty-two base pairs of pJD5 was obtained, which corresponds to coordinates 352 to 636 of pJD4 (Figure 26). Comparison of this sequence to that of pJD4 found that it was identical. Because the primary sequence of pJD4 was identical to that of TnA at the corresponding area, the primary sequence of pJD5 was therefore, identical to that of TnA. The nucleotide sequence of pJD7 obtained (201-bp) in this region corresponded to coordinates 371 to 571 of pJD4 (Figure 26). This primary sequence (pJD7 sequence in the IR-R region) was identical to both pJD4 and TnA. Therefore, in the IR-R region of TnA, the nucleotide sequences of pJD4, pJD5, pJD7 and TnA were identical.

III.9.2. Region homologous to tnpR of TnA in pJD4, pJD5 and pJD7

By comparing to Tn1, Tn2 and Tn3, Chen and Clowes (1987b) found that the nucleotide sequences homologous to TnA in pFA3 and pFA7 were more identical to Tn2 than to Tn1 or Tn3. They also reported only one base pair difference between nucleotide sequences in the region homologous to TnA in pFA3 (an Asia-type plasmid) and pFA7 (an Africa-type plasmid), and that of Tn2. Therefore, they concluded that the TnA present in the β-lactamase plasmids of N. gonorrhoeae pFA3 and pFA7 was Tn2.
The \textit{tnpR} region of Tn2, the one homologous to Tn2 in pFA3, starts at coordinate 6235 of pJD4, while the region homologous to Tn2 in pFA7 starts at coordinate 6236 of pJD4. I would like to know whether the nucleotide sequences homologous to TnA/Tn2 in pJD4, pJD5 and pJD7 are different. Since pJD4, pJD5 and pJD7 are \(\beta\)-lactamase plasmids from \textit{N. gonorrhoeae}, it is very likely that the TnA sequence present in these plasmids is also Tn2.

To establish the region homologous to TnA in pJD4, pJD5 and pJD7, nucleotide sequences of pJD4, pJD5 and pJD7 in the \textit{tnpR} region were compared to that of Tn2. The regions homologous to TnA in pFA3 and pFA7 (Chen and Clowes, 1987b) were used to estimate the location of the end homology between TnA (in the \textit{tnpR} region), and pJD5 and pJD7.

The primary sequence of the 3.2 kb \textit{BamH}I-\textit{Hind}III fragment of pJD4 was obtained by sequencing the DNA insert of pSTD41 (Results, section III.5.). Comparison of this sequence to the \textit{tnpR} sequence of Tn2 found that from coordinates 6235 to 6518 indicated that the sequence was identical (Figure 27). The homology of pJD4 to Tn2 starts at coordinate 6235 of pJD4. Coordinate 6518 was the end of sequence determined in pJD4 (in this study). The sequence from coordinates 6519 to 7426 was not determined in this study.

The primary sequence of pJD5 in the \textit{tnpR} region was obtained by sequencing the DNA insert of pATA3, a recombinant plasmid DNA containing the 3.1-kb \textit{BamH}I
Figure 27. Comparison of the primary sequence of pJD4, pJD5, pJD7, and Tn2 (Chen and Clowes, 1987a) in the tnpR region. tnpR sequence is underlined; *, there is no nucleotide at a given coordinate. \( n \), the nucleotide differences among Tn2, pJD4, pJD5, and pJD7. The nucleotide differences were written in bold. End, end of sequence determination. The coordinates are based on the sequence of pJD4.
Tn2
pJD4 ATATCTAGTTGAGGCACAAC CCGCACAAGTCATTTGCCCA ACCAAGAGGCGCATATAACCG
pJD5 ATATCTAGTTGAGGCACAAC CCGCACAAGTCATTTGCCCA ACCAAGAGGCGCATATAACCG
pJD7 CAAAGTCATTGCCCCCA ACCAAGAGGCGCATATAACCG

6250 6260 6270 6280 6290 6300
Tn2 TATATTACCCATAGGGATAT CCGCAGTTCAACAGAGCCGA GAAGGGGCTGGACATTTCGAG
pJD4 TATATTACCCATAGGGATAT CCGCAGTTCAACAGAGCCGA GAAGGGGCTGGACATTTCGAG
pJD5 TATATTACCCATAGGGATAT CCGCAGTTCAACAGAGCCGA GAAGGGGCTGGACATTTCGAG
pJD7 TATATTACCCATAGGGATAT CCGCAGTTCAACAGAGCCGA GAAGGGGCTGGACATTTCGAG

6310 6320 6330 6340 6350 6360
Tn2 GATGAAAGTGAGGAGGAAGTTG ATGTCATTCTGCTGTTAGAAG CTCGACGCTCTGTGGCCGCCGA
pJD4 GATGAAAGTGAGGAGGAAGTTG ATGTCATTCTGCTGTTAGAAG CTCGACGCTCTGTGGCCGCCGA
pJD5 GATGAAAGTGAGGAGGAAGTTG ATGTCATTCTGCTGTTAGAAG CTCGACGCTCTGTGGCCGCCGA
pJD7 GATGAAAGTGAGGAGGAAGTTG ATGTCATTCTGCTGTTAGAAG CTCGACGCTCTGTGGCCGCCGA

6370 6380 6390 6400 6410 6420
Tn2 CACTGCGATATGATCCCAAC TGATAAAAGGAATTTGAGCT CAGGGCCTGGGCAAGTCCGTTT
pJD4 CACTGCGATATGATCCCAAC TGATAAAAGGAATTTGAGCT CAGGGCCTGGGCAAGTCCGTTT
pJD5 CACTGCGATATGATCCCAAC TGATAAAAGGAATTTGAGCT CAGGGCCTGGGCAAGTCCGTTT
pJD7 CACTGCGATATGATCCCAAC TGATAAAAGGAATTTGAGCT CAGGGCCTGGGCAAGTCCGTTT

6430 6440 6450 6460 6470 6480
Tn2 CATGGATAGCAGGGATCTAGTA CCGCAGTGGGATATGCGGCCATGTGTA CCGAGTGGCATGATATGGGGGAA ATGCTGTCACCATCTCCTGTCC
pJD4 CATGGATAGCAGGGATCTAGTA CCGCAGTGGGATATGCGGCCATGTGTA CCGAGTGGCATGATATGGGGGAA ATGCTGTCACCATCTCCTGTCC
pJD5 CATGGATAGCAGGGATCTAGTA CCGCAGTGGGATATGCGGCCATGTGTA CCGAGTGGCATGATATGGGGGAA ATGCTGTCACCATCTCCTGTCC
pJD7 CATGGATAGCAGGGATCTAGTA CCGCAGTGGGATATGCGGCCATGTGTA CCGAGTGGCATGATATGGGGGAA ATGCTGTCACCATCTCCTGTCC

6490 6500 6510 6520 6530 6540
Tn2 GCCGTGCGCACACGGCTGAC GCCCGAGGATCTGGAGCCGC ACCAATGGGAGGGCCGCACAGA
pJD4 GCCGTGCGCACACGGCTGAC GCCCGAGGATCTGGAGCCGC ACCAATGGGAGGGCCGCACAGA
pJD7 GCCGTGCGCACACGGCTGAC GCCCGAGGATCTGGAGCCGC ACCAATGGGAGGGCCGCACAGA
fragment of pJD5, using primers Tn.2 and T7. Two hundred and ninety four bp were obtained, which corresponded to coordinates 6181 to 6474 of pJD4 (Figure 27). Comparison of this sequence to that of pJD4 showed one base pair different, which corresponded to coordinate 6235. The nucleotide at coordinate 6235 is A on pJD4, while on pJD5 the nucleotide is not present. The primary sequence of pJD5 is identical to that of Tn2 in the tnpR from coordinates 6236 to 6474 (Figure 27). Homology to Tn2 in pJD5 starts at coordinate 6236, one bp different from pJD4, which starts at coordinate 6235 of pJD4.

To establish the homology between pJD7 and Tn2 in the tnpR region, recombinant plasmid DNA pATA4 containing the 2.7 kb BamH1 fragment of pJD7 was constructed (Materials and Methods, Figure 8). The primary sequence of pJD7 in the tnpR region was obtained by sequencing pATA4 using primers Tn.2 and T7. The nucleotide sequence obtained was 309-bp corresponding to coordinates 6205 to 6518 of pJD4 (Figure 27). Comparison of this sequence to that of pJD4 showed one bp different, which corresponded to coordinate 6340, i.e., G on pJD4, and A on pJD7. Comparison of this sequence to that of Tn2, from coordinates 6235 to 6218, was similar, except at coordinate 6240. Homology to Tn2 in pJD7 was similar to that of pJD4, starting from coordinate 6235. Therefore, in the tnpR region, pJD4 and pJD7 were found to be similar to pFA3 (an Asia-type plasmid).
III.10. Primary sequence of the insertion of pGF1 (the Nimes plasmid).

The 1.2-kb insertion of pGF1 was located on the 2.4-kb BamHI fragment of the Africa-type plasmid, outside the region homologous to TnA (Gouby et al., 1986; Dillon and Yeung, 1989). To further specify the location of the insertion on pGF1 prior to DNA sequencing, the plasmid was digested with PstI and Spel and compared to a similar restriction endonuclease digest of pJD4. PstI and Spel were selected because both have a single site in pJD4 and pJD5 and were located within the 2.4-kb BamHI fragment of pJD5 and pJD4 (Dillon and Yeung, 1989; Gouby et al., 1986). Digestion of pJD4 with PstI-Spel produced two fragments, 6.8-kb and 0.6-kb in size (data not shown). When pGF1 was digested with the same restriction endonuclease (PstI and Spel; Figure 28), two fragments were produced, 4.8 kb and 1.8 kb in size. Because the small PstI-Spel fragment of pGF1 was 1.8-kb, 1.2 kb bigger than that of pJD4, therefore, the 1.2-kb insertion of pGF1 is located between the PstI and the Spel sites.

The primary sequence of the insertion of pGF1 was obtained by sequencing the DNA insert of pATA2, which was a recombinant plasmid containing the 1.8-kb PstI-Spel fragment of pGF1. Nested deletions were constructed from the PstI and the Spel sites (Figure 29). Nested deletions from the PstI site produced subclones pATA2P3, pATA2P5, pATA2P6, and pATA2P12. Nested deletion from the Spel site produced subclones pATA2S3, pATA2S4, pATA2S5, pATA2S7, pATA2S8, pATA2S11, pATA2S12, and pATA2S14. These subclones were numbered sequentially and ordered by size.
Figure 28. Sizing of pGF1. Lane 1, 1KbDNA ladder; Lane 2, pGF1 (undigested); Lane 3, pGF1 digested with PstI and SphI; Lane 4, pGF1 digested with BamHI.
Figure 29. Strategy to determine the primary sequence of the insert of pATA2. Sequencing reactions from each of the sites are indicated in the top and the bottom of the figure. The length of the arrows corresponds to the number of nucleotides actually sequenced from each primer site. The arrows indicate the direction of the sequence from 5’ to 3’.
The DNA sequence of the *PstI*-SpeI insert of pATA2 from the *PstI* site was generated from overlapping sequences of ATA2P0, ATA2PTn3.3, Nimes.1, ATA2P3, ATA2P5, ATA2P6, and ATA2P12. Sequence ATA2P0 was generated from pATA2 using T3 primer, and sequences ATA2PTn3.3 and Nimes.1 from pATA2 using Tn3.3 and Nimes.1 primers. The remaining sequences were generated from the subclones using T3 primer. The DNA sequence of the *PstI*-SpeI insert of pATA2 from the SpeI site consisted of overlapping sequences of ATA2S0, ATA2S3, ATA2S4, ATA2S5, ATA2S7 and ATA2S7, ATA2S8, ATA2S11, ATA2S12 and ATA2S14. Sequence ATA2S0 was generated from pATA2 with T7 primer. The remaining sequences were generated from the subclones using T7 primer.

The entire *PstI*-SpeI fragment of pGF1 is 1836 bp (Figure 31). The nucleotide sequences from coordinates 1 to 604 and 1804 to 1836 were identical to that of pJD4. The insertion of pGF1 was 1199 bp in length, 605-bp to 1803-bp from *PstI* site as compared to pJD4. The insertion was located between coordinates 604 and 605 of pJD4. The insertion was flanked by a 4-bp direct repeat sequence 5'-TTAA-3'. The G-C content of the insertion is 53.2% (mole fraction), much higher than the G-C contents of pJD4.

Comparison of the sequence with the Data Bank found a 100% homology to the entire nucleotide sequence of IS5 (Schoener and Kahn, 1981; Kroeger and Hobom, 1982; Engler and Bree, 1981).
Figure 30. The primary sequence of the insertion of the Nimes plasmid (pGF1), was written in 5' to 3' direction. 5'-TTAA-3', duplication in the host sequence; ---- IR ----, terminal inverted repeats of IS5. The coordinate 1 is the PstI site. Sequence homologous to pJD4 was underlined.
PstI

CTGCAGCAATGGCAACAACGGTGGGCAAACTATTTAAGCTCTTGCGCAGACTACTTCTACTCTAGCTT

pJD4

61

CCCGGCAAACAATTAATAGACTGAGTTGGAGGGCAATTAAAATGTGACGAGACCTCTCTC

pJD4

121

GGGCCCCTTCTGCTGGCTGGTTATATTCTGATGAAATCTGGAGCCGGTTGAGCGTGCTGGGTC

pJD4

181

GCGGTACATTGCGACGGACTGGGCCCAGATGTAAGCCCTCTCCGTATGATTATATCACA

pJD4

241

CGACGGGGAGTGACGCAATTATGGAATGAAACGAATAGACAGATGGCTGAGATAGGTGCTCT

pJD4

301

CACTGATTAAGCATTGGTAAAGTCACGAGCCAAATGTTAATTATCATTATATACATTTAGATTGAT

pJD4

361

TAAACCTTCTATTTTTAAATTAAAAAGGATCTAGGTGAAGATTTCTTTTGTATAATCTCAGA

pJD4

421

CCAAAATCTCCCTAACTGGAGATTTTCTGTTTCCACTGAGGCTAGACCCCTATCCTATAAAAATC

pJD4

481

TGGGCTTGTTCTAATATCCCTACTCAAAGGATTATTATATTACTAATAGCCTGCTTAAACGCTTTCT

pJD4

541

TCGGCTTTAATTTTTCTGCCTCTGTTATAAAATGCGTATTTTATTTTCTTCTCTTTTCAAAAA

pJD4

605

AAAGTGTAAAGGGAGGTTGGAATAAGGCGGGAAATTCTCTCTGGCTGACTGCTCATTTTCA

pJD4

661

TTCTCTCATCCTTTTCTGAGCGGCTTTTTTTTCTCCGGTTAAAAGCTCCTGTGAATCAGGCTTTAT

pJD4

721

GGTTCTCGCCTATTTAAGGGCTTATCCCAAGTTTTTATTGTGAGATCTCTTCCACCTGACGTA

pJD4

781

TCATTGGTCGGGCAACACAGTTGGCCAGCGTGAATACATCGCCAGTTGGTTATCTG

pJD4

841

TTTTCAAGAAACCCTTTGTATCTGCTTTTCAGAAGCGAGCACTGTCCTTGATGATGCGGA

pJD4

901

ATGGGTGTCCACCCTCGGCGGATGTGCTCATTCTGATTGTTGATGTTGGCCTGTTT

pJD4

961

TGTTCTTCGCGTGGATGCTTTTCAGGGTTCTACACCTCCTGCGCGGCGGCTGCAGGCAGC

pJD4

1021

AGTCCACATCCACCTCGGCCAGCTCTCGCGCTTGCGCGCCCTTGTGAGCCGGCCTC

pJD4

1081

CTGAGACAATTTGCTCTCTTCATCTAGCACAGATTACCCAGCTGATTGAGGTGCTATGTGCTG

pJD4

1141

TTGGCCGGTGGTGCAGACCCAGCCTGCTGTTGGTACGCGGCACTCTTGGCATGACAACAAATGTTGG

pJD4

1201

CCTTGATGCGGAAAGGTCACGTATTCCCTTTTCTTCTGTCTGATTGATCTCTTGGGCTGCTGGT

pJD4

1261

GCTGTCTTTGTTTCTTGAGGTGGCTGACTCAATGATGTTGCGATCGACCAAGGTTG

pJD4

1321

CTTGAGCTCATCTGAGCGCCCTGCTCGGCCAGCCAGCGATGGATGGCTTCTGGAACAATTGCG

pJD4

1381

GGGGAGTTGGAGTCTGCTCCAGCACGATGGCGGAAATTCTATGATGTTGCTGTTGGCGCTCGGCC

pJD4

1441

AGGGCGCTATCAGGGATAAACCAGGGCAACGACGCACTGGGAGGCGGAGTTTCTGTACAGACACT
1501 CTTCCATCGCGCCATCGCTCAGGTTGTACCAATGCTGATGCAGTGATGGCATGG
1561 TTTCCACGGATAAGGTCCGCGCCATTACACGCCCTTGGGTAAAGCGGCTCGATGACTT
1621 CCCACATGTTTTTGGCCATAGCAAATCTGCTCCATGCGGGGACAGAAATCTCTTTTCCTGG
1681 TCTGACCGCGCCTTACTGCTGAAATTCACGTGCCTGCGCAGGTAAGTTGATGACTCATGATGA
1741 ACCCTGTTTCTATGGCTCCAGATGACAAACATGATCTCATATCGGACCTTGGTTCGACCT

----- IR -----  

1801 TCCTTAAGTAAAAATACCTACCTAAAATTTTTTACTAGT

SpeI

pJD4
CHAPTER IV: DISCUSSION

IV.1. Structure and location of various deletions of the $\beta$-lactamase plasmids of *N. gonorrhoeae* pJD4, pJD5, pJD7, Toronto type plasmids and Rio type plasmids

The reported sizes of the Asia-type $\beta$-lactamase-producing plasmids of *N. gonorrhoeae*, determined by restriction endonuclease and heteroduplex analyses, varied from 7.1-kb (4.4-Mdal) to 7.5-kb (4.7-MDal) (Roberts *et al.*, 1977; Elwell *et al.*, 1977; Dickgiesser *et al.*, 1982; Fayet *et al.*, 1982; Chen and Clowes, 1987b). The primary sequence of the entire pJD4 (an Asia-type plasmid), excluding 0.9-kb *BamHl*-PstI fragment which is homologous to TnA, was determined. The primary sequence of the 0.9-kb *BamHl*-PstI fragment of TnA was determined previously (Heffron *et al.*, 1979); the remaining region, 745-bp homologous to TnA, 745-bp was determined in this study. Assuming that the 0.9-kb *BamHl*-PstI fragment of TnA in pJD4 is identical to TnA, the size of pJD4 should be 7426-bp (approximately 7.4-kb). This result is in agreement with previous reports using restriction endonuclease and electron microscopic analyses (Elwell *et al.*, 1977; ; Fayet *et al.*, 1982; Dickgiesser *et al.*, 1982).

The reported sizes of the Africa-type plasmid of varied from 5.1 (3.2-Mdal) to 5.5-kb (3.4-Mdal) (Roberts *et al.*, 1977; Elwell *et al.*, 1977; McNicol *et al.*, 1983;
Mayer and Robbins, 1983; Dickgiesser et al., 1982; Yeung and Dillon 1985; Yeung et al., 1986; Chen and Clowes, 1987b; Aalen and Gundersen, 1987; Dillon and Yeung, 1989). By determining the nucleotide sequence around the deletion of pJD5 (an Africa-type plasmid), this study located this deletion, which is 1827-bp, at coordinates 1881 to 3707 of pJD4 down stream of the PstI site. Assuming that there is no other deletion or insertion, the size of pJD5 is 5,599-bp (5.6-kb). This result, however, does not agree with most of the previous reports. The closest value was observed by Aalen and Gundersen (1987) who, using restriction endonuclease analysis, reported that the size of the plasmid was 5.5-kb; Chen and Clowes (1987b) reported the size to be 5.4-kb.

My result shows that the deletion of pJD5 relative to pJD4 was 1.88-kb downstream of the PstI site. This value is very close to those found in two previous reports. Based on electron microscopic analysis of heteroduplex formed between Asia-type (PJD4) and Africa-type (pJD5) plasmids, Yeung and Dillon (1985) located the deletion at 1.74-kb downstream of the PstI site. Aalen and Gundersen (1987) located it at approximately 1.85-kb downstream of the PstI site. In previous studies it was very difficult to pinpoint the location of deletion using only several restriction endonucleases for analysis.

Based on electron microscopic analysis of the heteroduplex formed between pNG10 (an Asia-type plasmid) and pNG18 (an Africa-type plasmid) digested with PstI, Dickgiesser (1984) reported that the deletion was located 1.74-kb upstream of the PstI site. Dickgiesser (1983b) hypothesized that approximately 300-bp inverted
repeats flanked the deleted sequence. The primary sequence of pJD4, however, does not have any 300-bp inverted repeats. Dickgiesser (1983b) might have observed one of the inverted repeats, i.e., IR-7, IR-8, IR-9 or IR-10, upstream of the deleted fragment and one of two inverted repeats (IR-16 or IR-17) immediately downstream of the fragment.

The reported size of deletion of the Africa-type plasmid varied between 1.8 kb and 2.1 (McNicol et al., 1983; Mayer and Robbins, 1983; Dickgiesser et al., 1982; Yeung and Dillon 1985; Aalen and Gundersen, 1987). My result found that the deletion was 1,827-bp (1.8-kb) in size, which agrees with one of the previously reported values, 1.8-kb (Aalen and Gundersen, 1987), determined by restriction endonuclease and electron microscopic analysis. The variation in the reported deletion size may have contributed to the discrepancies in the reported size of the Africa-type plasmid.

Analysis of the primary sequence of pJD4 identified a (507-509) direct repeat DR-30, comprising DR-30A (coordinates 1881 to 2387) and DR-30B (coordinates 3709 to 4216). DR-30A is located at the beginning of the fragment, which is deleted in pJD5. The second sequence (DR-30B) is located immediately downstream of the deleted fragment. The primary sequence of pJD5 contains a sequence homologous to both DR-30A and DR-30B. The difference between the nucleotide sequences of pJD5 and DR-30A is 8 nucleotides, while only two nucleotide differences were identified between the primary sequence of pJD5 and DR-30B. The presence of DR-30 may have contributed to the controversy concerning the exact location of the
deletion of the Africa-type plasmid, because the primary sequence of pJD5, during heteroduplex studies could hybridize to either DR-30A or DR-30B. If the corresponding sequence of pJD5 hybridizes to DR-30A, the deletion of pJD5 will be at coordinates 2388 to 4725 of pJD4.

Restriction endonuclease analysis has shown that the Toronto-type plasmid (pJD7) is identical to the Asia-type plasmid (pJD4), except for the 2.3-kb absent in pJD7 (Yeung and Dillon, 1985; Yeung et al., 1986). A previous study using restriction endonuclease and electron microscopic analyses reported that the deletion of pJD7 was located at 3.7-kb downstream of the PstI site and approximately 0.5-kb from one of the BamHI sites (Yeung and Dillon, 1985; Yeung et al., 1986). By comparing the primary sequence of pJD7 to pJD4, the deletion of pJD7 was determined to be 2,272-bp (2.3-kb), corresponding to coordinates 3803 to 6074 of pJD4, and 433-bp from one of the BamHI sites. This value agrees with that found previously (Yeung and Dillon, 1985; Yeung et al., 1986). Assuming that there is no other deletion, the size of pJD7 is 5,154-bp (approximately 5.15-kb).

The primary sequences around the deletion of Toronto-type plasmids, i.e., pGC1213, pGC4538, pGC5221, pGC5228, and pGC5230, were found to be identical. The deletion of all these Toronto-type plasmids was 2,272-bp, identical to the deletion of pJD7 and corresponding to coordinates 3803 to 6074 of pJD4. Therefore, I propose that β-lactamase-producing plasmids of the same size are identical even though they are from different isolates from different countries.
Comparing the primary sequence of pGO4717, a Rio-type plasmid, to pJD4 revealed that the fragment deleted from pJD4 to produce this plasmid was 2,272-bp, corresponding to coordinates 3803 to 6074 of pJD4. This result is identical to that of pJD7 and supports that found in a previous study (Dillon and Yeung, 1989). van Embden et al. (1985) reported that the deletion of this plasmid occurs in two locations: one which is 2.5-kb in size, is located within the 3.2-kb BamHI fragment; and the second, 0.1-kb in size, is located within the 1.8-kb BamHI-HindIII fragment of the Asia-type plasmid. Dillon and Yeung (1989) did not report the 0.1-kb deletion. Only DNA sequencing can clarify the presence of the 0.1-kb deletion.

The deletion of pJD5 (an Africa-type plasmid) begins at coordinate 1881, which is adjacent to a region with a cluster of direct and inverted repeats. The deletion of pJD7, all of the other Toronto-type plasmids studied, and the Rio-type plasmid begin at coordinate 3803, which is also adjacent to the region with a cluster of direct and inverted repeats. It seems that both regions are "hot spots" for deletion, and both deletions may use a similar mechanism, which involves the cluster of direct and inverted repeats. This fact suggests that the Africa-, the Toronto- and Rio-type plasmids are probably deletion derivatives of the Asia-type plasmid.
IV.2. Regions homologous to TnA in the β-lactamase-producing plasmids pJD4, pJD5 and pJD7

Previous heteroduplex analyses have established that approximately 40% of the TnA sequence is present in the β-lactamase plasmids of *N. gonorrhoeae* (Roberts *et al.*, 1977; Fayet *et al.*, 1982). By sequencing regions homologous to TnA of pFA3 (an Asia-type) and pFA7 (an Africa-type) plasmids, Chen and Clowes (1987b) found that the TnA/Tn2 sequences in pFA3 and pFA7 were identical except for one base pair. My analysis found that the TnA sequence of pJD4 begins at coordinate 6235 (Figure 31), while that of pJD5 begins at 6236 of pJD4. My results agree with the previous study, which reported that the TnA sequence of pFA3 begins at coordinate 6235 (similar to pJD4), while that of pFA7 begins at 6236 of pJD4 (similar to pJD5) (Chen and Clowes, 1987b). In addition, similar to the TnA sequence in pJD4, the TnA sequence in pJD7 (Toronto-type plasmid) begins at coordinate 6235 of pJD4.

I also found that in the IR-R region of TnA, the sequence of pJD4 is identical to TnA. This result agrees with the previous study by Chen and Clowes (1987b). The DNA sequences of pJD5 and pJD7 in the IR-R region are also identical to TnA. Assuming that the 0.9-kb *Bam*H1-*Pst*I fragments of pJD4, pJD5 and pJD7 are identical to that of TnA (Heffron *et al.*, 1979); therefore, the region homologous to TnA in pJD4, identical to that of in pJD7, is 1659-bp, while the region homologous to that of pJD5 is 1658-bp.
Figure 31. Region homologous to TnA in pJD4, pJD5 and pJD7. Map of pJD4 was used as comparison. —, region homologous to TnA. IR-R, right hand of the terminal inverted repeat of TnA. TnpR, part of tnpR gene of TnA.
IV.3. The evolution of the $\beta$-lactamase plasmids of *N. gonorrhoeae*

Roberts *et al.*, (1977) proposed that the $\beta$-lactamase plasmids in *N. gonorrhoeae* were acquired from a foreign origin since the Asia-type and the Africa-type plasmids had a G+C content (40%) different from that of the chromosomal DNA (50%) of *N. gonorrhoeae*. The Asia-type and the Africa-type plasmids have G+C contents similar to pRSF0885, a 4.1-MDal $\beta$-lactamase plasmid in *H. influenzae* (Roberts *et al.*, 1977). Brunton *et al.* (1982) suggested that gonococcal plasmids might have arisen through the transformation of plasmid DNA from *H. ducreyi* or other *Haemophilus* spp. to *N. gonorrhoeae*. The Asia-type and the Africa-type $\beta$-lactamase-producing plasmids of *N. gonorrhoeae* were homologous to a variety of $\beta$-lactamase-producing plasmids isolated from *H. ducreyi*, *H. influenzae* and *H. parainfluenzae* (Brunton *et al.*, 1986; Chen and Clowes, 1987b; McNicol *et al.*, 1983). Based on restriction endonuclease analysis and Southern blot hybridization, two plasmids from *H. ducreyi*, 8.6-kb (5.7-MDal; pJB1) and 10.5-kb (7.0-MDal; pHDI31) in size, were identical to the Africa-type and the Asia-type plasmids, except that *Haemophilus* plasmids carried a complete Tn2 sequence (Brunton *et al.*, 1982; McNicol *et al.*, 1983; Chen and Clowes, 1987b). Nucleotide sequences of the region homologous to Tn2 of the $\beta$-lactamase plasmids found in *H. ducreyi* and *N. gonorrhoeae* are almost identical (Chen and Clowes, 1987b).
Dickgiesser et al., (1982) suggested that the Asia-type plasmid might have been generated from the Africa-type plasmid by transposition acquisition of an insertion element. They proposed that the Asia-type plasmid has an insertion element bounded by approximately 300-bp inverted repeats. Computer analysis of the primary sequence of pJD4 (an Asia-type plasmid) showed that the 300-bp inverted repeats did not exist. Dickgiesser et al., (1982) might have observed other inverted repeats upstream and downstream of the fragment, which is absent in the Africa-type plasmid, that are much smaller. Therefore, my results do not support their hypothesis.

Other researchers (Sox et al., 1979; Yeung and Dillon, 1985) suggested that the Africa-type plasmid might have been a derivative of the Asia-type plasmid through a transformation-associated deletion. Furthermore, Yeung and Dillon (1985) proposed that the Africa-type and the Toronto-type plasmids are derivatives of the Asia-type plasmid based on the homology between the Asia-type and the Africa-type plasmids and between the Asia-type and the Toronto-type plasmids. Sequence analysis of pJD4, pJD5 and pJD7 in the region homologous to TnA revealed that the primary sequence of pJD4 is similar to that of pJD5 except for one base pair at coordinate 6235 of pJD4, while the primary sequence of pJD4 is similar to that of pJD7 except for one base pair at coordinate 6340 of pJD4. Therefore, all of these plasmids may have derived from the same origin. It also suggest that separate deletion events may have taken place for each plasmid. It is possible that the Africa-type and the Toronto-type plasmids are derivatives of the Asia-type plasmid.
The fact that both plasmids appeared in *N. gonorrhoeae* at the same time might have supported the hypothesis that both were acquired from a foreign origin. However, the fact that a) the Toronto-type and Rio plasmids are structurally related to the Asia-type but not the Africa-type plasmid, and b) based on the structural analysis, the mechanism of deletion of the Africa- and the Toronto-type plasmids indicated similarities, both involving a cluster of direct and inverted repeats, suggests that both the Africa- and the Toronto-type plasmids may have been derivatives of the Asia-type plasmid.

Several β-lactamase plasmids isolated after the first isolation of the Asia- and Africa-type plasmids, i.e., the New Zealand, Toronto-type and Rio-type plasmids, are structurally related to the Asia-type plasmid. The Nimes plasmid (pGF1) is structurally related to the Africa-type plasmid. This may indicate that the β-lactamase plasmids in *N. gonorrhoeae* continue to evolve.

IV.4. Potential protein coding regions in pJD4

Analysis of the primary sequence of pJD4 in this study found 30 potential protein coding regions. The coding regions found in pJD4 and the proteins reported from *in vitro* transcription and translation studies were compared (Table 11; Yeung and Dillon, 1985; Tenover *et al.*, 1985; Biswas *et al.*, 1986). Most proteins reported in these studies were found in the analysis of pJD4 (Table 11). Several of these
proteins previously reported, such as the one 17.5-Kdalton in size, were not identified. Some of these reported proteins had varied in their intensity and appearance from one experiment to another (Yeung and Dillon, 1985). This may indicate that they represent precursors of other proteins or incomplete proteins (Yeung and Dillon, 1985). Some proteins can exist as aggregates of many copies of a similar polypeptide chain (Creighton, 1993), while others can exist as aggregates of one or more copies of different polypeptide chains (Creighton, 1993). This was likely the reason for the existence of some proteins in the transcription and translation studies, that were not found in the analysis of pJD4. Another possibility was that the region which coded several proteins, such as a 17.5-Kdal one, was not identified in the analysis of pJD4. This possibility is very small since this method has been used to identify protein coding regions in procaryote, and has identified 96 % of the known regions (Kolaskar and Reddy, 1985).

ORF8 has the potential to encode the 31.5-Kdal protein (286 amino acids), which is identical to the β-lactamase gene reported previously (Heffron et al., 1979). This protein was found to be 100 % homologous to the β-lactamase precursor of E. coli plasmid pBR322 and showed significant homology to a variety of β-lactamases found in many bacteria. This provides further supports that this type of β-lactamase has spread to many species.
IV.5. Replication region of the \(\beta\)-lactamase plasmids of \(N.\) gonorrhoeae

McNicol et al. (1984) reported that the replication region of the Asia-type plasmid (pFA3) was located on the non TnA region of the 2.4-kb \(BamHI\) fragment, or at coordinates 468 to 1505 of pJD4 (Figure 6). Analysis of the primary sequence of pJD4 in this region did not find any sequence resembling known procaryotic replication regions. Other investigators also found that the 2.4-kb \(BamHI\) fragment of the Asia-type plasmid did not replicate by itself (Johnson, 1985; Yeung and Dillon, 1988; Gilbride and Brunton, 1990). Johnson (1985) located the replication region of the Asia-type plasmid on the 3.7-kb \(BamHI-PvuII\) fragment, corresponding to coordinates 1499 to 5360 of pJD4. Yeung and Dillon (1988) reported two regions: 1) replication region "a" located at a 1.5-kb fragment containing the \(HindIII\) site, and 2) replication region "b" located at a 1.6-kb \(BamHI-HinfI\) (Figure 6). On pJD4 replication region "b" was separated by "a". Gilbride and Brunton (1990) located the replication region of the Asia-type plasmid within the 3.3-kb \(PstI-HindIII\) fragment beginning 480-bp downstream of the \(BamHI\) site to \(PstI\), excluding the TnA region (Gilbride and Brunton 1990).

The DnaA protein (a \textit{trans}-acting element) and DnaA-box (a \textit{cis}-acting element) are the essential elements to initiate chromosomal replication of \(E.\) coli and other enteric bacteria (Yoshikawa and Ogasawara, 1991). In \(E.\) coli, the \textit{cis}-acting element (\textit{oriC}) consists of four 9-mer repeats (DnaA-boxes) and A-T rich 13-mer repeats (Bramhill and Kornberg, 1988a). The 9-mer and 13-mer repeats are highly conserved.
among organisms of enterobacterial origin (Zyskind et al., 1983). The origin of replication of the *Bacillus subtilis* chromosome is about 200-bp, comprised of a series of 9-mer Dna boxes that lie adjacent to three AT-rich 16-mer tandem repeats (Bramhill and Kornberg, 1988b). The *cis*-acting element required for replication of plasmid ColE1 is about 400-bp, consisting of a DnaA-box (Fuller et al., 1984), an 80-bp region containing two 13-bp repeats (Bramhill and Kornberg, 1988b), three 18-bp direct repeats, and a pair of inverted repeats (Vocke and Bastia, 1983). The *cis*-acting sequence of several other plasmids, such as F, P1, R1 and R6K, contains one or two DnaA-boxes and a series of tandem repeats within its AT-rich region (Bramhill and Kornberg, 1988b). The consensus sequence, TTAT(C/A)CA(C/A)A for the DnaA-box, is not present in pJD4.

Analysis of the primary sequence of pJD4 found two regions that might be the origin of replication. First, the region downstream of the *BamHI*, at coordinates 1607 to 1788, carries four tandem 22-mer direct repeats (DR-26) within an 88-bp (1701 to 1788) AT-rich region (78%). This region also contains the following pairs: 25-mer, 32-mer, 13-mer, and 22-mer inverted repeats (IR-6, IR-7, IR-8 and IR-9; Figure 16). This region encompasses nucleotide sequences, which are present in pJD4, pJD5 and pJD7, and is located within replication region "b" (Yeung and Dillon, 1988) and replication regions proposed by other investigators (Johson, 1985; Gilbride and Brunton 1990). It is very likely that this region is essential for the replication and maintenance of the plasmid. I propose that this region may be the essential *cis*-acting element for the replication of pJD4, pJD5 and pJD7. This region (coordinates 1607
to 1788) also resembles replication regions reported by Baum and Gilbert (1991), who identified replication origin sequences of three large B. thuringiensis plasmids, ori 43, ori 44 and ori 60. These sequences are characterized by the presence of overall A-T rich (67.9 % to 71.4 %) and a segment of exceptionally A-T rich (>80%). The ori 43 contained an 11-bp direct repeat and a 21- to 26-bp inverted repeat; the ori 44, a 10-bp direct and a 21-bp inverted repeat, and ori 60, three 11-bp direct and a 17-bp inverted repeat. Furthermore, the dominant features of enteric replication origin are the presence of a 9-bp direct and inverted repeat and three 13-bp direct repeats (Zyskind et al., 1983).

Second, the region at coordinates 3543 to 3801 contains a tandem direct repeat (3 X 22-mer), DR-48. This region carries inverted repeats IR-15 and IR-16 within an AT-rich (70 %) region. This region is located within the replication region "a" proposed by Yeung and Dillon (1985). It is possible that this region is the essential cis-acting element of the replication region "a". Further studies, such as site-directed mutagenesis are warranted.

IV.6. Mobilization region of the β-lactamase plasmids of N. gonorrhoeae

The region required for the mobilization of the Asia- and the Africa-type plasmids of N. gonorrhoeae have been studied. McNicol et al. (1983) located the origin of transfer (OriT) of the Asia-type plasmid (p22209) on the 1.8-kb BamHI-HindIII
fragment, corresponding to coordinates 1499-2387 of pJD4, and the oriT of the
Africa-type plasmid (p88557) on the 1.4-kb BamHl-AvaI fragment (McNicol et al.,
1983). Gauthier (MSc thesis, 1990) located the oriT of the Asia-type plasmid on the
1.8-kb BamHl-HindIII fragment, several hundred base pairs from the HindIII site. My
result showed that approximately 890-bp within the 1.8-kb BamHl-HindIII of pJD4
(coordinates 1499 to 2388) are homologous to the nucleotide sequence of pJD5 at
the corresponding area (within 3.1-kb BamHl fragment of pJD5). The 890-bp
fragment of pJD4 contains four x 22-bp direct (DR-26) and several inverted repeats.
This region is also A-T rich (79%). Therefore, I propose that the oriT of the Asia- and
Africa-type plasmids is located in the region within the 890-bp fragment commonly
present in both plasmids. The DNA sequence of oriT of the F plasmid and the oriT loci
from related Col and R plasmids all contained an 80-bp A-T rich region (80%) (Finlay
et al., 1986). In addition, the oriT loci of IncF plasmids contained a number of direct
or inverted repeats (Fu et al., 1991). McIntire and Dempsey (1987) reported that
integration host factor (IHF) is required in the transformation of plasmid R100. IHF
in this transformation recognizes the consensus sequence 5'-C/TAANNNNTTGA/T-
3’ or the complementary strand 5’-A/TATCAANNNNTTA/G-3’. The 1.8-kb BamHl-
HindIII fragment of pJD4 also carried a sequence (coordinates 1794 to 1816) which
is 100% homologous to the consensus sequence of the integration host factor. This
finding further supports the region at coordinates 1499 to 2388 as the essential
region required for the mobilization or the oriT of these plasmids. Further studies,
such as site-directed mutagenesis, are needed to further characterize this region. This
does not exclude other regions, that may be involved in the mobilization of these plasmids.

The mobilization of ColE1 requires a specific plasmid-encoded protein (16-Kdalton) that interacts with two other proteins at the basis of mobility (bom) site on the plasmid to initiate the formation of a relaxation complex believed to be in the mobilization (mob) region (Waren et al., 1978). Bom is a special rite on the plasmid DNA where mobility proteins must interact (Waren et al., 1978). Tenover et al., (1985) reported a mob region on the 1.9-kb Hinfl fragment, corresponding to coordinates 3381 to 5347 of pJD4, which encodes the 16 Kdal protein. Furthermore, Gauthier (1990) located the 0.6-kb PvuII-Aval fragment of pJD4, corresponding to coordinates 4769 to 5360 of pJD4 as the essential part of the mob region. This fragment is located within the 1.9-kb Hinfl fragment. However, it was not specified whether the 0.6-kb fragment encodes for all or part of the 16-Kdal protein implicated in the regulation of mobilization. Analysis of the 1.9-kb Hinfl fragment of pJD4 did not identify any potential coding region for the 16-Kdal protein.

Dillon and Yeung (1989) proposed that the mob region of the Asia-type plasmid was located in the non-Tn2 BamHI fragment designated "M", which corresponds to coordinates 468 to 1499 of pJD4. This hypothesis was supported by sequence analysis at the "M" region of pFA7, an Africa-type plasmid (Yeung and Dillon, 1988), which contained several consensus sequences of the integration host factor 5'-C/TAANNNTTGATA/T-3' which was required for the transfer R100 (McIntire and Dempsey, 1987). In the "M" region of pJD4, two sequences at coordinates 1057 to
1071 and 1476 to 1491) were 90% homologous to the consensus sequence of integration host factor. Two inverted repeat sequences (IR-2 and IR-3) and DR-7 were found in the "M" region. The role of these direct and inverted repeats remains to be determined.

It appears that mobilization of the β-lactamase-producing plasmids of *N. gonorrhoeae* by various conjugative plasmids involves different mechanisms of transfer. Differences in *mob* or *oriT* regions probably reflect the ability for a particular conjugative plasmid to mobilize a particular plasmid. This may suggest that the Asia-type plasmid has several *oriT* and *mob* regions. The possibility of the Asia-type plasmid having more than one *oriT* is also supported by a study carried out by Ikeda *et al.* (1986), which revealed that some *N. gonorrhoeae* isolates carrying the 39.2-kb conjugative plasmid and the Asia-type plasmid were able to transform the Asia-type plasmid into *N. meningitidis*. In the same study, the gonococcal isolates which could not mobilize the Asia-type plasmid into *N. meningitidis* could mobilize the Asia-type plasmid into *E. coli*.

IV.7. The insertion of the Nimes plasmid (pGF1)

By restriction endonuclease analysis, the Nimes plasmid (pGF1) is identical to the Africa-type plasmid except for the insertion of a 1.2-kb inside the 2.4-kb *Bam*HI fragment (Gouby *et al.*, 1985; Dillon and Yeung, 1989). The precise location of the
insertion was not previously identified. My analysis precisely locates the insertion between coordinates 604 and 605 of pJD4 and determines it to be 1199-bp in size. Assuming that there is no other insertion or deletion, the size of Nimes plasmid is 6,798 bp. This is the first report to precisely establish the location and size of the insertion of pGF1.

Comparison of the primary sequence of the 1.2 kb insertion of pGF1 with DNA sequence in the Data Bank (PC gene and Microgenie®) found that the insertion is 100 % homologous to IS5 (Engler and van Bree, 1981; Kroger and Hobom, 1982). The IS5 in pGF1 is flanked by two 4 bp 5'-TTAA-3' sequences. The first sequence is located at coordinates 605 to 608 from PstI site, which is part of the insertion of pGF1 (Figure 30). The second sequence is located at coordinates 605 to 608 of pJD4 (present in pJD4; Figure 30). One of these 4-bp 5'-TTAA-3' sequences most probably resulted from the duplication of host DNA during transposition.

Many insertion sequences (IS) transpose to specific sequences (target sequences) in the genome (Galas and Chandler, 1989). Previous studies have identified the target sequence of IS5. Engler and van Bree (1981) found that the target sequences in bacteriophages Mu, pGP31, pGP33, and pGM1 were CTAA, CAAG and CTAG, respectively. Schooner and Kahn (1981) found about 10 copies of IS5, and their target sequences (CTAG) in the chromosome of E. coli K12 were similar. In addition, Lusky et al., (1981) observed duplication of the sequence CTAA in different IS5 insertions. Based on their finding and others, Engler and van Bree (1981) suggested that the consensus target sequence for IS5 is "C.T/A.A.G/A"; they
also suggested that IS5 may have specific target sequences. However, Kroger and Hobom (1982) found the target sequence was TTAG. My finding in this study indicates that the formulation of the target sequence of IS5 is "C/T.T/A.A.G/A". I propose that the target sequence of IS5 is host specific, and different hosts can have different specific target sequences.

The presence of IS5 in the β-lactamase plasmid in *N. gonorrhoeae* raises the question of its origin. It has been known that the *E. coli* chromosome has many copies of IS5 in its genome (Schooner and Kahn, 1981). It might be that pGF1, the 6.6-kb plasmid, is a derivative of the Africa-type plasmid with insertion of IS5 from *E. coli* or from multiple matings in nature. The possibility that the plasmid obtained the IS5 from *N. gonorrhoeae* can not be ruled out, since the G-C contents of the insertion is 53 % of the mole fraction, which is close to that of genomic *N. gonorrhoeae*. However, there is no report to date indicating that the genome of *N. gonorrhoeae* has the insertion sequence IS5. Another possibility is that the IS5 originated through a trip parental mating by contact in nature from *N. gonorrhoeae* to *E. coli* and then from *E. coli* to *N. gonorrhoeae*.

IV.8. Conclusion

This is the first study to determine the size of the Asia-, Africa- and Toronto-type, and Nimes plasmids based on DNA sequence analysis. The size of the Asia-,
Africa- and Toronto-type, and Nimes plasmids are 7426-bp, 5599-bp, 5154-bp and 6798-bp, respectively. The size and location of the deletion of the Africa- and the Toronto-type plasmids and the size and location of the insertion of Nimes plasmid in comparison to pJD4 were also determined by DNA sequence analysis. The size of the deletion of the Africa- and the Toronto-type plasmids, and the size of the insertion of the Nimes plasmid were 1823-bp, 2272-bp and 1199-bp, respectively. The deletions of the Africa- and the Toronto-type plasmids are at coordinates 1881 to 3704 and 3803 to 6074 of pJD4, respectively. The insertion of the Nimes plasmid is located between coordinates 604 and 605 of pJD4.

The Africa-, the Toronto-, and the Rio-type plasmids are probably deletion derivatives of the Asia-type plasmid, while the Nimes plasmid is probably an insertion derivative of the Africa-type plasmid. Plasmid pJD4 may have two replication regions. One of these regions, which is present in pJD5 and pJD7, might be the main replication region for pJD4 (the Asia-plasmid), pJD5 (the Africa-type plasmid) and pJD7 (the Toronto-type plasmid). Several regions may be involved in the mobilization of the β-lactamase plasmids of *N. gonorrhoeae*, using different mechanisms.
APPENDIX 1:

DNA SEQUENCE OF DIRECT REPEATS FOUND IN THE PRIMARY SEQUENCE
OF pJD4, INCLUDING THE REGION HOMOLOGOUS TO TnA/Tn2

1.  312  CATTTGGTAACTGTC  325
    759  CATTTGGTAACTTTCC  772
    *

2.  502  TAAACGATTATTATCAATAGCGCTCTAACCAGCTTTTTTCGCTTATTTTT
    2386  TAAACGATTATTATCAATAGCGCTCTAACCAGCTTTTTTCGCTTATTTTT
    555  CTGTCTCTGTATAAAAATTGCTATTCATCTGTTCCTTCT  593
    2439  CTGTCTCTGTATAAAAATTGCTATTCATCTGTTCCTTCT  2477

3.  a.  527  CTAACCGCTTTTTT  539
    b.  2411  CTAACCGCTTTTTT  2423
    c.  7321  CTAACCGCTTTTTT  7333

4.  608  AGTAAAATAC  617
    5428  AGTAAAATAC  5437

5.  a.  665  TTTTTCAATT  674
    b.  2279  TTTTTCAATT  2288
    c.  4108  TTTTTCAATT  4121

6.  677  TTTAAAAATCAGA  690
    2534  TTTAAAAATCAGA  2547
    *

7.  683  AAATCAGATTT  693
    1019  AAATCAGATTT  1029

8.  687  CAGATTTTGA  696
    5489  CAGATTTTGA  5498

9.  703  TTTTGATCTA  712
    2560  TTTTGATCTA  2569

The number beside the DNA sequences indicate the coordinates of each sequence. Sequences were written 5' to 3' direction. * indicates a mismatch.
10. 822 ATAAACCGTA 831
     6233 ATAAACCGTA 6242
11. 838 ATTGCTCATC 847
     6635 ATTGCTCATC 6644
12. 875 AACTTCCCATACTTGTGTGTTATATGTGAAAT 904
     2732 AACTTCCCATACTTGTGTGTTATATGCGAAAT 2761
     *    *
13. 913 TTTTGCAACT 922
     2522 TTTTGCAACT 2531
14. 986 CGCCCACGCTTGATTTGTTCACCTCAAC 1014
     2834 CGCCCACGCTTGATTTGTTCACCTCAAC 2862
     ** *  
15. 1000 TTGTTCAACC 1009
     6024 TTGTTCAACC 6033
16. 1074 ATGAATTAATCTA 1087
     3175 ATGAATTAAGCTA 3188
     *  
17. 1168 AATTAGCTCAT 1178
     3115 AATTAGCTCAT 3125
18.  a. 1387 AATCCGTTTTATA 1399
     b. 2163 AATCCGGTTTTTA 2175
     c. 3992 AATCCGTTTTTTA 4004
     *  
19. 1406 TGAATTTGGCTT 1416
     4788 TGAATTTGGCTT 4798
20. 1535 ATTTCATCAA 1544
     3389 ATTTCATCAA 3398
21. 1542 CAATACTCAA 1551
     4547 CAATACTCAA 4556
22. 1594 TAAATCATTTTGCCATA 1609
     3448 TAAATCATTTTGTCATA 3463
     *  
23. 1629 TAAACACACACAAG 1640
     1665 TAAACACACACAAG 1676
| 24. | 1696 TATAACAGGAAATTTGTTGTC 1716 |
|     | 1718 TATAACAGGAAATTTGTTGTC 1738 |
| 25. | 1701 CAGGAAATTGGTTGTCCTTATAACAGGAAATTTGTTGTCGTATAA 1744 |
|     | 1745 CAGGAAATTGGTTGTCGTATAACAGGAAATTTGTTGTCGTATAA 1788 |
| 26. | 1701 CAGGAAATTGGTTGTCCTTATAA 1722 |
|     | 1723 CAGGAAATTGGTTGTCGTATAA 1744 |
|     | 1745 CAGGAAATTGGTTGTCGTATAA 1766 |
|     | 1767 CAGGAAATTGGTTGTCGTATAA 1788 |
| 27. | a. 1701 CAGGAAATTGGTT 1713 |
|     | b. 1723 CAGGAAATTGGTT 1735 |
|     | c. 1745 CAGGAAATTGGTT 1757 |
|     | d. 1767 CAGGAAATTGGTT 1779 |
|     | e. 6148 CAGGAAATTGGTT 6160 |
| 28. | 1836 TAAAAAACAAA 1846 |
|     | 3527 TAAAAAACAAA 3537 |
| 29. | 1871 TCCCTTTTTTG 1881 |
|     | 6918 TCCCTTTTTTG 6928 |
| 30. | a. 1881 ....GGGCTTTTCAGCCCTAAATTTTTCTTTTTCTTTTTTTGTATAAAATTTACAAA |
|     | b. 3708 ....GGGCTTTTCAGCCCTAAATTTTTCTTTTTCTTTTTTTCTTTTTTGAGGATTTAAAAATTTACAAA |
| 1929 | ACCCTTACAGGACAGGACTAAACTGTGTTTCTGTCTCAGGGTTTCAGCAACCCGAAAGCCG |
| 3757 | ACCCTTACAGGACAGGACTAAACTGTGTTTCTGTCTCAGGGTTTCAGCAACCCGAAAGCCG |
| 1989 | TTAGGGCTAGG CGGTAAGCTATAAAGCCATTTAAATTTTATCTTTAAATTTTTCCGTTAA |
| 3817 | TCGAGGGCTAGGCGGTAAGCTATAAAGCCATTTAAATTTTATCTTTAAATTTTTCCGTTAA |
| 2048 | ATGCTTTTGAAGTGTTGCTTTTTATCGTACTCATCAATCCCCCTTTTTCTTTGATTTG |
| 3877 | ATGCTTTTGAAGTGTTGCTTTTTATCGTACTCATCAATCCCCCTTTTTCTTTGATTTG |
| 2108 | CTTTGTGATCGCAATTTTTGAAATAAGATTTTTCCATTCTATCTAAACATTCTATCAATCC |
| 3937 | CTTTGTGATCGCAATTTTTGAAATAAGATTTTTCCATTCTATCTAAACATTCTATCAATCC |
| 2168 | GTTTTTTATGTGTTGCCATTTCAGGTTAACATAAAAACCTTAGCAAATAAAGAGCAAAATAC |
| 3997 | GTTTTTTATGTGTTGCCATTTCAGGTTAACATAAAAACCTTAGCAAATAAAGAGCAAAATAC |
| 2228 | ATACATTGGAAAATGATTGTTACATTTTCGCTCACAGTTATTTTTTACCTTTTTTCAT |
| 4057 | ATACATTGGAAAATGATTGTTACATTTTCGCTCACAGTTATTTTTTACCTTTTTTCAT |
2288 TTCTTCAATTGATAAAATGCACTCAATTCATCAAATTTCTTGTCATCATGATAAAATTTACG
4117 TTCTTCAATTGATAAAATGCACTCAATTCATCAAATTTCTTGTCATCATGATAAAATTTACG

2348 CAACTTAAAGGAAATTTCTATCTACATCTAAAGAGGGTTA 2387
4177 CAACTTAAAGGAAATTTCTATCTACATCTAAAGAGGGTTA 4216

31. 1911 AGGATTTAAAA 1921
     3352 AGGATTTAAAA 3362

32. 1955 TGCTTGTCTT 1964
     4285 TGCTTGTCTT 4294

33. 2085 CCTTTTTTGC 2094
     6920 CCTTTTTTGC 6929

34. 2086 CTTTTTTTGCA 2095
     7328 CTTTTTTTGCA 7337

35. 2207 TAGCAATAAAA 2216
     4872 TAGCAATAAAA 4881

36. 2279 TTTTTCAATTTCCTT 2292
     4108 TTTTTCAATTTCCTT 4121

37. a. 2292 TCATGGATAAAT 2303
    b. 2331 TCATGGATAAAT 2342
    c. 4121 TCATGGATAAAT 4132
    d. 4160 TCATGGATAAAT 4171

38 2296 TGATAAATGC 2305
   4125 TGATAAATGC 4134
   6850 TGATAAATGC 6859

29. 2331 TCATGGATAAAT 2343
    4121 TCATGGATAAAT 4133

39. 2603 TGCCGTAATCT 2614
    6165 TGCCGTAATGT 6176
    *

40. 2655 GGATTTTTAGG 2665
    3344 GGATTTTTAGG 3354

41. 2885 ATTTCCATTAA 2894
    4223 ATTTCCATTAA 4232
42. 2915 TTTTTAAAT  2924  
    3155 TTTTTAAAT  3164 
43. 3278 TCAGGATTTA  3287  
    3737 TCAGGATTTA  3746 
44. 3280 AGGATTACATCG  3292  
    5597 AGGATTACAGCG  5609  
    *  
45. 3514 AAATGGTGGTCA  3525  
    6459 AAATGGTGGTCA  6470 
46. 3542 ACTATAACAA  3551  
    5621 ACTATAACAA  5630 
47. 3543 CTATAACAATAAATTTTGTTCCACCTATAA  3570  
    3587 CTATAACAATAAATTTTGTTCCACCTATAA  3614 
48. a. 3543 CTATAACAATAAATTTTGTTCCAC  3564  
    b. 3565 CTATAACAATAAATTTTGTTCCAC  3586  
    c. 3587 CTATAACAATAAATTTTGTTCCAC  3608 
49. 3652 TACAAAAACAGAA  3663  
    3664 TACAAAAACAGAA  3675 
50. 3783 TGCTTGTTCCT  3792  
    4235 TGCTTGTTCCT  4294 
51. 3915 CTTTTTTGCA  3924  
    7328 CTTTTTTGCA  7337 
52. 4121 TCATTGATAAAAT  4132  
    4160 TCATTGATAAAAT  4171 
53. 4231 TAGCCAAAAAA  4240  
    5766 TAGCCAAAAAA  5775 
54. 4249 TCTGCTTGTGTT  4258  
    4383 TCTGCTTGTGTT  4392 
55. 4306 CGCACGAATT  4315  
    5133 CGCACGAATT  5112 
56. 4597 AAAAGCAAAAG  4606  
    5682 AAAAGCAAAAG  5691
57. 4950 AGAACGCCAA 4959
    5238 AGAACGCCAA 5247

58. 5185 AGAGCAAGAA 5194
    5229 AGAGCAAGAA 5238

59. 5188 GCAAGAATTGA 5198
    5211 GCAAGAATTGA 5221

60. 5286 CAAAGAAAA 5295
    5687 CAAAGAAAA 5696

61. 5316 AGAAAAACAG 5325
    5931 AGAAAAACAG 5940

62. 5324 AGGCTCAAGG 5333
    5888 AGGCTCAAGG 5897
APPENDIX 2:

DNA SEQUENCE OF INVERTED REPEATS OF THE PRIMARY SEQUENCE OF pJD4, INCLUDING THE REGION HOMOLOGOUS TO TnA/Tn2

1. 397  AGATCCTTTTTGAT   410
    390  TCTAGGAAAATTTA   377

2. 1201 GTTGAAA TAA   1210
    1104 CAACCTTTATTT   1094
    *

3. 1282 CATGGCAATTCTAA   1295
    1194 GTAA GTTAAAGCTT   1182
    *   *

4. 1652 TGTGTTT TTAT   1661
    1617 ACAACAGAATA   1607
    *

5. 1663 TATAAACAACAAAGTATTT   1682
    1662 ATATTT GTTGTCATATTTA   1644
    *   *   *

6. 1696 TATAACAGGAAAATTGTTGTTCAT   1720
    1630 AT TTGTCCTTTAAACAACAGAATA   1610
    *

7. 1711 GTTGTCCTATAACAGGAAAATTGTTGTTCGTAT   1742
    1637 CAACAAAT TTGTCCTTTAAACAACAGAATA   1607
    *   *

8. 1709 TTGTTGTCTTTATA   1721
    1675 ACAACAAAATAT   1663
    *

The number beside the DNA sequences indicate the coordinates of each sequence. Sequences were written 5' to 3' direction. * indicates a mismatch.
9.  1743 AACAGGAATTTGTGTGTCGTAT 1764
    1628 TTTGCCTTTAAACACAGAAATA 1607
     *
10. 1828 AAAAAAAGTAAA 1840
    1817 TTTTGCTCTTTTT 1805
     *
11. 1898 TTTTTCTTTTT 1909
    1848 AAAAAAACAATAAA 1837
     *
12. 1962 TCTGCAAGGGTT 1973
    1939 AGACATTTCCCAA 1928
     *
13. 3556 TT TGTCACCT 3566
    3481 AATACAGGTGGA 3470
     *
14. 3578 TT TGTCACCT 3588
    3481 AATACAGGTGGA 3470
     *
15. 3696 AGGCTCTTTTT 3706
    3693 TCCGAGATAAA 3683
     *
16. 3790 TCTGCAAGGGTT 3801
    3767 AGACATTTCCCAA 3756
     *
17. 3848 TT TAAATTTTA 3857
    3755 AACATTAAAT 3745
     *
18. 4473 TAAAAATACG TAAT 4486
    4431 ATTTTATTGCTATTA 4417
     *
19. 4478 TAACGTAATGCCTCA 4491
    4474 ATAGCATTACTTTGT 4461
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LITERATURE CITED


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