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STRUCTURAL STUDIES OF THE β-LACTAMASE-PRODUCING PLASMIDS OF *NEISSERIA GONORRHOEAE.*

By

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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

ISBN 0-612-00443-0

Canada

UNIVERSITÉ D'OTTAWA
UNIVERSITY OF OTTAWA

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 piasmids pJD5, pJD7 and pGC4717 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 *Pst*I 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 Tn*A* 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 *Pst*I 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 Tn*A* in pJD4 is similar to that of pJD7 and pFA3. The region homologous to Tn*A* 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.

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.

ACKNOWLEDGEMENTS.

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.

! would like to thank to Dr. Lai-King Ng. (Head Research Section, National Laboratory for Sexually Transmitted Diseases - NLSTD) for being very patient in helping me through this study, Dr. Kwok-Him Yeung (Head, *N. gonorrhoeae* Section, NLSTD) for his advice, help and friendship. I would like also to thank to Drs. Rob Charlebois, Dave Brown and Bob Iyer for being members of my thesis advisory committee. Their critical comments and suggestions throughout this work are greatly appreciated.

I am thankful to my colleagues, Jim Ng, Stephane Bernatchez, Fiona Lawson and Hui Li for their support and friendship.

I would like also to thank to my mother and a friend of mine, Trilistya, who always encouraged me while I was away from my country for such a long time.

Finally, I would like to thank to Eleanor Paulson (Editor-in-Chief, Canada Communicable Disease Report - LCDC) for critical comments.

DEDICATION

To the most gracious and the most merciful

To my mom and dad.

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LIST OF ABREVIATIONS

ATP	adenosine triphosphate
bla	β-lactamase gene
bp	base pair(s)
C	Celsius
CMRNG	chromosomally resistant *Neisseria gonorrhoeae* (isolates)
dATP	deoxyadenosine 5'triphosphate
dCTP	deoxycytidine 5'triphosphate
dGTP	deoxyguanosine 5'triphosphate
dITP	deoxyinosine 5'triphosphate
DNA	deoxyribonucleic acid
DR	direct repeat
ds	double stranded
dTTP	deoxythimidine 5'triphosphate
EDTA	ethylenediamine tetracetate
g	gram
GCMB	GC medium base
IHF	integration host factor
Inc	incompatibility group
IR	inverted repeat
IS	insertion sequence

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

US	uptake sequence
uv	ultraviolet
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

CHAPTER I: INTRODUCTION

I.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 - itermediate (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 exceded 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 (5×10^6 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 occured in Tennesse, 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).

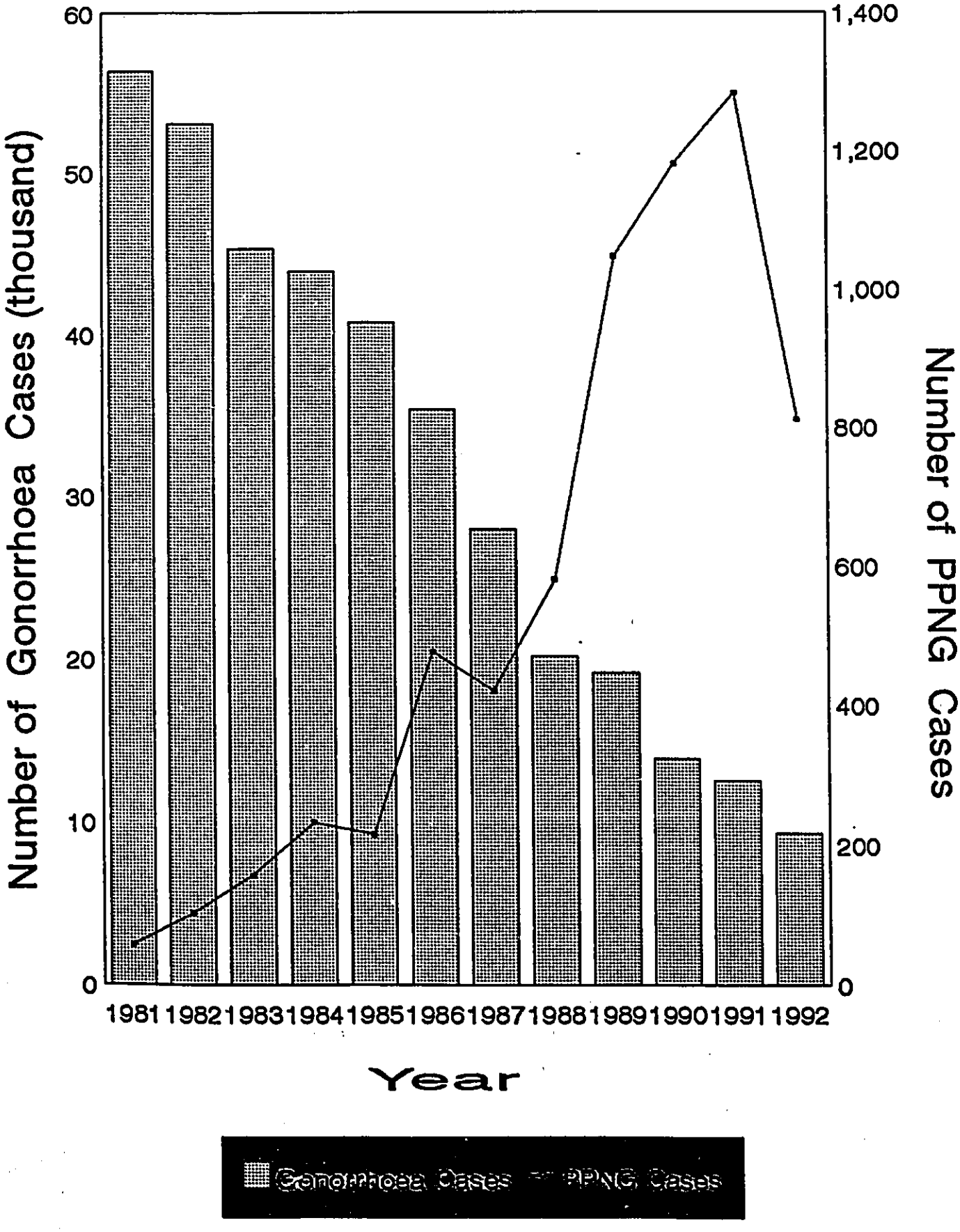
I.1.2. Plasmid-mediated penicillinase-producing *Neisseria gonorrhoeae* (PPNG)

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 Tn*2,* 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

Figure 1. Reported cases of gonorrhoea and PPNG in Canada, 1981 - 1992. Data derived from Health Canada (1992).


Number of Gonorrhoea Cases (thousand)
60
50
40
30
20
10
0
1,400
1,200
1,000
800
600
400
200
0
Number of PPNG Cases
1981 1982 1983 1984 1985 1986 1987 1988 1989 1990 1991 1992
Year
Gonorrhoea Cases
PPNG Cases

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 (Elghoul 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)(Centre

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; Klingeren *et el.*, 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-MDal) 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).

I.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

conjugative 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 liked 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.

I.3. Molecular characterization of β-lactamase-producing plasmids of *N. gonorrhoeae*

The β-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 Md). 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 *Bam*HI cut pJD4 into two fragments: 2.4-kb and 4.8-kb. Aalen and Gundersen (1987) used the *Hin*cII 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 *Bam*HI 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 β-lactamase-producing plasmids of *N. gonorrhoeae* as reported in the literature.

Plasmid	Reported plasmid size	Reference
pJD4	7.2-kb	Yeung and Dillon, 1985
pRosB	7.3-kb	Aalen and Gundersen, 1987
pFA3	7.5-kb	Chen and Clowes, 1987
pFA3	7.1-kb	Sox *et al.*, 1979
pFA3	7.1-kb	Brunton *et al.*, 1982
p22209	7.1-kb	McNicol *et al.*, 1983
pFT1	7.1-kb	Tenover *et al.*, 1985
pNG10	7.4-kb	Dickgiesser, 1982
pMR0360	7.1-kb	Roberts *et al.*,1977
pMR0360	7.1-kb	Mayer and Robbins, 1983
pCDC66	7.1-kb	Elwell *et al.*, 1977
pPJ102	7.4-kb	Fayet *et al.*, 1982

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 *Hin*cII 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 *Bam*HI as the zero reference position reported pFA7 as 5.4 kb in size. Tenover *et al.*(1985) used *Hin*cII 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.

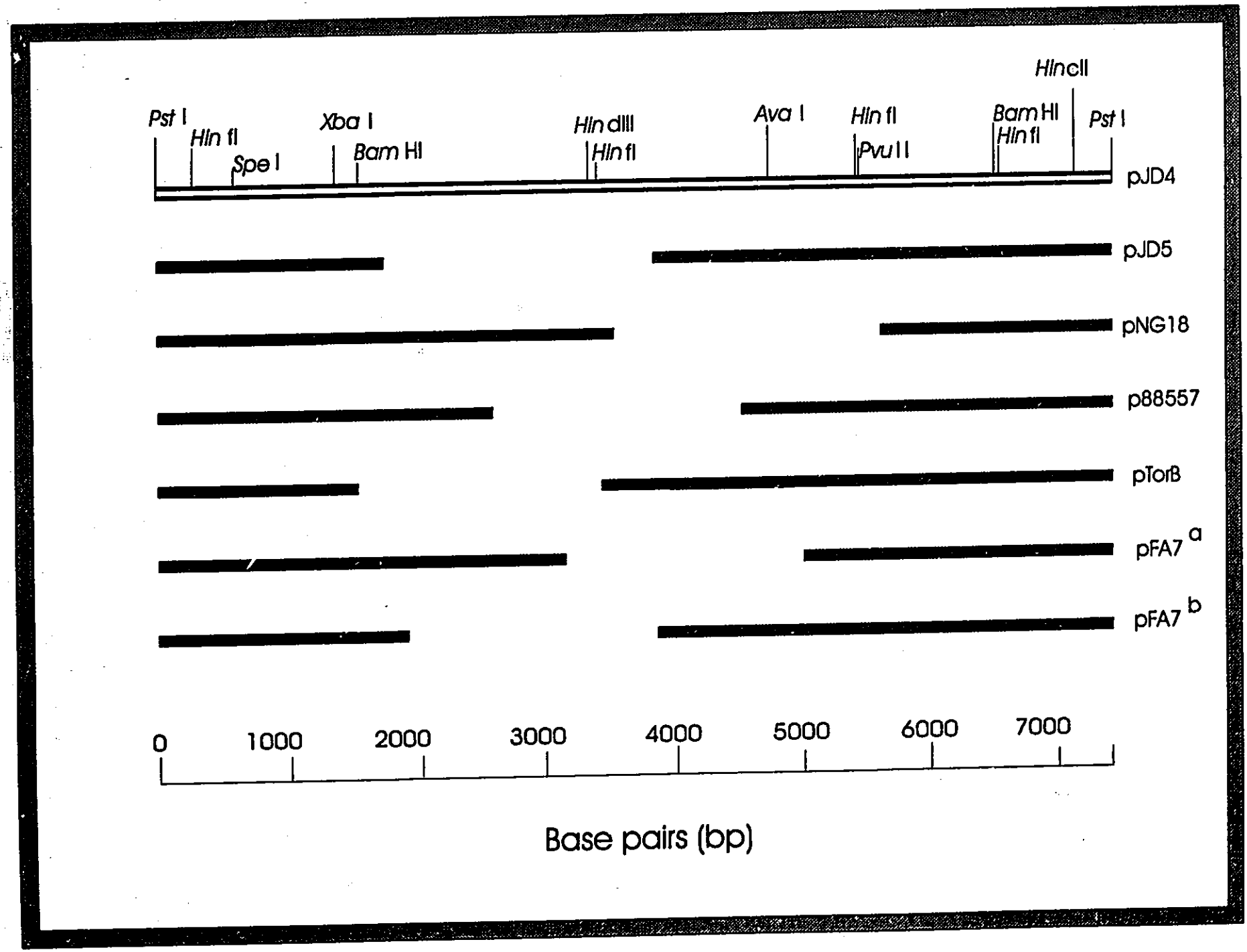
Plasmid	Reported plasmid size	Reported deletion size	References
pJD5	5.1-kb	2.1-kb	Dillon and Yeung, 1985
pTorB	5.5-kb	1.8-kb	Aalen and Gundersen, 1987
pFA7	5.4-kb	2.1-kb	Chen and Clowes, 1987
pFA7	5.1-kb	2.0-kb	Brunton *et al.*, 1981
pFA7	5.1-kb	2.0-kb	Sox *et al.*, 1979
pFT300	5.2-kb	1.9.-kb	Tenover *et al.*, 1985
p88557	5.1-kb	2.0-kb	McNicol *et al.*, 1983
pNG18	5.3-kb	2.1-kb	Dickgiesser, 1984
pMR0200	5.1-kb	2.0-kb	Roberts *et al.*, 1977
pLPL	5.1-kb	2.0-kb	Elwell *et al.*, 1977

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 *Pst*I 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 *Pst*I 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 *Pst*I site. Brunton *et al.*(1981) located a 1.87-kb (1.2 Mdal) deletion of pFA7 at 1.87-kb downstream of the *Pst*I site. Sox *et al.* (1979) located a 2.0-kb deletion of pFA7 at 2.77-kb downstream of the *Pst*I 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 *Pst*I 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 *Bam*HI

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); pFA7[a] (Sox *et al.*, 1979); pFA7[b] (Brunton *et al.*, 1981).


Pst I
Hin fI
Spe I
Xba I
Bam HI
Hin dIII
Hin fI
Ava I
Hin fI
Pvu II
Bam HI
Hin fI
Hinc II
Pst I
pJD4
pJD5
pNG18
p88557
pTorB
pFA7 a
pFA7 b
0
1000
2000
3000
4000
5000
6000
7000
Base pairs (bp)

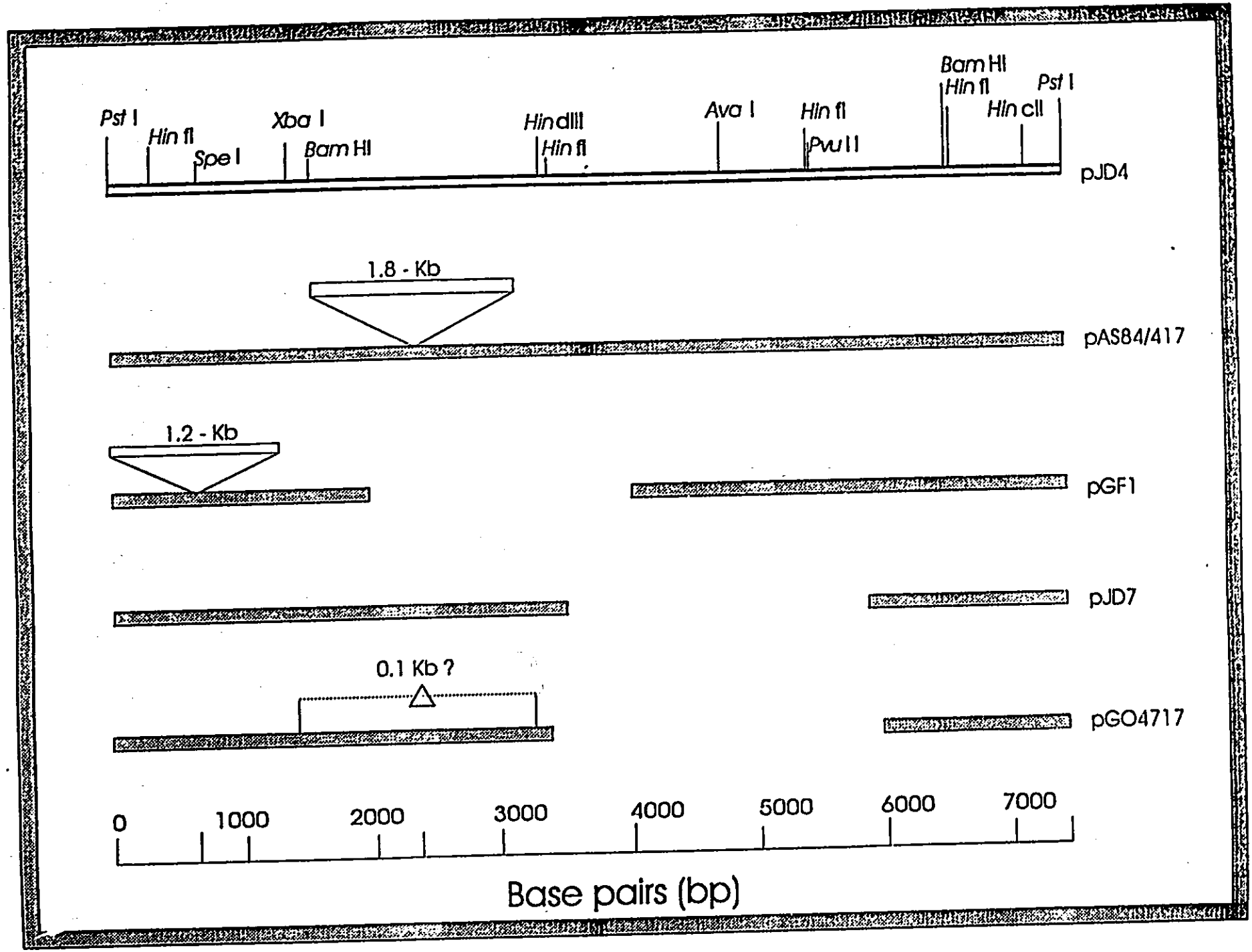
jd4001

Table 3. Size of the Toronto, Rio, Nimes and New Zealand plasmids of *N. gonorrhoeae* as reported in the literature.

Plasmid	Reported size[a]	Reference
pGF1 (Nimes)	6.6-kb	Gouby *et al.*, 1986
pGF1 (Nimes)	6.6-kb	Dillon and yeung, 1989
pJD7 (Toronto)	4.9-kb	Yeung *et al.*, 1986
pGO4717 (Rio)	4.6-kb	vanEmbden, 1985
pGO4717 (Rio)	9.0-kb	Yeung *et al.*, 1991
pAS84/417 (New Zealand)	9.0-kb	Brett, 1989

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.


Pst I
Hin fI
Spe I
Xba I
Bam HI
Hindlll
Hin fI
Ava I
Hin fI
Pvu II
Bam HI
Hin fI
Pst I
Hin cII
pJD4
1.8 - Kb
pAS84/417
1.2 - Kb
pGF1
pJD7
0.1 Kb ?
pGO4717
0
1000
2000
3000
4000
5000
6000
7000
Base pairs (bp)

jd4002

and *Hin*dIII, van Embden *et al* (1985) reported that the region homologous to the small (1.8-kb) *Bam*HI-*Hin*dIII 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 *Bam*HI-*Hin*dIII fragment of pGO181. The claim that the small *Bam*HI-*Hin*dIII 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 *Bam*HI and *Hin*dIII. 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 *Bam*HI-*Hin*dIII 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 *Bam*HI fragment (Gouby *et al.*, 1986; Dillon and Yeung, 1989). However, the exact location or the nature of the insertion was not established (Gouby *et al.*, 1986; Dillon and Yeung, 1989).

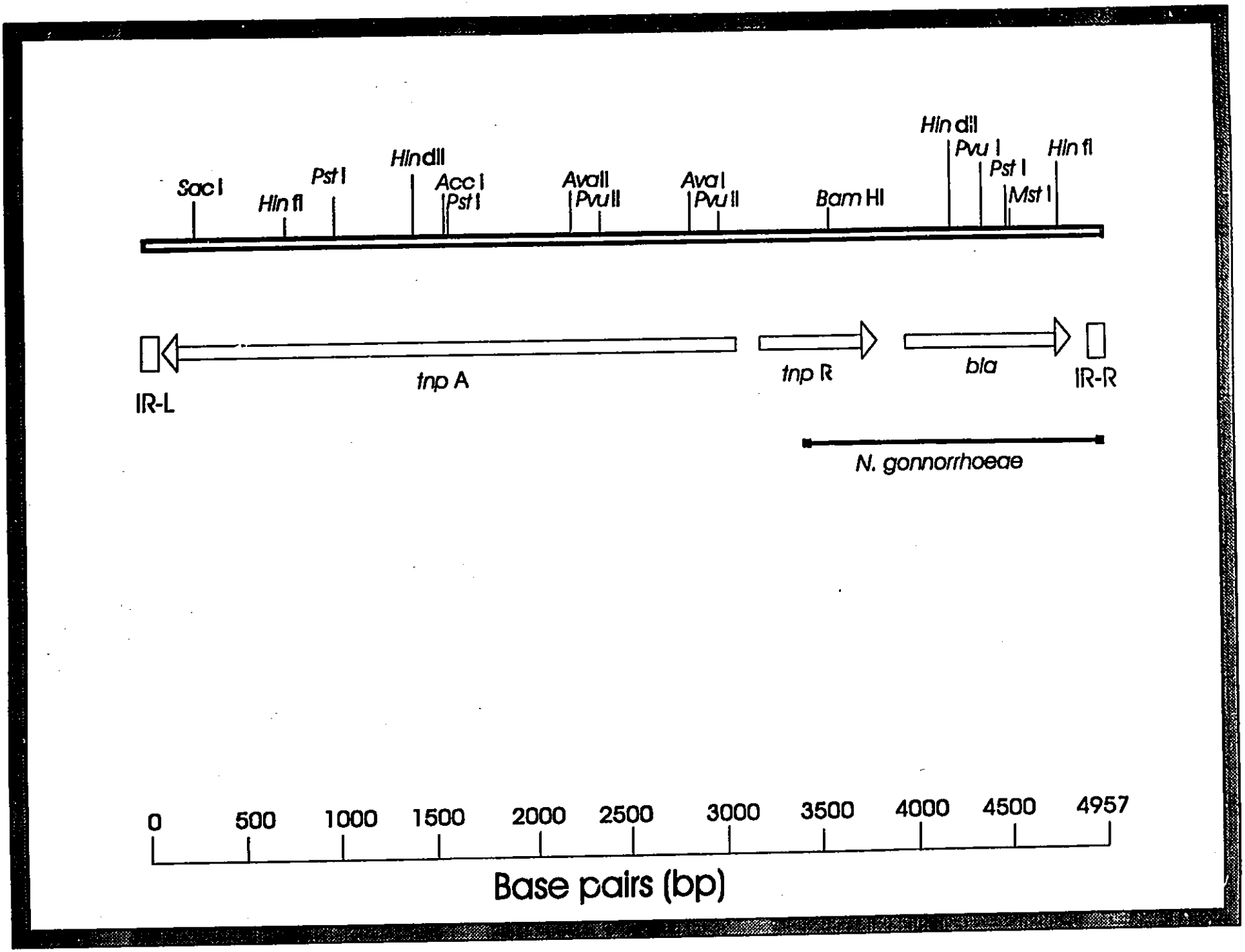
Restriction endonuclease analysis indicated that the New Zealand plasmid was identical to the Asia plasmid except for the addition of 1.8-kb (Yeung *et al.*, 1991a), which is located at approximately 2.4-kb downstream of the *Pst*I site (Bigelow and

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 (Tn*A*) is widely distributed in prokaryotic genera (Heffron *et al.*, 1977). It comprises closely related transposons that were designated as Tn*1*, Tn2, and Tn*3* (Heffron *et al.*, 1977; Calos and Miller, 1980). Tn*3* is the first transposable element encoding antibiotic resistance to be reported (Hedges and Jacob, 1974). Tn*3* is derived from IncF plasmid R1*drd*19, while its very similar relatives, Tn*1* 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).

Tn*3* 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 *tnp*A and *tnp*R genes, and between the *tnp*R 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 Tn*3* (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*.


Hin dII
Pvu I
Hin fI
Pst I
Hin dIII
Sac I
Pst I
Acc I
Hin fI
Pst I
AvaII
Pvu II
AvaI
Pvu II
Bam HI
Mst I
tnp A
tnp R
bla
IR-L
IR-R
N. gonnorrhoeae
0
500
1000
1500
2000
2500
3000
3500
4000
4500
4957
Base pairs (bp)

jd4009

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, Tn*2* (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 Tn*3*-like transposon Tn*2301*. The 1.6-kb segment is not transposable, however, it became a functional transposon when linked to the left part of Tn*2301*. Chen and Clowes (1987b) proposed that the Tn*A* sequence in plasmid pFA3, an Asia-type plasmid, and pFA7, an Africa-type plasmid of *N. gonorrhoeae* are derived from Tn*2*, since they are more similar to Tn*2* than to Tn*1* or Tn*3* (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 Tn*A* 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).

I.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.

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

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-MDal; pJB1) and 10.5-kb (7.0-MDal; 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 Tn*2* sequence, while the gonococcal plasmids carry about 40% of Tn*2* (Brunton *et al.*, 1982; McNicol *et al.*, 1983; Chen and Clowes, 1987b).

Laufs *et al* (1979) reported a 7.1-kb (4.4-MDal) 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 Tn*2*. 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 Tn*2* 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-MDal (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 Tn*2* 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 β-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-MDal β-lactamase plasmid in *H. influenzae* (Roberts *et al.*, 1977). The 7.4-kb and 5.3-kb β-lactamase-producing plasmids of *N. gonorrhoeae* were also homologous to a variety of β-lactamase-producing plasmids isolated from *H. ducreyi, H. influenzae* and *H. parainfluenzae* (Brunton *et al,* 1986; Chen and Clowes, 1987b; Mc Nicol *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 β-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).

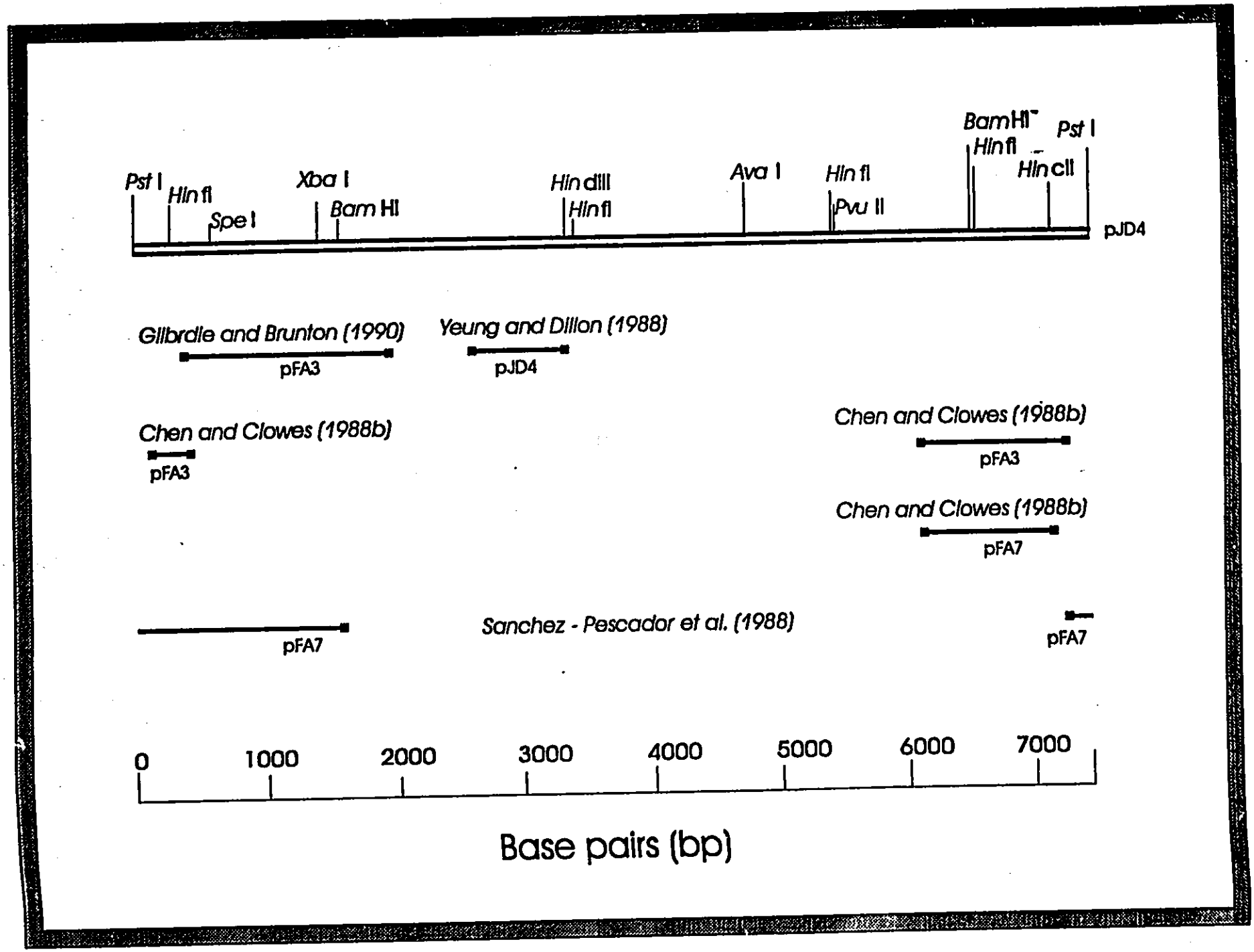
The 4.9-kb (Toronto) plasmid (Yeung *et al.*, 1986) and the 4.6-kb (Rio) plasmid (van Embden *et al.*, 1985), structurally related to the Asia-type but not to the Africa-type plasmid, favour the hypothesis proposed by Sox *et al.* (1979), and may indicate that transformation- associated deletion may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

I.5. DNA sequence of the β-lactamase producing plasmids of *N. gonorrhoeae*

None of the β-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 β-lactamase (*bla*) gene and 1031 bp of pFA7 from one of the *Bam*HI sites outside Tn*A* to the right hand of inverted repeat (IR-R) of Tn*A* (including the entire IR-R) (Figure 5). Chen and Clowes (1987b) sequenced 1447-bp of the region homologous to Tn*A* 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* (β-lactamase) gene, and 305-bp from the end of IR-R of Tn*A* (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 Tn*A*. Gilbride and Brunton (1990) sequenced approximately 1.6-kb of the 3.4-kb *Hind*III-*Pst*I fragment of pFA3, an Asia-type plasmid, starting at the 480-bp proximal of a *Bam*HI site toward the *Pst*I site, including the IR-R of Tn*A*, 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.


Pst I
Hin fI
Spe I
Xba I
Bam HI
Hin dIII
Hin fI
Ava I
Hin fI
Pvu II
Bam HI
Hin fI
Pst I
Hin cII
pJD4
Gilbrdie and Brunton (1990)
pFA3
Yeung and Dillon (1988)
pJD4
Chen and Clowes (1988b)
pFA3
Chen and Clowes (1988b)
pFA3
Chen and Clowes (1988b)
pFA7
pFA7
Sanchez - Pescador et al. (1988)
pFA7
0
1000
2000
3000
4000
5000
6000
7000
Base pairs (bp)

jd6005

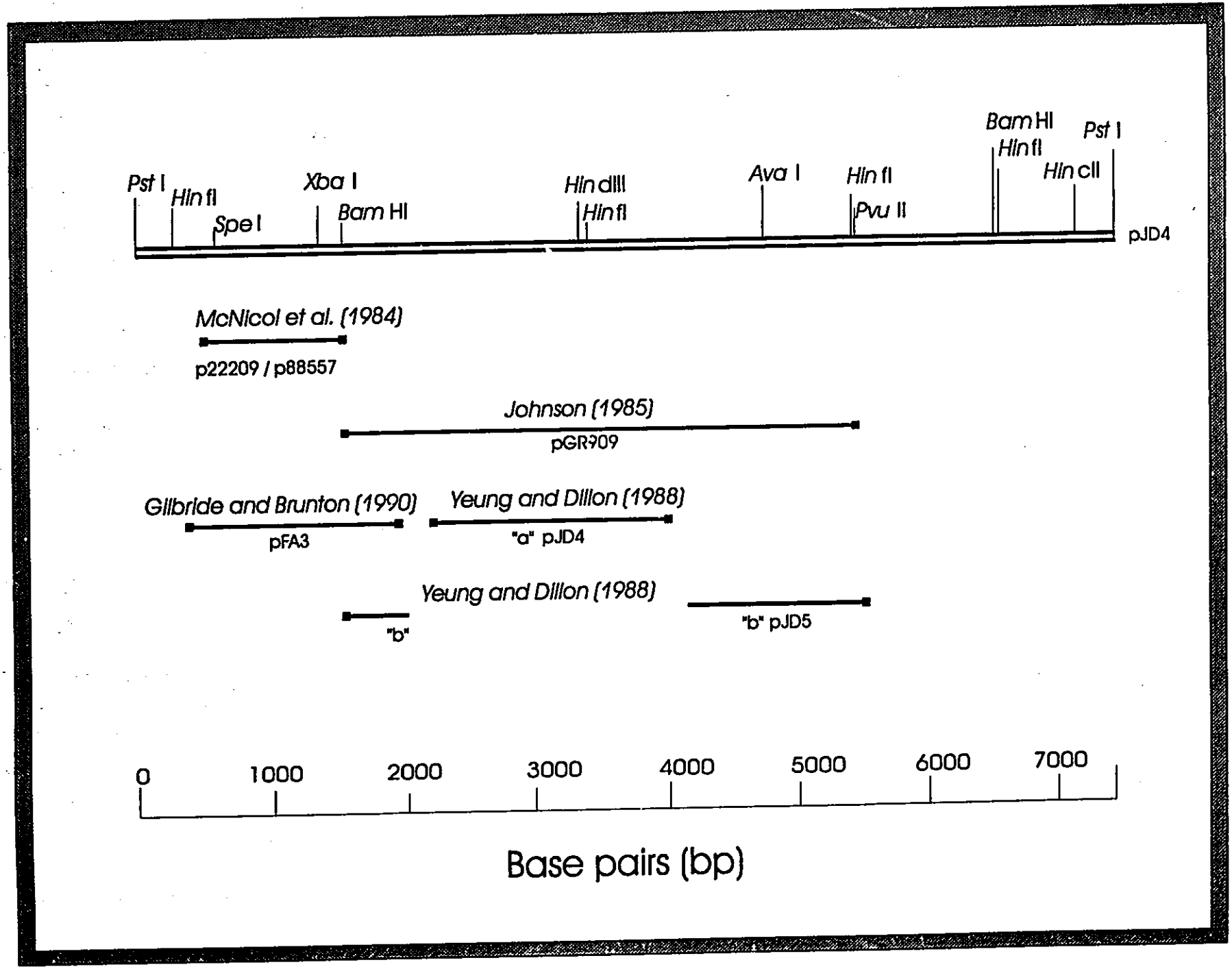
I.6. Studies to identify phenotype and function on the β-lactamase plasmids of *N. gonorrhoeae*

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 β-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 β-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 *Bam*HI fragment and the large *Bam*HI 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-Tn*2* portion of the 2.4-kb *Bam*HI fragment, 0.5-kb downstream of the *Pst*I site (Figure 6). Studies by Johnson (1985) did not support this conclusion. Johnson (1985) cloned the 2.4-kb *Bam*HI fragment, the 1.1-kb and the 3.8-kb *Bam*HI-*Pvu*II fragments of an Asia-type plasmid

Figure 6. Reported replication regions of the Asia-type and the Africa-type plasmids of *N. gonorrhoeae*.


Pst I
Hin fI
Spe I
Xba I
Bam HI
Hin dIII
Hin fI
Ava I
Hin fI
Pvu II
Bam HI
Hin fI
Pst I
Hin cII
pJD4
McNicol et al. (1984)
p22209 / p88557
Johnson (1985)
pGR909
Gilbride and Brunton (1990)
pFA3
Yeung and Dillon (1988)
"a" pJD4
Yeung and Dillon (1988)
"b"
"b" pJD5
0
1000
2000
3000
4000
5000
6000
7000
Base pairs (bp)

jd6004

pGR9091 into pBR322 or pMB8, and found that recombinant plasmids containing either the 2.4-kb *Bam*HI or the 1.1-kb *Bam*HI-*Pvu*II 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 *BamH*I-*Pvu*II 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 *Pst*I site. Second, a 1.5-kb region, designated "b", was located in the 3.1-kb *Bam*HI fragment of pJD5, 1.5-kb downstream of the *Pst*I 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 *Pst*I-*Hind*III fragment, which starts from approximately 460-bp proximal to the *Bam*HI site and includes all non-Tn2 portions of the 2.4-kb *Bam*HI fragment. Gilbride and Brunton (1990) also confirmed the previous report by Yeung and Dillon (1988) and Johnson (1985) that the 2.4-kb *Bam*HI 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 *Pst*I 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).

I.6.2. Regions required for the mobilization

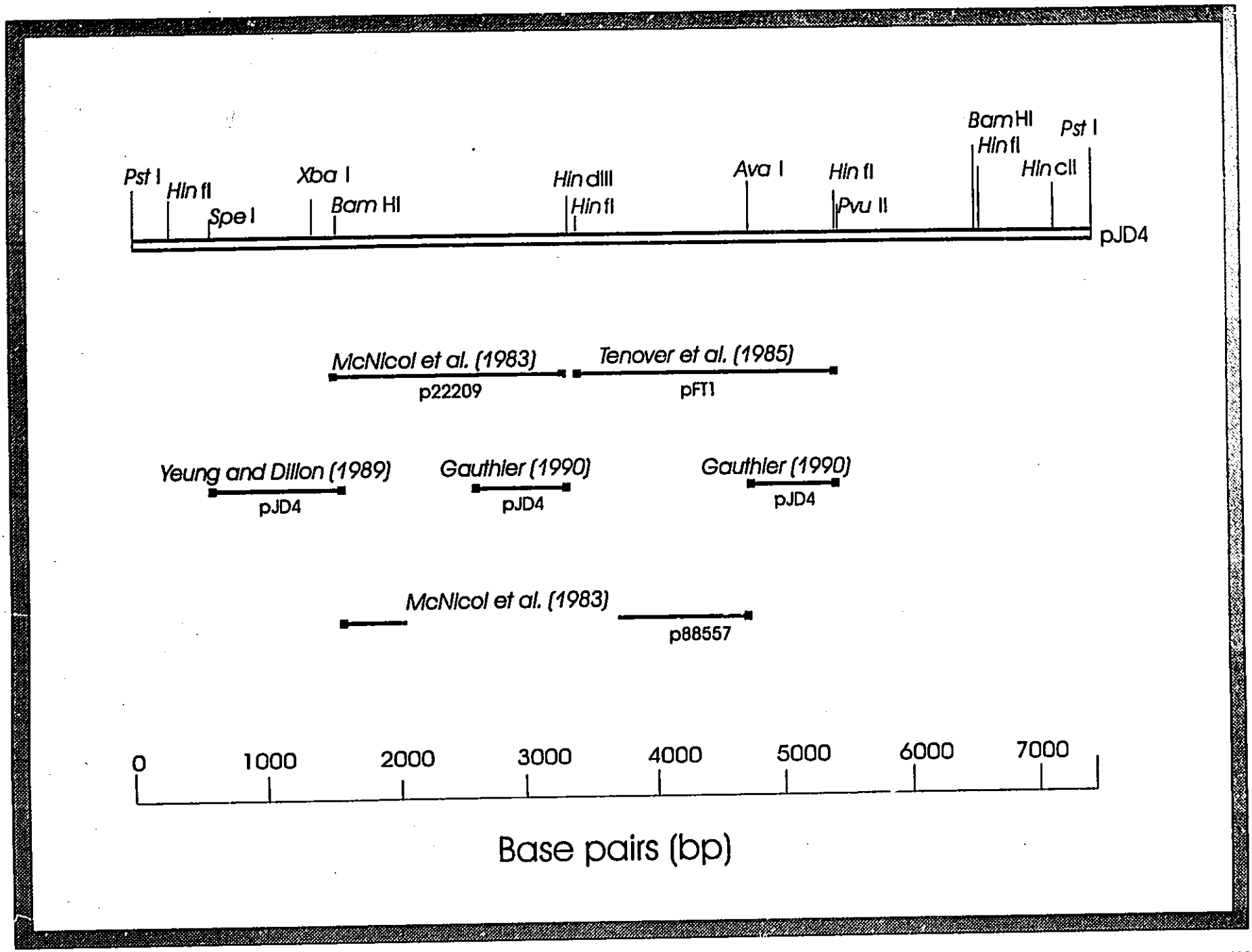
Conjugation is the process of genetic exchange that requires intimate cell to cell contact (Guiney and Lanka, 1989). In most cases, the DNA transfer from the donor to the recipient is a plasmid (Dale, 1989). Plasmids capable of promoting their own transfer are called conjugative, while those that can 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 plamids such as pRK231, pRK2013, and pR751), and Incl*α* (R64*drd*-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 (IncFII), pR124 (IncFIV), and pBG791 (IncI) 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 *Bam*HI-*Hind*III fragment, and the *oriT* of the Africa-type plasmid (p88557) in the 1.4-kb *Bam*HI-*Ava*I fragment (McNicol *et al.*, 1983).

Figure 7. Location of the putative mobilization regions of the β-lactamase plasmids of *N. gonorrhoeae*.


Pst I
Hin fI
Spe I
Xba I
Bam HI
Hin dIII
Hin fI
Ava I
Hin fI
Pvu II
Bam HI
Hin fI
Hin cII
Pst I
pJD4
McNicol et al. (1983)
p22209
Tenover et al. (1985)
pFT1
Yeung and Dillon (1989)
pJD4
Gauthier (1990)
pJD4
Gauthier (1990)
pJD4
McNicol et al. (1983)
p88557
0
1000
2000
3000
4000
5000
6000
7000
Base pairs (bp)

jd6001

Tenover *et al.* (1985) found that a recombinant plasmid pFT2, which comprised pFT1, an Asia-type plasmid lacking its 1.9-kb *Hinf*I fragment (3.7-kb downstream of the *Pst*I 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 *ori*T 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 *Hinf*I fragment, 3.7-kb downstream of the *Pst*I site.

Dillon and Yeung (1989) cloned three fragments of pJD4 (an Asia-type plasmid): the 2.4-kb *Bam*HI, the 1.8-kb *Bam*HI-*Hind*III and the 3.2-kb *Bam*HI-*Hind*III fragment, into pACY184. Only the recombinant plasmid containing the 2.4-kb *Bam*HI fragment could be mobilized by pBG791 (Inclα) (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 *Bam*HI fragment contained the β-lactamase gene, it was concluded that the mobilization region was located in the 0.8-kb non-Tn*A* *Bam*HI 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 ori*T* in the 1.8-kb *Bam*HI-*Hind*III fragment of pJD4. Gauthier (1990) also proposed the 0.6-kb *Pvu*II-*Ava*I fragment of pJD4 as

the essential part of the mob region, since a deletion of the derivative plasmid lacking the 0.6-Kb *Pvu*II-*Ava*I 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 *Pvu*II-*Ava*I fragment.

I.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 Tn*A* (the 0.9-kb *Bam*HI-*Pst*I 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, Tn*A*, in pJD4, pJD5 and pJD7 and to compare the regions flaking Tn*A* in the various β-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 β-lactamase-producing plasmids of *N. gonorrhoeae* in general.

6. To analyze the structure of the β-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 *leuB*6 *thr*-1 *lacY*1 *tonA*21 *supE*44) (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 Nationalo de la Santé et de la Recherche Medicalé, Nimes, France). Plasmids pGF1 had been transformed (Table 5) previously into *E. coli* HB101 (*supE*44 *supF*58 *hsdS*3 ($r_b^-m_b^-$) *recA*13 *ara*-14 *proA*2 *lacY*1 *galK*2 *rpsL*20 *xyl*-5 *mtl*-1) (Gouby *et al.*, 1986; Bolivar and Backman, 1979).

Table 5. Bacterial strains and plasmids

E. coli strain	PPNG strains	Plasmid content	Description/reference
C600-JD4		pJD4	pJD4 transformed into *E. coli* C600 (Yeung and Dillon, 1985)
C600-JD5		pJD5	pJD5 transformed into *E. coli* C600 (Yeung and Dillon, 1985)
C600-JD7		pJD7	pJD7 transformed into *E. coli* C600 (Yeung and Dillon, 1985)
	GC1213	Toronto-type	PPNG strain containing Toronto-type plasmid from NLSTD, LCDC
	GC4538	Toronto-type	PPNG strain containing Toronto-type plasmid from NLSTD, LCDC
	GC5221	Toronto-type	PPNG strain containing Toronto-type plasmid from NLSTD, LCDC
	GC5228	Toronto-type	PPNG strain containing Toronto-type plasmid from Dr. J. van Embden
	GC5230	Toronto-type	PPNG strain containing Toronto-type plasmid from Dr. J. van Embden
HB101-GF1		pGF1	Gouby *et al.*, 1986
JM83-STD32		pSTD32	1.5-kb *Bam*HI-*Pst*I fragment of pJD4 cloned into pBluescript II KS (+) (Hutnik and Dillon, unpublished data).
JM83-STD41		pSTD41	3.2-kb *Bam*HI fragment of pJD4 cloned into pBluescript II KS (+) (Hutnik and Dillon, unpublished data).
C600-GO4717		Rio plasmid	Rio plasmid transformed into *E. coli* C600 (Yeung, Ph. D. Thesis, 1990).

A recombinant plasmid, pSTD32, which contained a 1.5 kb *Bam*HI/*Pst*I fragment and pSTD41, which contained a 3.2 kb *Bam*HI/*Hin*dIII 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*, Δ*lac pro*, *thi*, *str*A, *p*80d*lac*ZΔM15) (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-4hloro-4indolyl-β-D-galactoside (X-gal) was purchased from Boehringer Mannheim Canada Ltd. (BMC, Laval, Quebec). Restriction buffers, restriction endonuclease enzymes (*Ava*I, *Bam*HI, *Hin*dIII, *Hin*fI, *Kpn*I, *Pst*I, *Pvu*II, *Sac*I, *Spe*I, *Taq*I and *Xba*I), 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 CO_2 (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 $CaCl_2$ 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 $CaCl_2$.

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 loopfull 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 (ddH_2O).

B. To prepare plasmid DNA for sequencing, the Magic Minipreps™ DNA purification system was used, as described in the manufacture's protocal (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 mililitres 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 Maxipreps™ 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 Minipreps™ 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 *Hin*dIII (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 $MgCl_2$; 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 % agarose 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 % agarose 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-^{35}S]-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 diveloped 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 *Bam*HI-*Hind*III 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 Tn*A* 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^{R}, restriction endonuclease sites, direct and inverted repeats, open reading frames (ORFs) and other structural features by Microgenie^{R} Sequence Analysis Program (Beckman Instrument Inc., Palo Alto, California) and PC gene (Stratagene, Canada).

Table 6. Primers used for DNA sequencing

No	Primers	Sequence (5'-3')	Position[a]
1	JD4.1	AAGGATTGAT GAGTACG	2088-2072
2	JD4.3	CCACGCTTGA TAGGTTC	2837-2853
3	JD4.4	GGCAATCTCT CGCATAG	2664-2680
4	JD4.5	CTGACCGCTT ACCAAGT	2544-2560
5	JD4.6	TTACGCAACT TAGGGAA	2343-2359
6	JD4.7	CGTTTGCTTT GTGATCG	2102-2118
7	JD4.9	GGTTCAGCAA CCGAAGC	1970-1986
8	JD4.11	TTACAAAACC CTTACAG	1922-1938
9	JD5.1	GCTACGCATT ACAAGGT	4269-4253
10	JD5.2	GTGAGCGAAA TTGTAAC	4093-4077
11	JD5.5	GATTGATGAG TACGATA	3914-3898
12	JD7.4	TCTCGCAAGC CTTGTGTAAC	3616-3635
13	JD7.5	CATATCATCA AGGTGA	3460-3476
14	JD7.7	TATCGCCGTT CTGGTTGG	6235-6218
15	Tn3.1	GCTCGTAGAT TGCGAACT	652-635
16	Tn3.2	CCGGCAGTTC AACAGACC	6261-6278
17	Tn3.3	GACAGATCGC TGAGATAG	277-294
18	Tn3.5	TGTGCCACAG CCGACAGGAT	6492-6473
19	Tn3.6	CGTTCAGCCT GTGCCACAGC	6501-6482
20	Nimes.1	TCAATAGCCG CTCTAACC	514-532[b]

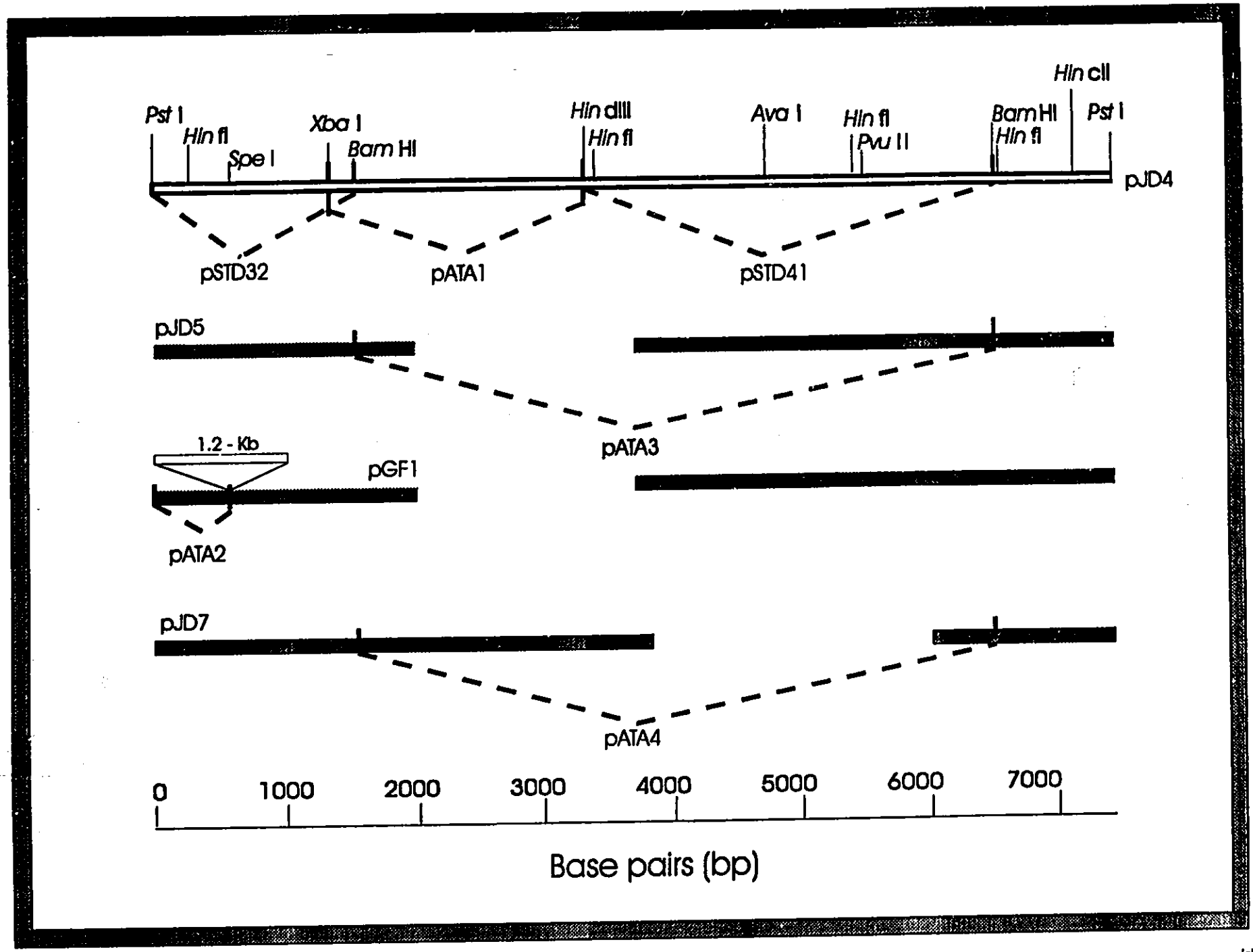
[a]. The coordinates of primers are calculated according to the primary sequence of pJD4 with the *Pst*I as coordinate 1.
[b]. Coordinate of the Nimes.1 primer is based on the primary sequence of pGF1, with the *Pst*I site as coordinate 1.

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 *Bam*HI-*Pst*I, the 3.2-kb *Bam*HI-*Hind*III and the 2.0-kb *Xba*I-*Hind*III 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 *Spe*I/*Pst*I fragment of pGF1, the 3.1 kb *Bam*HI fragment of pJD5 and the 2.7 kb *Bam*HI 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.


Hin cII
Pst I
Hin fI
Spe I
Xba I
Bam HI
Hin dIII
Hin fI
Ava I
Hin fI
Pvu II
Bam HI
Hin fI
Pst I
pJD4
pSTD32
pATA1
pSTD41
pJD5
pATA3
1.2 - Kb
pGF1
pATA2
pJD7
pATA4
0
1000
2000
3000
4000
5000
6000
7000
Base pairs (bp)

jd4003

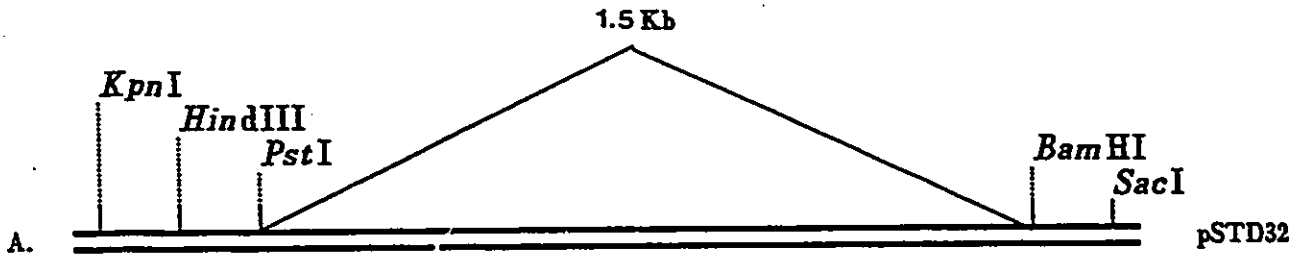
III.2. Construction of nested deletion in recombinant DNA

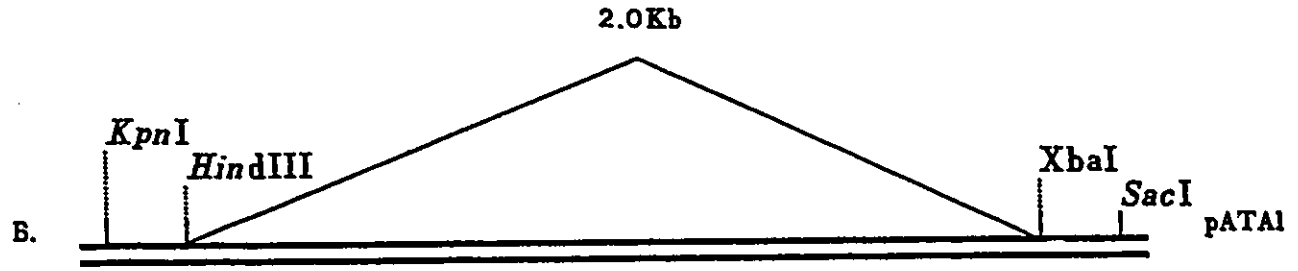
Nested deletions of pSTD32 were constructed in both directions. Restriction endonucleases *Kpn*I (3' overhang) and *Hin*dIII (5' overhang) were used to produce nested deletions from the *Pst*I site toward the *Bam*HI site (Figure 9a), and restriction endonucleases *Sac*I (3' overhang) and *Bam*HI (5' overhang) were used to produce nested deletions from the *Bam*HI site toward the *Pst*I site.

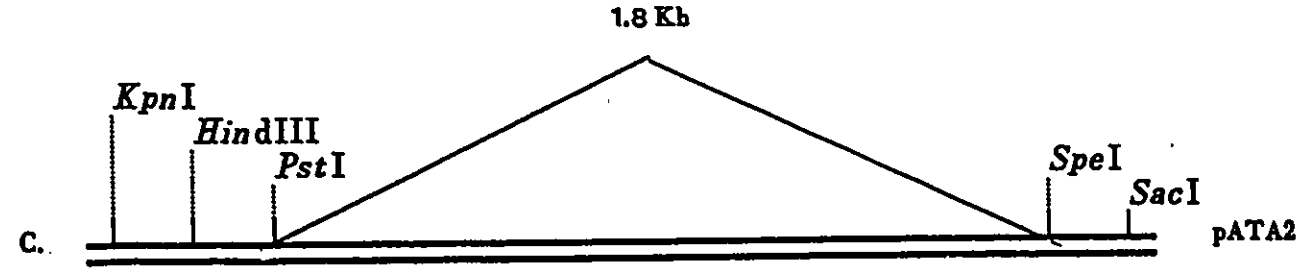
Nested deletions of pATA1 were constructed in both directions, from the *Xba*I site and from the *Hin*dIII site. Restriction endonucleases *Sac*I (3' overhang) and *Xba*I (5' overhang) were used to produce nested deletions from the *Xba*I site toward the *Hin*dIII site (Figure 9b). Restriction endonucleases *Sac*I (3' overhang) and *Xba*I (5' overhang) were used to produce nested deletions from the *Hin*dIII site toward the *Xba*I site.

Nested deletions of pATA2 were constructed in both directions, from the *Pst*I site and from the *Spe*I site. Restriction endonucleases *Kpn*I (3' overhang) and *Hin*dIII (5' overhang) were used to produce nested deletions from the *Pst*I site toward the *Spe*I site (Figure 9c). Restriction endonucleases *Sac*I (3' overhang) and *Spe*I (5' overhang) were used to produce nested deletions from the *Spe*I site toward the *Pst*I site.

Figure 9. Restriction endonucleases used to generated nested deletions of pSTD32, pATA1, and pATA2.


1.5 Kb
KpnI
HindIII
PstI
BamHI
SacI
A.
pSTD32


2.0Kb
KpnI
HindIII
XbaI
SacI
B.
pATA1


1.8 Kb
KpnI
HindIII
PstI
SpeI
SacI
C.
pATA2

III.3. DNA sequence of pJD4

The primary sequence of pJD4 excluding a 0.9-kb *Bam*HI-*Pst*I fragment, which is homologous to Tn*A*, 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 *Bam*HI 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 *Bam*HI-*Pst*I insert of pSTD32 from the *Bam*HI 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 *Bam*HI site of pSTD32 on an 1% agarose gel. Subclones were digested with *Pvu*II. 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.

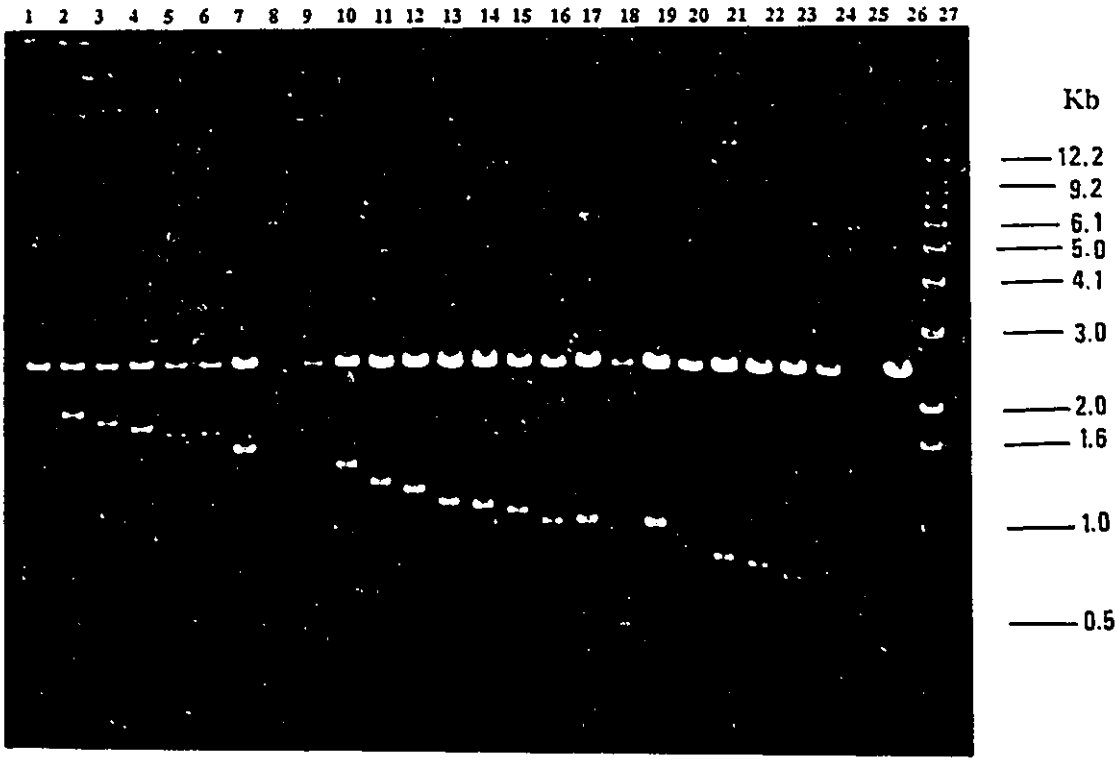
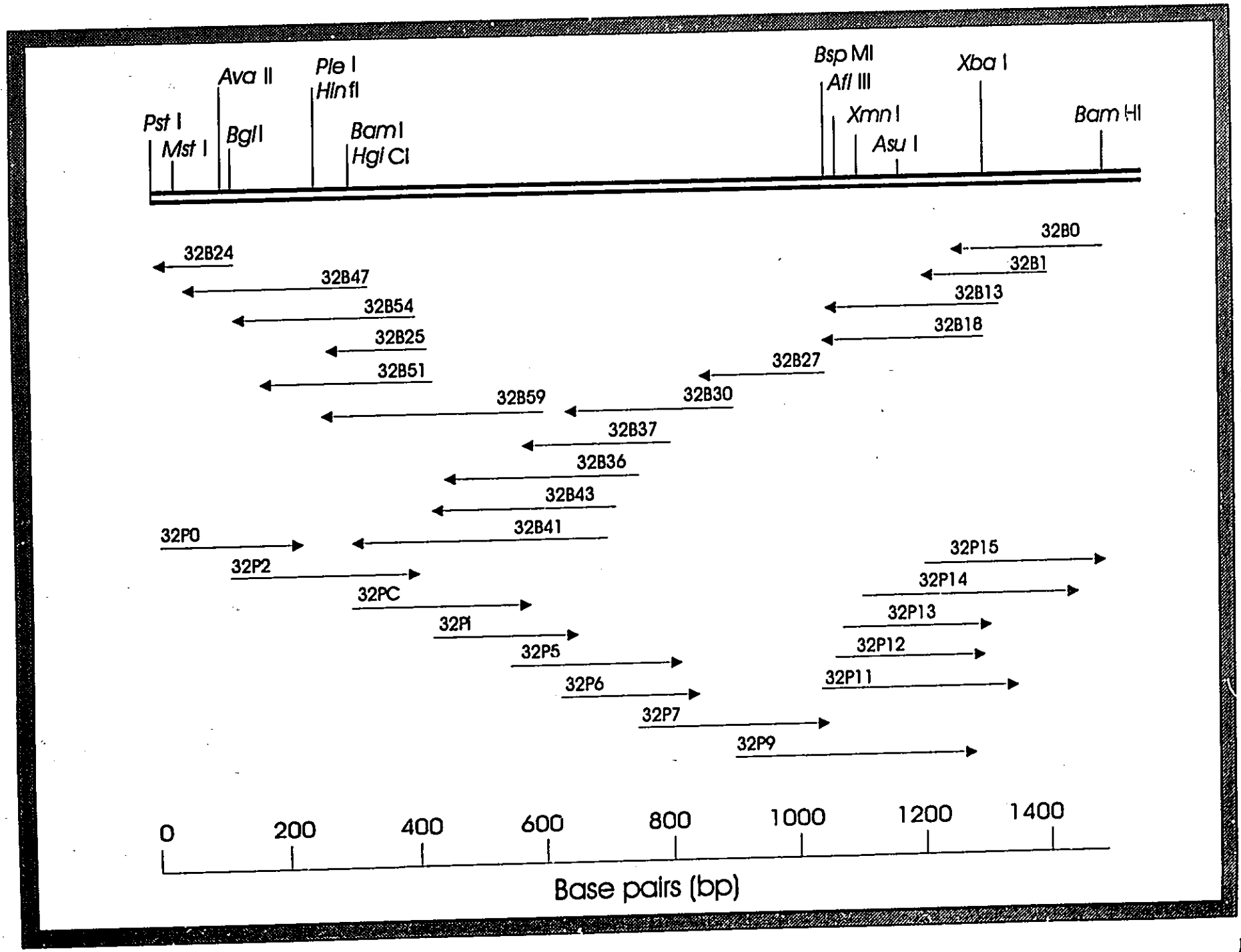

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27
Kb
12.2
9.2
6.1
5.0
4.1
3.0
2.0
1.6
1.0
0.5

Figure 11. Strategy used to determine the primary sequence of the 1.5-kb *Pst*I-*Bam*HI insert of pSTD32. The length of the arrows corresponds to the number of nucleotides actually sequenced from each subclone.

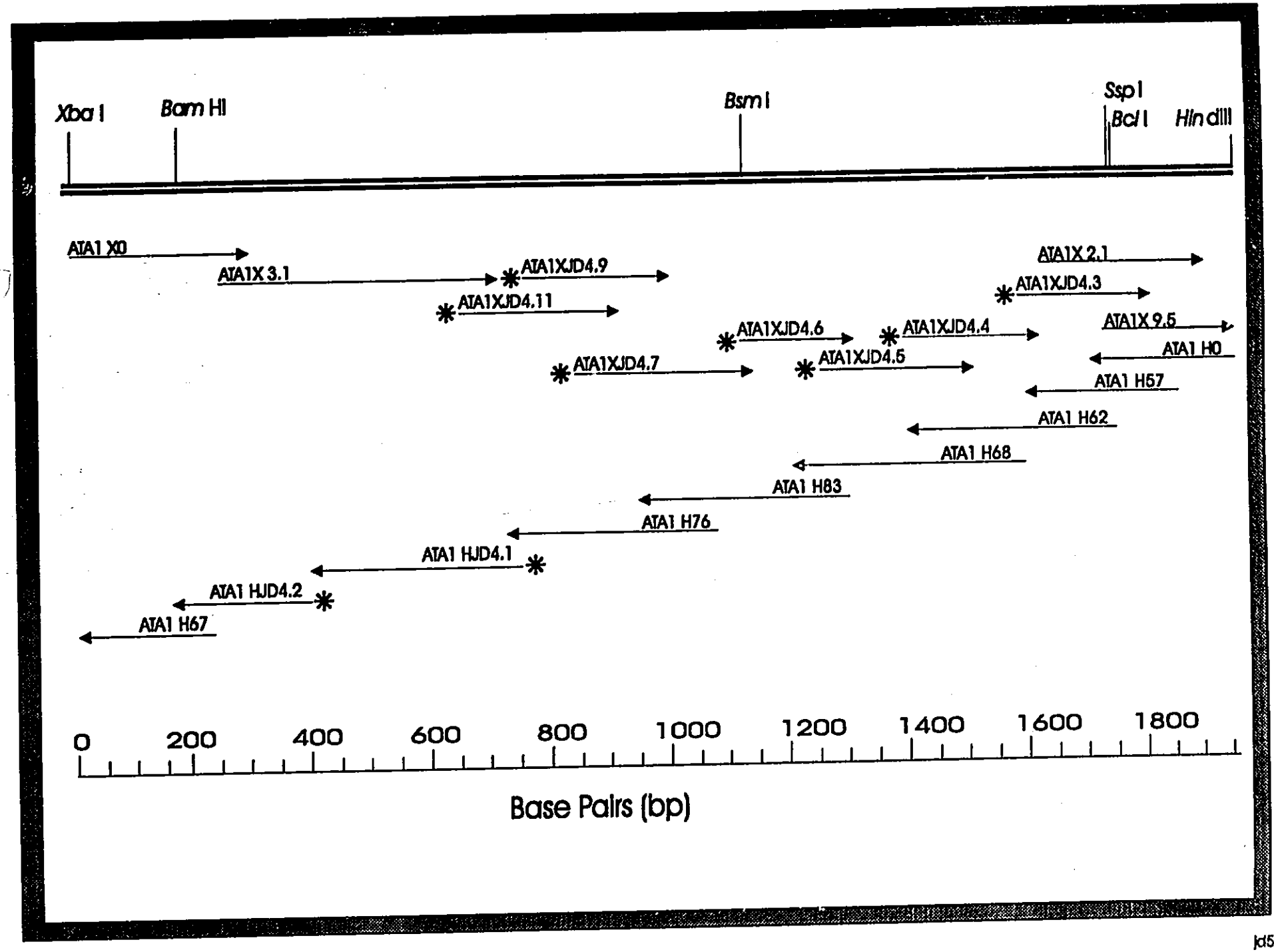

Pst I
Mst I
Ava II
Bgl I
Ple I
Hinf I
Bam I
Hgi CI
Bsp MI
Afl III
Xmn I
Asu I
Xba I
Bam HI
32B0
32B1
32B13
32B18
32B27
32B30
32B24
32B47
32B54
32B25
32B51
32B59
32B37
32B36
32B43
32B41
32P0
32P2
32PC
32Pi
32P5
32P6
32P7
32P9
32P15
32P14
32P13
32P12
32P11
0
200
400
600
800
1000
1200
1400
Base pairs (bp)

jd4004

1.5-kb *Bam*HI-*Pst*I insert of pSTD32 from the *Pst*I 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 *Xba*I-*Hind*III 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 *Hind*III site produced subclones pATA1H57, pATA1H62, pATA1H68, pATA183, pATA1H76, and pATA1H67. Nested deletions from the *Xba*I site produced subclones pATA1X3.1, pATA1X2.1, and pATA1X9.5. The sequence from the *Hind*III 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 *Xba*I 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 *Xba*I-*Hin*dIII 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 *Xba*I as zero reference position.

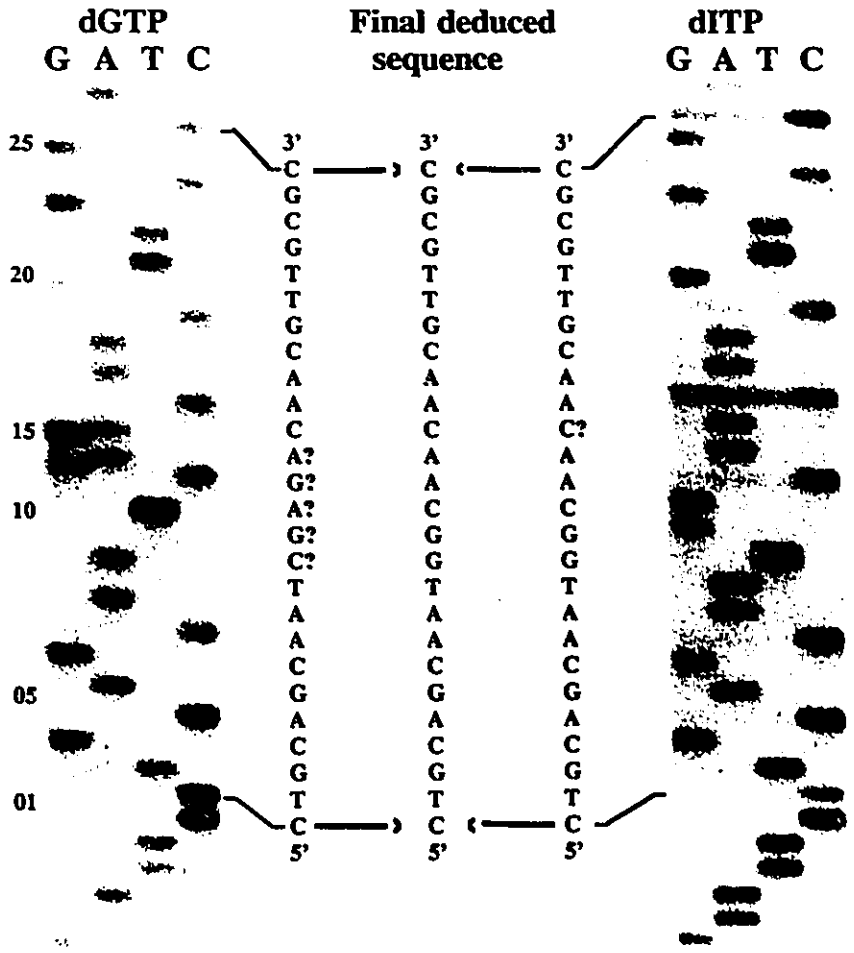

Xba I
Bam HI
Bsm I
Ssp I
Bcl I
Hin dIII
ATA1 X0
ATA1X 3.1
ATA1XJD4.9
ATA1XJD4.11
ATA1X 2.1
ATA1XJD4.3
ATA1X 9.5
ATA1XJD4.6
ATA1XJD4.4
ATA1 H0
ATA1XJD4.7
ATA1XJD4.5
ATA1 H57
ATA1 H62
ATA1 H68
ATA1 H83
ATA1 H76
ATA1 HJD4.1
ATA1 HJD4.2
ATA1 H67
0
200
400
600
800
1000
1200
1400
1600
1800
Base Pairs (bp)

jd5003

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^{R} 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'-CTGCAGCAAT GGCAACAACG TTGCG C-3'.

DNA sequences were determined in both directions except for nucleotide sequences from coordinates 1321 to 1504 (a 185-bp *Xba*I-*Bam*HI 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 *Pst*I site using dGTP and dITP at coordinates 1 to 26.


dGTP
G A T C
Final deduced sequence
dITP
G A T C
25
20
15
10
05
01
3' C G C G T T G C A A C A? G? A? G? C? T A A C G A C G T C 5'
3' C G C G T T G C A A C A A C G G T A A C G A C G T C 5'
3' C G C G T T G C A A C? A A C G G T A A C G A C G T C 5'

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 *Pst*I site as coordinate 1. The nucleotides from coordinates 6514 to 7426 were taken from the Tn*3* 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 *Bam*HI-*Pst*I fragment comprising Tn*3*, is 38.4 %, and the GC content of pJD4 excluding the region homologous to Tn*A*, 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-*Pst*I fragment of Tn*3* (Heffron et al., 1979), as identified using the Micro Genie^{R} program, are listed in Table 7. Twenty-three restriction endonucleases (*Aat*II, *Afl*III, *Apa*LI, *Asu*II, *Ava*I, *Ban*I, *Bgl*I, *Bsp*MI, *Bst*EII, *Drd*I,

Figure 14. The primary sequence of pJD4. It was written in 5'to 3' direction. Coordinate 1 is the *Pst*I site. Coordinates 6514 to 7426 were taken from the Tn*3* sequence (Heffron *et al*, 1979). The sequence homologous to Tn*A* 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.

```
        10         20         30         40         50         60         70         80         90        100
5'-CTGCAGCAAT GGCAACAACG TTGCGCAAAC TATTAACTGG CGAACTACTT ACTCTAGCTT CCCGGCAACA ATTAATAGAC TGGATGGAGG CGGATAAAGT

       110        120        130        140        150        160        170        180        190        200
TGCAGGACCA CTTCTGCGCT CGGCCCTTCC GGCTGGCTGG TTTATTGCTG ATAAATCTGG AGCCGGTGAG CGTGGGTCTC GCGGTATCAT TGCAGCACTG
       210        220        230        240        250        260        270        280        290        300
GGGCCAGATG GTAAGCCCTC CCGTATCGTA GTTATCTACA CGACGGGGAG TCAGGCAACT ATGGATGAAC GAAATAGACA GATCGCTGAG ATAGGTGCCT
       310        320        330        340        350        360        370        380        390        400
CACTGATTAA GCATTGGTAA CTGTCAGACC AAGTTTACTC ATATATACTT TAGATTGATT TAAAACTTCA TTTTTAATTT AAAAGGATCT AGGTGAAGAT
       410        420        430        440        450        460        470        480        490        500
CCTTTTTGAT AATCTCATGA CCAAAATCCC TTAACGTGAG TTTTCGTTCC ACTGAGCGTC AGACCCCTAT CTATAAACTC TTGGCTTGGT TCTAATCCCT
       510        520        530        540        550        560        570        580        590        600
CTAAACGATT ATTATCAATA GCCGCTCTAA CCGCTTTTTC TCGGCTTAAT TTTTCTGTCT CTGTTATAAA ATTGCTATTC ATCTTGTTCT TCTTCAAAAA
       610        620        630        640        650        660        670        680        690        700
AAAGTTAAGT AAAATACCTA CCTAAATTTT TACTAGTTCG CAATCTACGA GCTTATAACC TCGTTTTTTC AATTCATTTA AAAAATCAGA TTTTGAGCCT
       710        720        730        740        750        760        770        780        790        800
AATTTTGATC TATTGCTATC GTTACCCGCT AGAAATACCC AGTAATTACG CAAATCTTCA TTGGTAACTT TCGTAATATC GGTGTAATGA TCTTCGAGTA
       810        820        830        840        850        860        870        880        890        900
TTTTTAAGCA ATCTCTAGCC CATAAACCGT ACTCGTGATT GCTCATCTTA GGGTTTTGCT TATCGAGTTT GACGAACTTC CCATACTTGT TTTTATGTGG
       910        920        930        940        950        960        970        980        990       1000
AAATACTGGC CGTTTTGCAA CTTCTTCAAT TTTTTGAGCT GTTCGTTTTT TACTACCAAT CACAAAATTT AAAGAGTGAA TAGTACGCCC ACGCTTGATT
      1010       1020       1030       1040       1050       1060       1070       1080       1090       1100
TGTTCAACCT CAACGACTAA ATCAGATTTC TCGTTAATCT CAGTTATTGC AGGTTCCAAA ACACGTTGAT TTAATGAATT AAATCTAGGG TATTTATTTT
                                                                 IHF (92%)
      1110       1120       1130       1140       1150       1160       1170       1180       1190       1200
CAACCTGAAG CCATTCTTTT AGTTTTTCTA CTGTAATTTC ACGACTACCA ACAGAGCGAT ATTGTGTAAT TAGCTCATAA ATTCGAATTG AATGTACACT
      1210       1220       1230       1240       1250       1260       1270       1280       1290       1300
GTTGAAATAA GCGATATGTT TGAGTTGATA TTGCGTGAAT TGCCCTTTAA GTTGCGTTAG GTATGGCATA ACTTCATCAG TCATTGCAAT TCTAAAACGC
      1310       1320       1330       1340       1350       1360       1370       1380       1390       1400
CCCTCTTTCT TGAAATATGT TCTAGAGGAA ACCCAACGAA ATTCAGTTAC ACGGTCTTTA TCTTCAGTTT TAACACTTCG GTCATAAATC CGTTTTATAG
      1410       1420       1430       1440       1450       1460       1470       1480       1490       1500
CCGCCTGAAT TTGCTTATAG GCGTTATCTT GGCTTATTTC TGGAAACTCA CGGACAAAAT CAGCCACCGT AAAATCAAAA ATCTTTTGAT TAGATTTCGG
                                                                                 IHF (92%)
US (90 %)
      1510       1520       1530       1540       1550       1560       1570       1580       1590       1600
ATCCATAGTC CCAATAGTTA AAGCTAAAAT TCTGATTTCA TCAATACTCA ATCGGTAATT GGCTTCAATA AGGCTATTAG CCTTTACAAC AACTAAATCA
      1610       1620       1630       1640       1650       1660       1670       1680       1690       1700
TTTGGCATAA GACAACAAAT TTCCTGTTTA AAACAACAAG CAAAATATAC CTGTTGTTTA TATATAAAAC AACAAGTATT TTCTTAAAAG TTGTCTATAA
      1710       1720       1730       1740       1750       1760       1770       1780       1790       1800
CAGGAAATTT GTTGTCTTAT AACAGGAAAT TTGTTGTCGT ATAACAGGAA ATTTGTTGTC GTATAACAGG AAATTTGTTG TCGTATAAGT TTGTAACTTA
                                                                                                      IHF
      1810       1820       1830       1840       1850       1860       1870       1880       1890       1900
TTGATTTTAC TGGTTTTAAA AACGCCGAAA ACAAGTAAAA AACAAAAATA TAAAAATATA GGGACTTTCG TCCCTTTTTT GGGCTTTCAG CCCTAATTTT
                                                                                          DR-30A
      1910       1920       1930       1940       1950       1960       1970       1980       1990       2000
TTCTTTTTTC AGGATTAAAA ATTACAAAAC CCTTACAGAG CAAGTAAACT TGTTTGCTTG TTCTGCAAGG GTTCAGCAAG CGAAGCCGTT AGGCGTAGGC
DR-30A
      2010       2020       2030       2040       2050       2060       2070       2080       2090       2100
GGTAGCCTAT AAAAGCCATT TAATTTTATC TTTAAATTTC CGTTTAAATG CTTTGAGTGG GTGTCTTTTA TCGTACTCAT CAATCCTTTT TTGCATTCTT
DR-30A
      2110       2120       2130       2140       2150       2160       2170       2180       2190       2200
TCGTTTGCTT TGTGATCGGC AAATTTTGAA TAAGATTTTT CCATCTCATC TAACATTCTA TCAATCCGTT TTTTATGTTG CCATTTCAGG TAAACATAAA
                                                                 IHF (92%, Compl)
DR-30A
      2210       2220       2230       2240       2250       2260       2270       2280       2290       2300
CACTTATAGC AATAAAAGAC AATATCAATA CATTGTAAAA AATGATTGTT ACAATTTCGC TCACAGTTAT TTTTTACCTT TTTCAATTTC TTCATTGATA
DR-30A                    IHF      IHF (92%, Compl)
      2310       2320       2330       2340       2350       2360       2370       2380       2390       2400
AATGCACTCA ATTCATCAAA TTTCTTGTCA TCATTGATAA ATTTACGCAA CTTAGGGAAG TTTCTATCTA CATCTAAAAG AGGGTTAAAC GATTATTATC
DR-30A
      2410       2420       2430       2440       2450       2460       2470       2480       2490       2500
AATAGCCGCT CTAACCGCTT TTTCTCGGCT TAATTTTTCT GTCTCTGTTA TAAAATTGCT ATTCATCTTG TTCTTCTCTC ACACTTTAAC TAATTCACAG
      2510       2520       2530       2540       2550       2560       2570       2580       2590       2600
TTCACAATCT TATACCCTCG ATTTTGCAAC TCGTTTAAAA AATCTGACCG CTTACCAAGT TTTGATCTAA AACTAGCATT GCTAGTCAAA AAAACCCAAT
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      2610        2620        2630        2640        2650        2660        2670        2680        2690        2700
AATGCCGTAA ATCTTCTGTT GTAACATCGG CAAGATCAGA ATAAAAATCT TCAAGGATTT TTAGGCAATC TCTCGCATAG TTTCCATATT CAGCATTACT
      2710        2720        2730        2740        2750        2760        2770        2780        2790        2800
CATTTTGGGA TTTTGAGTAT CCAATTTCAC AAACTTCCCG TACTTGTTTT TATGCGGAAA TGCAGGGCGT TTCTGTTCGA TTTTTACCGC ACTTTTCTTA
      2810        2820        2830        2840        2850        2860        2870        2880        2890        2900
CTCTTGATCG TGAATTTTAA TGCTACGATT GTTCGCCCAC GCTTGATAGG TTCAACATCA ACAAGCAGAT CGGATTTAGC ATTAATTTCA TTTATGGATG
      2910        2920        2930        2940        2950        2960        2970        2980        2990        3000
GAGTTAATAC TCGCTTTTTA AAATCCTTAA ACAGTGGGTA CTTATCAGAG ATACTTAACC AACTTTTAAT ATCTTCTACG CTTGTTTGTC GCCAACCTGT
      3010        3020        3030        3040        3050        3060        3070        3080        3090        3100
ATCACGATAT TGAGAACACA ATTCATAAAG GCGAATAGCG TGCGTACTAC CCAAAGCCCC AATATTGATC AATTTATATT TTGTGTAGTT ATCGTGTAAT
      3110        3120        3130        3140        3150        3160        3170        3180        3190        3200
TCAGAAATGT AAGGAATTAG CTCATCGTGG AACTCGATAT AAAATCGCCC TTCTTTTTTA AAATAGGAAC GCTTATGAAT TAAAGCTACT TCTGTTAATT
      3210        3220        3230        3240        3250        3260        3270        3280        3290        3300
CGTGTTCGTT ATCAACCAGT GTAACCCAAC GCTTTGAGAT TTTTAAAACG GCATTTCTAA CTTGTGTGTA AGCTATATCA GGATTTACAT CGGGGAAGCT
                                                      US (80 %)
      3310        3320        3330        3340        3350        3360        3370        3380        3390        3400
TTTACAAAAA TCTGCCACCG TGAAATCAAA TCCACGCTTA GACGGATTTT TAGGATTAAA AACCCCCAAA GTTAAAGCCA GAATCCGCAT TTCATCAAGT
                                          US (80 %)
      3410        3420        3430        3440        3450        3460        3470        3480        3490        3500
GTCATTGAAT AGCTGGCTTG TACAAAATTG TTAGCTTTAT GGACTGTTAA ATCATTTGTC ATATCATCAA GGTGGACATA AAATAAAGAT TGTCCCATTA
      3510        3520        3530        3540        3550        3560        3570        3580        3590        3600
TAACCATACA GTTAAATGGT GGTCAATAAA AAACAAAGAC CACTATAACA ATAAATTTGT CCACCTATAA CAATAAATTT GTCCACCTAT AACAATAAAT
      3610        3620        3630        3640        3650        3660        3670        3680        3690        3700
TTGTCCACCT ATAAATCTCG CAAGCCTTGT GTAACAAGGG GAGCCAGAGC CTACAAACAA GAATACAAAC AAGAATACAA AAAAATAGAG CCTAAAGGCT
      3710        3720        3730        3740        3750        3760        3770        3780        3790        3800
CTTTTTGGGG CTTTCAGCCC TAATTTTTTC TTTTTTTCAG GATTTAAAAT TACAAAACCC TTACAGAGCA AGTAAACTTG TTTGCTTGTT CTGCAAGGGT
      DR-30B
      3810        3820        3830        3840        3850        3860        3870        3880        3890        3900
TCAGCAACCG TAGCCGTCAG GCGTAGGGCG GTAGCCTATA AAAGCCATTT AATTTTATCT TTAAACTTCC TTTTAAATGC TTTGAGTGGG TGTCTTTTAT
DR-30B
      3910        3920        3930        3940        3950        3960        3970        3980        3990        4000
CGTACTCATC AATCCTTTTT TGCATTCTTT CGTTTGCTTT GTGATCGGCA AATTTTGAAT AAGATTTTTC CATCTCATCT AACATTCTAT CAATCCGTTT
DR-30B                                                                                                IHF (92%, Compl)
      4010        4020        4030        4040        4050        4060        4070        4080        4090        4100
TTTATGTTGC CATTTCAGGT AAACATAAAC ACTTATAGCA ATTAAAGACA ATATCAATAC ATTGTAAAAA ATGATTGTTA CAATTTCGCT CACAGTTATT
DR-30B                                                      IHF     IHF (92 %)
      4110        4120        4130        4140        4150        4160        4170        4180        4190        4200
TTTTACCTTT TTCAATTTCT TCATTGATAA ATGCACTCAA TTCATCAAAT TTCTTGTCAT CATTGATAAA TTTACGCAAC TTAGGGAAGT TTCTATCTAC
DR-30B
      4210        4220        4230        4240        4250        4260        4270        4280        4290        4300
ATCTAAAAGA GGGTTATTTA TTATTTCATT TAGCCAAAAA GCCCCTAATA AAACCTTGTA ATGCGTAGCT TTCTTACGCT TTTCTGCTTG TTCTTTTGAC
DR-30B
      4310        4320        4330        4340        4350        4360        4370        4380        4390        4400
TTAATCGCAC GAATTTTCGC TTTGATTTCG TCCTGCTTGC GTTGTAAATC TGCTTGTTGC TGTTCCAATC TTGTAAGTTT TTCGCTTGCC ATACTAGCCC
      4410        4420        4430        4440        4450        4460        4470        4480        4490        4500
CTTTATATAG TTAGAAATTA TCGTTATTTT ATTCAGTAGG TGCTAGGCTT GCAAGTGTTC TGTTCATTAC GTTAAAATAA CGTAATGCCC ACTTATCAGT
      4510        4520        4530        4540        4550        4560        4570        4580        4590        4600
TTCTCTTCGA GAAACTGGTG GGCAAGCGTA CCGCTTGACC GTTTCGCAAT ACTCAACACT ATGGCAATCT ATCATTTAAA CGTTCGCTAT TGCAGTAAAA
      4610        4620        4630        4640        4650        4660        4670        4680        4690        4700
GCAAAGGGCA ATCAGCTCAA GCCAAAAACG ACTACATCAA CCGCAATGAT AAATATTCAA AGCGGTTAGA TGATTTACAG TTTTCAGGCT ATGGTAATAT
      4710        4720        4730        4740        4750        4760        4770        4780        4790        4800
GCCAAAATTT GCCGAAGATA ATCCGCAAGA ATTTTGGCGA TTGTCAGATA TTTACGAGCG AGCTAATGCC CGAGTTTGTA CTGAAATTGA ATTTGCTTTA
      4810        4820        4830        4840        4850        4860        4870        4880        4890        4900
CCTAGAGAAT TAACCCTAGA ACAACAGCAA AAATTAGTAA GTTCGTTTAT AGAAAATACG GTTGATAGCG GTAGCAATAA ACTACCCTAC TCTTTCGCTA
                                                      US (80 %)
      4910        4920        4930        4940        4950        4960        4970        4980        4990        5000
TCCATACCGA TAAAAATAAT CATAATCCCC ATTGTCATTT GATATTTTCA GAACGCCAAC TTGACGGCAT AGACCGTACA GCCGAGCAGT TTTTTAAACG
                                                                  US (80 %)
      5010        5020        5030        5040        5050        5060        5070        5080        5090        5100
TGCTAATACT AAATCCCCAG AAAAGGGCGG AGCGATGAAA ACGGCAGATT TTCGAGATCG TGAGTTTATC CAATCTGTCC GAAAAACGTG GAGAGAGCAA
      5110        5120        5130        5140        5150        5160        5170        5180        5190        5200
GCTAATCAAG CCTTAGAGCA ATACGGATAT GCCGCACGAA TTGACGAACG TAGCTACAAG GAACAAGGCA TAGAGCAAGC CCCAAGAGCA AGAATTGACA
      5210        5220        5230        5240        5250        5260        5270        5280        5290        5300
GGGTAACGTG GCAAGAATTG AACCGATTAG AGCAAGAAGA ACGCCAAATC GTGCAAGAGC TTGCACTTAA AGGACAAGAA ATTAACAAAG AAAAATCCTA
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      5310        5320        5330        5340        5350        5360        5370        5380        5390        5400
CTTGCAGAAA ATCGAAGAAA AACAGGCTCA AGGAATGGGC AAATATGAAT CCAAATTCGC AGCTGCGTTT TCTAAATTAT CGGAAAGTGC CCTAAAACAC
      5410        5420        5430        5440        5450        5460        5470        5480        5490        5500
GATTTAAGCA ACGAAAAAGA AAAAGACAGT AAAATACACA CTCAAGAAGA AAAAGTGCCT CAAAATCGCA TTCAGGGGCT TTCTCAAGCA GATTTTGATC
      5510        5520        5530        5540        5550        5560        5570        5580        5590        5600
AGTTTTTAAT TGATGAATGG CTACCTCAAA TAGAAAAATA CGTTAAAGCC CAAGAAAAGC GGGACGGAAT GGAAGTAGAG ATCACGCAAT ACGACAAGGA
      5610        5620        5630        5640        5650        5660        5670        5680        5690        5700
TTTACAGCGT ATTCAGGGAG ACTATAACAA GCTCACAGAT AAAAATCAGG GTTTTCTCGG TTTATGGGAA ACTAAAGAGC AAAAAGCAAA GAAAAAAGAG
      5710        5720        5730        5740        5750        5760        5770        5780        5790        5800
CTTGAAGATG AATACAAACA TACAGCAGAG CAACGGAACG CTAAAAGCCA AGAATTAGCC GAGTATAGCC AAAAAATAAA AGCATACGAA CAGAAAACGC
      5810        5820        5830        5840        5850        5860        5870        5880        5890        5900
TAGAGCCAAT CAACGAGAAG ATTGCCAAAT ATCAAGCTGA CAACCCTGAA ATAAAAATGC GGAGCTTAGG ATTTGTGAAA AAAATTAAGG CTCAAGGGGC
      5910        5920        5930        5940        5950        5960        5970        5980        5990        6000
ATATAAAGCG GCTCAAGAGC GAATGGAGCG AGAAAAACAG CACCAACAGG AAAAACAACA GAGACATTTA GAGCGAGAGA GTGGTTTGAG CTTGTAGCTA
      6010        6020        6030        6040        6050        6060        6070        6080        6090        6100
ACGCCCTACG CCTACGGCTT CGGTTGTTCA ACCCTTAAAG AACTCGCAAC AAGTTGCAAA TTCTTTAAGG GTTCGCAATA AAAACAACCG CTAAACATTT
      6110        6120        6130        6140        6150        6160        6170        6180        6190        6200
CTGCCCAGCG GTTGAAAATT TACCTATTCA CCATTACAAT GATCAAGCAG GAAATTTTTT TGATTGCCGT AAATGTCCGT ATATCTAGTT GAGGCACAAC
      US (80 %)
      6210        6220        6230        6240        6250        6260        6270        6280        6290        6300
CCGCCAAAGT CATTGCCCCA ACCAGAACGG CGATAAACCG TATATTTACC GATAAGGCAT CCGGCAGTTC AACAGACCGG GAAGGGCTGG ATTTGCTGAG
      6310        6320        6330        6340        6350        6360        6370        6380        6390        6400
GATGAAGGTG GAGGAAGGTG ATGTCATTCT GGTTAAGAAG CTCGACCGTC TTGGCCGCGA CACTGCCGAT ATGATCCAAC TGATAAAGGA ATTTGACGCT
      6410        6420        6430        6440        6450        6460        6470        6480        6490        6500
CAGGGCGTGG CAGTCCGGTT CATTGATGAC GGGATCAGTA CCGACGGTGA TATGGGGCAA ATGGTGGTCA CCATCCTGTC GGCTGTGGCA CAGGCTGAAC
      6510        6520        6530        6540        6550        6560        6570        6580        6590        6600
GCCGGAGGAT CCTAGAGCGC ACGAATGAGG GCCGACAGGA AGCAAAGCTG AAAGGAATCA AATTTGGCCG CAGGCGTACC GTGGACAGGA ACGTCGTGCT
      6610        6620        6630        6640        6650        6660        6670        6680        6690        6700
GACGCTTCAT CAGAAGGGCA CTGGTGCAAC GGAAATTGCT CATCAGCTCA GTATTGCCCG CTCCACGGTT TATAAAATTC TTGAAGACGA AAGGGCCTCG
      6710        6720        6730        6740        6750        6760        6770        6780        6790        6800
TGATACGCCT ATTTTTATAG GTTAATGTCA TGATAATAAT GGTTTCTTAG ACGTCAGGTG GCACTTTTCG GGGAAATGTG CGCGGAACCC CTATTTGTTT
      6810        6820        6830        6840        6850        6860        6870        6880        6890        6900
ATTTTTCTAA ATACATTCAA ATATGTATCC GCTCATGAGA CAATAACCCT GATAAATGCT TCAATAATAT TGAAAAAGGA AGAGTATGAG TATTCAACAT
      6910        6920        6930        6940        6950        6960        6970        6980        6990        7000
TTCCGTGTCG CCCTTATTCC CTTTTTTGCG GCATTTTGCC TTCCTGTTTT TGCTCACCCA GAAACGCTGG TGAAAGTAAA AGATGCTGAA GATCAGTTGG
      7010        7020        7030        7040        7050        7060        7070        7080        7090        7100
GTGCACGAGT GGGTTACATC GAACTGGATC TCAACAGCGG TAAGATCCTT GAGAGTTTTC GCCCCGAAGA ACGTTTTCCA ATGATGAGCA CTTTTAAAGT
      7110        7120        7130        7140        7150        7160        7170        7180        7190        7200
TCTGCTATGT GGCGCGGTAT TATCCCGTGT TGACGCCGGG CAAGAGCAAC TCGGTCGCCG CATACACTAT TCTCAGAATG ACTTGGTTGA GTACTCACCA
      7210        7220        7230        7240        7250        7260        7270        7280        7290        7300
GTCACAGAAA AGCATCTTAC GGATGGCATG ACAGTAAGAG AATTATGCAG TGCTGCCATA ACCATGAGTG ATAACACTGC GGCCAACTTA CTTCTGACAA
      7310        7320        7330        7340        7350        7360        7370        7380        7390        7400
CGATCGGAGG ACCGAAGGAG CTAACCGCTT TTTTGCACAA CATGGGGGAT CATGTAACTC GCCTTGATCG TTGGGAACCG GAGCTGAATG AAGCCATACC
      7410        7420   7426
AAACGACGAG CGTGACACCA CGATGC-3'
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Table 7. Location of restriction endonuclease sites on pJD4.

Number	Restriction endonuclease	Number of sites	Coordinate(s) of recognition site
1	*Aat*II	1	6750
2	*Acy*I	2	6750, 7132
3	*Afl*III	1	1061
4	*Aha*II	2	6750, 7132
5	*Aha*III	18	359, 378, 677, 968, 1627, 1815, 2031, 2043, 2534, 2917, 3157, 3242, 3743, 3860, 3872, 4575, 4993, 7093
6	*Alu*I	29	56, 650, 937, 1172, 1522, 3119, 3184, 3271, 3297, 3411, 3433, 4267, 4614, 4761, 5100, 5152, 5258, 5361, 5630, 5699, 5835, 5863, 5989, 5996, 6339, 6546, 6645, 7319, 7382
7	*Alw*I	11	385, 398, 1499, 1500, 6373, 6432, 6507, 6508, 7026, 7044, 7347
8	*Apa*LI	1	7001
9	*Ase*I	2	71, 2881
10	*Asu*II	1	1182
11	*Ava*I	1	4769
12	*Ava*II	2	105, 7309
13	*Bam*HI	2	1499, 6507
14	*Ban*I	1	294
15	*Bbv*I	5	3, 192, 5359, 5362, 7252
16	*Bcl*I	3	3066, 5496, 6140
17	*Bgl*I	1	123
18	*Bin*I	11	385, 398, 1499, 1500, 6373, 6432, 6507, 6508, 7026, 7044, 7347
19	*Bsm*I	3	2093, 3922, 5468

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

Number	Restriction endonuclease	Number of sites	Coordinate(s) of recognition site
20	*Bsm*AI	6	176, 557, 2441, 5618, 5961, 6837
21	*Bsp*1286	4	5587, 6616, 7001, 7086
22	*Bsp*HI	3	415, 6728, 6833
23	*Bsp*MI	1	1049
24	*Bsr*I	11	36, 79, 197, 739, 905, 1809, 3216, 4514, 6620, 7023, 7198
25	*Bst*EII	1	6466
26	*Dde*I	14	286, 452, 847, 1039, 2351, 3337, 4180, 5112, 5865, 6296, 6399, 6647, 6746, 7172
27	*Drd*I	1	6584
28	*Eae*I	4	907, 6352, 6565, 7280
29	*Ear*I	2	4503, 6879
30	*Eco*0109	1	6692
31	*Fnu*4H1	15	3, 192, 521, 1400, 2405, 5131, 5359, 5362, 5908, 6354, 6567, 6928, 7157, 7252, 7279
32	*Fnu*DII	4	180, 6356, 6781, 7113.
33	*Fok*I	7	82, 263, 2896, 6258, 6300, 6472, 7221
34	*Gdi*II	4	907, 6352, 6565, 7280
35	*Hae*III	8	122, 202, 908, 6353, 6530, 6566, 6694, 7281
36	*Hga*I	4	456, 6395, 6601, 7132
37	*Hgi*AI	2	7001, 7086
38	*Hgi*CI	1	294
39	*Hha*I	5	23, 116, 6517, 6780, 7112
40	*Hin*cII	1	7129

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

Number	Restriction endonuclease	Number of sites	Coordinate(s) of restriction sites
41	*Hin*dIII	1	3236
42	*Hin*fI	4	248, 3381, 5347, 6555
43	*Hpa*II	9	62, 129, 163, 6261, 6277, 6415, 6502, 7136, 7378
44	*Hph*I	9	165, 392, 6128, 6317, 6446, 6468, 6954, 6969, 7195
45	*Mbo*II	24	395, 588, 591, 755, 791, 923, 1361, 2289, 2472, 2612, 2648, 2972, 4118, 4504, 4714, 5236, 5314, 5446, 5704, 5817, 6683, 6879, 6988, 7066
46	*Mnl*I	20	87, 217, 298, 498, 659, 1008, 1302, 1325, 2380, 2516, 4209, 5458, 5524, 6191, 6298, 6311, 6505, 6527, 6696, 7307
47	*Mse*I	65	33, 72, 307, 360, 374, 379, 431, 546, 605, 678, 804, 969, 1034, 1071, 1079, 1247, 1370, 1518, 1628, 1684, 1816, 1915, 2020, 2032, 2044, 2385, 2430, 2486, 2535, 2817, 2882, 2904, 2918, 2927, 2955, 2966, 3158, 3180, 3195, 3243, 3356, 3372, 3447, 3512, 3744, 3849, 3861, 3873, 4042, 4301, 4472, 4576, 4810, 4994, 5267, 5282, 5404, 5506, 5543, 5885, 6035, 6065, 6333, 6722, 7094
48	*Mst*I	1	22
49	*Nci*I	3	61, 6277, 7136
50	*Nla*III	7	416, 6729, 6834, 7227, 7263, 7341, 7351
51	*Nla*IV	9	159, 200, 294, 1052, 1499, 3640, 6507, 6784, 7374
52	*Nsp*BII	3	5360, 6106, 7035

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

Number	Restriction endonuclease	Number of sites	Coordinate(s) of restriction sites
53	*Ple*I	1	248
54	*Pst*I	1	1
55	*Pvu*I	1	7301
56	*Pvu*II	1	5360
57	*Rru*I	1	7190
58	*Rsa*I	15	829, 983, 1194, 2073, 2740, 2938, 3044, 3420, 3902, 4528, 4778, 4976, 6438, 6576, 7191
59	*Sau*IIIA	26	281, 386, 398, 707, 789, 1500, 2114, 2564, 2634, 2806, 2868, 3067, 3943, 5056, 5497, 5580, 6141, 6373, 6433, 6508, 6991, 7027, 7044, 7302, 7348, 7366
60	*Sau*96	6	105, 122, 201, 6529, 6693, 7309
61	*Sca*I	1	7190
62	*Scr*FI	3	61, 6277, 7136
63	*Sdu*I	4	5387, 6616, 7001, 7086
64	*Sfa*NI	4	6257, 6982, 7212, 7422
65	*Spe*I	1	632
66	*Ssp*I	3	3061, 4652, 6866
67	*Taq*I	11	794, 863, 1183, 2518, 2777, 3134, 4507, 5052, 5312, 6342, 7019
68	*Tth*111II	2	1148, 5943
69	*Xba*I	1	1321
70	*Xho*II	6	385, 397, 1499, 6507, 7026, 7043
71	*Xmn*I	2	1107, 7069

*Eco*0109, *Hgi*CI, *Hin*CII, *Hin*dIII, *Mst*I, *Ple*I, *Pst*I, *Pvu*I, *Pvu*II, *Rru*I, *Sca*I, *Spe*I, and *Xba*I) had only one recognition site, nine restriction endonucleases (*Acy*I, *Aha*II, *Ase*I, *Ava*II, *Bam*HI, *Ear*I, *Hgi*AI, *Tth*111II, and *Xmn*I) had two, seven restriction endonucleases (*Bcl*I, *Bsm*I, *Bsp*HI, *Nci*I, *Nsp*BII, *Scr*FI, and *Ssp*I) had three, and eight restriction endonucleases (*Bsp*1286, *Eae*I, *Fnu*DII, *Gdi*II, *Hga*I, *Hin*fI, *Sdu*I, and *Sfa*NI) had four recognition sites. Twenty-six other restriction endonucleases (*Aha*II, *Alu*I, *Alw*I, *Bbv*I, *Bin*I, *Bsm*AI, *Bsr*I, *Dde*I, *Fnu*4H1, *Fok*I, *Hae*III, *Hha*I, *Hpa*II, *Hph*I, *Mbo*II, *Mnl*I, *Mse*I, *Nla*II, *Nla*IV, *Rsa*I, *Sau*IIIA, *Sau*96, *Taq*I and *Xho*II) each had $\geq$ five recognition sites (Table 7). The following restriction endonuclease sites were not present in pJD4: *Acc*I, *Afl*II, *Alw*NI, *Apa*I, *Ava*III, *Avr*II, *Bba*II, *Ban*II, *Bgl*II, *Bsa*AI, *Bsp*MII, *Bss*HII, *Bst*NI, *Bst*XI, *Cla*I, *Dra*III, *Eco*NI, *Eco*RI, *Eco*RV, *Hae*I, *Hae*II, *Hgi*JII, *Hpa*I, *Kpn*I, *Mlu*I, *Mst*II, *Nae*I, *Nar*I, *Nco*I, *Nde*I, *Nhe*I, *Not*I, *Nru*I, *Nsi*I, *Nsp*CI, *Pfl*MI, *Pml*I, *Ppu*MI, *Rsr*II, *Sac*I, *Sac*II, *Sal*I, *Sac*I, *Sfi*I, *Sma*I, *Sna*I, *Sna*BI, *Sph*I, *Stu*I, *Sty*I, *Tth*111I, *Xcm*I, *Xho*I, and *Xma*III.

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 *Pst*I 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 ≥12 recognition sites (*Aha*II, *Alu*I, *Dde*I, *Fnu*4HI, *Mbo*II, *Mnl*I, *Mse*I, *Rse*I, *Rsa*I, and *Sau*IIIA). ▬, region homologous to Tn*A*.

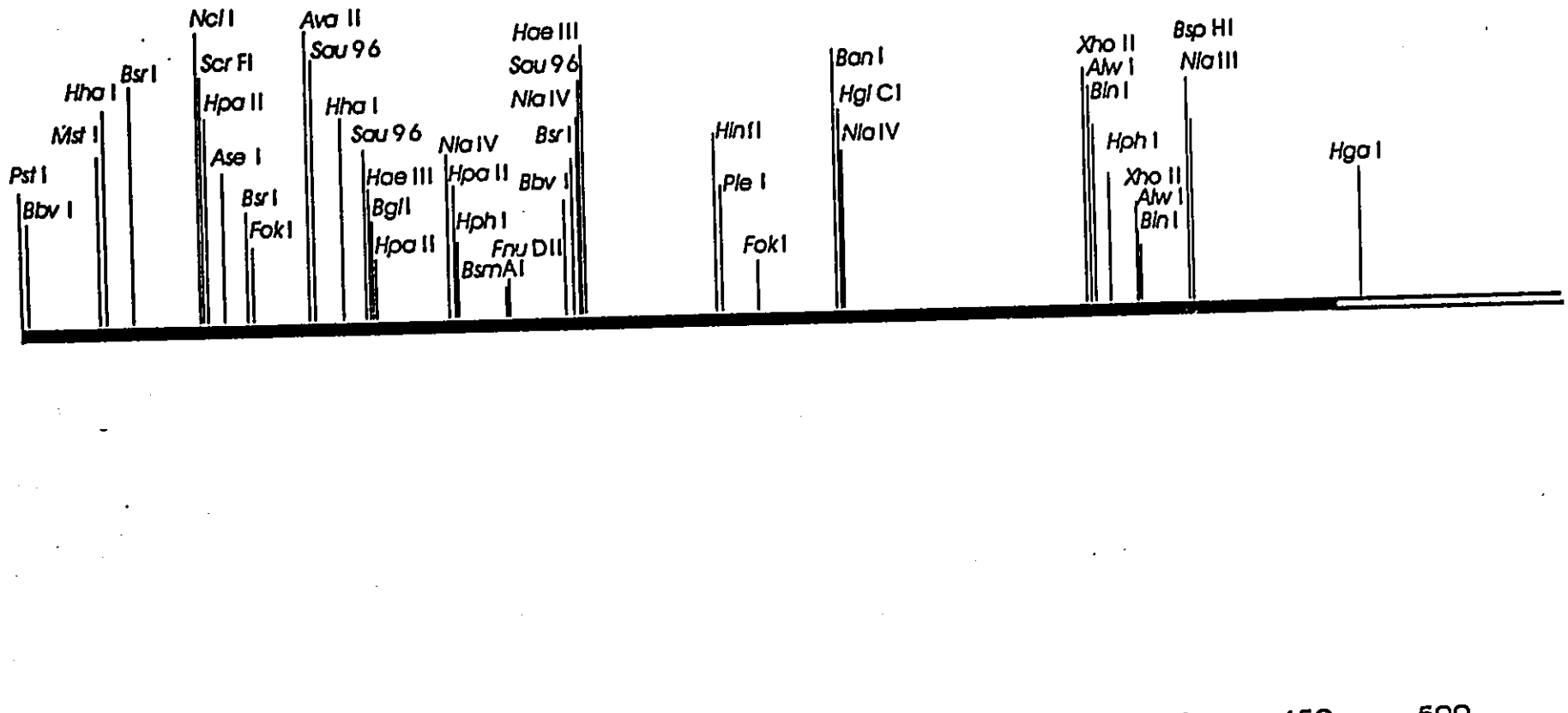


Figure 15a

JD4BIGx1

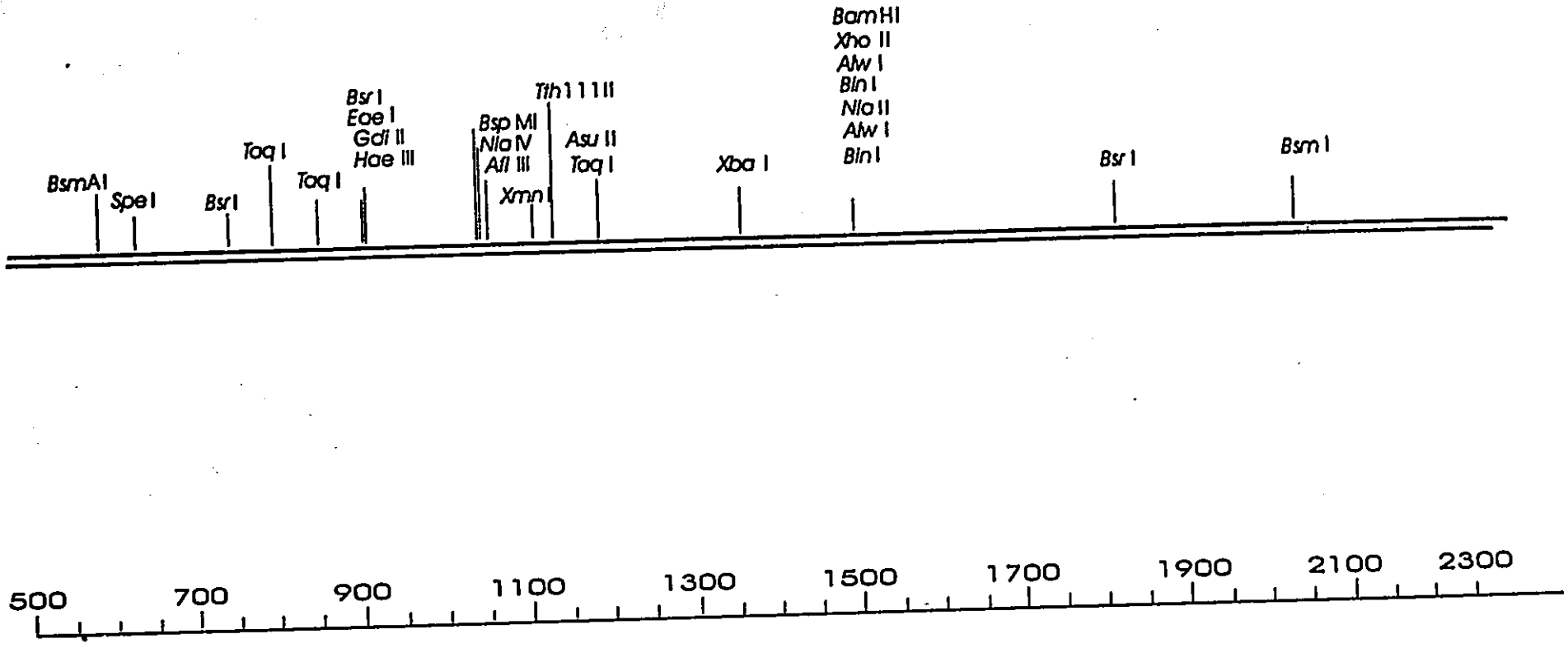


Figure 15b

JD4BIGx2

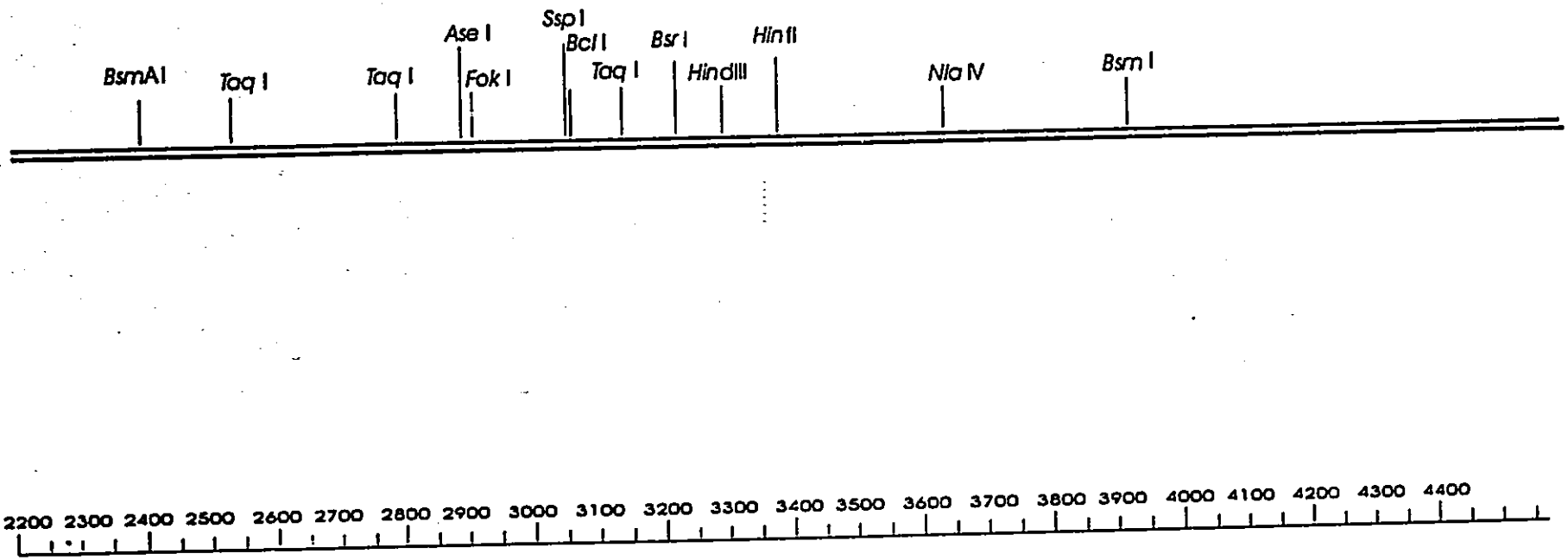


Figure 15c

JD4BIGx3

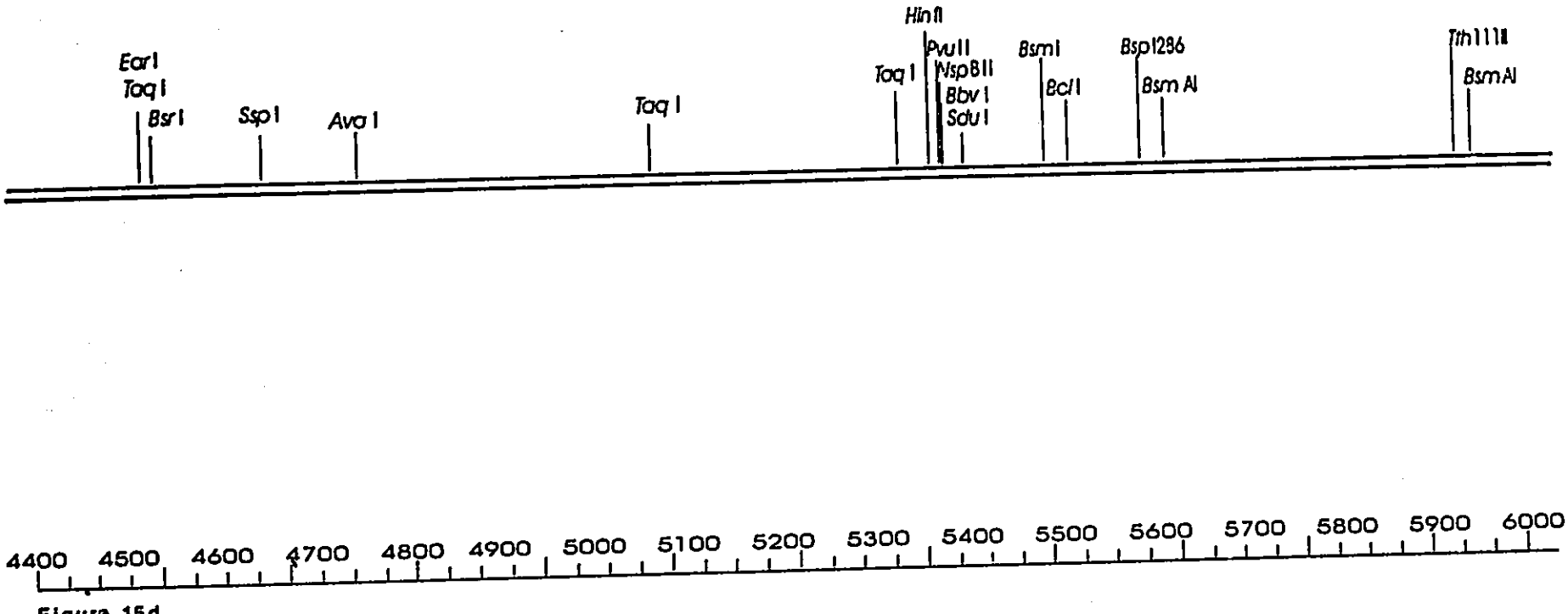


Figure 15d

JD48IGx4

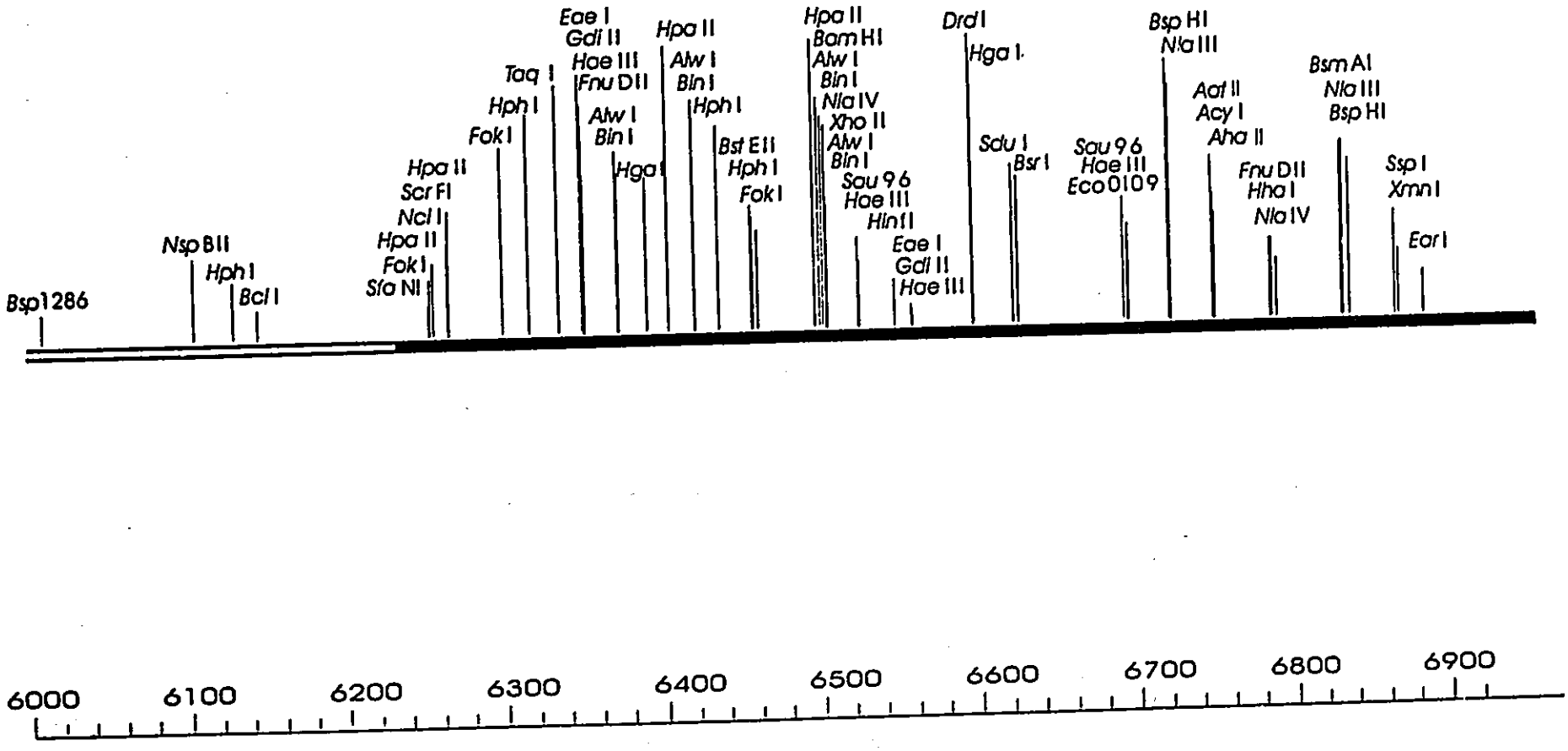
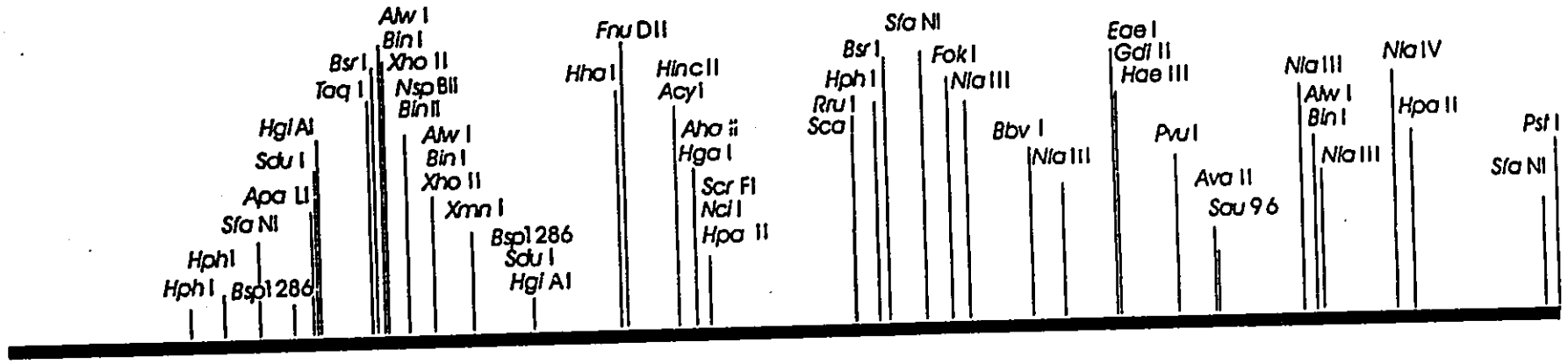


Figure 15e

JD4BIGx5



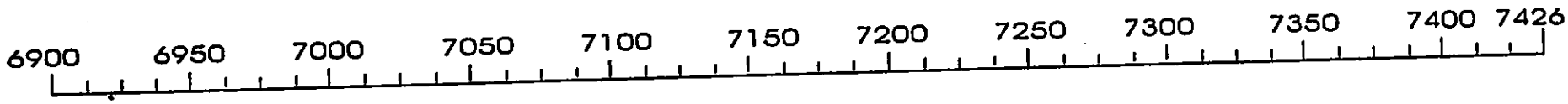


Figure 15f

JD48IGx6

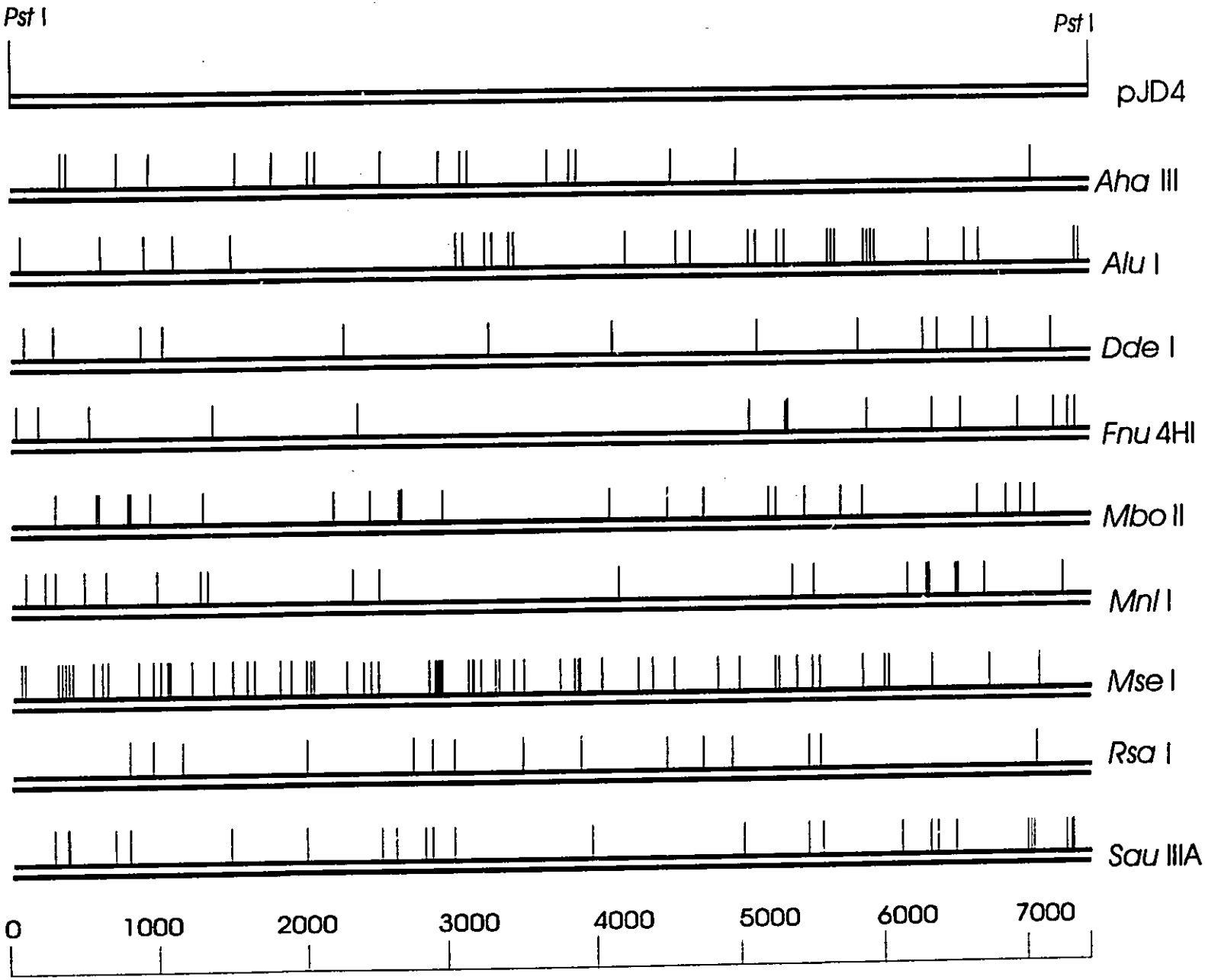

Pst I
Pst I
pJD4
Aha III
Alu I
Dde I
Fnu 4HI
Mbo II
Mnl I
Mse I
Rsa I
Sau IIIA
0
1000
2000
3000
4000
5000
6000
7000
Base pairs (bp)
JD6006

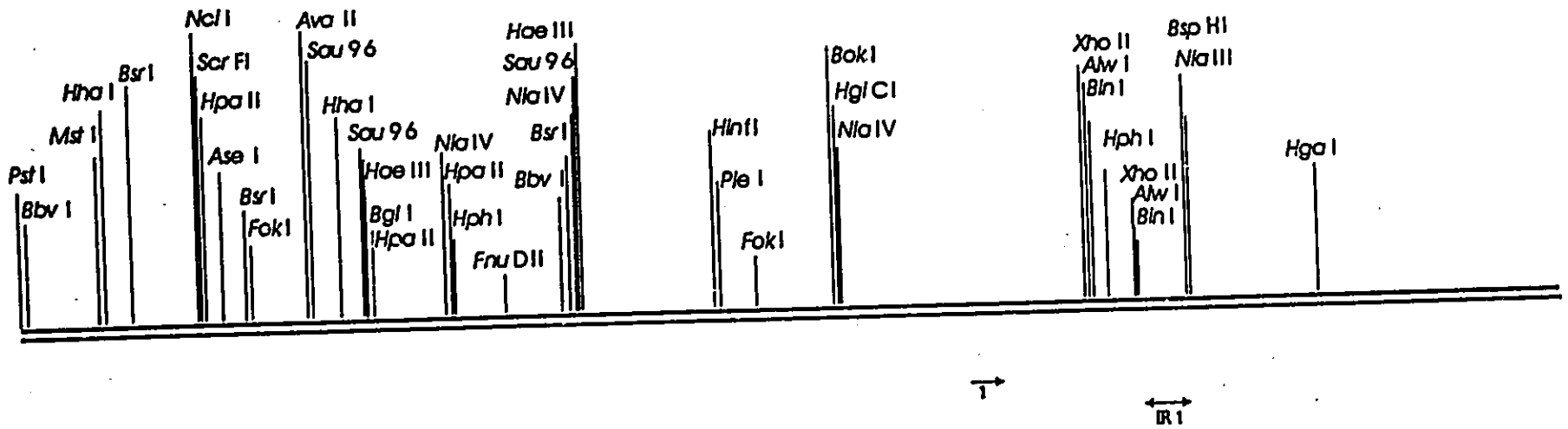
Figure 15g

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 ≥ 10-bp in length. The direct repeats (Table 8; Appendix 1) are ordered chronologically by coordinates, and called DR-1 to DR-62. Fourty-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 ($\geq$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.


Pst I
Bbv I
Mst I
Hha I
Bsr I
Ncl I
Scr FI
Hpa II
Ase I
Bsr I
Fok I
Ava II
Sau 96
Hha I
Sau 96
Bgl I
Hpa II
Hoe III
Nla IV
Hpa II
Hph I
Fnu DII
Hoe III
Sau 96
Nla IV
Bsr I
Bbv I
Hinf I
Ple I
Fok I
Bok I
Hgi CI
Nla IV
Xho II
AIw I
Bln I
Hph I
Xho II
AIw I
Bln I
Bsp HI
Nla III
Hga I
1
IR 1

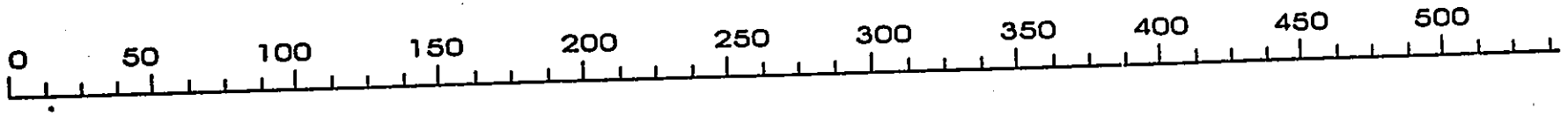

0
50
100
150
200
250
300
350
400
450
500

Figure 16a

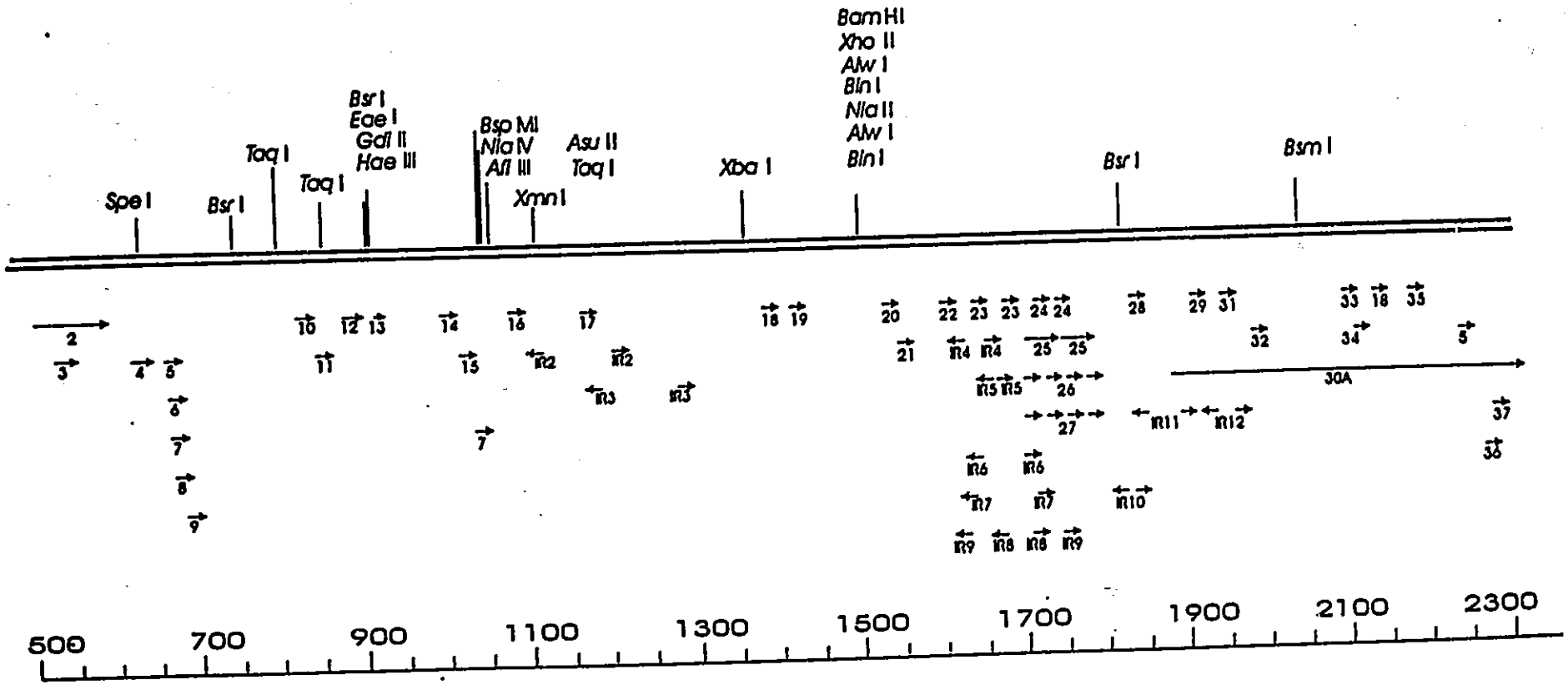

Spe I
Bsr I
Taq I
Taq I
Bsr I
Eae I
Gdi II
Hae III
Bsp MI
Nla IV
Afl III
Xmn I
Asu II
Taq I
Xba I
BamHI
Xho II
AIw I
Bln I
Nla III
AIw I
Bln I
Bsr I
Bsm I
30A
500
700
900
1100
1300
1500
1700
1900
2100
2300

Figure 16b

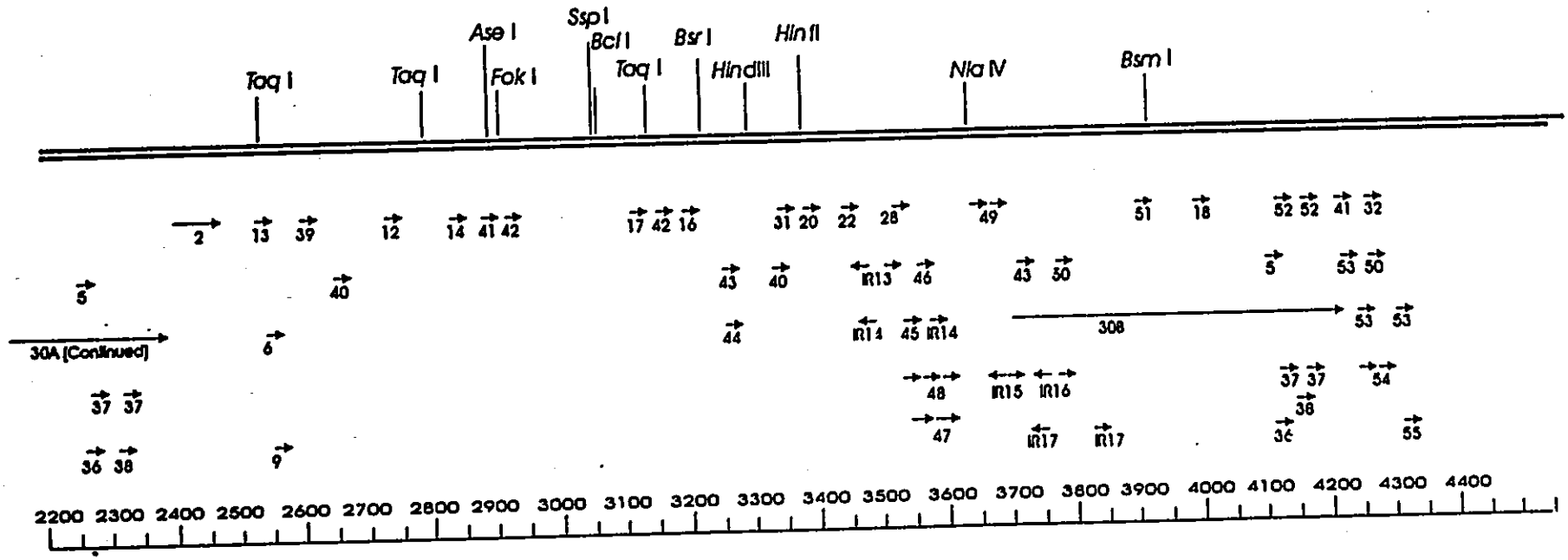

Taq I
Taq I
Ase I
Fok I
Ssp I
Bcl I
Taq I
Bsr I
Hind III
Hin fI
Nla IV
Bsm I
2
13
39
12
14
41
42
17
42
16
31
20
22
28
49
51
18
52
52
41
32
5
40
43
40
IR13
46
43
50
5
53
50
30A (Continued)
6
44
IR14
45
IR14
308
53
53
48
IR15
IR16
37
37
54
37
37
38
47
IR17
IR17
36
55
36
38
9
2200 2300 2400 2500 2600 2700 2800 2900 3000 3100 3200 3300 3400 3500 3600 3700 3800 3900 4000 4100 4200 4300 4400

Figure 16c

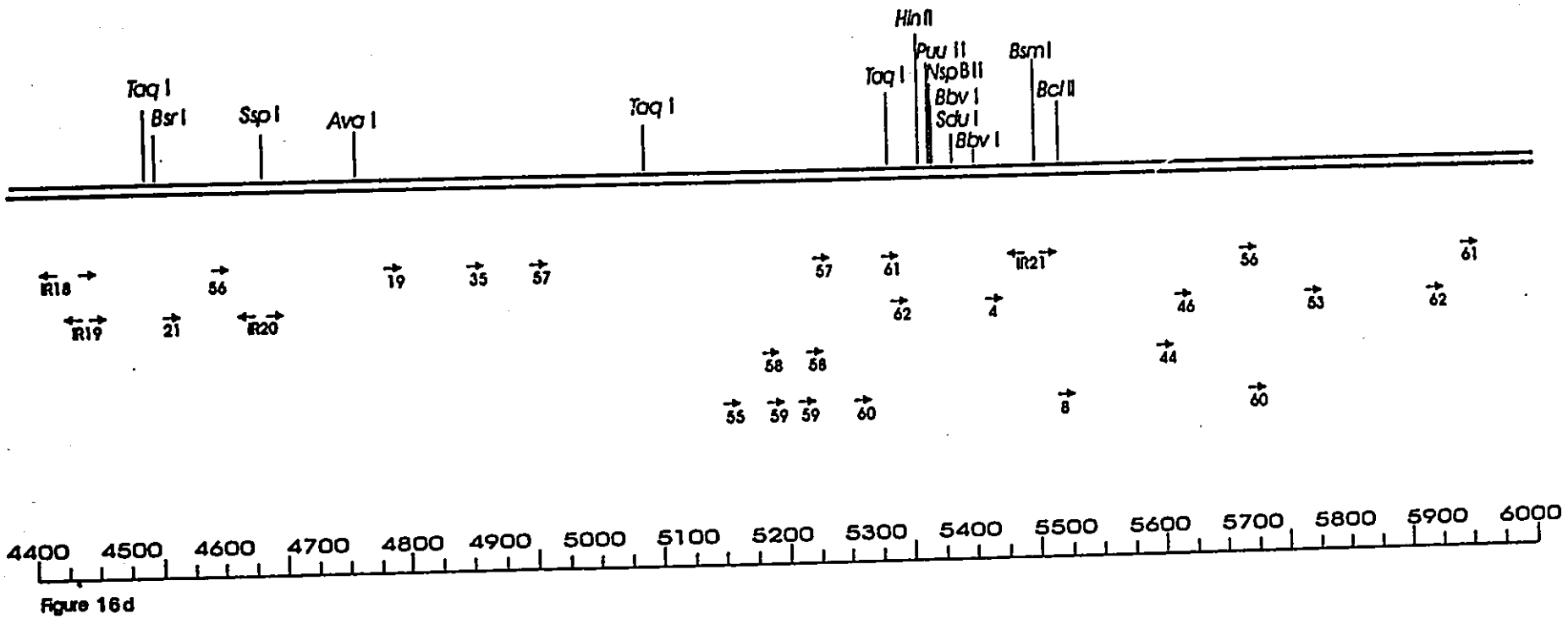

Taq I
Bsr I
Ssp I
Ava I
Taq I
Hin II
Taq I
Puu II
NspB II
Bbv I
Sdu I
Bbv I
Bsm I
Bcl I
R18
56
19
35
57
57
61
R21
56
61
R19
21
R20
62
4
46
53
62
44
58
58
55
59
59
60
8
60
4400
4500
4600
4700
4800
4900
5000
5100
5200
5300
5400
5500
5600
5700
5800
5900
6000

Figure 16d

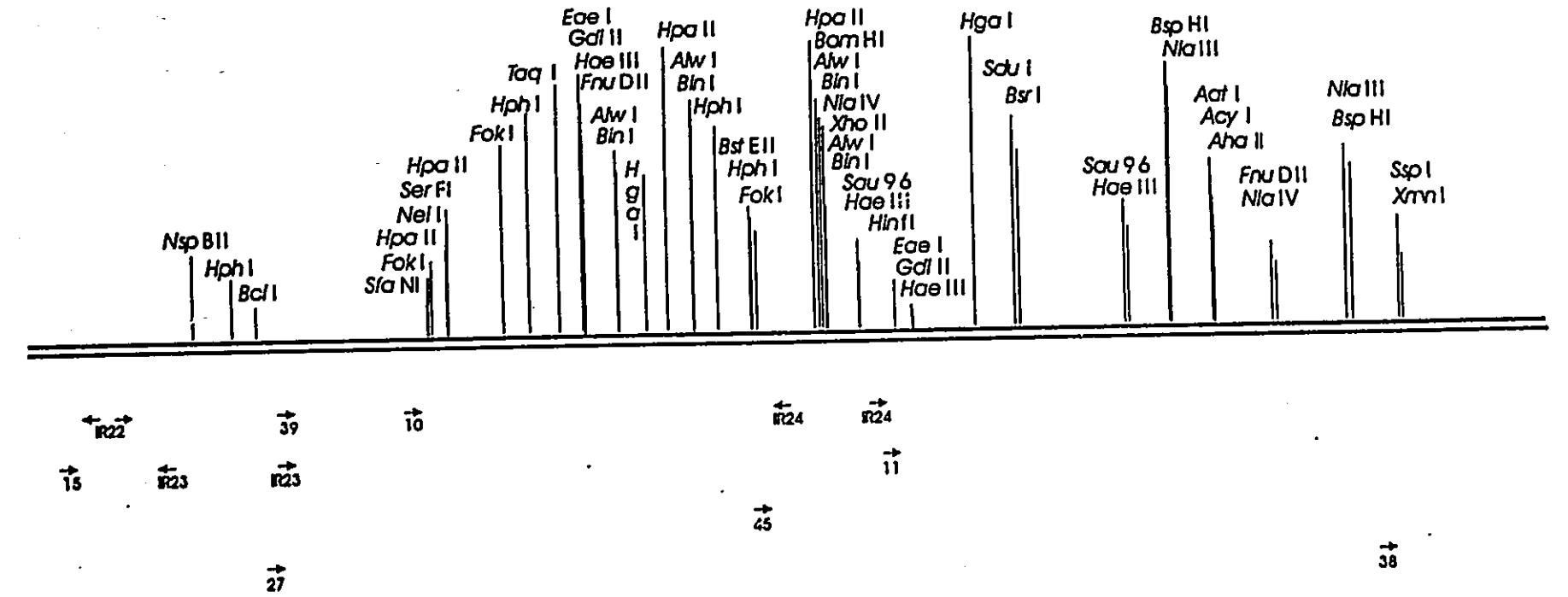
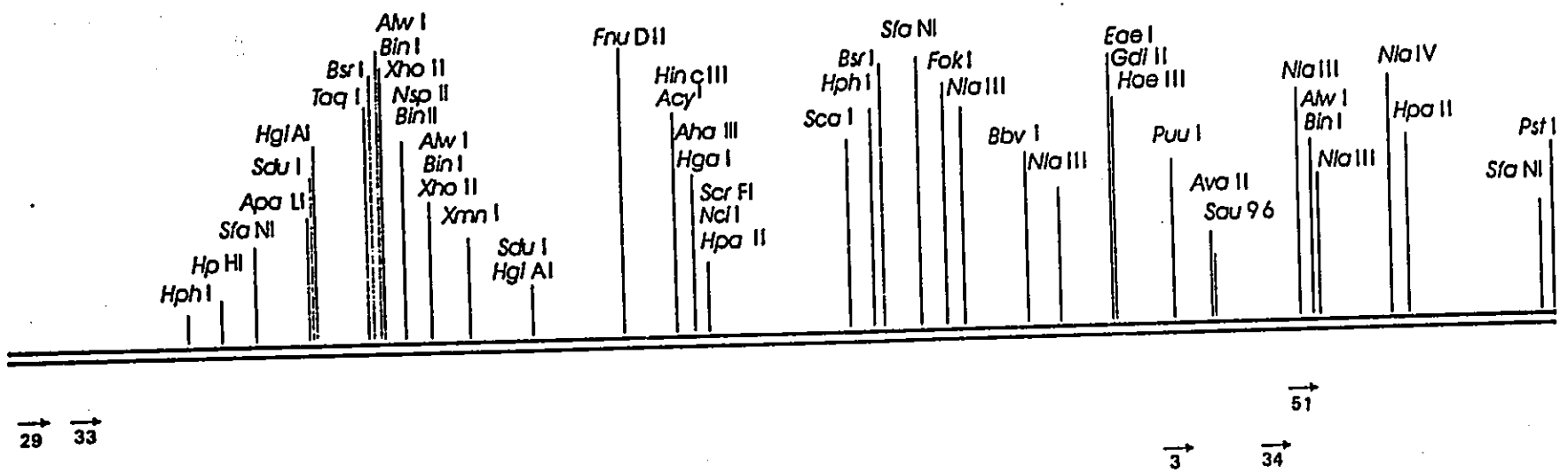


Figure 16e

JD4DNA5


Hph I
Hp HI
Sfa NI
Apa LI
Sdu I
HgiAI
Taq I
Bsr I
AIw I
Bin I
Xho II
Nsp II
Bin II
AIw I
Bin I
Xho II
Xmn I
Sdu I
HgiAI
Fnu DII
Hin cIII
Acy I
Aha III
Hga I
Scr FI
Ncl I
Hpa II
Sca I
Bsr I
Hph I
Sfa NI
Fok I
Nla III
Bbv I
Nla III
Eae I
Gdi II
Hae III
Puu I
Ava II
Sau 96
Nla III
AIw I
Bin I
Nla III
Nla IV
Hpa II
Pst I
Sfa NI
29
33
51
3
34

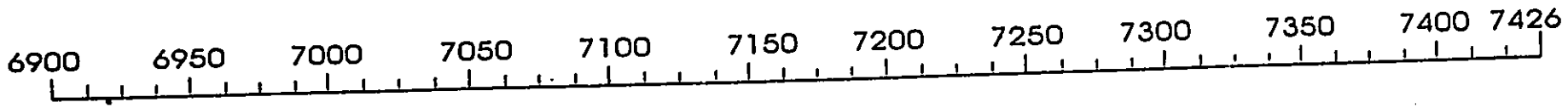

6900
6950
7000
7050
7100
7150
7200
7250
7300
7350
7400
7426

Figure 16 f

Table 8. Location of direct repeats on the primary sequence of pJD4[a].

Direct repeats	Coordinate of the first sequences	Coordinate of the other sequences	Number of nucleotides	Number of mismatched nucleotides
DR-1	312 to 325	759 to 772	14	0
DR-2	502 to 593	2386 to 2477	92	0
DR-3	527 to 539	2411 to 2423	23	0
		7321 to 7333	23	0
DR-4	608 to 617	5428 to 5437	10	0
DR-5	a. 665 to 674	b. 2279 to 2288	10	0
		c. 4108 to 4117	10	0
DR-6	677 to 690	2534 to 2547	14	1
DR-7	683 to 693	1019 to 1029	11	0
DR-8	687 to 696	5489 to 5498	10	0
DR-9	703 to 712	2560 to 2569	10	0
DR-10	822 to 831	6233 to 6242	10	0
DR-11	838 to 847	6635 to 6644	10	0
DR-12	875 to 904	2732 to 2761	30	2
DR-13	913 to 922	2522 to 2531	10	0
DR-14	986 to 1014	2834 to 2862	29	3
DR-15	1000 to 1009	6024 to 6033	10	0
DR-16	1074 to 1087	3175 to 3188	14	1
DR-17	1168 to 1178	3115 to 3125	11	0
DR-18	a. 1387 to 1399	b. 2163 to 2175	13	1
		c. 3992 to 4004	13	1
DR-19	1406 to 1416	4788 to 4798	11	0

a) DNA sequence of direct repeats found in Appendix 1.

Table 8 cont'd. Location of direct repeats on the primary sequence of pJD4[a].

DR	Coordinate of the first sequence	Coordinate of the other sequences	Number of nucleotide	Number of mismatched nucleotides
DR-20	1535 to 1544	3389 to 3398	10	0
DR-21	1542 to 1551	4547 to 4556	10	0
DR-22	1594 to 1609	3448 to 3463	15	1
DR-23	1629 to 1640	1665 to 1676	12	0
DR-24	1696 to 1716	1718 to 1738	21	0
DR-25	1701 to 1744	1745 to 1788	44	1
DR-26	a. 1701 to 1722	b. 1723 to 1744	22	1
		c. 1745 to 1766	22	1
		d. 1767 to 1788	22	1
	b. 1723 to 1744	c. 1745 to 1766	22	0
		d. 1767 to 1788	22	0
DR-27	a. 1701 to 1713	b. 1723 to 1735	13	0
		c. 1745 to 1757	13	0
		d. 1767 to 1779	13	0
		e. 6148 to 6160	13	1
DR-28	1836 to 1846	3527 to 3537	11	0
DR-29	1871 to 1881	6918 to 6928	10	0
DR-30	1881 to 2387	3708 to 4216	507-509	8
DR-31	1911 to 1921	3352 to 3362	11	0
DR-32	1955 to 1964	4285 to 4294	10	0

a) DNA sequence of direct repeats found in Appendix 1.

Table 8 cont'd. Location of direct repeats on the primary sequence of pJD4[a].

Name of DR	Coordinate of the first sequence	Coordinate of the other sequences	Number of nucleotides	Number of mismatched nucleotides
DR-33	2085 to 2094	6920 to 6929	10	0
DR-34	2086 to 2095	7328 to 7337	10	0
DR-35	2207 to 2216	4872 to 4881	10	0
DR-36	2279 to 2292	4108 to 4121	14	0
DR-37	a. 2292 to 2303	b. 2331 to 2342	12	0
		c. 4121 to 4232	12	0
		d. 4160 to 4171	12	0
DR-38	2296 to 2305	4125 to 4134	10	0
		6850 to 6859	10	0
DR-39	2603 to 2614	6165 to 6176	12	1
DR-40	2655 to 2665	3344 to 3354	11	0
DR-41	2885 to 2894	4223 to 4232	10	0
DR-42	2915 to 2924	3155 to 3164	10	0
DR-43	3278 to 3287	3737 to 3746	10	0
DR-44	3280 to 3292	5597 to 5609	13	1
DR-45	3514 to 3525	6459 to 6470	12	0
DR-46	3542 to 3551	5621 to 5630	10	0
DR-47	3543 to 3570	3587 to 3614	28	0
DR-48	a. 3543 to 3564	b. 3565 to 3586	22	0
		c. 3587 to 3608	22	0

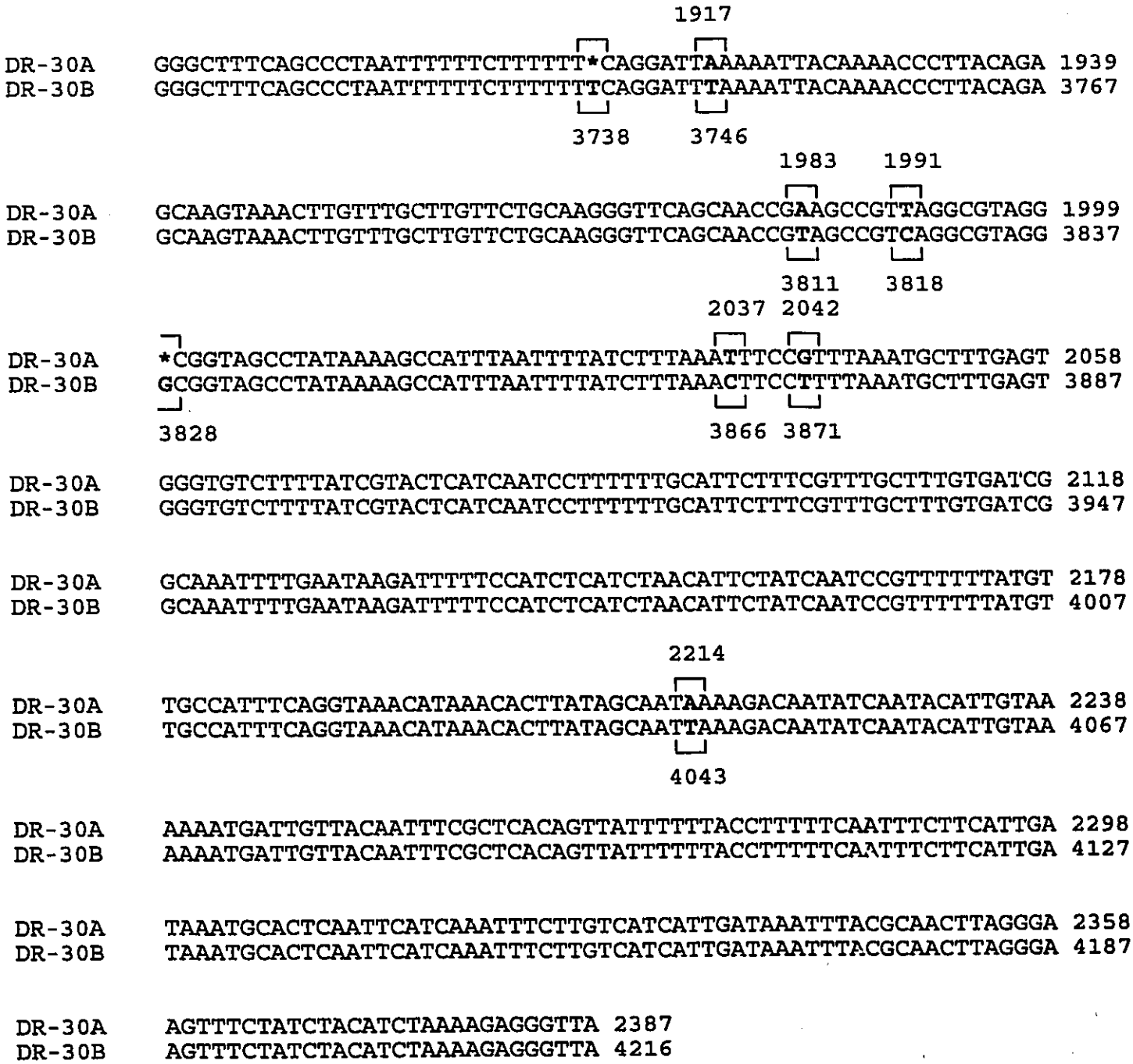
a) DNA sequence of direct repeats found in Appendix 1.

Table 8 cont'd. Location of direct repeats on the primary sequence of pJD4[a].

Name of DR	Coordinate of the first sequence	Coordinate of the other sequences	Number of nucleotides	Number of mismatched nucleotides
DR-49	3652 to 3663	3664 to 3675	12	0
DR-50	3783 to 3792	4285 to 4294	10	0
DR-51	3915 to 3924	7328 to 7337	10	0
DR-52	4121 to 4132	4160 to 4171	12	0
DR-53	4231 to 4240	5766 to 5775	10	0
DR-54	4249 to 4258	4283 to 4292	10	0
DR-55	4306 to 4315	5133 to 5142	10	0
DR-56	4597 to 4606	5682 to 5691	10	0
DR-57	4950 to 4959	5238 to 5247	10	0
DR-58	5185 to 5194	5229 to 5238	10	0
DR-59	5188 to 5198	5211 to 5221	11	0
DR-60	5286 to 5295	5687 to 5696	10	0
DR-61	5316 to 5325	5931 to 5940	10	0
DR-62	5324 to 5333	5888 to 5897	10	0

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. ⊓, indicates locations of nucleotide differences.


1917
DR-30A GGGCTTTCAGCCCTAATTTTTTCTTTTTT*CAGGATTAAAAATTACAAAACCCTTACAGA 1939
DR-30B GGGCTTTCAGCCCTAATTTTTTCTTTTTTTCAGGATTTAAAATTACAAAACCCTTACAGA 3767
3738 3746
1983 1991
DR-30A GCAAGTAAACTTGTTTGCTTGTTCTGCAAGGGTTCAGCAACCGAAGCCGTTAGGCGTAGG 1999
DR-30B GCAAGTAAACTTGTTTGCTTGTTCTGCAAGGGTTCAGCAACCGTAGCCGTCAGGCGTAGG 3837
3811 3818
2037 2042
DR-30A *CGGTAGCCTATAAAAGCCATTTAATTTTATCTTTAAATTTCCGTTTAAATGCTTTGAGT 2058
DR-30B GCGGTAGCCTATAAAAGCCATTTAATTTTATCTTTAAACTTCCTTTTAAATGCTTTGAGT 3887
3828 3866 3871
DR-30A GGGTGTCTTTTATCGTACTCATCAATCCTTTTTTGCATTCTTTCGTTTGCTTTGTGATCG 2118
DR-30B GGGTGTCTTTTATCGTACTCATCAATCCTTTTTTGCATTCTTTCGTTTGCTTTGTGATCG 3947
DR-30A GCAAATTTTGAATAAGATTTTTCCATCTCATCTAACATTCTATCAATCCGTTTTTTATGT 2178
DR-30B GCAAATTTTGAATAAGATTTTTCCATCTCATCTAACATTCTATCAATCCGTTTTTTATGT 4007
2214
DR-30A TGCCATTTCAGGTAAACATAAACACTTATAGCAATAAAAGACAATATCAATACATTGTAA 2238
DR-30B TGCCATTTCAGGTAAACATAAACACTTATAGCAATTAAAGACAATATCAATACATTGTAA 4067
4043
DR-30A AAAATGATTGTTACAATTTCGCTCACAGTTATTTTTTACCTTTTTCAATTTCTTCATTGA 2298
DR-30B AAAATGATTGTTACAATTTCGCTCACAGTTATTTTTTACCTTTTTCAATTTCTTCATTGA 4127
DR-30A TAAATGCACTCAATTCATCAAATTTCTTGTCATCATTGATAAATTTACGCAACTTAGGGA 2358
DR-30B TAAATGCACTCAATTCATCAAATTTCTTGTCATCATTGATAAATTTACGCAACTTAGGGA 4187
DR-30A AGTTTCTATCTACATCTAAAAGAGGGTTA 2387
DR-30B AGTTTCTATCTACATCTAAAAGAGGGTTA 4216

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 Tn*A* coding region, carries 24 inverted repeats which are $\geq$ 10bp, 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)[a] of the primary sequence of pJD4.

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

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 Tn*A*, was analyzed for potential open reading frames (ORFs) using the software Micro Genie^{R} 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.

ORF	Coordinates	Start/Stop codon	Size of amino acids	Molecular weight	Initiation score	Coding score
1	1192-1260	ATG/TAG	22	2.745	26	7
2	2894-3013	ATG/TGA	39	4.418	26	8
3	4561-5997	ATG/TAG	478	56.212	27	8
4	4699-5997	ATG/ATG	432	50.867	26	10
5	5035-5997	ATG/TAG	320	37.943	31	10
6	5335-5997	ATG/TAG	220	25.917	27	9
7	5569-5997	ATG/TAG	142	16.876	28	11
8	6886-318	ATG/TAA	286	31.519	35	8
9	7084-320	ATG/TAA	220	24.000	26	8
10	7264-320	ATG/TAA	160	17.377	26	8
11	4575-4429	ATG/TAA	48	5.653	29	8
12	4392-4096	ATG/TAA	98	11.615	33	9
13	4124-4002	ATG/TAA	40	4.757	28	7
14	3978-3817	ATG/TGA	53	6.199	32	7
15	3498-2479	ATG/TGA	339	39.453	28	9
16	2466-2374	ATG/TAG	30	3.483	32	6
17	2295-2173	ATG/TAA	40	4.801	28	7
18	2149-1973	ATG/TGA	58	6.655	32	6
19	2019-1933	ATG/TAA	28	3.106	24	7
20	1269-622	ATG/TAG	215	25.400	26	10

Table 10 cont'd. Summary of the most probable ORFs in pJD4 identified by the method of Kolaskar and Reddy.

ORF	Coordinates	Start/Stop codon	Size of amino acids	Molecular weight	Initiation score	Coding score
21	3886-4038	GTG/TAG	50	6.010	27	8
22	5208-5270	GTG/TAA	20	2.308	27	6
23	5251-5997	GTG/TAG	248	29.130	27	9
24	5455-5997	GTG/TAG	180	21.363	26	10
25	4559-4467	GTG/TAA	30	3.312	29	6
26	4310-4245	GTG/TAG	21	2.649	31	8
27	4093-3836	GTG/TAG	85	10.491	32	10
28	3608-3543	GTG/TAG	21	2.515	30	8
29	3586-3512	GTG/TAA	24	2.668	27	9
30	2264-2007	GTG/TAG	85	10.525	32	9

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.

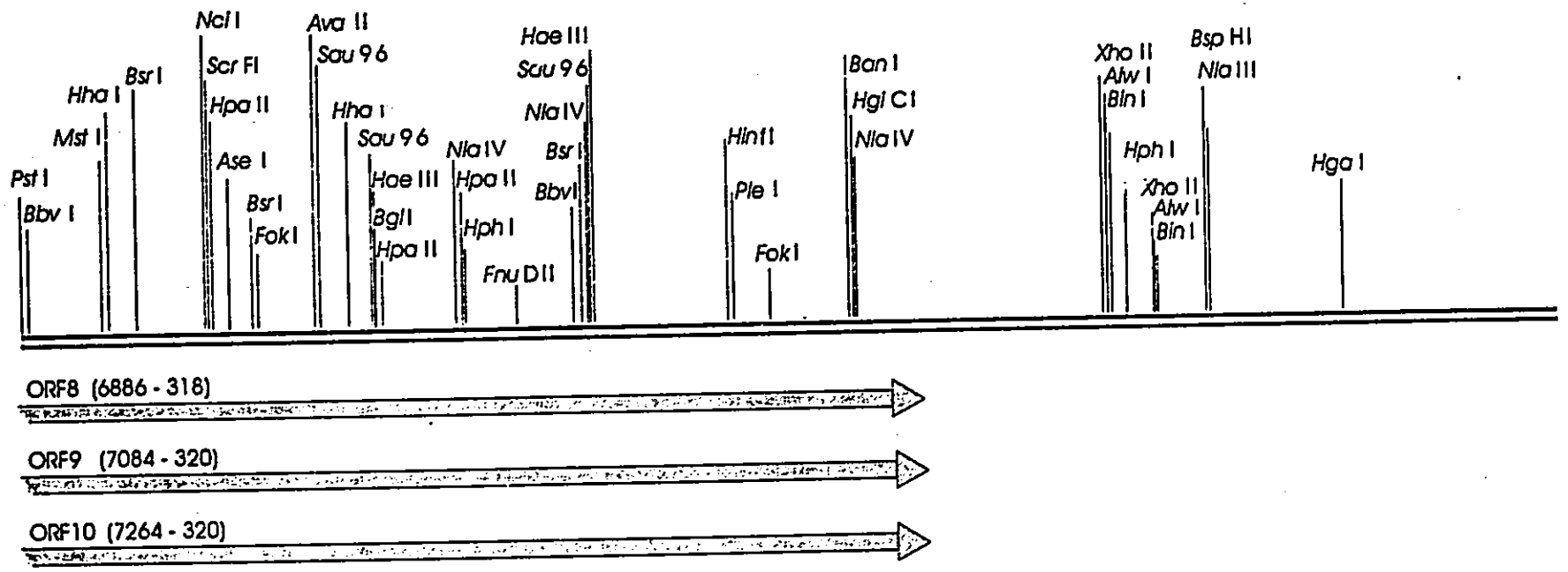
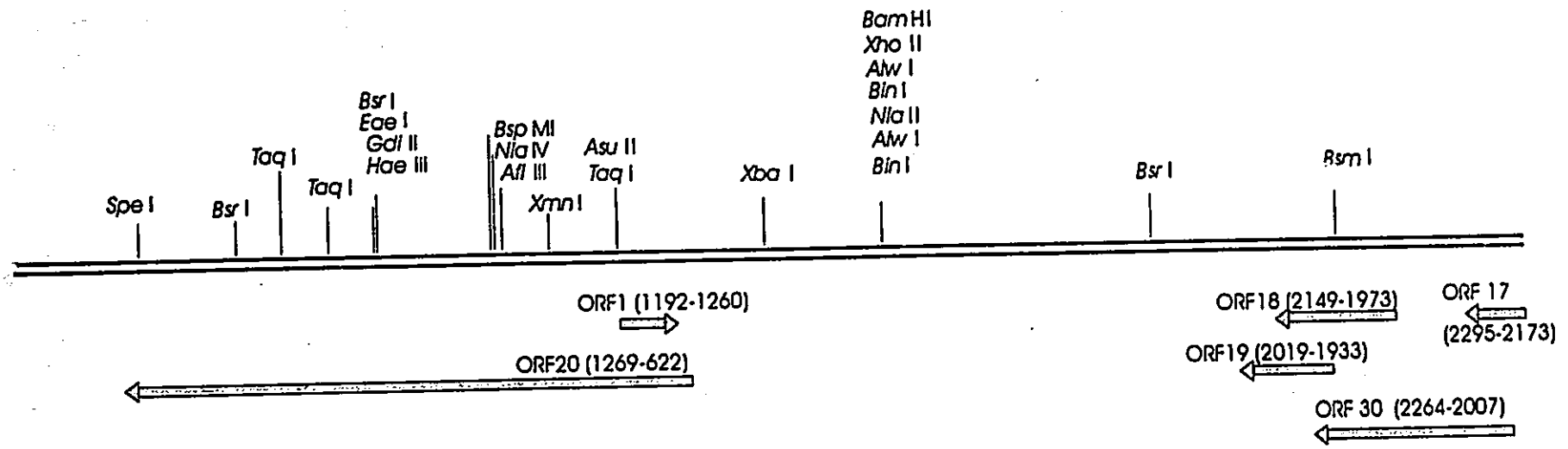


Figure 18a

JD48IG51



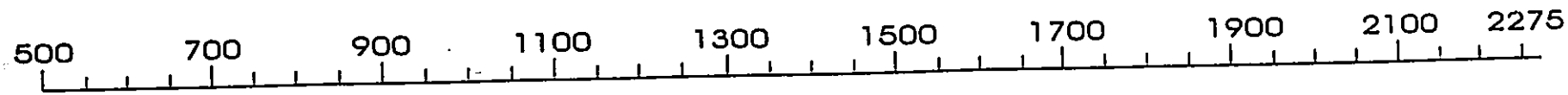
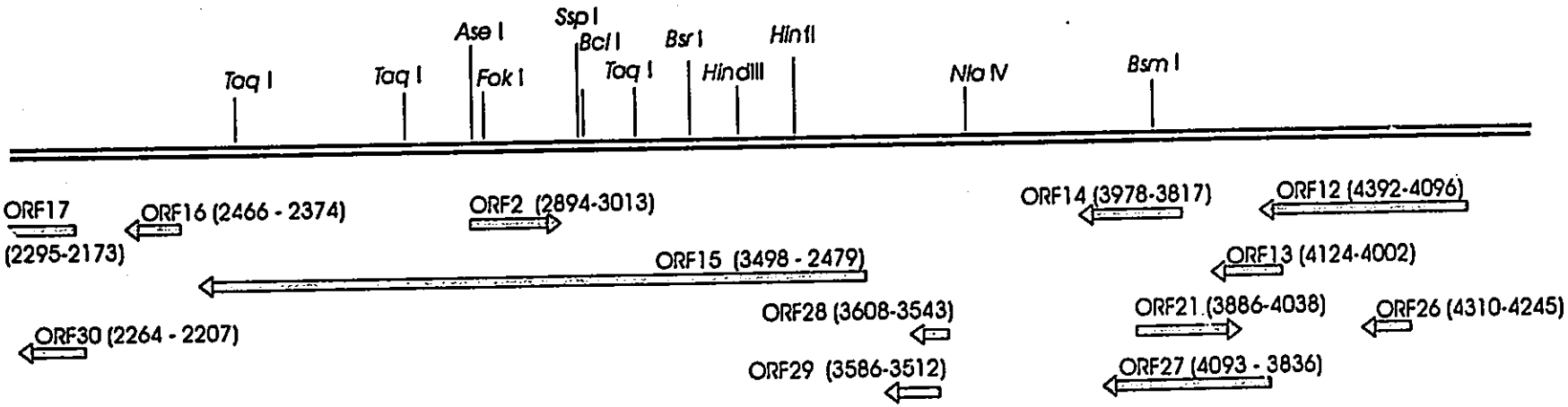


Figure 18b

JD4BIG52



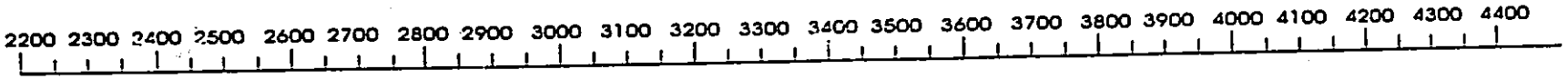


Figure 18c

JD4BIG53

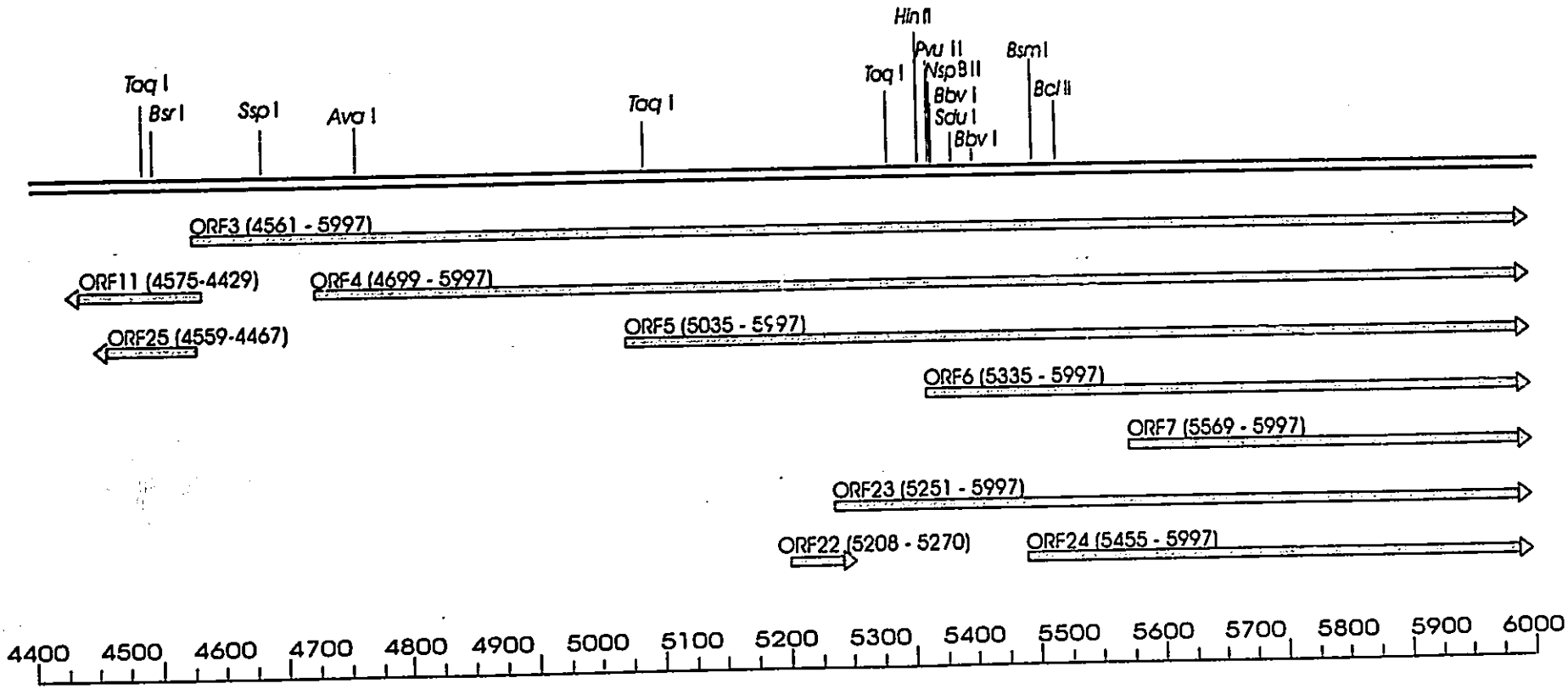
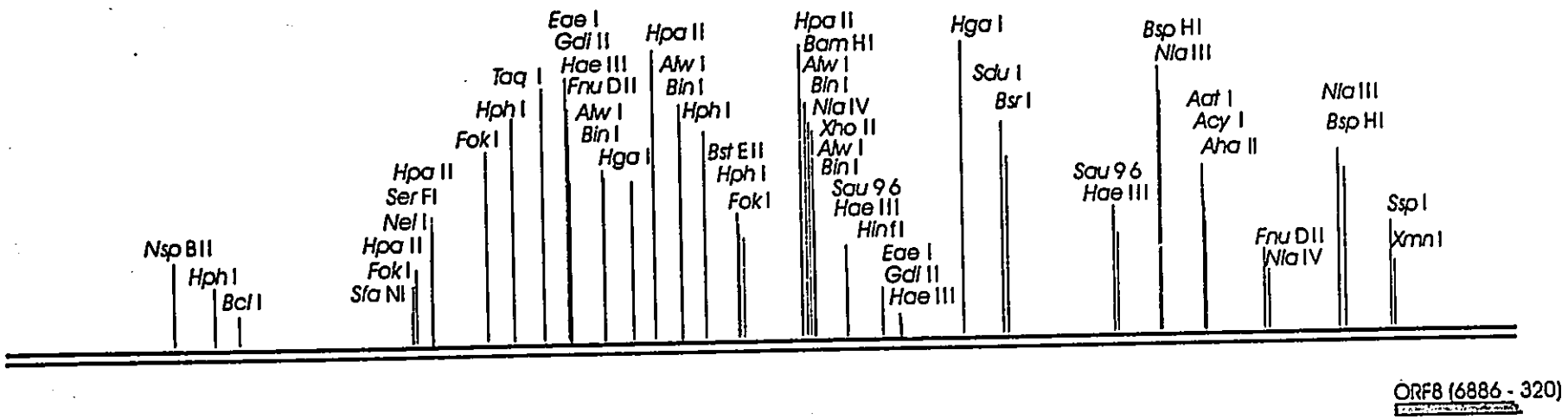


Figure 18d

JD4BIG54



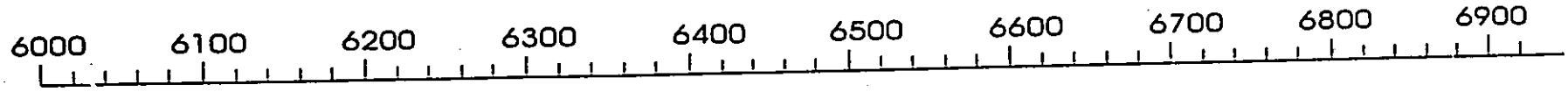


Figure 18e

JD48IG55

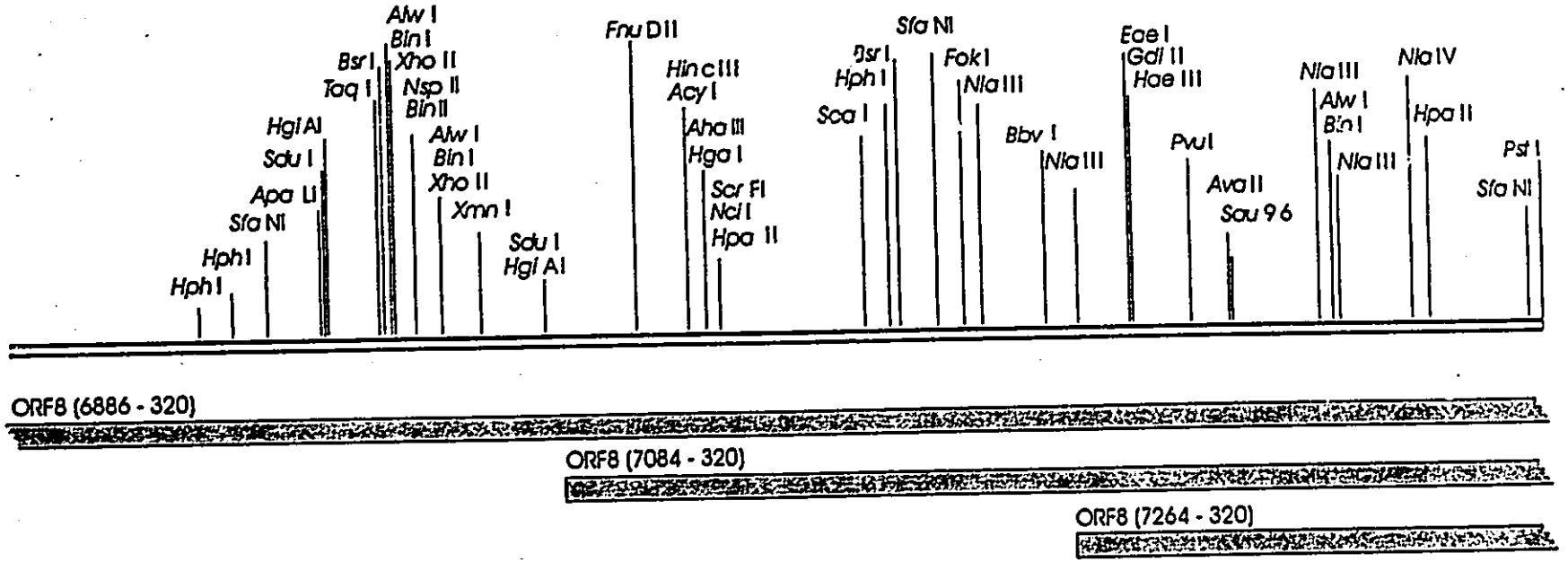


Figure 18f

JD4BIG56

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 Tn*A* sequences was compared to the sequences in the Data Bank of MicroGenie^R 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.

ORF number	ORFs of PJD4[a]	Yeung and Dillon (1985)	Tenover *et al.* (1985)	Biswas *et al.* (1986)
3	56.2	56.5		55
4	50.9	43	43	
15	39.5	41	41	41
				40
5	37.9	36		36.5
		33		
8	31.5	31	30	30
23	29.1	28	28	29
6	25.9	26	26	
20	25.4			
9	24.0	24	24	
24	21.4	22	20	18
10	17.4	17.5		17
7	16.9	16.5	16	
			15	
			14	14
12	11.6	12.8		12
30	10.5	10.8		
27	10.5	8.8		
18	6.7	7.8		
14	6.2	6.5		
21	6.0			
11	5.7	5.5		

[a] ORFs that have the potential to encode protein with moleculer 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.

```
      300                                                                359
pJD4  TCACTGATTAAGCATTGGTAACTGTCAGACCAAGTTTACTCATATATACTTTAGATTGAT
pFA3  ------------------------------------------------------------
pFA7  TCACTGATTAAGCATTGGTAACTGTCAGACCAAGTTTACTCATATATACTTTAGATTGAT

                369                 378
                ⌐                   ⌐                              419
pJD4  TTAAAACTTCATTTTTAATTTAAAAGGATCTAGGTGAAGATCCTTTTTGATAATCTCATG
pFA3  -------------------------GA*CTAGGTGAAGATCCTTTTTGATAATCTCATG
pFA7  TTAAAACTTAATTTTTAATTTAAAAGGATCTAGGTGAAGATCCTTTTTGATAATCTCATG
                ⌙                   ⌙

                                                                   479
pJD4  ACCAAAATCCCTTAACGTGAGTTTTCGTTCCACTGAGCGTCAGACCCCTATCTATAAACT
pFA3  ACCAAAATCCCTTAACGTGAGTTTTCGTTCCACTGAGCGTCAGACCCCTATCTATAAACT
pFA7  ACCAAAATCCCTTAACGTGAGTTTTCGTTCCACTGAGCGTCAGACCCCTATCTATAAACT

                                                                   539
pJD4  CTTGGCTTGGTTCTAATCCCTCTAAACGATTATTATCAATAGCCGCTCTAACCGCTTTTT
pFA3  CTTGGCTTGGTTCTAATCCCTCTAAACGATTATTATCAATAGCCGCTCTAACCGCTTTTT
pFA7  CTTGGCTTGGTTCTAATCCCTCTAAACGATTATTATCAATAGCCGCTCTAACCGCTTTTT

                                          576     582
                                          ⌐       ⌐                597
pJD4  CTCGGCTTAATTTTTCTGTCTCTGTTATAAAATTGCT*ATTCAT*CTTGTTCTTCTTCAA
pFA3  CTCGGCTTAATTTTTCTGTCTCTGTTATAAAATTGCT*ATTCAT*CTTGTTCTTCTTCAA
pFA7  CTCGGCTTAATTTTTCTGTCTCTGTTATAAAATTGCTTATTCATTCTTGTTCTTCTTTCA
                                          ⌙       ⌙                ⌙

          603
          ⌐                                                        656
pJD4  AAAAAA*GTTAAGTAAAATACCTACCTAAATTTTTACTAGTTCGCAATCTACGAGCTTAT
pFA3  AAAAAA*GTTAAGTAAAATACCTACCTAAATTTTTACTAGTTCGCAATCTACGAGCTTAT
pFA7  AAAAAAAGTTAAGTAAAATACCTACCTAAATTTTTACTAGTTCGCAATCTACGAGCTTAT
          ⌙

                                                    706
                                                    ⌐          716
pJD4  AACCTCGTTTTTTCAATTCATTTAAAAAATCAGATTTTGAGCCTAATTTTGATCTATTGC
pFA3  AACCTCGTTTTTTCAATTCATTTAAAAAATCAGATTTTGAGCCTAATTTTGATCTATTGC
pFA7  AACCTCGTTTTTTCAATTCATTTAAAAAATCAGATTTTGAGCCTAATTT*GATCTATTGC
                                                    ⌙

                                                               776
pJD4  TATCGTTACCCGCTAGAAATACCCAGTAATTACGCAAATCTTCATTGGTAACTTTCGTAA
pFA3  TATCGTTACCCGCTAGAAATACCCAGTAATTACGCAAATCTTCATTGGTAACTTTCGTAA
pFA7  TATCGTTACCCGCTAGAAATACCCAGTAATTACGCAAATCTTCATTGGTAACTTTCGTAA

        781
        ⌐                                                          836
pJD4  TATCGGTGTAATGATCTTCGAGTATTTTTAAGCAATCTCTAGCCCATAAACCGTACTCGT
pFA3  TATCGGTGTAATGATCTTCGAGTATTTTTAAGCAATCTCTAGCCCATAAACCGTACTCGT
pFA7  TATCTGTGTAATGATCTTCGAGTATTTTTAAGCAATCTCTAGCCCATAAACCGTACTCGT
        ⌙
```

```
                                                                      896
pJD4 GATTGCTCATCTTAGGGTTTTGCTTATCGAGTTTGACGAACTTCCCATACTTGTTTTTAT
pFA3 GATTGCTCATCTTAGGGTTTTGCTTATCGAGTTTGACGAACTTCCCATACTTGTTTTTAT
pFA7 GATTGCTCATCTTAGGGTTTTGCTTATCGAGTTTGACGAACTTCCCATACTTGTTTTTAT

                          916
                           ⌐                                          956
pJD4 GTGGAAATACTGGCCGTTTTGCAACTTCTTCAATTTTTTGAGCTGTTCGTTTTTTACTAC
pFA3 GTGGAAATACTGGCCGTTTTGCAACTTCTTCAATTTTTTGAGCTGTTCGTTTTTTACTAC
pFA7 GTGGAAATACTGGCCGTTT*GCAACTTCTTCAATTTTTTGAGCTGTTCGTTTTTTACTAC
                           ⌙

                                                                     1016
pJD4 CAATCACAAAATTTAAAGAGTGAATAGTACGCCCACGCTTGATTTGTTCAACCTCAACGA
pFA3 CAATCACAAAATTTAAAGAGTGAATAGTACGCCCACGCTTGATTTGTTCAACCTCAACGA
pFA7 CAATCACAAAATTTAAAGAGTGAATAGTACGCCCACGCTTGATTTGTTCAACCTCAACGA

                                                                     1076
pJD4 CTAAATCAGATTTCTCGTTAATCTCAGTTATTGCAGGTTCCAAAACACGTTGATTTAATG
pFA3 CTAAATCAGATTTCTCGTTAATCTCAGTTATTGCAGGTTCCAAAACACGTTGATTTAATG
pFA7 CTAAATCAGATTTCTCGTTAATCTCAGTTATTGCAGGTTCCAAAACACGTTGATTTAATG

                  1090 1095 1099/1100              1220   1227
                   ⌐    ⌐    ⌐                      ⌐      ⌐      1136
pJD4 AATTAAATCTAGGGTATTTATTTTCAACCTGAAGCCATTCTTTTAGTTTTTCTACTGTAA
pFA3 AATTAAATCTAGGGTATTTATTTTCAACCTGAAGCCATTCTTTTAGTTTTTCTACTGTAA
pFA7 AATTAAATCTAGG*TATT*ATT**CAACCTGAAGCCATTCTTT*AGTTTT*CTACTGTAA
                   ⌙    ⌙    ⌙                      ⌙      ⌙

                                                                     1196
pJD4 TTTCACGACTACCAACAGAGCGATATTGTGTAATTAGCTCATAAATTCGAATTGAATGTA
pFA3 TTTCACGACTACCAACAGAGCGATATTGTGTAATTAGCTCATAAATTCGAATTGAATGTA
pFA7 TTTCACGACTACCAACAGAGCGATATTGTGTAATTAGCTCATAAATTCGAATTGAATGTA

                                                                     1256
pJD4 CACTGTTGAAATAAGCGATATGTTTGAGTTGATATTGCGTGAATTGCCCTTTAAGTTGCG
pFA3 CACTGTTGAAATAAGCGATATGTTTGAGTTGATATTGCGTGAATTGCCCTTTAAGTTGCG
pFA7 CACTGTTGAAATAAGCGATATGTTTGAGTTGATATTGCGTGAATTGCCCTTTAAGTTGCG

                                                          1311
                                                           ⌐1316
pJD4 TTAGGTATGGCATAACTTCATCAGTCATTGCAATTCTAAAACGCCCCTCTTTCTTGAAAT
pFA3 TTAGGTATGGCATAACTTCATCAGTCATTGCAATTCTAAAACGCCCCTCTTTCTTGAAAT
pFA7 TTAGGTATGGCATAACTTCATCAGTCATTGCAATTCTAAAACGCCCCTCTTTCT*GAAAT
                                                           ⌙

                                                                     1376
pJD4 ATGTTCTAGAGGAAACCCAACGAAATTCAGTTACACGGTCTTTATCTTCAGTTTTAACAC
pFA3 ATGTTCTAGAGGAAACCCAACGAAATTCAGTTACACGGTCTTTATCTTCAGTTTTAACAC
pFA7 ATGTTCTAGAGGAAACCCAACGAAATTCAGTTACACGGTCTTTATCTTCAGTTTTAACAC
```

```
                                                                    1436
pJD4  TTCGGTCATAAATCCGTTTTATAGCCGCCTGAATTTGCTTATAGGCGTTATCTTGGCTTA
pFA3  TTCGGTCATAAATCCGTTTTATAGCCGCCTGAATTTGCTTATAGGCGTTATCTTGGCTTA
pFA7  TTCGGTCATAAATCCGTTTTATAGCCGCCTGAATTTGCTTATAGGCGTTATCTTGGCTTA

                                                        1483
                                                          ⌐ 1496
pJD4  TTTCTGGAAACTCACGGACAAAATCAGCCACCGTAAAATCAAAAATCTTTTGATTAGATT
pFA3  TTTCTGGAAACTCACGGACAAAATCAGCCACCGTAAAATCAAAAATCTTTTGATTAGATT
pFA7  TTTCTGGAAACTCACGGACAAAATCAGCCACCGTAAAATCAAAAATTTTTTGATTAGATT
                                                          ⌙
                                                                    1556
pJD4  TCGGATCCATAGTCCCAATAGTTAAAGCTAAAATTCTGATTTCATCAATACTCAATCGGT
pFA3  TCGGATCCATAGTCCCAATAGTTAAAGCTAAAATTCTGATTTCATCAATACTCAATCGGT
pFA7  TCGGATCC[End]

                                                                    1616
pJD4  AATTGGCTTCAATAAGGCTATTAGCCTTTACAACAACTAAATCATTTGGCATAAGACAAC
pFA3  AATTGGCTTCAATAAGGCTATTAGCCTTTACAACAACTAAATCATTTGGCATAAGACAAC

                                                                    1676
pJD4  AAATTTCCTGTTTAAAACAACAAGCAAAATATACCTGTTGTTTATATATAAAACAACAAG
pFA3  ACAACAACTAAATCATTTGGCATAAGACATATACCTGTTGTTTATATATAAAACAACAAG

                                                                    1736
pJD4  TATTTTCTTAAAAGTTGTCTATAACAGGAAATTTGTTGTCTTATAACAGGAAATTTGTTG
pFA3  TATTTTCTTAAAAGTTGTCTATAACAGGAAATTTGTTGTCTTATAACAGGAAATTTGTTG

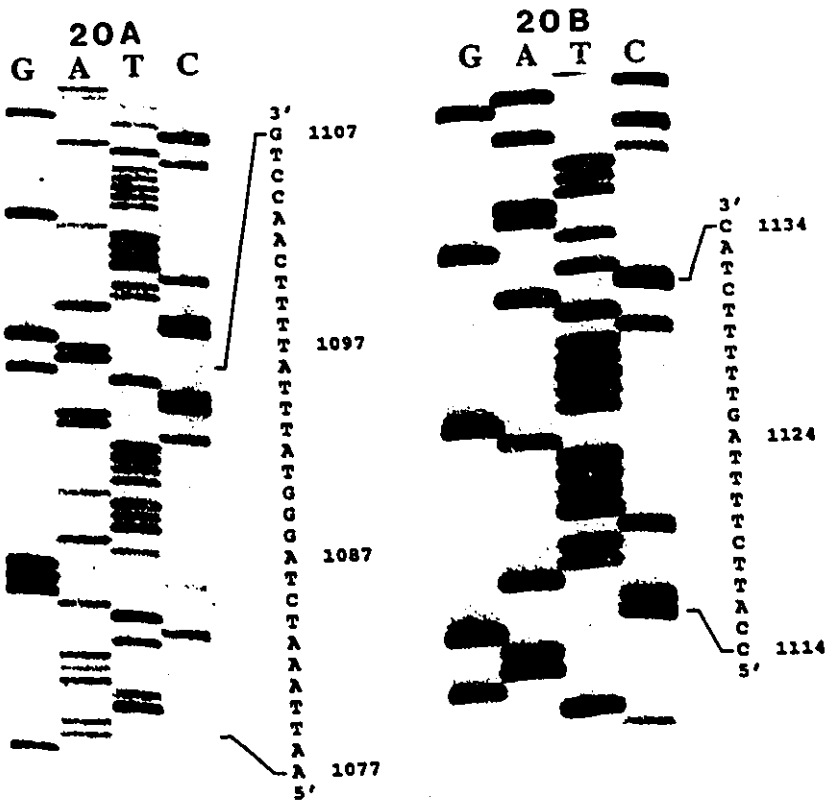
                                                                    1796
pJD4  TCGTATAACAGGAAATTTGTTGTCGTATAACAGGAAATTTGTTGTCGTATAAGTTTGTAA
pFA3  TCGTATAACAGGAAATTTGTTGTCGTATAACAGGAAATTTGTTGTCGTATAAGTTTGTAA

                                                                    1856
pJD4  CTTATTGATTTTACTGGTTTTAAAAACGCCGAAAACAAGTAAAAAACAAAAATATAAAAA
pFA3  CTTATTGATTTTACTGGTTTTAAAAACGCCGAAAACAAGTAAAAAACAAAAATATAAAAA

                      1873/74          1888 1892/93
                        ⌐ ⌐              ⌐ ⌐ ⌐                       1916
pJD4  TATAGGGACTTTCGTCCCTTTTTTGGGCTTTCAGCCCTAATTTTTTCTTTTTTCAGGATT
pFA3  TATAGGGACTTTCGTC**TTTTTTGGGCTTTGAGC**TAATTTTTTCTTTTTTCAGGATT
                        ⌙ ⌙              ⌙ ⌙ ⌙
                                                                    1966
pJD4  AAAAATTACAAAACCCTTACAGAGCAAGTAAACTTGTTTGCTTGTTCTGCAAGGGTTCAG
pFA3  AAAAATTACAAAACCCTTACAGAGCAAGTAAACTTGTTTGCTTGTTCTGCAAGGGTTCAG

                1987
pJD4  CAACCGAAGCC.....
pFA3  CAACCGAAGCC[End]
```

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.


20A
G A T C
3'
1107
1097
1087
1077
5'
20B
G A T C
3'
1134
1124
1114
5'

The primary sequence of pJD4 also showed homology to pFA3 (an Asia-type plasmid)(Gilbride and Bruntor., 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'-TTAAAACGGC-3'), 3338 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/TAANNNNTTGATA/T-3' or the complementary strand 5'-A/TATCAANNNNTTA/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'-AATCATTTTTTA-3'), 4000 to 3988 (5'-AAACGGATTGATA-3'), and 4065 to 4076 (5'-TAAAAAATGATT-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

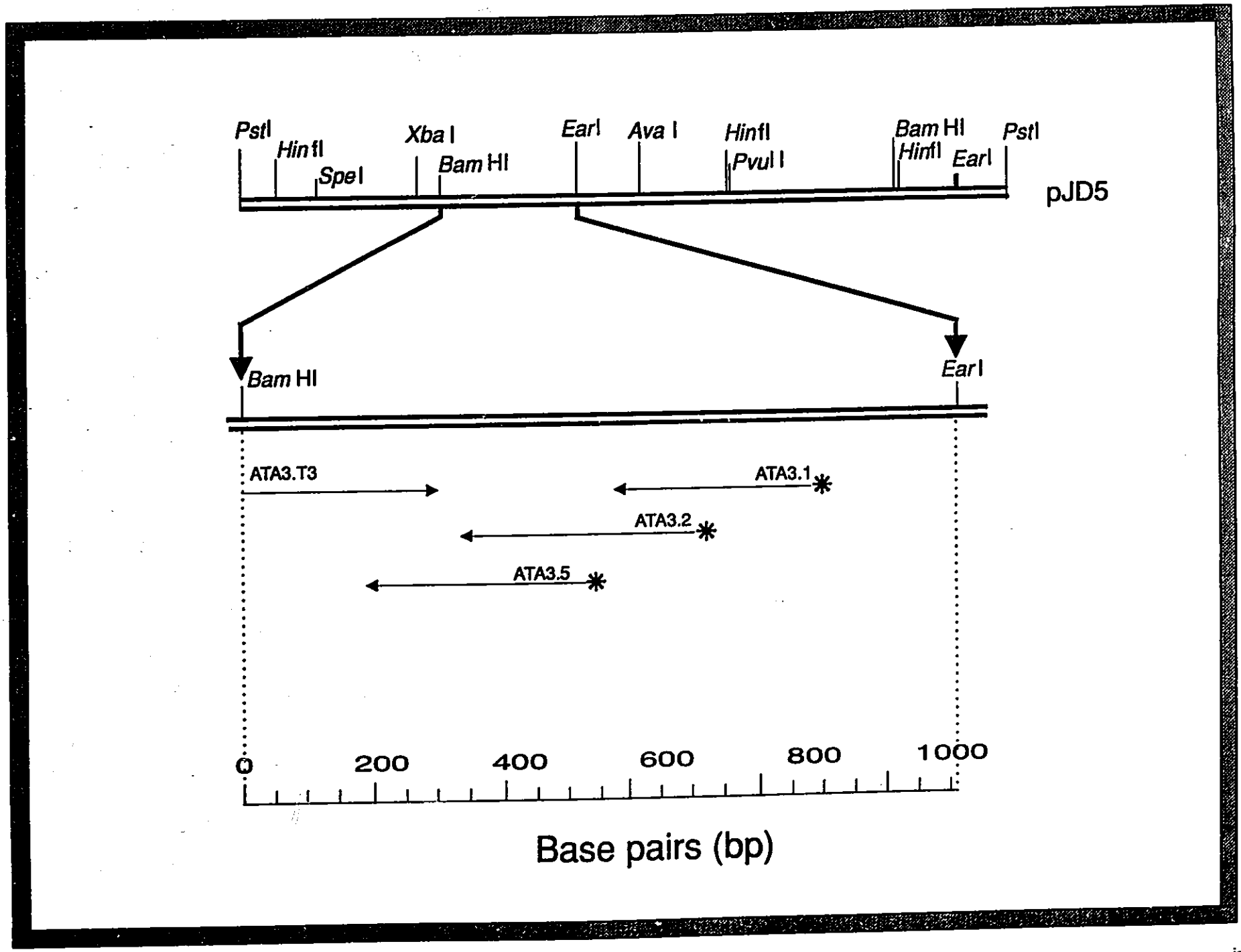
It has been shown by restriction endonuclease analysis and by limited DNA sequence analysis that the Asia-type plasmid of *N. gonorrhoeae* is homologous to the Africa-type plasmid except for a deletion reported from 1.8 to 2.1-kb by various groups (Yeung and Dillon, 1985; Yeung et al., 1986; Dickgiesser, 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 obtaine the primary sequence around the deletion of pJD5, a recombinant plasmid pATA3 which was constructed from pJD5. To construct pATA3, the 3.1-kb *Bam*HI fragment of pJD5 was cloned into the appropriate site of pBluscript 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 *Bam*HI 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 *Bam*HI-*Ear*I 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′.


PstI
HinfI
SpeI
XbaI
BamHI
EarI
AvaI
HinfI
PvuII
BamHI
HinfI
EarI
PstI
pJD5
BamHI
EarI
ATA3.T3
ATA3.1
ATA3.2
ATA3.5
0
200
400
600
800
1000
Base pairs (bp)

jd5001

Figure 22. Primary sequence around the deletion of pJD5 (bottom) and the primary sequence of pJD4 (top). Coordinate 1 being the *Pst*I site of pJD4. ^{end}, the end of the nucleotide determined from pJD5. DR-30B is underlined. Nucleotide differences between pJD4 and pJD5 were written in bold. ⊓, indicates location of nucleotide differences. *, indicates that there is nucleotide at a given coordinate.

```
      1524                                                        1583
pJD4  CTAAAATTCTGATTTCATCAATACTCAATCGGTAATTGGCTTCAATAAGGCTATTAGCCT
pJD5  CTAAAATTCTGATTTCATCAATACTCAATCGGTAATTGGCTTCAATAAGGCTATTAGCCT

                                                                  1643
pJD4  TTACAACAACTAAATCATTTGGCATAAGACAACAAATTTCCTGTTTAAAACAACAAGCAA
pJD5  TTACAACAACTAAATCATTTGGCATAAGACAACAAATTTCCTGTTTAAAACAACAAGCAA

                                                                  1703
pJD4  AATATACCTGTTGTTTATATATAAAACAACAAGTATTTTCTTAAAAGTTGTCTATAACAG
pJD5  AATATACCTGTTGTTTATATATAAAACAACAAGTATTTTCTTAAAAGTTGTCTATAACAG

                  1717                1739
                   ⌐                   ⌐                          1763
pJD4  GAAATTTGTTGTCTTATAACAGGAAATTTGTTGTCGTATAACAGGAAATTTGTTGTCGTA
pJD5  GAAATTTGTTGTCGTATAACAGGAAATTTGTTGTCTTATAACAGGAAATTTGTTGTCGTA
                   ⌙                   ⌙
                                                                  1823
pJD4  TAACAGGAAATTTGTTGTCGTATAAGTTTGTAACTTATTGATTTTACTGGTTTTAAAAAC
pJD5  TAACAGGAAATTTGTTGTCGTATAAGTTTGTAACTTATTGATTTTACTGGTTTTAAAAAC

                                                                  1883
pJD4  GCCGAAAACAAGTAAAAAACAAAAATATAAAAATATAGGGACTTTCGTCCCTTTTTTGGG
pJD5  GCCGAAAACAAGTAAAAAACAAAAATATAAAAATATAGGGACTTTCGTCCCTTTTTT...

                                                                  1943
pJD4  CTTTCAGCCCTAATTTTTTCTTTTTTCAGGATTAAAAATTACAAAACCCTTACAGA....
      -------------- sequence does not exist in pJD5--------------

                                                3737    3745
      3707                                       ⌐       ⌐          3758
pJD4  ..CTTTTTGGGGCTTTCAGCCCTAATTTTTTCTTTTTTTCAGGATTTAAAATTACAAAAC
pJD5  ---------GGGCTTTCAGCCCTAATTTTTTCTTTTTTT*CAGGATTAAAAATTACAAAAC
                                               ⌙       ⌙
                                                                  3818
pJD4  CCTTACAGAGCAAGTAAACTTGTTTGCTTGTTCTGCAAGGGTTCAGCAACCGTAGCCGTC
pJD5  CCTTACAGAGCAAGTAAACTTGTTTGCTTGTTCTGCAAGGGTTCAGCAACCGTAGCCGTC

                                                                  3878
pJD4  AGGCGTAGGGCGGTAGCCTATAAAAGCCATTTAATTTTATCTTTAAACTTCCTTTTAAAT
pJD5  AGGCGTAGGGCGGTAGCCTATAAAAGCCATTTAATTTTATCTTTAAACTTCCTTTTAAAT

                                                                  3938
pJD4  GCTTTGAGTGGGTGTCTTTTATCGTACTCATCAATCCTTTTTTGCATTCTTTCGTTTGCT
pJD5  GCTTTGAGTGGGTGTCTTTTATCGTACTCATCAATCCTTTTTTGCATTCTTTCGTTTGCT

                                                                  3998
pJD4  TTGTGATCGGCAAATTTTGAATAAGATTTTTCCATCTCATCTAACATTCTATCAATCCGT
pJD5  TTGTGATCGGCAAATTTTGAATAAGATTTTTCCATCTCATCTAACATTCTATCAATCCGT
```

```
                                                                    4058
pJD4   TTTTTATGTTGCCATTTCAGGTAAACATAAACACTTATAGCAATTAAAGACAATATCAAT
pJD5   TTTTTATGTTGCCATTTCAGGTAAACATAAACACTTATAGCAATTAAAGACAATATCAAT

                                                                    4118
pJD4   ACATTGTAAAAAATGATTGTTACAATTTCGCTCACAGTTATTTTTTACCTTTTTCAATTT
pJD5   ACATTGTAAAAAATGATTGTTACAATTTCGCTCACAGTTATTTTTTACCTTTTTCAATTT

                                                                    4178
pJD4   CTTCATTGATAAATGCACTCAATTCATCAAATTTCTTGTCATCATTGATAAATTTACGCA
pJD5   CTTCATTGATAAATGCACTCAATTCATCAAATTTCTTGTCATCATTGATAAATTTACGCA

                                                  4216              4234
pJD4   ACTTAGGGAAGTTTCTATCTACATCTAAAAGAGGGTTATTTATTATTTCATTTAGC
pJD5   ACTTAGGGAAGTTTCTATCTACATCTAAAAGAGGGTTATTTATTATTTCATTTAGC[End]
```

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.

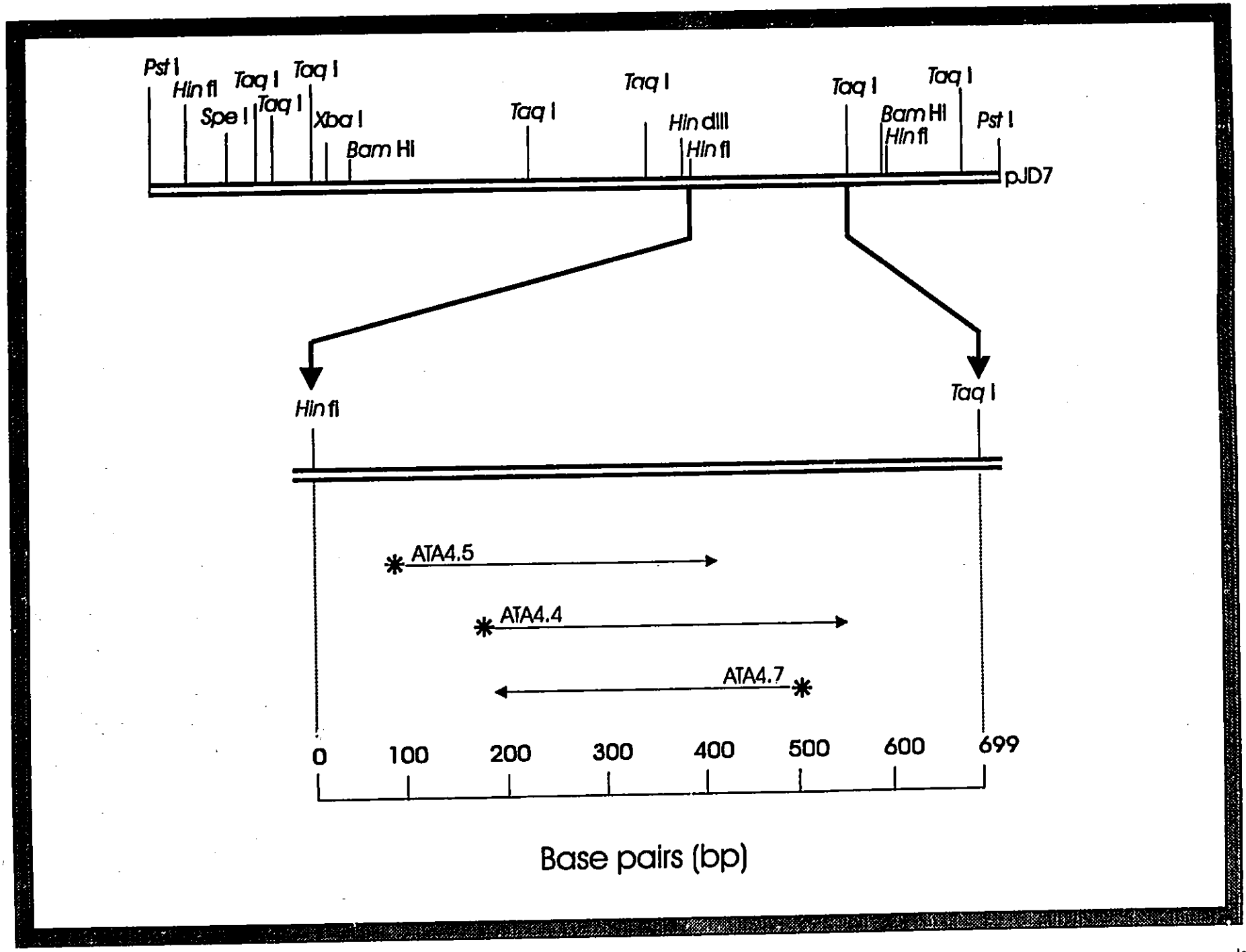
III.7. Primary sequence around the deletion of pJD7

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'.


Pst I
Hin fI
Taq I
Spe I
Taq I
Taq I
Xba I
Bam HI
Taq I
Taq I
Hin dIII
Hin fI
Taq I
Taq I
Bam HI
Hin fI
Pst I
pJD7
Hin fI
Taq I
ATA4.5
ATA4.4
ATA4.7
0
100
200
300
400
500
600
699
Base pairs (bp)

jd7001

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.

```
                3515       3525       3535       3545       3555       3565
pJD4    ATACAGTTAAATGGTGGTCA ATAAAAAACAAAGACCACTA TAACAATAAATTTGTCCACC
pJD7    ATACAGTTAAATGGTGGTCA ATAAAAAACAAAGACCACTA TAACAATAAATTTGTCCACC
                                                  DR-48

                3575       3585       3595       3605       3615       3625
pJD4    TATAACAATAAATTTGTCCA CCTATAACAATAAATTTGTC CACCTATAAATCTCGCAAGC
pJD7    TATAACAATAAATTTGTCCA CCTATAACAATAAATTTGTC CACCTATAAATCTCGCAAGC
        DR48                      DR-48
                3635       3645       3655       3665       3675       3685
pJD4    CTTGTGTAACAAGGGGAGCC AGAGCCTACAAACAAGAATA CAAACAAGAATACAAAAAAA
pJD7    CTTGTGTAACAAGGGGAGCC AGAGCCTACAAACAAGAATA CAAACAAGAATACAAAAAAA
                                   DR-49      DR-49

                3695       3705       3715       3725       3735       3745
pJD4    TAGAGCCTAAAGGCTCTTTT TGGGGCTTTCAGCCCTAATT TTTTCTTTTTTTCAGGATTT
pJD7    TAGAGCCTAAAGGCTCTTTT TGGGGCTTTCAGCCCTAATT TTTTCTTTTTTTCAGGATTT

                3755       3765       3775       3785       3795       3805
pJD4    AAAATTACAAAACCCTTACA GAGCAAGTAAACTTGTTTGC TTGTTCTGCAAGGGTTCAGC
pJD7    AAAATTACAAAACCCTTACA GAGCAAGTAAACTTGTTTGC TTGTTCTGCAAGGGTTC---

                3815       3825       3835       3845       3855       3865
pJD4    AACCGTAGCCGTCAGGCGTA GGGCGGTAGCCTATAAAAGC CATTTAATTTTATCTTT...
        ------------------- sequence not present in pJD7 -------------

                6070       6080       6090       6100       6110       6120
pJD4    ...TTTAAGGGTTCGCAATA AAAACAACCGCTAAACATTT CTGCCCAGCGGTTGAAAATT
pJD7    --------------GCAATA AAAACAACCGCTAAACATTT CTGCCCAGCGGTTGAAAATT

                6130       6140       6150       6160       6170       6180
pJD4    TACCTATTCACCATTACAAT GATCAAGCAGGAAATTTTTT TGATTGCCGTAAATGTCCGT
pJD7    TACCTATTCACCATTACAAT GATCAAGCAGGAAATTTTTT TGATTGCCGTAAATGTCCGT

                6190       6200       6209
pJD4    ATATCTAGTTGAGGCACAAC CCGCCAAAG
pJD7    ATATCTAGTTGAGGCACAAC CCGCCAAAG[End]
```

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 subsequentiy 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.

```
                3635       3645       3655       3665       3675       3685
pJD7    CTTGTGTAACAAGGGGAGCC AGAGCCTACAAACAAGAATA CAAACAAGAATACAAAAAAA
pGC1213 ----------------------------------------------------------------
pGC5438 ---------------------------------------------------- TACAAAAAAA
pGC5221 ----------------------------------------------------------------
pGC5228 ----------------------------------------------------------------
pGC5230 ----------------------------------------------------------------
pGO4717 ----------------------------------------------------------------
                3695       3705       3715       3725       3735       3745
pJD7    TAGAGCCTAAAGGCTCTTTT TGGGGCTTTCAGCCCTAATT TTTTCTTTTTTTCAGGATTT
pGC1213 -------------------- ------TTTCAGCCCTAATT TTTTCTTTTTTTCAGGATTT
pGC4538 TAGAGCCTAAAGGCTCTTTT TGGGGCTTTCAGCCCTAATT TTTTCTTTTTTTCAGGATTT
pGC5221 -------------------- -------------------- --------------------
pGC5228 --------AAAGGCTCTTTT TGGGGCTTTCAGCCCTAATT TTTTCTTTTTTTCAGGATTT
pGC5230 -------------------- -------------------- ----------GGATTT
pGO4717 -------------------- ------------------TT TTTTCTTTTTTTCAGGATTT
                3755       3765       3775       3785       3795   3802...
pJD7    AAAATTACAAAACCCTTACA GAGCAAGTAAACTTGTTTGC TTGTTCTGCAAGGGTTC...
pGC1213 AAAATTACAAAACCCTTACA GAGCAAGTAAACTTGTTTGC TTGTTCTGCAAGGGTTC...
pGC4538 AAAATTACAAAACCCTTACA GAGCAAGTAAACTTGTTTGC TTGTTCTGCAAGGGTTC...
pGC5221 ---------AAACCCTTACA GAGCAAGTAAACTTGTTTGC TTGTTCTGCAAGGGTTC...
pGC5228 AAAATTACAAAACCCTTACA GAGCAAGTAAACTTGTTTGC TTGTTCTGCAAGGGTTC...
pGC5230 AAAATTACAAAACCCTTACA GAGCAAGTAAACTTGTTTGC TTGTTCTGCAAGGGTTC...
pGO4717 AAAATTACAAAACCCTTACA GAGCAAGTAAACTTGTTTGC TTGTTCTGCAAGGGTTC...
                     6074  6080       6090       6100       6110       6120
pJD7    ..............GCAATA AAAACAACCGCTAAACATTT CTGCCCAGCGGTTGAAAATT
pGC1213 ..............GCAATA AAAACAACCGCTAAACATTT CTGCCCAGCGGTTGAAAATT
pGC4538 ..............GCAATA AAAACAACCGCTAAACATTT CTGCCCAGCGGTTGAAAATT
pGC5221 ..............GCAATA AAAACAACCGCTAAACATTT CTGCCCAGCGGTTGAAAATT
pGC5228 ..............GCAATA AAAACAACCGCTAAACATTT CTGCCCAGCGGTTGAAAATT
pGC5230 ..............GCAATA AAAACAACCGCTAAACATTT CTGCCCAGCGGTTGAAAATT
pGO4717 ..............GCAATA AAAACAACCGCTAAACATTT CTGCCCAGCGGTTGAAAATT
                6130       6140       6150       6160       6170       6180
pJD7    TACCTATTCACCATTACAAT GATCAAGCAGGAAATTTTTT TGATTGCCGTAAATGTCCGT
pGC1213 TACCTATTCACCATTACAAT GATCAAGCAGGAAATTTTTT TGATTGCCGTAAATGTCCGT
pGC4538 TACCTATTCACCATTACAAT GATCAAGCAGGAAATTTTTT TGATTGCCGTAAATGTCCGT
pGC5221 TACCTATTCACCATTACAAT GATCAAGCAGGAAATTTTTT TGATTGC[End]----------
pGC5228 TACCTATTCACCATTACAAT GATCAAGCAGGAAATTTTTT TGATTGCCGTAAATGTCCGT
pGC5230 TACCTATTCACCATTACAAT GATCAAGCAGGAAATTTTTT TGATTGCCGTAAATGTCCG[End]
pGO4717 TACCTATTCACCATTACAAT GATCAAGCAGGAAATTTTTT T[End]---------------
                6190       6200       6209
pJD7    ATATCTAGTTGAGGCACAAC CCGCCAAAG[End]
pGC1213 ATATCTAGTTGAGGCACAAC CC[End]----
pGC4538 ATATCTAGTTGAGGCACAAC CC[End]----
pGC5221 ------------------------------
pGC5228 ATATCTAGTTGAGGCACAAC CC[End]----
pGC5230 ------------------------------
pGO4717 ------------------------------
```

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 Tn*A* in pJD4, pJD5 and pJD7

The ampicillin resistance transposon (Tn*A*) comprises closely related transposons that were designated Tn*1*, Tn2, and Tn*3* (Heffron *et al.*, 1977). Tn*3* has been completely sequenced (Heffron *et al.*, 1979). Comparison of the primary sequence of pFA3 and pFA7 to that of Tn*1* and Tn*3* 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 Tn*A* present in pFA3 and pFA7 is Tn2 (Chen and Clowes, 1987b).

The Tn*A* sequence present in pFA3 and in pFA7 is different (Chen and Clowes, 1987b). I would like to know whether the Tn*A*/Tn*2* sequence is present in pJD4, pJD5 and pJD7. To find out the exact location of the region homologous to Tn*A*/Tn*2* in pJD4, pJD5 and pJD7, the primary sequences of pJD4, pJD5 and pJD7 were compared to the primary sequences of Tn*A*/Tn*2* (Heffron *et al.*, 1979; Chen and Clowes, 1987a).

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

Members of Tn*A* were similar on their inverted repeat (IR) termini and their mode of transmission (Sherratt, 1989). Since Tn*3* 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 Tn*A* (Tn*3*). The primary sequence of pJD4 in the IR-R region of Tn*A* is located within 1.5-kb *Pst*I-*Bam*HI 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 Tn*A* from coordinates 4991 (within *bla* gene) to 4957 (the end of IR-R sequence)(Figure 26). The 38-bp IR-R sequence of Tn*A* (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 Tn*A*.

Figure 26. Primary sequences of pJD4, pJD5, and pJD7 as compared to the primary sequence of Tn*A* (Tn*3*). The coordinates correspond to position in pJD4, with the *Pst*I site as coordinate 1. [a], end of IR-R of Tn*A*. IR-R sequence were underlined. ____, not sequenced.

```
pJD4 CTGCAGCAATGGCAACAACGTTGCGCAAACTATTAACTGGCGAACTACTTACTCTAGCTT  60
TnA  CTGCAGCAATGGCAACAACGTTGCGCAAACTATTAACTGGCGAACTACTTACTCTAGCTT
pJD5 ------------------------------------------------------------
pJD7 ------------------------------------------------------------

pJD4 CCCGGCAACAATTAATAGACTGGATGGAGGCGGATAAAGTTGCAGGACCACTTCTGCGCT 120
TnA  CCCGGCAACAATTAATAGACTGGATGGAGGCGGATAAAGTTGCAGGACCACTTCTGCGCT
pJD5 ------------------------------------------------------------
pJD7 ------------------------------------------------------------

pJD4 CGGCCCTTCCGGCTGGCTGGTTTATTGCTGATAAATCTGGAGCCGGTGAGCGTGGGTCTC 180
TnA  CGGCCCTTCCGGCTGGCTGGTTTATTGCTGATAAATCTGGAGCCGGTGAGCGTGGGTCTC
pJD5 ------------------------------------------------------------
pJD7 ------------------------------------------------------------

pJD4 GCGGTATCATTGCAGCACTGGGGCCAGATGGTAAGCCCTCCCGTATCGTAGTTATCTACA 240
TnA  GCGGTATCATTGCAGCACTGGGGCCAGATGGTAAGCCCTCCCGTATCGTAGTTATCTACA
pJD5 ------------------------------------------------------------
pJD7 ------------------------------------------------------------

pJD4 CGACGGGGAGTCAGGCAACTATGGATGAACGAAATAGACAGATCGCTGAGATAGGTGCCT 300
TnA  CGACGGGGAGTCAGGCAACTATGGATGAACGAAATAGACAGATCGCTGAGATAGGTGCCT
pJD5 ------------------------------------------------------------
pJD7 ------------------------------------------------------------
                                                          353
pJD4 CACTGATTAAGCATTGGTAACTGTCAGACCAAGTTTACTCATATATACTTTAGATTGATT 360
TnA  CACTGATTAAGCATTGGTAACTGTCAGACCAAGTTTACTCATATATACTTTAGATTGATT
pJD5 ----------------------------------------------------GATTGATT
pJD7 ------------------------------------------------------------

          371
pJD4 TAAAACTTCATTTTTAATTTAAAAGGATCTAGGTGAAGATCCTTTTTGATAATCTCATGA 420
TnA  TAAAACTTCATTTTTAATTTAAAAGGATCTAGGTGAAGATCCTTTTTGATAATCTCATGA
pJD5 TAAAACTTCATTTTTAATTTAAAAGGATCTAGGTGAAGATCCTTTTTGATAATCTCATGA
pJD7 ----------TTTTTAATTTAAAAGGATCTAGGTGAAGATCCTTTTTGATAATCTCATGA
                                                      467
pJD4 CCAAAATCCCTTAACGTGAGTTTTCGTTCCACTGAGCGTCAGACCCCTATCTATAAACTC 480
TnA  CCAAAATCCCTTAACGTGAGTTTTCGTTCCACTGAGCGTCAGACCCC[a]
pJD5 CCAAAATCCCTTAACGTGAGTTTTCGTTCCACTGAGCGTCAGACCCCTATCTATAAACTC
pJD7 CCAAAATCCCTTAACGTGAGTTTTCGTTCCACTGAGCGTCAGACCCCTATCTATAAACTC
                          IR-R

pJD4 TTGGCTTGGTTCTAATCCCTCTAAACGATTATTATCAATAGCCGCTCTAACCGCTTTTTC 540
TnA  ------------------------------------------------------------
pJD5 TTGGCTTGGTTCTAATCCCTCTAAACGATTATTATCAATAGCCGCTCTAACCGCTTTTTC
pJD7 TTGGCTTGGTTCTAATCCCTCTAAACGATTATTATCAATAGCCGCTCTAACCGCTTTTTC
```

```
                                                571
pJD4 TCGGCTTAATTTTTCTGTCTCTGTTATAAAATTGCTATTCATCTTGTTCTTCTTCAAAAA 600
TnA  ------------------------------------------------------------
pJD5 TCGGCTTAATTTTTCTGTCTCTGTTATAAAATTGCTATTCATCTTGTTCTTCTTCAAAAA
pJD7 TCGGCTTAATTTTTCTGTCTCTGTTATAAAA[End]-------------------------

                                                636
pJD4 AAAGTTAAGTAAAATACCTACCTAAATTTTTACTAGTTCGCAATCTACGAGCTTATAACC 660
TnA  ----------------------------------
pJD5 AAAGTTAAGTAAAATACCTACCTAAATTTTTACTAG[End]
pJD7 ----------------------------------
```

The primary sequences around the region homologous to IR-R of Tn*A* 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 Tn*A* at the corresponding area, the primary sequence of pJD5 was therefore, identical to that of Tn*A*. 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 Tn*A*. Therefore, in the IR-R region of Tn*A*, the nucleotide sequences of pJD4, pJD5, pJD7 and Tn*A* were identical.

III.9.2. Region homologous to *tnpR* of Tn*A* in pJD4, pJD5 and pJD7

By comparing to Tn*1*, Tn*2* and Tn*3*, Chen and Clowes (1987b) found that the nucleotide sequences homologous to Tn*A* in pFA3 and pFA7 were more identical to Tn*2* than to Tn*1* or Tn*3*. They also reported only one base pair difference between nucleotide sequences in the region homologous to Tn*A* in pFA3 (an Asia-type plasmid) and pFA7 (an Africa-type plasmid), and that of Tn*2*. Therefore, they concluded that the Tn*A* present in the β-lactamase plasmids of *N. gonorrhoeae* pFA3 and pFA7 was Tn*2*.

The *tnpR* region of Tn*2*, the one homologous to Tn*2* in pFA3, starts at coordinate 6235 of pJD4, while the region homologous to Tn*2* in pFA7 starts at coordinate 6236 of pJD4. I would like to know whether the nucleotide sequences homologous to Tn*A*/Tn*2* in pJD4, pJD5 and pJD7 are different. Since pJD4, pJD5 and pJD7 are β-lactamase plasmids from *N. gonorrhoeae*, it is very likely that the Tn*A* sequence present in these plasmids is also Tn*2*.

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

The primary sequence of the 3.2 kb *Bam*HI-*Hin*dIII fragment of pJD4 was obtained by sequencing the DNA insert of pSTD41 (Results, section III.5.). Comparison of this sequence to the *tnpR* sequence of Tn*2* found that from coordinates 6235 to 6518 indicated that the sequence was identical (Figure 27). The homology of pJD4 to Tn*2* 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 *tnpR* region was obtained by sequencing the DNA insert of pATA3, a recombinant plasmid DNA containing the 3.1-kb *Bam*HI

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. ⊓, 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.

```
                6190       6200       6210       6220       6230       6240
Tn2                                                                    AAACGG
pJD4  ATATCTAGTTGAGGCACAAC CCGCCAAAGTCATTGCCCCA ACCAGAACGGCGATAAACCG
pJD5  ATATCTAGTTGAGGCACAAC CCGCCAAAGTCATTGCCCCA ACCAGAACGGCGAT*AACCG
pJD7                       CAAAGTCATTGCCCCA ACCAGAACGGCGATAAACCG

                6250       6260       6270       6280       6290       6300
Tn2   TATATTTACCGATAAGGCAT CCGGCAGTTCAACAGACCGG GAAGGGCTGGATTTGCTGAG
pJD4  TATATTTACCGATAAGGCAT CCGGCAGTTCAACAGACCGG GAAGGGCTGGATTTGCTGAG
pJD5  TATATTTACCGATAAGGCAT CCGGCAGTTCAACAGACCGG GAAGGGCTGGATTTGCTGAG
pJD7  TATATTTACCGATAAGGCAT CCGGCAGTTCAACAGACCGG GAAGGGCTGGATTTGCTGAG

                6310       6320       6330       6340       6350       6360
Tn2   GATGAAGGTGGAGGAAGGTG ATGTCATTCTGGTTAAGAAG CTCGACCGTCTTGGCCGCGA
pJD4  GATGAAGGTGGAGGAAGGTG ATGTCATTCTGGTTAAGAAG CTCGACCGTCTTGGCCGCGA
pJD5  GATGAAGGTGGAGGAAGGTG ATGTCATTCTGGTTAAGAAG CTCGACCGTCTTGGCCGCGA
pJD7  GATGAAGGTGGAGGAAGGTG ATGTCATTCTGGTTAAGAAA CTCGACCGTCTTGGCCGCGA

                6370       6380       6390       6400       6410       6420
Tn2   CACTGCCGATATGATCCAAC TGATAAAGGAATTTGACGCT CAGGGCGTGGCAGTCCGGTT
pJD4  CACTGCCGATATGATCCAAC TGATAAAGGAATTTGACGCT CAGGGCGTGGCAGTCCGGTT
pJD5  CACTGCCGATATGATCCAAC TGATAAAGGAATTTGACGCT CAGGGCGTGGCAGTCCGGTT
pJD7  CACTGCCGATATGATCCAAC TGATAAAGGAATTTGACGCT CAGGGCGTGGCAGTCCGGTT

                6430       6440       6450       6460       6470       6480
Tn2   CATTGATGACGGGATCAGTA CCGACGGTGATATGGGGCAA ATGGTGGTCACCATCCTGTC
pJD4  CATTGATGACGGGATCAGTA CCGACGGTGATATGGGGCAA ATGGTGGTCACCATCCTGTC
pJD5  CATTGATGACGGGATCAGTA CCGACGGTGATATGGGGCAA ATGGTGGTCACCAT[End]
pJD7  CATTGATGACGGGATCAGTA CCGACGGTGATATGGGGCAA ATGGTGGTCACCATCCTGTC

          6490       6500       6510       6520       6530       6540
Tn2   GGCTGTGGCACAGGCTGAAC GCCGGAGGATCCTAGAGCGC ACGAATGAGGGCCGACAGGA
pJD4  GGCTGTGGCACAGGCTGAAC GCCGGAGGATCC[End]
pJD7  GGCTGTGGCACAGGCTGAAC GCCGGAGGATCC[End]
```

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 BamHI 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 *Bam*HI fragment of the Africa-type plasmid, outside the region homologous to Tn*A* (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 *Pst*I and *Spe*I and compared to a similar restriction endonuclease digest of pJD4. *Pst*I and *Spe*I were selected because both have a single site in pJD4 and pJD5 and were located within the 2.4-kb *Bam*HI fragment of pJD5 and pJD4 (Dillon and Yeung, 1989; Gouby *et al.*, 1986). Digestion of pJD4 with *Pst*I-*Spe*I produced two fragments, 6.8-kb and 0.6-kb in size (data not shown). When pGF1 was digested with the same restriction endonuclease (*Pst*I and *Spe*I; Figure 28), two fragments were produced, 4.8 kb and 1.8 kb in size. Because the small *Pst*I-*Spe*I 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 *Pst*I and the *Spe*I 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 *Pst*I-*Spe*I fragment of pGF1. Nested deletions were constructed from the *Pst*I and the *Spe*I sites (Figure 29). Nested deletions from the *Pst*I site produced subclones pATA2P3, pATA2P5, pATA2P6, and pATA2P12. Nested deletion from the *Spe*I 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 *Pst*I and *Spe*I; Lane 4, pGF1 digested with *Bam*HI

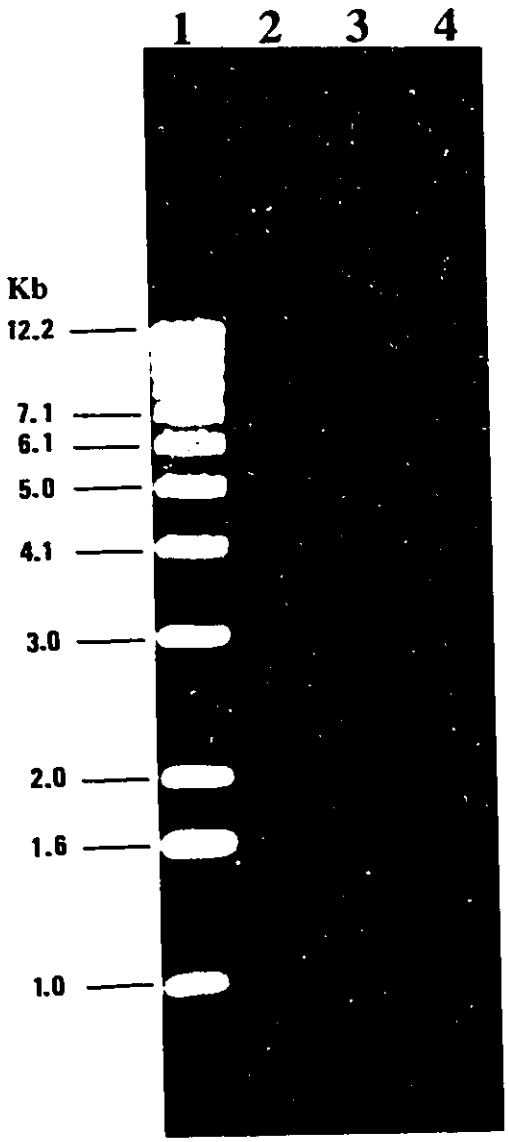
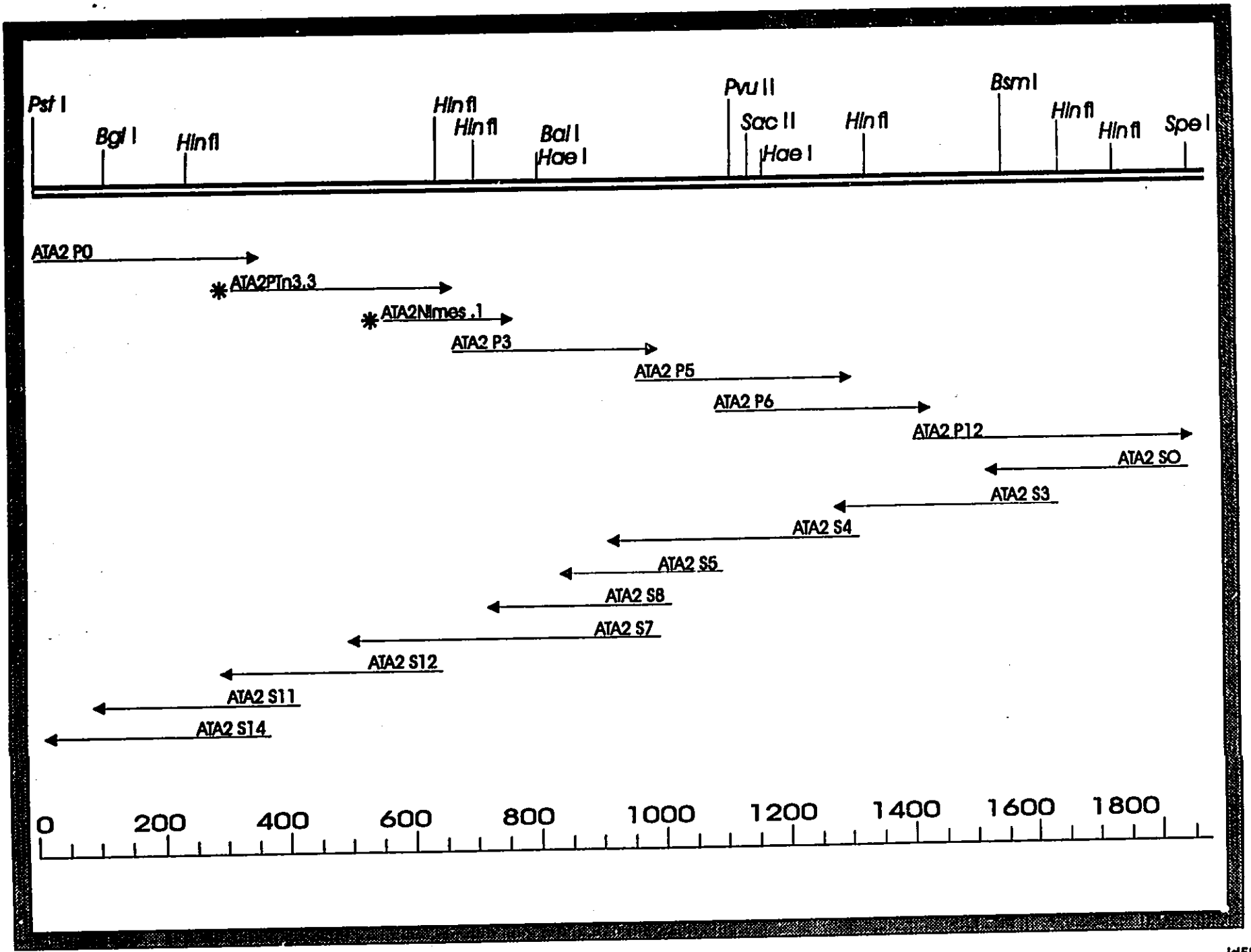

1
2
3
4
Kb
12.2
7.1
6.1
5.0
4.1
3.0
2.0
1.6
1.0

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′.


Pst I
Bgl I
Hinf I
Hinf I
Hinf I
Bal I
Hae I
Pvu II
Sac II
Hae I
Hinf I
Bsm I
Hinf I
Hinf I
Spe I
ATA2 P0
ATA2PTn3.3
ATA2Nimes .1
ATA2 P3
ATA2 P5
ATA2 P6
ATA2 P12
ATA2 SO
ATA2 S3
ATA2 S4
ATA2 S5
ATA2 S8
ATA2 S7
ATA2 S12
ATA2 S11
ATA2 S14
0
200
400
600
800
1000
1200
1400
1600
1800

jd5002

The DNA sequence of the *Pst*I-*Spe*I insert of pATA2 from the *Pst*I 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 *Pst*I-*Spe*I insert of pATA2 from the *Spe*I 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 *Pst*I-*Spe*I 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 *Pst*I 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 IS*5* (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 *Pst*I site. Sequence homologous to pJD4 was underlined.

PstI

1 CTGCAGCAATGGCAACAACGTTGCGCAAACTATTAACTGGCGAACTACTTACTCTAGCTT

pJD4

61 CCCGGCAACAATTAATAGACTGGATGGAGGCGGATAAAGTTGCAGGACCACTTCTGCGCT

pJD4

121 CGGCCCTTCCGGCTGGCTGGTTTATTGCTGATAAATCTGGAGCCGGTGAGCGTGGGTCTC

pJD4

181 GCGGTATCATTGCAGCACTGGGGCCAGATGGTAAGCCCTCCCGTATCGTAGTTATCTACA

pJD4

241 CGACGGGGAGTCAGGCAACTATGGATGAACGAAATAGACAGATCGCTGAGATAGGTGCCT

pJD4

301 CACTGATTAAGCATTGGTAACTGTCAGACCAAGTTTACTCATATATACTTTAGATTGATT

pJD4

361 TAAAACTTCATTTTTAATTTAAAAGGATCTAGGTGAAGATCCTTTTTGATAATCTCATGA

pJD4

421 CCAAAATCCCTTAACGTGAGTTTTCGTTCCACTGAGCGTCAGACCCCTATCTATAAACTC

pJD4

481 TTGGCTTGGTTCTAATCCCTCTAAACGATTATTATCAATAGCCGCTCTAACCGCTTTTTC

pJD4

541 TCGGCTTAATTTTTCTGTCTCTGTTATAAAATTGCTATTCATCTTGTTCTTCTTCAAAAA

605 pJD4

601 AAAGTTAAGGAAGGTGCGAATAAGCGGGGAAATTCTTCTCGGCTGACTCAGTCATTTCAT

----- IR -------

661 TTCTTCATGTTTGAGCCGATTTTTTCTCCCGTAAATGCCTTGAATCAGCCTATTTAGACC

721 GTTTCTTCGCCATTTAAGGCGTTATCCCCAGTTTTTAGTGAGATCTCTCCCACTGACGTA

781 TCATTTGGTCCGCCCGAAACAGGTTGGCCAGCGTGAATAACATCGCCAGTTGGTTATCGT

841 TTTTCAGCAACCCCTTGTATCTGGCTTTCACGAAGCCGAACTGTCGCTTGATGATGCGAA

901 ATGGGTGCTCCACCCTGGCCCGGATGCTGGCTTTCATGTATTCGATGTTGATGGCCGTTT

961 TGTTCTTGCGTGGATGCTGTTTCAAGGTTCTTACCTTGCCGGGGCGCTCGGCGATCAGCC

1021 AGTCCACATCCACCTCGGCCAGCTCCTCGCGCTGTGGCGCCCCTTGGTAGCCGGCATCGG

1081 CTGAGACAAATTGCTCCTCTCCATGCAGCAGATTACCCAGCTGATTGAGGTCATGCTCGT

1141 TGGCCGCGGTGGTGACCAGGCTGTGGGTCAGGCCACTCTTGGCATCGACACCAATGTGGG

1201 CCTTCATGCCAAAGTGCCACTGATTGCCTTTCTTGGTCTGATGCATCTCCGGATCGCGTT

1261 GCTGCTCTTTGTTCTTGGTCGAGCTGGGTGCCTCAATGATGGTGGCATCGACCAAGGTGC

1321 CTTGAGTCATCATGACGCCTGCTTCGGCCAGCCAGCGATTGATGGTCTTGAACAATTGGC

1381 GGGCCAGTTGATGCTGCTCCAGCAGGTGGCGGAAATTCATGATGGTGGTGCGGTCCGGCA

1441 AGGCGCTATCCAGGGATAACCGGGCAAACAGACGCATGGAGGCGATTTCGTACAGAGCAT

1501 CTTCCATCGCGCCATCGCTCAGGTTGTACCAATGCTGCATGCAGTGAATGCGTAGCATGG

1561 TTTCCAGCGGATAAGGTCGCCGGCCATTACCAGCCTTGGGGTAAAACGGCTCGATGACTT

1621 CCACCATGTTTTGCCATGGCAGAATCTGCTCCATGCGGGACAAGAAAATCTCTTTTCTGG

1681 TCTGACGGCGCTTACTGCTGAATTCACTGTCGGCGAAGGTAAGTTGATGACTCATGATGA

1741 ACCCTGTTCTATGGCTCCAGATGACAAACATGATCTCATATCAGGGACTTGTTCGCACCT
------ IR ---

SpeI
1801 TCC**TTAA**GTAAAATACCTACCTAAATTTTTACTAGT
--- pJD4

CHAPTER IV: DISCUSSION

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

The reported sizes of the Asia-type β-lactamase-producing plasmids of *N. gonorrhoeae*, determined by restriction endonuclease and heterodulpex 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 *Bam*HI-*Pst*I fragment which is homologous to Tn*A*, was determined. The primary sequence of the 0.9-kb *Bam*HI-*Pst*I fragment of Tn*A* was determined previously (Heffron *et al.*, 1979); the remaining region, 745-bp homologous to Tn*A*, 745-bp was determined in this study. Assuming that the 0.9-kb *Bam*HI-*Pst*I fragment of Tn*A* in pJD4 is identical to Tn*A*, 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 *Pst*I 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 *Pst*I 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 *Pst*I site. Aalen and Gundersen (1987) located it at approximately 1.85-kb downstream of the *Pst*I 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 *Pst*I, Dickgiesser (1984) reported that the deletion was located 1.74-kb upstream of the *Pst*I 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 *Pst*I site and approximately 0.5-kb from one of the *Bam*HI 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 *BamH*I 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 *Bam*HI fragment; and the second, 0.1-kb in size, is located within the 1.8-kb *Bam*HI-*Hin*dIII 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.

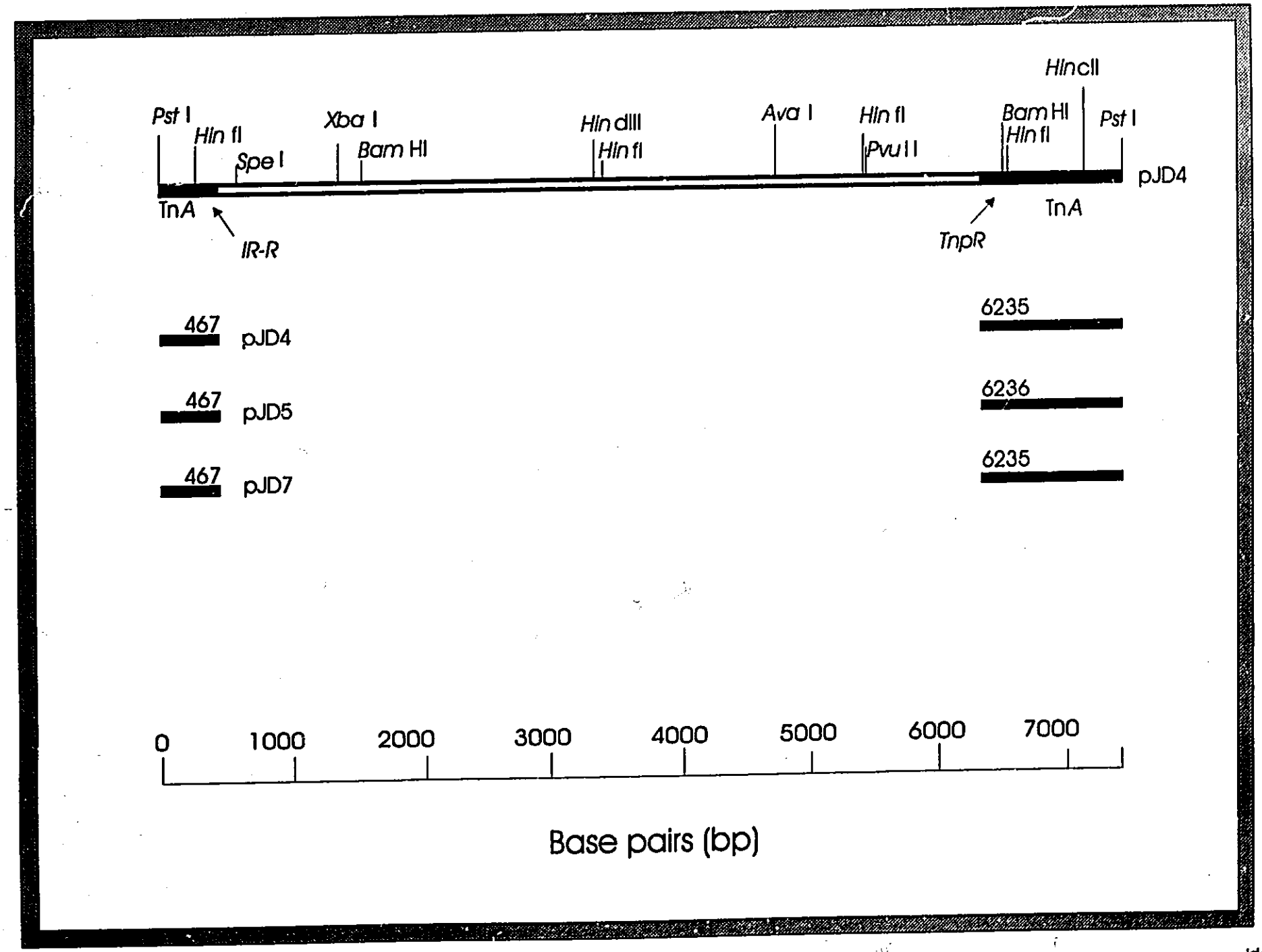
The deletion of pJD5 (an Africa-type plasmid) begins at coordinate 1881, which is adjacent to a region with a cluster of direct and inverted repeats. The deletion of pJD7, all of the other Toronto-type plasmids studied, and the Rio-type plasmid begin at coordinate 3803, which is also adjacent to the 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 Tn*A* in the β-lactamase-producing plasmids pJD4, pJD5 and pJD7

Previous heteroduplex analyses have established that approximately 40% of the Tn*A* sequence is present in the β-lactamase plasmids of *N. gonorrhoeae* (Roberts *et al.*, 1977; Fayet *et al.*, 1982). By sequencing regions homologous to Tn*A* of pFA3 (an Asia-type) and pFA7 (an Africa-type) plasmids, Chen and Clowes (1987b) found that the Tn*A*/Tn*2* sequences in pFA3 and pFA7 were identical except for one base pair. My analysis found that the Tn*A* 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 Tn*A* 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 Tn*A* sequence in pJD4, the Tn*A* sequence in pJD7 (Toronto-type plamid) begins at coordinate 6235 of pJD4.

I also found that in the IR-R region of Tn*A*, the sequence of pJD4 is identical to Tn*A*. 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 Tn*A*. Assuming that the 0.9-kb *Bam*HI-*Pst*I fragments of pJD4, pJD5 and pJD7 are identical to that of Tn*A* (Heffron et al., 1979); therefore, the region homologous to Tn*A* 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 Tn*A* in pJD4, pJD5 and pJD7. Map of pJD4 was used as comparison. ▬, region homologous to Tn*A*. IR-R, right hand of the terminal inverted repeat of Tn*A*. *TnpR*, part of *tnpR* gene of Tn*A*.


Hin cII
Pst I
Hin fI
Xba I
Hin dIII
Ava I
Hin fI
Bam HI
Pst I
Spe I
Bam HI
Hin fI
Pvu II
Hin fI
pJD4
TnA
TnA
IR-R
TnpR
6235
467
pJD4
6236
467
pJD5
6235
467
pJD7
0
1000
2000
3000
4000
5000
6000
7000
Base pairs (bp)

jd4010

IV.3. The evolution of the β-lactamase plasmids of *N. gonorrhoeae*

Roberts *et al.*, (1977) proposed that the β-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 β-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 β-lactamase-producing plasmids of *N. gonorrhoeae* were homologous to a variety of β-lactamase-producing plasmids isolated from *H. ducreyi, H. influenzae* and *H. parainfluenzae* (Brunton *et al,* 1986; Chen and Clowes, 1987b; Mc Nicol *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; pHD131) in size, were identical to the Africa-type and the Asia-type plasmids, except that *Haemophilus* plasmids carried a complete Tn*2* sequence (Brunton *et al.*, 1982; McNicol *et al.*, 1983; Chen and Clowes, 1987b). Nucleotide sequences of the region homologous to Tn*2* of the β-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 plamid 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 Tn*A* 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 placed 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 β-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 Tn*A* region of the 2.4-kb *Bam*HI 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 *Bam*HI 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 *Bam*HI-*Pvu*II 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 *Hin*dIII site, and 2) replication region "b" located at a 1.6-kb *Bam*HI-*Hin*fI (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 *Pst*I-*Hin*dIII fragment beginning 480-bp downstream of the *Bam*HI site to *Pst*I, excluding the Tn*A* region (Gilbride and Brunton 1990).

The DnaA protein (a *trans*-acting element) and DnaA-box (a *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 *cis*-acting element (*ori*C) 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 all., 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 *Bam*HI, 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 (Jonhson, 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 *Bam*HI-*Hin*dIII

fragment, corresponding to coordinates 1499-2387 of pJD4, and the *oriT* of the Africa-type plasmid (p88557) on the 1.4-kb *Bam*HI-*Ava*I fragment (McNicol *et al.*, 1983). Gauthier (MSc thesis, 1990) located the ori*T* of the Asia-type plasmid on the 1.8-kb *Bam*HI-*Hin*dIII fragment, several hundred base pairs from the *Hin*dIII site. My result showed that approximately 890-bp within the 1.8-kb *Bam*H-*Hin*dIII of pJD4 (coordinates 1499 to 2388) are homologous to the nucleotide sequence of pJD5 at the corresponding area (within 3.1-kb *Bam*HI 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 ori*T* 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 *ori*T 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/TAANNNNTTGATA/T-3' or the complementary strand 5'-A/TATCAANNNNTTA/G-3'. The 1.8-kb *Bam*HI-*Hin*dIII 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 site 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 *Hin*fI fragment, corresponding to coordinates 3381 to 5347 of pJD4, which encodes the 16 Kdal protein. Furthermore, Gauthier (1990) located the 0.6-kb *Pvu*II-*Ava*I 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 *Hin*fI 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 *Hin*fI 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-Tn*2* *Bam*HI fragment designated "M", which correspondes 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/TAANNNNTTGATA/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^{R}) found that the insertion is 100 % homologous to IS5 (Engler and van Bree, 1981; Kroger and Hobom, 1982). The IS5 in pGF1 is flaked by two 4 bp 5'-TTAA-3' sequences. The first sequence is located at coordinates 605 to 608 from *Pst*I 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 triparental 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 plamid). 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 Tn*A*/Tn*2*

1. 312 CATTGGTAACTGTC 325
   759 CATTGGTAACTTTC 772
   *

2. 502 TAAACGATTATTATCAATAGCCGCTCTAACCGCTTTTTCTCGGCTTAATTTTT
   2386 TAAACGATTATTATCAATAGCCGCTCTAACCGCTTTTTCTCGGCTTAATTTTT

   555 CTGTCTCTGTTATAAAATTGCTATTCATCTTGTTCTTCT 593
   2439 CTGTCTCTGTTATAAAATTGCTATTCATCTTGTTCTTCT 2477

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

4. 608 AGTAAAATAC 617
   5428 AGTAAAATAC 5437

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

6. 677 TTTAAAAAATCAGA 690
   2534 TTTAAAAAATCTGA 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   AACTTCCCATACTTGTTTTTATGTGGAAAT   904
     2732  AACTTCCCGTACTTGTTTTTATGCGGAAAT  2761
                  *                 *

13.  913   TTTTGCAACT   922
     2522  TTTTGCAACT  2531

14.  986   CGCCCACGCTTGATTTGTTCAACCTCAAC  1014
     2834  CGCCCACGCTTGATAGGTTCAACATCAAC  2862
                       **       *

15.  1000  TTGTTCAACC  1009
     6024  TTGTTCAACC  6033

16.  1074  ATGAATTAAATCTA  1087
     3175  ATGAATTAAAGCTA  3188
                    *

17.  1168  AATTAGCTCAT  1178
     3115  AATTAGCTCAT  3125

18.  a. 1387  AATCCGTTTTATA  1399
     b. 2163  AATCCGTTTTTTA  2175
     c. 3992  AATCCGTTTTTTA  4004
                       *

19.  1406  TGAATTTGCTT  1416
     4788  TGAATTTGCTT  4798

20.  1535  ATTTCATCAA  1544
     3389  ATTTCATCAA  3398

21.  1542  CAATACTCAA  1551
     4547  CAATACTCAA  4556

22.  1594  TAAATCATTTGGCATA  1609
     3448  TAAATCATTTGTCATA  3463
                     *

23.  1629  TAAAACAACAAG  1640
     1665  TAAAACAACAAG  1676
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24.  1696  TATAACAGGAAATTTGTTGTC  1716
     1718  TATAACAGGAAATTTGTTGTC  1738

25.  1701  CAGGAAATTTGTTGTCTTATAACAGGAAATTTGTTGTCGTATAA  1744
     1745  CAGGAAATTTGTTGTCGTATAACAGGAAATTTGTTGTCGTATAA  1788
                              *
26.  1701 CAGGAAATTTGTTGTCTTATAA  1722
     1723 CAGGAAATTTGTTGTCGTATAA  1744
     1745 CAGGAAATTTGTTGTCGTATAA  1766
     1767 CAGGAAATTTGTTGTCGTATAA  1788
                              *

27.  a. 1701   CAGGAAATTTGTT  1713
     b. 1723   CAGGAAATTTGTT  1735
     c. 1745   CAGGAAATTTGTT  1757
     d. 1767   CAGGAAATTTGTT  1779
     e. 6148   CAGGAAATTTTTT  6160
                           *

28.  1836  TAAAAAACAAA  1846
     3527  TAAAAAACAAA  3537

29.  1871  TCCCTTTTTTG  1881
     6918  TCCCTTTTTTG  6928

30.a. 1881 ....GGGCTTTCAGCCCTAATTTTTTCTTTTTT CAGGATTAAAAATTACAAA
   b. 3708 ....GGGCTTTCAGCCCTAATTTTTTCTTTTTTTCAGGATTTAAAATTACAAA
                                                *       *
1929 ACCCTTACAGAGCAAGTAAACTTGTTTGCTTGTTCTGCAAGGGTTCAGCAACCGAAGCCG
3757 ACCCTTACAGAGCAAGTAAACTTGTTTGCTTGTTCTGCAAGGGTTCAGCAACCGTAGCCG
                                                           *
1989 TTAGGCGTAGG CGGTAGCCTATAAAAGCCATTTAATTTTATCTTTAAATTTCCGTTTAA
3817 TCAGGCGTAGGGCGGTAGCCTATAAAAGCCATTTAATTTTATCTTTAAACTTCCTTTTAA
      *          *                                      *     *
2048 ATGCTTTGAGTGGGTGTCTTTTATCGTACTCATCAATCCTTTTTTGCATTCTTTCGTTTG
3877 ATGCTTTGAGTGGGTGTCTTTTATCGTACTCATCAATCCTTTTTTGCATTCTTTCGTTTG

2108 CTTTGTGATCGGCAAATTTTGAATAAGATTTTTCCATCTCATCTAACATTCTATCAATCC
3937 CTTTGTGATCGGCAAATTTTGAATAAGATTTTTCCATCTCATCTAACATTCTATCAATCC

2168 GTTTTTTATGTTGCCATTTCAGGTAAACATAAACACTTATAGCAATAAAAGACAATATCA
3997 GTTTTTTATGTTGCCATTTCAGGTAAACATAAACACTTATAGCAATTAAAGACAATATCA
                                                      *
2228 ATACATTGTAAAAAATGATTGTTACAATTTCGCTCACAGTTATTTTTTACCTTTTTCAAT
4057 ATACATTGTAAAAAATGATTGTTACAATTTCGCTCACAGTTATTTTTTACCTTTTTCAAT
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2288 TTCTTCATTGATAAATGCACTCAATTCATCAAATTTCTTGTCATCATTGATAAATTTACG
4117 TTCTTCATTGATAAATGCACTCAATTCATCAAATTTCTTGTCATCATTGATAAATTTACG

2348 CAACTTAGGGAAGTTTCTATCTACATCTAAAAGAGGGTTA  2387
4177 CAACTTAGGGAAGTTTCTATCTACATCTAAAAGAGGGTTA  4216

31.  1911  AGGATTAAAAA  1921
     3352  AGGATTAAAAA  3362

32.  1955  TGCTTGTTCT  1964
     4285  TGCTTGTTCT  4294

33.  2085  CCTTTTTTGC  2094
     6920  CCTTTTTTGC  6929

34.  2086  CTTTTTTGCA  2095
     7328  CTTTTTTGCA  7337

35.  2207  TAGCAATAAA  2216
     4872  TAGCAATAAA  4881

36.  2279  TTTTTCAATTTCTT  2292
     4108  TTTTTCAATTTCTT  4121

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

38   2296  TGATAAATGC  2305
     4125  TGATAAATGC  4134
     6850  TGATAAATGC  6859

29.  2331  TCATTGATAAAT  2343
     4121  TCATTGATAAAT  4133

39.  2603  TGCCGTAAATCT  2614
     6165  TGCCGTAAATGT  6176
                    *

40.  2655  GGATTTTTAGG  2665
     3344  GGATTTTTAGG  3354

41.  2885  ATTTCATTTA  2894
     4223  ATTTCATTTA  4232
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42.  2915  TTTTTAAAAT  2924
     3155  TTTTTAAAAT  3164

43.  3278  TCAGGATTTA  3287
     3737  TCAGGATTTA  3746

44.  3280  AGGATTTACATCG  3292
     5597  AGGATTTACAGCG  5609
                    *

45   3514  AAATGGTGGTCA  3525
     6459  AAATGGTGGTCA  6470

46.  3542  ACTATAACAA  3551
     5621  ACTATAACAA  5630

47.  3543  CTATAACAATAAATTTGTCCACCTATAA  3570
     3587  CTATAACAATAAATTTGTCCACCTATAA  3614

48.  a. 3543  CTATAACAATAAATTTGTCCAC  3564
     b. 3565  CTATAACAATAAATTTGTCCAC  3586
     c. 3587  CTATAACAATAAATTTGTCCAC  3608

49.  3652  TACAAACAAGAA  3663
     3664  TACAAACAAGAA  3675

50.  3783  TGCTTGTTCT  3792
     4235  TGCTTGTTCT  4294

51.  3915  CTTTTTTGCA  3924
     7328  CTTTTTTGCA  7337

52.  4121  TCATTGATAAAT  4132
     4160  TCATTGATAAAT  4171

53.  4231  TAGCCAAAAA  4240
     5766  TAGCCAAAAA  5775

54.  4249  TCTGCTTGTT  4258
     4383  TCTGCTTGTT  4392

55.  4306  CGCACGAATT  4315
     5133  CGCACGAATT  5112

56.  4597  AAAAGCAAAG  4606
     5682  AAAAGCAAAG  5691
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57.	4950	AGAACGCCAA	4959
	5238	AGAACGCCAA	5247
58.	5185	AGAGCAAGAA	5194
	5229	AGAGCAAGAA	5238
59.	5188	GCAAGAATTGA	5198
	5211	GCAAGAATTGA	5221
60.	5286	CAAAGAAAAA	5295
	5687	CAAAGAAAAA	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 Tn*A*/Tn*2*

```
1.   397   AGATCCTTTTTGAT   410
     390   TCTAGGAAAATTTA   377

2.   1201  GTTGAAA TAA  1210
     1104  CAACTTTTATT  1094
                 *

3.   1282  CATTGCAATTCTAA  1295
     1194  GTAA GTTAAGCTT  1182
               *      *

4.   1652  TGTTGT TTAT  1661
     1617  ACAACAGAATA  1607
                 *

5.   1663  TATAAAACAACAAGTATTTT  1682
     1662  ATATTT GTTGTCCATATAA  1644
                 *     *    *

6.   1696  TATAACAGGAAATTTGTTGTCTTAT  1720
     1630  AT TTGTCCTTTAAACAACAGAATA  1610
             *

7.   1711  GTTGTCTTATAACAGGAAATTTGTTGTCGTAT  1742
     1637  CAACAAAAT TTGTCCTTTAAACAACAGAATA  1607
                 *   *                    *

8.   1709  TTGTTGTCTTATA  1721
     1675  AACAACAAAATAT  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  AACAGGAAATTTGTTGTCGTAT  1764
     1628  TTGTCCTTTAAACAACAGAATA  1607
                          *

10.  1828  AAAACAAGTAAAA  1840
     1817  TTTTGGTCATTTT  1805
               *

11.  1898  TTTTTCTTTTTT  1909
     1848  AAAAACAAAAAA  1837
               *

12.  1962  TCTGCAAGGGTT  1973
     1939  AGACATTCCCAA  1928
               *

13.  3556  TT TGTCCACCT  3566
     3481  AATACAGGTGGA  3470
             *

14.  3578  TT TGTCCACCT  3588
     3481  AATACAGGTGGA  3470
             *

15.  3696  AGGCTCTTTTT  3706
     3693  TCCGAGATAAA  3683
                 *

16.  3790  TCTGCAAGGGTT  3801
     3767  AGACATTCCCAA  3756
               *

17.  3848  TT TAATTTTA  3857
     3755  AACATTAAAAT  3745
             *

18.  4473  TAAAATAACG TAAT  4486
     4431  ATTTTATTGCTATTA  4417
                     *

19.  4478  TAACGTAATGCCCA  4491
     4474  ATTGCATTACTTGT  4461
                     **
```

```
20.  4662  GCGGTTAGATG  4672
     4644  CGCCAA CTAC  4635
                *

21.  5488  GCAGATTTTGA  5498
     5469  CG CTAAAACT  5460
             *

22.  6053  GTTGCAAATTCTTTAAGGGTT  6073
     6050  CAACGCTCAAGAAATTCCCAA  6030
                * *

23.  6157  TTTTTGATTGC  6167
     6084  AAAAA TAACG  6075
                *

24.  6531  GCCGACAGGA  6540
     6483  CGGCTGTCCT  6474
```

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