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GENETIC AND MOLECULAR CHARACTERIZATION OF ORIGINS OF REPLICATION FROM β-LACTAMASE-PRODUCING PLASMIDS OF *NEISSERIA GONORRHOEAE*

A Thesis

Presented to

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of

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by

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Department of Biochemistry, Microbiology and Immunology

Faculty of Medicine

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ABSTRACT

Plasmids are extra-chromosomal, self-replicating DNA molecules found in both Gram negative and Gram positive bacteria, including some yeasts and fungi. The β-lactamase-producing plasmids of the sexually transmitted disease pathogen, *Neisseria gonorrhoeae*, were first discovered in 1976 simultaneously in Asia and Africa. While there have been many epidemiological investigations, there has always existed controversy with respect to the nature of their structural organization and evolutionary relationships. This work represents a comprehensive analysis of the precise structural relationship and organization existing amongst the gonococcal β-lactamase-producing plasmids. The second major aspect of this work deals with investigations into the genetic and molecular characterization of the multiple origins of replication of the gonococcal β-lactamase-producing plasmids.

The Asia-plasmid, pJD4, a prototype gonococcal β-lactamase-producing plasmid, is 7,426 bp and contains two large, direct repeats (DR-30A, 507 bp and DR-30B, 509 bp) which are implicated in the formation of deletion variant plasmids, such as the naturally-occurring Africa-type plasmid. The deletion observed in Africa-type plasmids, represented by pJD5, is 1,827 bp. One of the DR-30 repeats is also missing in the formation of Africa plasmids. The deletion in the Rio-type and several Toronto-type is 2,273 bp and the sequence spanning the deletion was identical irrespective of geographic or temporal origin. Thus, the Rio and Toronto-type plasmids are identical. The Nîmes-type plasmid is proposed to be identical to the Africa-type but contains an IS*5* insertion sequence. Since IS*5* has not been identified in gonococcal isolates and is not present in the gonococcal genome, it is suggested that this sequence was inserted after the original gonococcal plasmid was

transformed into a strain of *Escherichia coli*. The New Zealand plasmid was shown to be an Asia-type plasmid which contains an endogenous tandem duplication of 1,883 bp. The direct repeat DR-2 is implicated in this duplication.

Branch-point analysis by electron microscopy indicated that the Asia-type plasmid contains three origins of replication, named *ori1*, *ori2*, and *ori3*. Although pJD4 belongs to the incompatibility (Inc) group IncW, it also carries a silent IncFII determinant which is expressed when *ori2* and *ori3* are absent. The Africa-type plasmid was shown to carry only *ori1*, belongs to the IncFII group, and, in contrast to pJD4, requires DNA polymerase I for replication. Plasmid constructs from pJD4 lacking *ori1* but carrying *ori2* and *ori3* are incompatible with IncW plasmids, suggesting the *ori2/ori3* region contains the IncW determinant. A novel replication initiation protein, RepB, was identified and shown to be necessary for *ori2* and *ori3* to function. The RepB is distinct from RepA, the replication initiation protein required for plasmids carrying *ori1*. Plasmid pJD4 is the smallest plasmid characterized containing three origins of replication.

Using DNA sequence analysis, important regions have been identified relating to origin of replication usage. Unusually, nine integration host factor (IHF) binding sites (versus one for plasmids requiring IHF) were located on pJD4. Several IHF binding domains were altered using site-directed mutagenesis and their effects were examined *in vivo*. Deletion of IHF binding sites 3 and 8/9 were found not to significantly alter the plasmid copy number relative to wild-type pJD4, whereas, deleting binding domains 4 and 7 caused an apparent increase in copy number. In contrast, collectively deleting binding domains 4, 7, 8 and 9 caused a 20% decrease in copy number. IHF binding domains 5 and 6 appeared essential, as no plasmid replication was possible when these sites were removed. Gel

retardation assays, using purified gonococcal IHF protein, further confirmed IHF binding at the replication origins of pJD4. Overall, the data demonstrate that the gonococcal IHF protein is an essential component for efficient replication of gonococcal β-lactamase-producing plasmids.

Genetic manipulation of the gonococcus has been limited to natural transformation, with the incoming DNA recombining into the chromosome via allelic-replacement. Using data collected during this work, the first shuttle vectors capable of replicating in *N. gonorrhoeae*, *Haemophilus influenzae*, *Haemophilus ducreyi*, and *E. coli* were constructed. The gonococcal *proAB* genes were cloned into the vector to complement proline-requiring *N. gonorrhoeae* strain F62 and *E. coli* HB101 *in trans*. Equally, the vector was used to show, for the first time, the expression of the green fluorescent protein (GFP) in both the gonococcus and in *H. ducreyi*.

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RÉSUMÉ

Les plasmides sont des molécules d'ADN extra-chromosomiques autoréplicatives qui sont présentes dans les bactéries Gram-négatives et Gram-positives de même que chez certaines levures et moisissures. Les plasmides producteurs de β-lactamase de *Neisseria gonorrhoeae*, un agent pathogène transmissible sexuellement, furent découverts simultanément en Asie et en Afrique en 1976. Bien qu'il y ait eu de nombreuses enquêtes épidémiologiques, la nature de leur organisation structurale et de leurs liens évolutionnels ont toujours été source de controverse. Le présent travail constitue une analyse détaillée de la relation structurale et de l'organisation des plasmides gonococcaux producteurs de β-lactamase. Le second aspect principal du présent travail porte sur l'étude de l'organisation génétique et moléculaire des multiples origines de réplication des plasmides gonococcaux producteurs de β-lactamase.

Le plasmide asiatique, pJD4, l'archétype du plasmide gonococcal producteur de β-lactamase, a une taille de 7426 paires de bases (pb) incluant deux longues séquences répétitives consécutives (DR-30A, 507 pb et DR-30B, 509 pb) qui sont impliquées dans la formation de divers plasmides de délétion tel que le plasmide de type africain, un plasmide survenu naturellement. La délétion observée dans les plasmides de type africain, représenté par pJD5, consiste en 1827 pb. L'une des séquences répétitives DR-30 est également manquante chez les plasmides de type africain. La délétion dans le plasmide de type Rio et dans plusieurs plasmides de type Toronto a une taille de 2273 pb et la séquence flanquant les délétions est identique peu importe l'origine géographique ou temporelle du plasmide. Les plasmides de type Rio et de type Toronto sont donc identiques. Il est proposé que le plasmide

de type Nîmes est identique au plasmide de type africain mais il contient une séquence d'insertion IS5. Puisque l'IS5 n'a été identifié dans aucune souche de *N. gonorrhoeae* et qu'elle n'est pas présente dans son génôme, il a été proposé que l'IS5 fut insérée à la suite de l'entrée par transformation du plasmide gonococcal originel chez *Escherichia coli*. Il a été démontré que le plasmide de type Nouvelle-Zélande est un plasmide de type asiatique contenant une duplication endogène en tandem de 1883 pb. La répétition directe DR-2 est impliquée dans cette duplication.

L'analyse de ramification ("branch-point analysis") par microscopie électronique a indiqué que le plasmide de type asiatique contient trois origines de réplication nommées *ori1*, *ori2* et *ori3*. Malgré le fait que pJD4 fasse partie du groupe d'incompatibilité (Inc) IncW, il contient néanmoins un déterminant IncFII qui est exprimé en l'absence d'*ori2* et d'*ori3*. Le plasmide de type africain contient seulement *ori1* et fait partie du groupe d'incompatibilité IncFII; contrairement à pJD4 il requiert l'ADN polymérase I pour sa réplication. Les plasmides construits à partir de pJD4 ne contenant pas *ori1* mais contenant *ori2* et *ori3* sont incompatibles avec les plasmides IncW, suggérant que la région *ori2/ori3* contienne le déterminant IncW. Une nouvelle protéine impliquée dans l'initiation de la réplication, RepB, a été identifiée et il a été démontré qu'elle est essentielle au bon fonctionnement de *ori2* et *ori3*. RepB est différente de RepA, la protéine initiatrice nécessaire à la réplication des plasmides contenant *ori1*. Le plasmides pJD4 est le plus petit plasmide contenant trois origines de réplication ayant été caractérisé.

L'analyse des séquences d'ADN des plasmides a mené à l'identification d'autres régions importantes liées à l'utilisation de l'origine de réplication. Fait inusité, neuf sites de liaison au facteur d'intégration de l'hôte ("integration host factor" ou IHF) furent identifiés

chez pJD4 comparativement à un seul chez les plasmides ayant besoin de l'IHF. Plusieurs sites de liaison de l'IHF furent modifiés par mutagénèse dirigée et l'effet de ces changements fut examiné *in vivo*. La délétion des sites de liaison à l'IHF 3 ou 8/9 n'affecte pas de façon significative le nombre de copies du plasmide en comparaison à celui de pJD4 tandis que la délétion des sites de liaison 4 et 7 affecte vraisemblablement à la hausse le nombre de copies du plasmide. Par contre, la délétion combinée des sites de liaison 4, 7, 8 et 9 provoque une baisse de 20% du nombre de copies du plasmide. Les sites de liaison à l'IHF 5 et 6 semblent essentiels puisque qu'il n'y a aucune réplication plasmidique si ces sites sont éliminés. Des expériences de changement de mobilité sur gel ("gel retardation assay"), effectués avec la protéine IHF gonococcale pure a également prouvé la liaison de la protéine IHF aux origines de réplication de pJD4. Dans l'ensemble, les résultats montrent que la protéine IHF gonoccale est une composante essentielle pour la réplication efficace des plasmides gonococcaux producteurs de β-lactamase.

Les manipulations génétiques du gonocoque sont limitées à la transformation naturelle au cours de laquelle l'ADN dit "arrivant" est recombiné dans le chromosome par remplacement allélique. A partir des résultats obtenus dans le cadre du présent travail, les premiers vecteurs navette pouvant de se répliquer chez *N. gonorrhoeae*, *Haemophilus influenzae*, *Haemophilus ducreyi* et *E. coli* ont été construits. Les gènes *proAB* du gonocoque furent clonés dans le vecteur afin de complémenter en *trans* les besoins en proline des souches *N. gonorrhoeae* F62 et *E. coli* HB101. De plus, à l'aide de ce vecteur, l'expression de la protéine verte fluorescente ("green fluorescent protein") a été démontrée pour la première fois dans le gonocoque et dans *H. ducreyi*.

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ABBREVIATIONS

bla	beta-lactamase
BHI	brain heart infusion
bp	base pair
CFU	colony forming unit
dH_20	distilled water
dNTPs	deoxyribonucleoside 5'-triphosphate
DNA	deoxyribonucleic acid
DR	direct repeat
EDTA	ethylenediaminetetra acetic acid
GCMB	GC-agar base
GFP	green fluorescent protein
HU	histone-like protein
IHF	integration host factor
Inc	incompatibility
IR	inverted repeat
IS	insertion sequence
kb	kilobase
kDa	kilodalton
LB	Luria Bertani broth
Mda	megadalton
Mdr	multidrug resistance

MIC	minimum inhibitory concentration
Mtr	multiple transferable resistance
MW	molecular weight
Ori	origin of replication
OriT	origin of transfer
ORF	open reading frame
PCR	polymerase chain reaction
PolA	DNA polymerase
PolI	DNA polymerase I
PPNG	penicillinase-producing *Neisseria gonorrhoeae*
RBS	ribosome binding site
R/E	restriction endonuclease
Rep	replication initiation protein
RNA	ribonucleic acid
RNAP	RNA polymerase
SDA	sodium dodecyl sulfate
STD	sexually transmitted disease
Tn	transposon
TnpR	resolvase
TSA	trypticase soy agar
TSB	trypticase soy broth
YT	yeast tryptone

CHAPTER 1.1

SPECI FIC AIMS, AND HYPOTHESIS

Despite the significant advances that have been made in our knowledge of the mechanisms of DNA replication, there still remains much unknown at the molecular level of the control of initiation of DNA replication in any organism . Because of experimental difficulties inherent in the genetic and molecular analysis of the processes of regulation of chromosomal DNA replication, considerable effort has been expended in the analysis of replication control using bacterial plasmids as model systems.

Plasmids are extrachromosomal self replicating DNA entities that evolved intrinsic regulatory pathways that ensure their stable maintenance at a defined copy-number in different hosts. Because plasmids are not essential for host survival, they are particularly well suited for genetic studies to investigate the control mechanisms of replication.

Penicillinase production in isolates of *Neisseria gonorrhoeae* is mediated by a related family of plasmids which carry deletions or insertions in relation to the 7.4 kb prototype Asia plasmid pJD4 (Dillon and Yeung, 1989; Pagotto *et al.*, 2000a). Gonococcal strains containing these plasmids comprise from 10 to over 50% of all gonococcal isolates worldwide, thereby necessitating the use of drugs other than penicillin for treating gonorrhoea (Lind, 1997).

Studies on the role and interaction of factors for replication initiation have mostly involved plasmids with single origins of replication (Korberg and Baker, 1992; Helinski *et al.*, 1996). In those rare plasmids with multiple origins of replication, a variety of factors, including host background, must determine which origin takes precedence. This thesis presents a detailed examination of a unique family of broad host range plasmids, each having a different host range which includes *Escherichia coli* as the common "lab" host. In the studies that follow, focus is placed on plasmid encoded factors and a major auxilliary, host-

encoded protein, the integration host factor (IHF), shown in the past to be intimately involved in a number of interactions with DNA, including replication, recombination and transcription.

By analyzing those factors needed for activating origins of multicomplex replicons, a better understanding of the mechanisms of replisome formation and the definition of molecular components participating in this process can be achieved. DNA replication is central to all growing organisms and many features are shared by non-plasmid replicons and bacteriophages. Certain molecular components are intrinsic and common to the replication process in general (Kornberg and Baker, 1992). Thus, knowledge obtained from such investigations may well be extended to other and seemingly distant replication systems, including bacterial chromosomes and their phages, viral replication and perhaps, replication of eukaryotic chromosomes. This work will also provide insight into the host mediated regulation of origin selection and usage. Broad host range plasmids are implicated as vehicles for the spread of newly re-emerged antibiotic resistance genes in bacteria. Therefore, development of a new generation of antimicrobial therapies targeted at various components of the replication machinery may also provide new avenues for treatment of microbial infections.

Our laboratory has focussed on all aspects of plasmid biology in the gonococcus. There are discrepancies with respect to the origin, structure and relationship among this family of plasmids (Dillon an Yeung, 1989; Roberts, 1989; Gilbride and Brunton, 1990). Little work previously had been done on the origins of replication of this family of plasmids. The study of plasmid replication has relied on one major assumption: that neighbouring origins of replication in multicomplex (i.e. plasmids with three origins of replication)

plasmids influence replication processes, disrupting the hierarchical relationship of components used for single origins. Thus, the specific hypotheses for this work are as follows:

1. The gonococcal β-lactamases-producing plasmids are structurally and physically related to each other, possibly derived from a common ancestor

2. The progenitor, Asia-type plasmid, pJD4, has more than one origin of replication, capable of functioning in isolation from each other

3. Based on the structural relationship between gonococcal plasmids, there exist two different replication initiation proteins that are required for each of the replication origins found on pJD4. These replication initiation proteins differ with respect to which of the origin regions they control

4. The integration host factor, while supplied by the host, is not an auxiliary, but a necessary component of gonococcal plasmid replication

5. A new shuttle vector, capable of replicating in the gonococcus as well as enteric bacteria such as *E. coli*, can be made from data obtained from the genetic and molecular characterization of gonococcal plasmids

CHAPTER 1.2

ANTIBIOTIC RESISTANCE IN *NEISSERIA GONORRHOEAE*

(Part of this chapter was used in the following publication: Dillon, J.R., and Pagotto, F. (2000) Importance of drug resistance in gonococci: from mechanisms to monitoring. *Curr Opin Infect Dis* **12:** 35-40.)

1.2.1 INTRODUCTION

The challenges posed by antibiotic resistant gonococci at the turn of the millennium remain similar to those when the first penicillinase-producing strain of *Neisseria gonorrhoeae* (PPNG) was isolated in 1976. Thirty regimens comprising 21 antimicrobial drugs are effective for treating gonococcal rectal and urogenital infections (Moran and Levine, 1995), including third generation cephalosporins, quinolone drugs and spectinomycin. However, their long term efficacy is now in doubt.

Although the number of gonorrhea cases in many developed and developing countries has declined since the mid-1980s, recent data indicated a reversal in annual decreases observed in past years (Division of STD Prevention, 1999). The percentage of antibiotic resistant gonococcal isolates has increased (Lind, 1997, Hiltunen-Back *et al.*, 1998; Ison *et al.*, 1998). Since the start of the antimicrobial era, treatment regimens using sulfa drugs, penicillin, tetracycline and aminoglycosides, have fallen by the wayside as gonococcal isolates have become resistant. Gonococcal resistance to antimicrobial drugs is caused primarily by chromosomal mutations or by the acquisition of plasmids carrying resistance determinants to penicillin or tetracycline (Jephcott, 1986; Dillon and Yeung, 1989; Lind, 1997; Ison *et al.*, 1998). Often several mechanisms are present in the same resistant strain. An often observed resistance in gonococcal isolates worldwide has been plasmid-mediated resistance to penicillin (PPNG isolates) and is encoded by a TEM-1-type β-lactamase which is carried on related plasmids (Jephcott, 1986; Dillon and Yeung, 1989; Roberts, 1989). In many regions, including many countries of the Western Pacific, Latin America and Africa, over 50% of all gonococcal isolates are PPNG (Dillon *et al.*, 1997; Lind, 1997; Tapsall *et al.*, 1997; Ison *et al.*, 1998). In industrialized countries, the percentage of PPNG is often high

enough to preclude penicillin for treatment (Moran and Levine, 1995). Recently, the prevalence of PPNG isolates has been slowly declining in many countries (Ison *et al.*, 1998); this has been attributed to a decrease in selective pressure through the use of drugs other than penicillin to treat gonorrhea, and, interestingly, a possible plasmid curing effect which may be mediated by the widespread use of quinolones (Ross, 1998).

Strains with high level plasmid-mediated tetracycline resistance contain the *tetM* determinant on a conjugative plasmid (Morse *et al.*, 1986; Roberts, 1989). Reports of high-level tetracycline resistance have increased during the 1990's and in many countries now comprise over 50% of the isolates (Adegbola *et al.*, 1997; Chalkley *et al.*, 1997; Dillon *et al.*, 1997; Ferreira *et al.*, 1997; Tapsall *et al.*, 1997; Djajakusumah *et al.*, 1998;). In addition, the total burden of chromosomal resistance to tetracycline is rising significantly in regions such as the Americas and the Caribbean (Figure 1.2.1). This increase is probably caused by the continual selective pressure exerted by the simultaneous treatment with tetracycline for possible chlamydial infections combined with the popularity of this drug as an over-the-counter, low cost remedy in many countries. This pressure may also account for the increase in chromosomal resistance to penicillin through the development of a multi-drug resistant phenotype. The extent of chromosomal resistance to antimicrobial drugs is often not appreciated since it can only be ascertained through antimicrobial susceptibility testing. A number of chromosomal mutations contribute to resistance, some specifying resistance or decreased susceptibility to several drugs simultaneously, and others producing low level

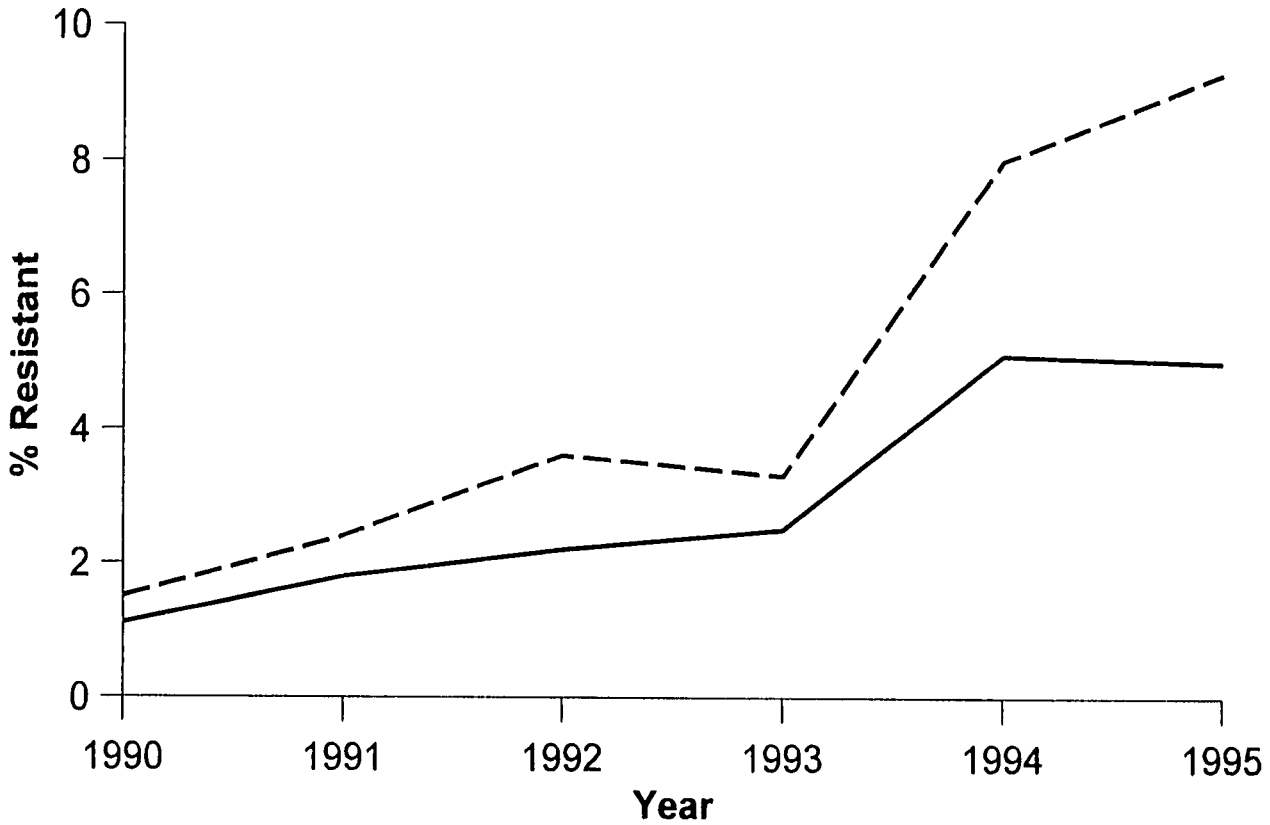

Figure 1.2.1. Chromosomal resistance of *N. gonorrhoeae* to penicillin and tetracycline in the Americas and the Caribbean from 1990 to 1995. Chromosomal resistance (includes single and multiresistant isolates): to penicillin (solid line) and tetracycline (broken line). n: 1990=18800, n: 1991=17900, n: 1992=15200, n: 1993=12200, n: 1994=11320 (pen), 11200 (tet), n: 1995=10400. Data for this figure were obtained through the GASP-Americas and Caribbean Network

decreases in susceptibility to individual drugs (Jephcott, 1986; Dillon and Yeung, 1989; Ison *et al.*, 1998; Table 1.2.1). The effects of these mutations can be additive (Jephcott, 1986). Other chromosomal mutations produce high level resistance of the type observed with on-going sporadic reports of resistance to spectinomycin (Dillon *et al.*, 1997; Tapsall *et al.*, 1997; Table 1.2.1).

1.2.2 MULTIDRUG RESISTANCE

Multidrug resistance (Mdr) is a term that is commonly used to describe those resistance mechanisms mediated by chromosomal mutations caused by the exposure of the organism to antibiotics in natural or clinical environments. The main characteristic of the Mdr phenotype is that there is no change or enzymatic breakdown of the drug(s) affected. Exposure to sub-lethal concentrations of a single drug usually leads to resistance to that drug and cross-resistance to many other drugs which are generally functionally and/or structurally unrelated (George, 1996). The only mechanism described for bacterial Mdr systems is drug efflux by membrane transporters, although many of these transporters have yet to be identified. Gram-positive bacteria have a single gene encoding an efflux transporter for unrelated drugs (Ahmed *et al.*, 1995), while the efflux genes in Gram-negative microorganisms generally comprise operons or regulons which encode repressors and transcriptional activators in addition to the efflux protein (George, 1996; Miller and Sulavik, 1996). In *N. gonorrhoeae*, the Mdr phenotype is encoded by the chromosomal *m*utiple *t*ransferable *r*esistance (*mtr*) locus which is organized as an operon and which forms an energy-dependent efflux pump (Pan and Spratt, 1994; Hagman *et al.*, 1995; Hagman and Shafer, 1995; Lucas *et al.*, 1995; Hagman *et al.*, 1997; Delahay *et al.*, 1997). The *mtrCDE*

Table 1.2.1. Chromosomal mutations in *Neisseria gonorrhoeae* resulting in antibiotic resistance.

Genotype	Phenotype	Reference(s)
ampA, ampB	low-level resistance to ampicillin	Jones *et al.*, 1985
ampC, ampD	4-fold increase in MIC of ampicillin when combined with *ampA/ampB*	Jones *et al.*, 1985
ery	low-level resistance to erythromycin	Maier *et al.*, 1975
env (*env*-1, *env*-2, *env*-10)	increased resistance to antibiotics, Triton-X100, fusidic acid, crystal violet, and phenotypic suppression of *mtr* and *penB*	Sparling *et al.*, 1975; Lysko and Morse, 1981; Shafer *et al.*, 1984
fus, tet, chl	4-fold increase in tetracycline, chloramphenicol, and fusidic acid resistance	Sparling *et al.*, 1975; Cannon and Sparling, 1984
mtr	low-level resistance to several unrelated antibiotics, dyes, fatty acids, and detergents	Maier *et al.*, 1975; Sparling *et al.*, 1975
mom	modifier of *mtr* (suppresses *mtr* phenotype)	Shinners and Catlin, 1988
penA	4-8-fold increase in resistance to β-lactams	Cannon and Sparling, 1984; Dougherty, 1985
penB, tem	low-level penicillin and tetracycline resistance if *mtr* present	Dougherty, 1985; Dougherty, 1985
rif	low-level resistance rifampin resistance	Sparling *et al.*, 1975; Cannon and Sparling, 1984
spc, str	high-level spectinomycin and streptomycin resistance	Maier *et al.*, 1975; Cannon and Sparling, 1984
vnc	hypersusceptibility to vancomycin	Koelbl and Catlin, 1986
vel	hypersusceptibility to vancomycin and erythromycin	Koelbl and Catlin, 1986

complex forms a single transcriptional unit encoding the efflux pump while an upstream regulatory (repressor) gene, *mtrR*, is transcribed in the opposite direction (Hagman *et al.*, 1995). The gonococcal efflux pump proteins are similar to proteins in *Pseudomonas aeruginosa* and *Escherichia coli*, except, while the efflux pump in *P. aeruginosa* enhances resistance to ciprofloxacin, this is not the case in gonococcal isolates (Zhanel *et al.*, 1995; Delahay *et al.*, 1997; Hagman *et al.*, 1997).

Mutants of *N. gonorrhoeae* with the *mtr* phenotype were first described in the early 1970's (Maness and Sparling, 1973). The *mtr* phenotype confers resistance to hydrophobic agents (HAs) such as detergent-like fatty acids, bile salts as well as a number of antibiotics including erythromycin, tetracycline, chloramphenicol, rifampicin, and penicillin (Maness and Sparling, 1973; Hagman *et al.*, 1995; Shafer *et al.*, 1995; Veal *et al.*, 1998). Fatty acids and bile salts are present in the rectum and are antimicrobial (Shafer *et al.*, 1995). Yet, rectal isolates are more resistant to HAs than isolates from other body sites (Morse *et al.*, 1982). In addition to the *mtr* locus, the outer membrane is an important structural determinant in Gram-negative bacteria in controlling the entry of hydrophobic agents. Gonococci have different lipooligosccharide (LOS) structural requirements for *mtr*-mediated resistance to different HAs on the basis of their lipophilic properties (Lucas *et al.*, 1995).

Recently, the role of the *mtr* locus in determining gonococcal susceptibility to antibacterial, cationic peptides produced in vertebrates has been investigated (Shafer *et al.*, 1998). Antibacterial peptides are present in some phagocytic cells and may also be synthesized in epithelial cells (Shafer *et al.*, 1998). Two classes of cationic peptides include defensins and protegrins. While defensins have no activity against gonococcal cells, protegrins, which are of porcine origin, affect gonococcal membrane structure and inhibit

gonococcal growth (Qu *et al.*, 1997). The protegrins also have activity against the elementary bodies of *Chlamydia trachomatis* and are considered promising topical microbicides for the control of STDs (Yasin *et al.*, 1996). This potential may now be limited in view of the results from a recent study indicating that the *mtr* efflux system influences gonococcal susceptibility to antibacterial peptides (Shafer *et al.*, 1998). Gonococcal isolates in which the *mtrC*, *mtrD* or *mtrE* genes were insertionally inactivated were more susceptible to protegrin PG-1 and to other structurally different antibacterial peptides, suggesting that expression of the *mtr* efflux pump in the gonococcus could reduce the activity of antimicrobial peptides produced at mucosal surfaces.

1.2.3 FLUOROQUINOLONE RESISTANCE

Fluoroquinolone drugs, such as ciprofloxacin and ofloxacin, are generally effective against gonococcal strains which are resistant to penicillin and tetracycline, irrespective of the mechanisms of resistance. These drugs act by binding to proteins involved in DNA replication and inhibiting replication. For these reasons, and because of their overall safety and patient acceptability, they have been recommended in most countries as one of the first-line therapies for treating gonococcal infections (Moran and Levine, 1995). Isolates with either decreased susceptibility or resistance to these agents have been reported throughout the 1990s (Fox *et al.*, 1997; Knapp *et al.*, 1997; Tapsall *et al.*, 1998). There is mounting concern that ciprofloxacin may eventually follow the path of penicillin and tetracycline and be sidelined as an effective drug for treating gonorrhoea (Tapsall *et al.*, 1997; Ross, 1998). The prevalence of strains with reduced susceptibility to fluoroquinolone drugs has been reported at over 50% in isolates recently tested in Hong Kong and China, the Phillipines, Korea and Cambodia (Tapsall *et al.*, 1997; Ison *et al.*, 1998) and fluoroquinolone resistant

gonococcal strains are increasing in other geographical areas (Fox *et al.*, 1997; Harnett *et al.*, 1997; Knapp *et al.*, 1997; Ross, 1998; Tapsall *et al.*, 1998). At the same time, the percentage of resistant isolates (MIC ≥ 1) has risen in most regions of the world including the United Kingdom (Ross, 1998), the United States (Fox *et al.*, 1997; Knapp *et al.*, 1997), and countries of the western pacific region (Tapsall *et al.*, 1997; Ng *et al.*, 1998). On the other hand, gonococcal quinolone resistance in Latin America and the Caribbean (Dillon *et al.*, 1997; Swanston *et al.*, 1997; Ison *et al.*, 1998) is rare. This may reflect the more limited use of these drugs in the region. Given the increasing percentage of gonococcal isolates resistant to quinolones, susceptibility monitoring is essential to detect resistance early and to develop appropriate treatment policies.

The major mechanism of resistance to fluoroquinolone drugs in *N. gonorrhoeae* includes the development of mutations in the genes encoding DNA gyrase or topoisomerase IV (Belland *et al.*, 1994; Knapp *et al.*, 1997; Ross, 1998). DNA gyrase is composed of two A subunits and two B subunits encoded by the *gyrA* and *gyrB* genes, and topisomerase IV is encoded by the *parC* gene (Belland *et al.*, 1994; Knapp *et al.*, 1997; Ross, 1998). Although mutations in *gyrB* confer low level resistance (Deguchi *et al.*, 1997), these mutations are rarely found in gonococcal isolates. Mutations in GyrA, especially those which occur in amino acids Ser-91 and Asp-95, have been the most commonly identified (Tanaka *et al.*, 1998; Trees *et al.*, 1998). A mutation at position 91 has been also implicated in resistance to newer quinolone drugs, such as pazufloxacin, thereby reducing its potential for the treatment of gonococcal infections (Tanaka *et al.*, 1998). No single mutation in the *parC* has been noted without a coexisting mutation in *gyrA* (Knapp *et al.*, 1997; Ross, 1998; Trees *et al.*, 1998). Mutations in ParC, which have been documented in amino acids at

positions 86-88 and 91, act to increase resistance to higher levels (Trees *et al.*, 1998). Additional mutations in ParC corresponding to amino acid changes at positions 85 and 116 have been identified in clinical isolates resistant to fluoroquinolone drugs and double mutations in *parC* were also identified (Trees *et al.*, 1998). Isolates with double mutations in *parC* were characterized by high level ciprofloxacin resistance (MIC 8- 64 μg/ml) (Trees *et al.*, 1998). Several different patterns of mutations in both *gyrA* and *parC* have been described and the level of resistance to ciprofloxacin and to other quinolones has been found to be determined by the type and number of mutations in these genes (Ross, 1998; Trees *et al.*, 1998). Other mechanisms may also act to confer resistance to quinolone drugs, including reduced accumulation in cells (Corkhill *et al.*, 1991; Deguchi *et al.*, 1997; Tanaka *et al.*, 1998a, b).

Gonococcal isolates which are resistant to quinolone drugs generally remain susceptible to third generation cephalosporins. Based on findings in other genera (for example, *Pseudomonas*), this situation may be temporary as resistance to fluoroquinolones through a multiple drug resistance phenotype included cross resistance or reduced susceptibility to cephalosporins (Zhanel *et al.*, 1995). This phenomenon has recently been observed with clinical isolates of *N. gonorrhoeae* (Carlyn *et al.*, 1995; Deguchi *et al.*, 1997). The association between quinolone resistance and reduced susceptibility to ceftriaxone is controversial (Moss, 1997) but must be closely monitored. It is now apparent that *N. gonorrhoeae* isolates have developed an array of mechanisms to resist the action antimicrobial drugs used for treatment. This proclivity was first noted after the introduction of sulfa drugs over 60 years ago and continues unabated with newer drugs for treatment such as the quinolone drugs. Although plasmid-mediated resistance mechanisms have abolished

considerations of using inexpensive drugs such as tetracycline or penicillin for treatment, resistance to the currently recommended drugs or even newer topical microbicides will probably be caused by chromosomal mutations, although the possibility of new plasmid-mediated mechanisms of resistance is also real. Since many resistant phenotypes can be created in the laboratory before their selection in a clinical setting, it might be advantageous to evaluate the possible emergence of such resistance mechanisms in vitro, thus predicting resistant arising in clinical settings, and permitting rational design of new antimicrobial agents or evaluation of drugs already developed. Regional and international surveillance programs to monitor the emergence of resistance will become a critical and key component in public health initiatives to control gonococcal disease.

1.2.4 B-LACTAMASE-PRODUCING PLASMIDS

Before the definitive isolation of the β-lactamase-producing plasmids from isolates of *N. gonorrhoeae*, it was thought that only chromosomal mutation caused resistance to antimicrobial agents (Sparling *et al.*, 1978). In 1976, the first β-lactamase-producing plasmids were isolated in North America and the United Kingdom (reviewed in Dillon and Yeung, 1989). The plasmids isolated from the United Kingdom were associated with gonococcal strains from Africa and those from North America were linked to strains from the Far East (Elwell *et al.*, 1977; Perine *et al.*, 1977). The plasmids were named Africa- (5.1 kb) and Asia-type (7.4 kb) to reflect their geographical point of origination.

The Africa and Asia-type plasmids have been shown to be endemic in isolates from diverse locations, including North America, the Caribbean, Europe, as well as Africa and Asia (Roberts, 1989). A third gonococcal plasmid, capable of producing the same β-lactamase, was recovered from strains in two Canadian provinces, and was named Toronto

(Yeung and Dillon, 1985; Yeung *et al.*, 1986). While the Africa, Asia and Toronto-type plasmids have been implicated in outbreaks, two other plasmids have been described that have not. The first of these was isolated in the Netherlands (van Embden *et al.*, 1985). Further analysis of this plasmid epidemiologically linked it to Rio de Janiero and the plasmid was name Rio. The second plasmid was isolated in Nîmes, France, and was designated Nîmes (Gouby *et al.*, 1986). The Rio plasmid was reported as having a 100 bp deletion in its *Bam*HI-*Hin*dIII fragment (van Embden *et al.*, 1985); however, this small deletion was not observed in our laboratory (Dillon, unpublished).

The β-lactamase-producing plasmids were compared to each other using molecular analysis and hybridization studies. They were shown to contain a TEM-1 β-lactamase gene as well as carrying approximately 40% of the transposon Tn*2* (Fayet *et al.*, 1982; Roberts *et al.*, 1977; Sanchez-Pescador *et al.*, 1988). The TEM-1 β-lactamase is capable of hydrolyzing cyclic amide bonds in β-lactam molecules and inactivates benzylpenicillin, ampicillin, and cephaloridin substrates. It has low activity, however, against substrates such as azolyl, oxacillin and methicillin (Heffron *et al.*, 1977).

Gonococcal β-lactamase-producing plasmids are related to small β-lactamase-producing plasmids in various *Haemophilus* species (Brunton *et al.*, 1986a; Chen and Clowes, 1987). They were shown to differ from each other by small deletions or insertions in either the Tn*2* region encoding the β-lactamase or in the non-Tn*2* region (Dillon and Yeung, 1989; Roberts, 1989). The gonococcal plasmids were shown to have a G + C content similar to the chromosomal G + C content of *Haemophilus* (approximately 40%) whereas the G + C content of *Neisseria* chromosomal DNA is approximately 50% (Roberts *et al.*, 1977; Roe *et al.*, 1997).

Data collected hinted that they evolved from a single ancestral plasmid (Brunton *et al.*, 1986a; Dickgiesser *et al.*, 1982; Dickgiesser, 1984). This was based on the evidence that the 7 and 5.7 Mda plasmids from *H. ducreyi* were identical to the Asia and Africa-type gonococcal plasmids, respectively, with the exception that *H. ducreyi* plasmids carried a complete Tn*2* (Brunton *et al.*, 1986a; Chen and Clowes, 1987). The *H. ducreyi* plasmids also contained a DNA sequence of 273 bp adjacent to the left inverted repeat of the Tn*2* (Chen and Clowes, 1987). The gonococcal Asia and Africa plasmids were shown to lack a 3.2 kb DNA fragment encompassing this same left inverted repeat and its proximal sequences (Chen and Clowes, 1987). Two hypothetical plasmids, proposed to have originated in *Haemophilus* species, evolved independently and gave rise to 7 and 5.7 Mda plasmids in *H. ducreyi* (Chen and Clowes, 1987; Dillon and Yeung, 1989). This was supported by observations that a 2.5 Mda plasmid from *H. parainfluenzae*, while not carrying Tn*2* DNA sequences, was homologous to β-lactamase-producing plasmids from both *N. gonorrhoeae* and *H. ducreyi* (Brunton *et al.*, 1986b). The model put forth proposed that β-lactamase plasmids were transferred from *Haemophilus* species to gonococci (reviewed in Dillon and Yeung, 1989 and Roberts, 1989). However, only one report appears in the literature demonstrating the transfer of a plasmid from *Haemophilus* to *Neisseria* (Sparling *et al.*, 1978), and repeated attempts have not been successful by other groups.

CHAPTER 1.3

REPLICATION OF PLASMID DNA FROM GRAM-NEGATIVE BACTERIA

AND

GONNOCOCCAL β-LACTMASE PRODUCING PLASMIDS

1.3.1 INTRODUCTION

Plasmids which are found in most bacteria are extrachromosomal genetic elements that replicate independently from the host chromosome. Thought originally to be circular, covalently intact, double-stranded DNA molecules, plasmids may also exist as linear molecules within cells, as shown for plasmids of *Borrelia* and *Streptomyces* (Summers, 1996). The following is a brief review of the two major classes of plasmid replication mechanisms observed in Gram-negative bacteria studied to date, followed by what was known on gonococcal plasmid replication at the time this study was undertaken. It is not possible to properly address all aspects of plasmid replication, and many reviews have been written describing circular replicating plasmids (most often seen in Gram-positive bacteria)(Novick, 1989; Helinski *et al.*, 1996) and linear plasmids (Actis *et al.*, 1998).

There are two broad models for the replication of plasmids in Gram-negative bacteria, both of which are controlled by a negative feedback mechanism (Kornberg and Baker, 1992; Kues and Stahl, 1989; Helinski *et al.*, 1996). The first mechanism, RNA-regulation, can be divided into two categories based on whether plasmids have a high or low copy number (Asano and Mizobuchi, 2000). High copy number plasmids (e.g. pMB1, ColE1) are typified by the antisense RNA molecule (approximately 100 bases) which they encode that binds the preprimer RNA for the synthesis of the leading strand of DNA (Fig. 1.3.1; Eguchi *et al.*, 1991). Other features of these ColE1-type plasmids includes unidirectional replication from a single origin and a reliance on host encoded proteins for replication, including RNA polymerase (RNAP), DNA polymerase I (Pol I), and RNase H (Itoh and Tomizawa, 1980; Itoh and Tomizawa, 1982). ColE1 does not require *de novo* protein synthesis for initiation of replication.

Low copy number plasmids, such as those belonging to the incompatibillity (Inc) group FII, IncIα and IncB family, control their copy numbers by encoding antisense RNA molecules that fold into stem-loops thereby controlling the expression of the plasmid-encoded replication initiator protein (Rep) (Fig. 1.3.2; Asano *et al.*, 1991). These antisense RNA molecules hybridize to the *rep* messenger RNA (mRNA), and prevent the translation of the Rep protein (Asano *et al.*, 1999). In IncFII plasmids, typified by R1, the antisense RNA molecule regulating the translation of RepA is named *copA* (Blomberg *et al.*, 1992).

The second mechanism of replication, based on iteron regulation, is exemplified by plasmids such as pSC101 and F (Kues and Stahl, 1989; Fig. 1.3.3). These plasmids have multiple binding sites that compete with the origin for the Rep protein, decreasing the initiation of replication frequency (Kues and Stahl, 1989). Common structural features of such plasmids include an A-T rich region, one or more clusters of direct repeats (iterons) acting in *cis*, and DnaA binding sequences, all part of the minimal origin of replication (Fig. 1.3.3; Linder *et al.*, 1985; Filutowicz *et al.*, 1987; Kues and Stahl, 1989). The Rep protein is the only plasmid encoded protein required for replication. DnaA is supplied by the host and acts in a cooperative fashion with Rep to activate the origin. Iteron-regulated plasmids do not require Pol I for replication initiation. The integration host factor (IHF), supplied by the host, may act as an auxilliary protein in the inititiation process (Kues and Stahl, 1989; del Solar *et al.*, 1998). The bending function of IHF allows certain proteins (e.g. Rep and DnaA) to be brought in close proximity, ensuring that denaturing events occur and plasmid replication begins (Fig. 1.3.4; Kues and Stahl, 1989; del Solar *et al.*, 1998; see Chapter 5).

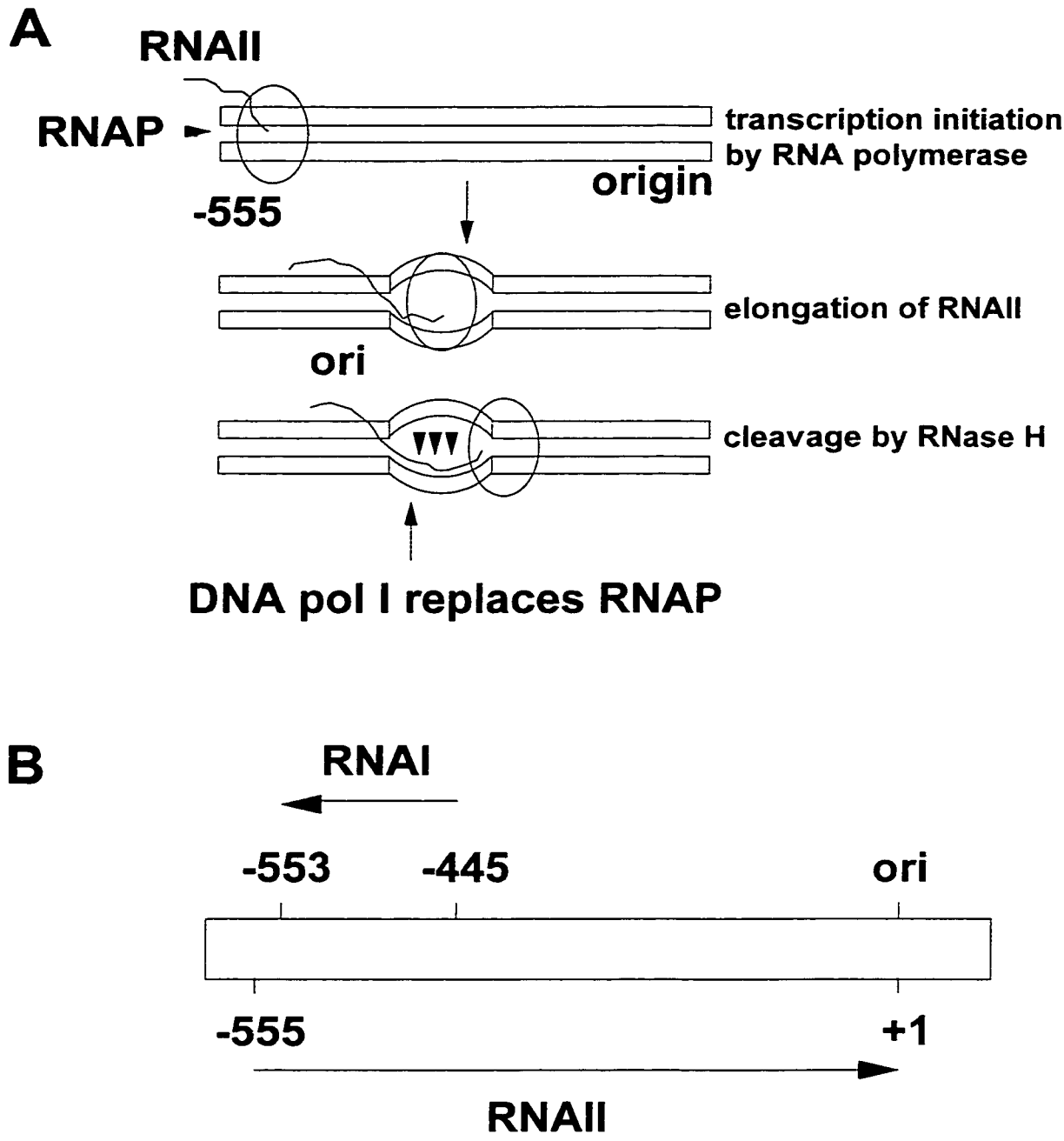

Figure 1.3.1 Replicon for the ColE1 plasmid and the mechanism of replication. A. Initiation of replication begins at position -555 with the transcription of RNAII by RNA polymerase (RNAP). Elongation of RNAII occurs to the *ori* where RNase recognizes the RNA/DNA hybrid and cleaves the RNA molecule, exposing the primer for DNA polymerase I (PolI). PolI is later replaced by PolIII where double-stranded replication on both strands occur. B. Incompatibility in these plasmids is due to an antisense molecule, RNAI, which is able to bind to the 5' end of RNAII. The resulting double stranded RNA molecule is inaccessible for the RNase protein. Thus, DNA polI is unable to bind and start DNA synthesis.

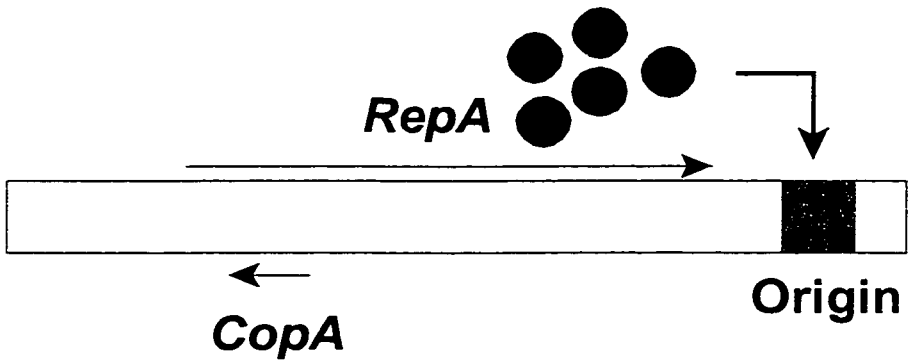

Figure 1.3.2 Basic mechamism for the control of the IncFII plasmid R1. Shown are the RepA and CopA RNA transcripts (arrows) and the RepA protein (circles) which bind to the origin of replication. The antisense *CopA* RNA molecule (80 bases) has homology to the RepA mRNA, and can form a specific RNA duplex. This duplex results in the post-transcriptional control of the synthesis of the RepA protein. Adapted from Helinski *et al.*, 1996.

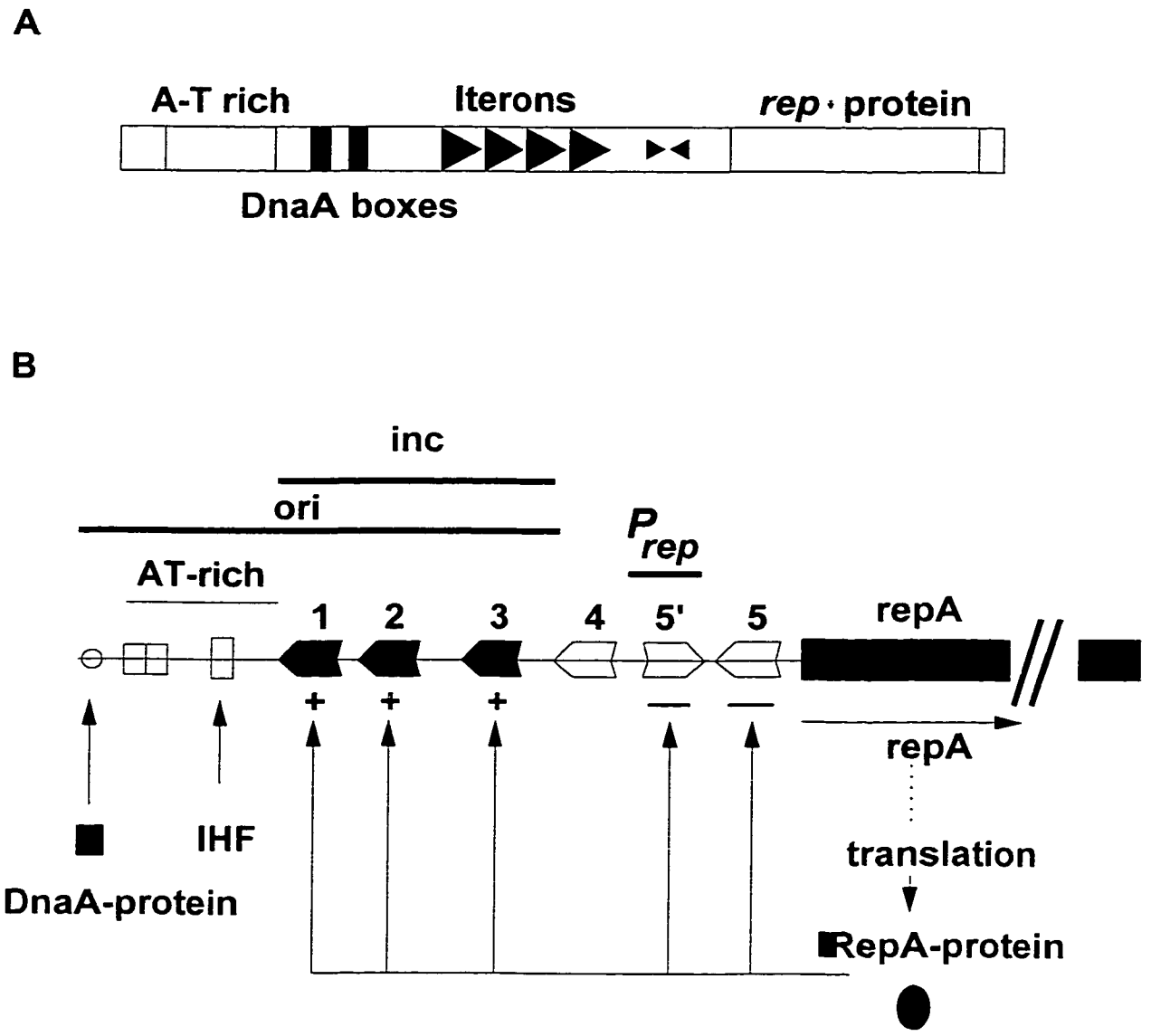

Figure 1.3.3 Iteron plasmid replication. A, schematic of the replicon of an idealized iteron-regulated plasmid. Shown are the *repA* gene and its promoter (inverted arrowheads), the iterons which bind the RepA protein (side-by-side arrowheads), the A-T rich region (shaded box) and the DnaA binding domains (small boxes). B, The mechanism of replication initiation for pSC101. The RepA protein is able to bind iterons in its promoter region as well as those within the origin of replication. Binding of the DnaA and IHF proteins to their consensus recognition sequences is believed to aid the RepA protein unwind the DNA at the A-T rich region, allowing for the DNA replication machinery to be loaded. Adapted from Kues and Stahl (1989) and Helinski *et al.*, 1996.

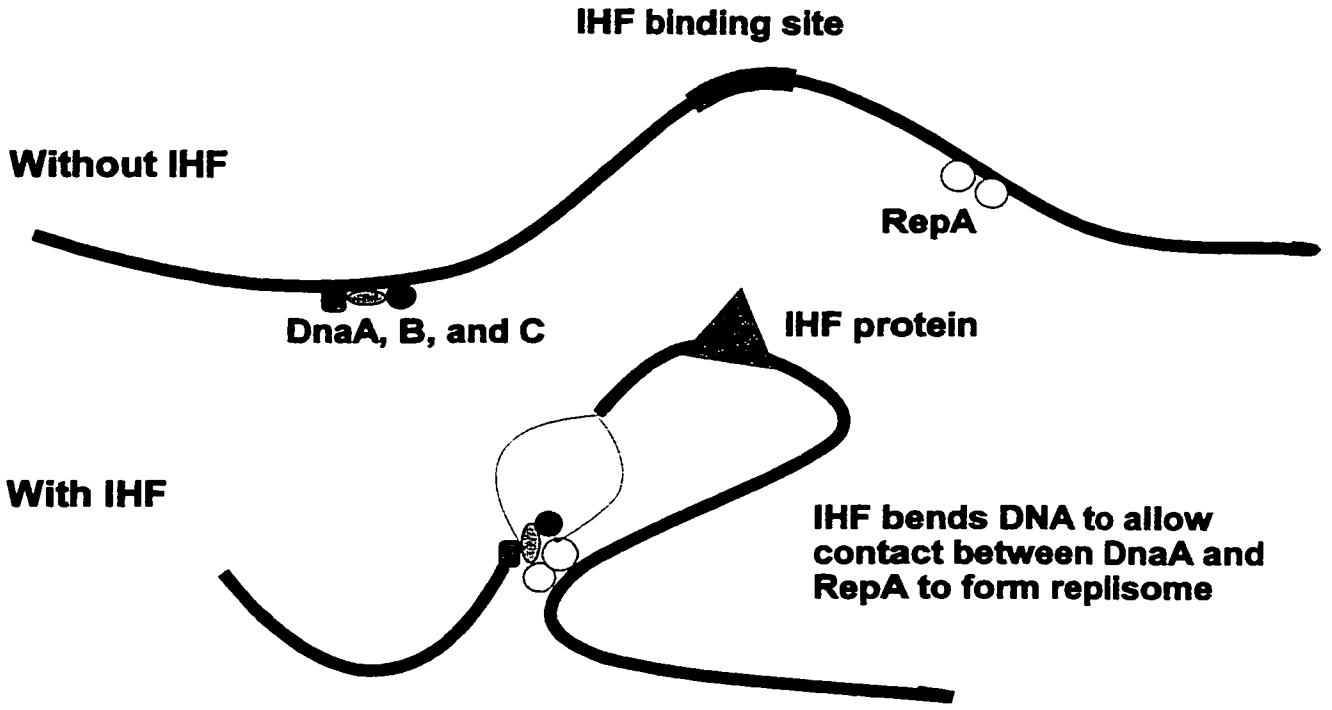

Figure 1.3.4. The postulated role of IHF in the initiation of iteron-based plasmid replication. The binding of IHF (blue triangle) to its specific site (green line) in the origin may bring DnaA (red square), DnaB (ellipse), DnaC (blue circle), and Rep proteins (yellow circles) together to form a functional replisome. Denaturation of the plasmid DNA yielding single stranded DNA in this region can then allow replication to proceed.

1.3.2 INCOMPATIBILITY

Incompatibility is referred to as the inability of two plasmids to be maintained in a cell under non-selective conditions (Bergquist, 1987; Couturier *et al.*, 1988). A cell containing, for example, plasmids A and B, will give rise to descendants which will contain either plasmid A or plasmid B, if they are incompatible. Incompatibility is a property of relatedness in the replication or partition mechanism in plasmids. Elements that are shared among plasmids will give rise to a strong or weak incompatible phenotype (Couturier *et al.*, 1988). In the early 1970s, a formal classification system was established, where plasmids that were incompatible with each other were assigned to the same incompatibility group (Datta and Hedges, 1972).

To test for incompatibility, a plasmid with a genetic marker A must be introduced into a strain carrying another plasmid with a different genetic marker, B. This is achieved using transformation, transduction or conjugation experiments. Having different markers allows for the segregation pattern to be easily observed. Selection is usually made for the incoming plasmid and the progeny is examined for the presence or absence of the resident plasmid (Bergquist, 1987; Novick, 1987). A new method, replicon typing, has been described, where hybridization with specific probes containing replication control genes, allows for incompatibility testing in those instances where technical problems occur (Couturier *et al.*, 1988).

1.3.3 GONOCOCCAL PLASMID REPLICATION

Penicillinase production in isolates of *Neisseria gonorrhoeae* is mediated by a related family of plasmids which carry deletions or insertions in relation to the 7.4 kb prototype Asia plasmid pJD4 (Dillon and Yeung, 1989; Pagotto *et al.*, 2000a). While there has been little

molecular characterization with respect to replication mechanisms, it appears that these plasmids contain different and multiple origins of replication as well. Initially, a 3.8 kb *Bam*HI-*Pvu*II fragment of Asia-type plasmids such as pJD4 had been shown to be essential for replication (Johnson, 1985). When this fragment was cloned into PolA dependent vectors, such as pBR322, pMB8, and pBluescript KS^+, these ColE1-type plasmids could be maintained in *polA*$^-$ hosts indicating that the replication was independent of *polA* (Dillon and Yeung, 1989). Subsequently, Yeung and Dillon (1988) constructed nested deletions of the Asia-type plasmid (pJD4) and deduced that this plasmid had two replication origin regions designated *a* and *b*. One replication region, characterized on plasmid pFA3 (Asia-type similar to pJD4) (Gilbride and Brunton, 1990), included a putative replication protein. This region coincided with the *b* origin of replication described previously (Yeung and Dillon, 1988; see Chapter 3).

Available data, mostly from the work done in our laboratory, set the starting point for the following chapters. The chapters represent a systematic approach to ascertain the relationships that exist among the gonococcal β-lactamase-producing plasmids (if such a relationship does exist). The location and number of origins and incompatibility classification for this family of plasmids were determined. Genetic data are presented to support the existence of a second Rep protein, distinct from the previously described Rep protein (Gilbride and Brunton, 1991). I was able to use the data from this part of the project to construct a series of simple plasmid cloning/shuttle vectors for use in the gonococcus, a first in this field. Of the components involved in the replication of plasmid DNA (i.e. DnaA, IHF), I focussed on the IHF protein and its possible role in the replication process of these plasmids.

CHAPTER 2

SEQUENCE ANALYSIS OF THE FAMILY OF PENICILLINASE-PRODUCING PLASMIDS OF *NEISSERIA GONORRHOEAE*

(Pagotto, F., Ng, L.-K., Aman, T., Brett, M., Yeung, K.-H., and Dillon, J.R. (2000) Structural analysis of the family of penicillinase-producing plasmids of *Neisseria gonorrhoeae* based on DNA sequencing. *Plasmid* **43:** 24-34)

2.1 INTRODUCTION

The family of gonococcal β-lactamase-producing plasmids is genetically related (Dillon and Yeung, 1989; Roberts, 1989). To date, six plasmid types, classified as Asia (generally reported as 4.2-4.4 MDa), Africa (3.2 MDa), Toronto (3.05 MDa), Rio (2.9 MDa), Nîmes (3.8 MDa) and New Zealand types (6.5 MDa) have been described (Brett, 1989; Dillon and Yeung, 1989; Gouby *et al.*, 1986; Van Embden *et al.*, 1985; Yeung *et al.*, 1986). Penicillinase production in these plasmids is mediated by a TEM1-type β-lactamase encoded by the Tn*A* transposon Tn*2* which is truncated and includes 84% of *tnpR*, non-coding sequences, the entire *bla* gene, and the right inverted repeat (IR-R) (Chen and Clowes, 1987; Fayet *et al.*, 1982).

A number of studies, based on restriction endonuclease (R/E) analysis, hybridization analysis, and heteroduplex analysis by electron microscopy have shown that the Africa, Toronto, Nîmes, Rio and New Zealand-type plasmids are deletion or insertion derivatives of the prototype Asia plasmid (Dickgiesser *et al.*, 1982; Van Embden *et al.*, 1985; Gouby *et al.*, 1986; Yeung *et al.*, 1986). However, the exact location and nature of either the reported deletions or insertions still have not been precisely determined, leading to discrepancies concerning the organization of these plasmids (Dillon and Yeung, 1989; Roberts, 1989; Gilbride and Brunton, 1990). Several reports have given partial DNA sequences for some gonococcal β-lactamase producing plasmids, but none has reported the sequencing of the entire plasmid nor has investigated the basis for their structural diversity (Chen and Clowes, 1987; Gilbride and Brunton, 1990; Sanchez-Pescador *et al.*, 1988; Yeung and Dillon, 1988).

In the present study, the entire DNA sequence and structural features of the Asian-type plasmid, pJD4 is reported. Based on the sequence of pJD4, regions surrounding

deletions or insertion of other plasmids in the gonococcal family of penicillinase plasmids have been sequenced. In doing so, the identification of repeated sequences in the Asia-type plasmid that are implicated in the formation of the deletions of the Africa and Toronto-type plasmids have been identified. It is shown that the insertion in the Nîmes plasmid is IS*5* and that the New Zealand plasmid carries a duplication of an endogenous DNA sequence. A discussion of other important structural features of the plasmids, such as integration host factor (IHF) and putative DnaA binding sites, putative functions of open reading frames, origins of transfer (*oriT*) and the nature of the truncation of Tn*2* present on the various plasmids is presented.

2.2 MATERIALS AND METHODS

Bacterial strains and plasmids

The bacterial strains and plasmids used in this study are described in Table 2.1. *E. coli* strains, including transformants, were cultured on tryptic soy agar (TSA) or tryptic soy broth (TSB; Difco, Detroit, MI), which was also supplemented with 100 μg ampicillin ml^{-1} (Sigma, St. Louis, MO), if required, for plasmid maintenance and incubated at 37^0C. Penicillinase-producing *N. gonorrhoeae* (PPNG) strains were grown on GC agar base (GCMB, Difco) supplemented with 1% modified Kellogg's defined supplement (40 g glucose, 1 g glutamine, 10 ml of a 0.5% ferric nitrate solution, 1 ml of 20% cocarboxylase) and ampicillin at 5 μg ml^{-1} and were incubated at 35^0C for 14-18 hours in a humid atmosphere supplemented with 5% CO_2. The identity of all gonococcal isolates was reconfirmed by standard biochemical methods, serological analysis and auxotyping (Dillon *et al.*, 1988).

DNA manipulations and sequencing

All plasmids were transformed into *E. coli* JM83 or C600 as previously described (Sambrook *et al.*, 1989). Nested deletions of clones were prepared using the Erase-a-base Kit as instructed by the manufacturer (Promega). Plasmid DNA was prepared using a modified alkaline-lysis method (Ng *et al.*, 1987). Plasmids used for DNA sequencing were prepared with the Promega Magic Minipreps™ DNA purification system (Promega, Madison, WI), according to the manufacturer's instructions. Large-scale plasmid DNA was isolated using a scaled up alkaline-SDS method followed by cesium chloride-ethidium bromide ultracentrifugation (Sambrook *et al.*, 1989) and DNA was precipitated and cleaned as described previously (Dillon *et al.*, 1985).

Table 2.1 Bacterial strains and plasmids used in this study.

E. coli strains	PPNG strains	Plasmid content	Genbank accession #	Description/reference
JM83				American Type Culture Collection
C600				Stratagene
		pBluescript KSII (+)		Stratagene
		pGO4717	U55934	J.D.A. van Embden
	GC1213	pGC1213	U20423	PPNG strain containing Toronto-type plasmid from NLSTD, LCDC
	GC4538	pGC4538	U20424	PPNG strain containing Toronto-type plasmid from NLSTD, LCDC
	GC5221	pGC5221	U20425	PPNG strain containing Toronto-type plasmid from NLSTD, LCDC
	GC5228	pGC5228	U20426	PPNG strain containing Toronto-type plasmid from B. van Klingeren
	GC5230	pGC5230	U20427	PPNG strain containing Toronto-type plasmid from B. van Klingeren
AS84/417		pAS84/417	U20422	Brett, 1989
HB101-GF1		pGF1	U20421	Gouby *et al.*, 1986
JM83-STD32		pSTD32		1.5-kb *Bam*HI-*Pst*I fragment of pJD4 cloned in pBluescript KSII (+)
JM83-STD41		pSTD41		3.2-kb *Bam*HI-*Hin*dIII fragment of pJD4 cloned in pBluescript KSII (+)

Table 2.1 (continued).

E. coli strains	PPNG strains	Plasmid content	Genbank accession #	Description/reference
JM83-STDATA1		pATA1		2.0 -kb *XbaI-Hin*dIII fragment of pJD4 cloned in pBluescript KSII (+)
JM83-ATA2		pATA2		1.8-kb *SpeI/Pst*I fragment of pJD4 cloned in pBluescript KSII (+)
JM83-ATA3		pATA3		3.1-kb *Bam*HI fragment of pJD5 cloned in pBluescript KSII (+)
JM83-ATA4		pATA4		2.7-kb *Bam*HI fragment of pJD7 cloned in pBluescript KSII (+)
JM83-STD82		pSTD82		4.8-kb *Bam*HI fragment of pAS84/417 cloned in pBluescript KSII (+)
JM83-STD83		pSTD83		2.4-kb *Bam*HI fragment of pAS84/417 cloned in pBluescript KSII (+)
JM83-STD84		pSTD84		1.8-kb *Bam*HI fragment of pAS84/417 cloned in pBluescript KSII (+)

DNA sequencing strategies included the use of nested sets of deletions from various clones and primer extension. All DNA sequences were obtained in both directions. Manual DNA sequencing was performed with the Sequenase® Version 2.0 kit (United States Biochemical Corporation, Cleveland, Ohio, USA), *Taq* DNA polymerase (Taq-Track ® Sequencing Kit, Fisher Scientific, Nepean, ON), and [α-^{35}S]-dATP (Dupont, Mississauga, ON), based on the dideoxy chain termination method of Sanger *et al.* (1977) and as described by the manufacturer. Automated DNA sequencing (Applied Biosystems Model 37A DNA Sequencing System, Applied Biosystems, Mississauga, ON) was performed using the PRISM Ready Reaction DyeDeoxy Terminator Cycle Sequencing Kit (Applied Biosystems) in conjunction with the Centri-Sep Spin Columns (Princeton Separations, Adelphia, NJ). DNA sequence anomalies caused by high G-C contents were resolved by replacing dGTP with dITP (Mizusawa *et al.*, 1986). Certain DNA sequences for pJD5, pJD7, pGF1, pAS84/417, as well as regions 5' to Tn*A* in pJD5 and Toronto-type plasmids (Aman, 1994) were obtained by primer extension (Sambrook *et al.*, 1989). Primers were designed based on the corresponding sequences present on pJD4 using the Primer Designer software (Scientific and Educational Software, Durham, NC) package and were purchased from General Synthesis and Diagnostics (Toronto, ON) and from the University of Ottawa Biotechnology Research Institute (Ottawa, ON).

DNA sequences were compared to sequences found in the Data Bank Microgenie^{R} as well as other databases, using Blast (Altschul *et al.*, 1990). DNA sequences were aligned with the DNA sequence of pJD4 using PC-Gene software (Intelligenetics, Mountain View, CA). Raw DNA sequences for pJD4 were further analyzed using homology searches. Restriction endonuclease sites, location of direct and inverted repeats, open reading frames,

and other structural features were ascertained using the Microgenie^{R} Sequence Analysis Program and PC-Gene software.

When I started my Ph.D., Tholib Aman had just completed his M.Sc. project with Dr. Dillon, where he sequenced most of plasmid pJD4 and surrounding areas of insertion/deletion points of related gonococcal plasmids, and his thesis served as an invaluable reference point throughout the completion of this chapter. Dr. Dillon's technician at Health Canada, Ms. Nancy Bigelow, was involved in obtaining the DNA sequence of the New Zealand plasmid corresponding to the duplicated region when aligned with pJD4. I would like to acknowledge their work which was of tremendous use during this aspect of the project.

2.3 RESULTS

Sequence analysis of the Asia-type plasmid pJD4

The entire DNA sequence of the gonococcal Asia-type plasmid, pJD4 was determined to be 7,426 bp (Fig. 2.1); the unique *Pst*I site within *bla* was arbitrarily assigned as coordinate 1. This plasmid contains 62 direct (DR) and 24 inverted repeats (IR) of ≥10 bp. One notable large direct repeat, designated DR-30 (Fig. 2.1), is present as two copies, DR-30A (507 bp) and DR-30B (509 bp). The only other large, direct repeat DR-2 is present as two identical 92 bp (Fig. 2.1). DR-30 and DR-2 repeats are proposed to be separately implicated in the formation of different deletion derivative plasmids of the Asia-type.

DNA sequence analysis confirmed the findings of Chen and Clowes (1987) in determining that the Tn*A* sequence of the gonococcal plasmid is similar to Tn*2*, but that the left inverted repeat, *tnpA* and the first 90 nucleotides of *tnpR* are deleted. Specifically, pJD4 contains *tnpR*, *bla* (99.9% identical to *bla* in Tn*2*) and the inverted right repeat (IR-R). The Tn*2* sequence obtained for pJD4 was identical to that reported by Chen and Clowes (1987) for the Asia-type plasmid pFA3, except for a single difference.

Plasmid pJD4, contains over 50 open reading frames (ORFs) coding for proteins of at least 20 amino acids (Aman, 1994; Aman *et al.*, 1994). These putative ORFs were compared with previously reported *in vitro* transcription/translation products of gonococcal Asia-type plasmids (Biswas *et al.*, 1986a; Tenover *et al.*, 1985; Yeung and Dillon, 1985) and we attempted to assign an ORF based on size and putative function with the reported protein

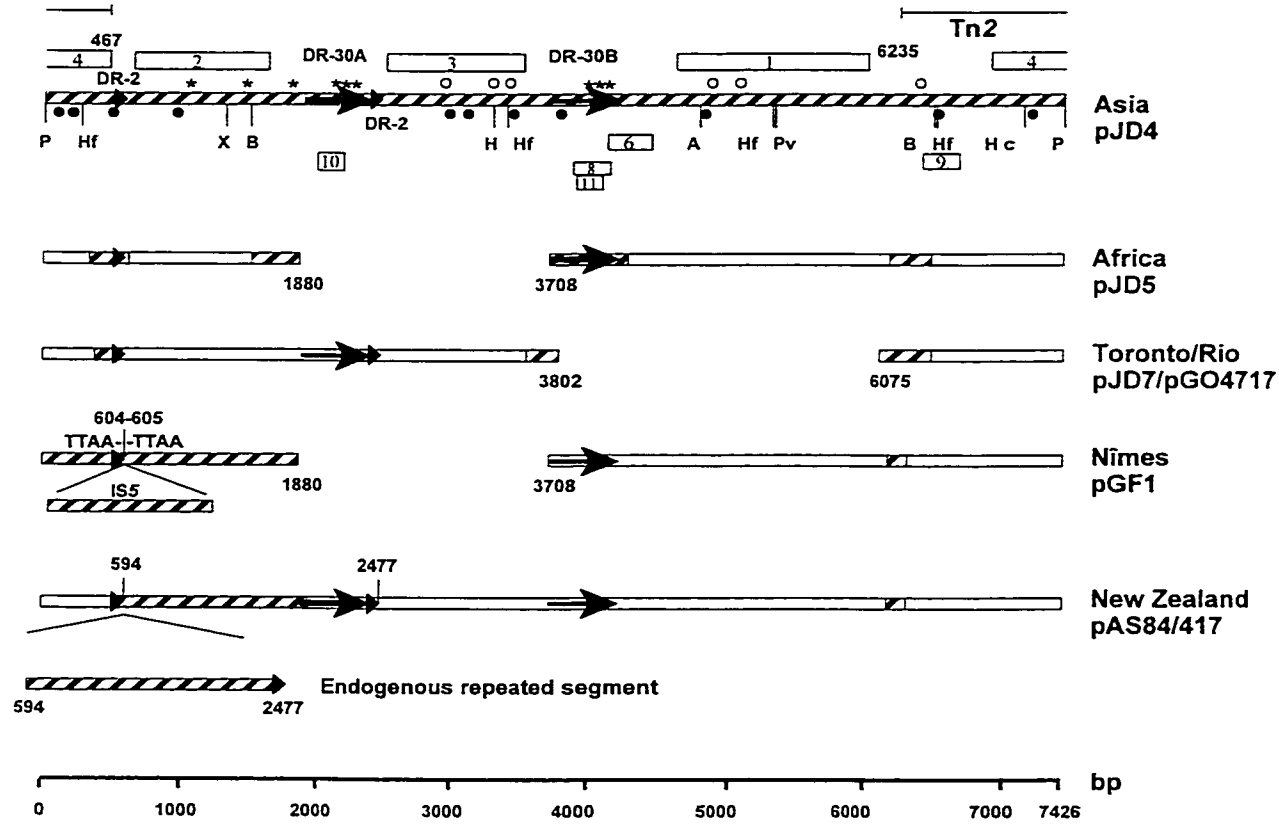

Figure 2.1 Linear map of the gonococcal β-lactamase-producing plasmids. Selected restriction sites are indicated to aid in orientation, as is the position of Tn*2*, and direct repeats DR-30 (arrow) and DR-2 (arrowhead). Cross-hatched boxes indicate that the DNA was sequenced on both strands using a number of strategies. Open, numbered boxes represent open reading frame products inferred from previously published *in vitro* transcription/translation experiments. Closed and open circles represent putative origins of transfer (*oriT*) and DnaA boxes, and stars represent the integration host factor binding sites. Coordinates are based on the sequence of pJD4, with spaces indicating deletions. The Nîmes-type plasmid has IS*5*. The New Zealand-type plasmid has a 1,883 bp duplication corresponding to coordinates 594-2477 of pJD4. Abbreviations: *A*, *Ava*I; *B*, *BamHI*· Hc *Hinc*II· Hf *Hin*fI· P *Pst*I· Pv *Pvu*II· X *Xba*I

products (Table 2.2). ORF number 2 (Table 2.2) corresponds to the putative replication initiation protein (Rep) of pFA3. We determined that the deduced sequence for the Rep protein of pFA3 is identical to the sequence obtained in that region for pJD4. We have also identified a second Rep protein (ORF number 3), consistent with the findings of Yeung and Dillon (1988) that the plasmid contains more than one origin of replication (Pagotto and Dillon, manuscript submitted). ORF4 encodes the TEM-1 β-lactamase. Tenover *et al.* (1985) reported a 16 kDa protein (probably ORF number 6) which was implicated in mobilization (Mob). Aside from these proteins, none of the ORFs on the Asia-type plasmid have been characterized genetically or functionally. BLAST searches using the ORFs in Table 2.2 did not reveal any significant homology with known proteins (data not shown).

DNA sequence analysis has allowed us to identify putative binding sites on pJD4 implicated with plasmid replication. The Asia-type plasmid contains 9 integration host factor (IHF) consensus recognition sequences (5'-C/T A A N N N N T T G A T A/T-3'). In addition, 6 putative DnaA recognition sequences (5'-T T A T C/A C A C/A C-3') were found. We have shown that some of the IHF binding sites are implicated in the regulation of copy number (Pagotto *et al.*, manuscript in preparation; see chapter 5).

The 10 bp gonococcal uptake sequence, 5'-GCCGTCTGAA-3' or 5'-TTCAGACGGC-3', which is required for gonococcal transformation (Goodman and Scocca, 1988), is not present on pJD4, thereby accounting for the difficulty in transforming competent gonococcal cells with this plasmid. However, one sequence with 90% identity was found at coordinates 1400 to 1409 and two other sites were 80% identical to the reported sequence (at positions 3243-3252; 4947-4956).

Gonococcal β-lactamase-producing plasmids have been studied with respect to their

ability to be mobilized using various conjugative plasmids (McNicol *et al.*, 1983; Tenover *et al.*, 1985, Dillon *et al.*, 1989; Piffaretti *et al.*, 1988; Gauthier, 1990). Our DNA sequencing analysis reveals the presence of a number of consensus nick sequences which are found in the *oriT* region. Based on the reported nick sequence (5'-C C/T G T C C T A T/C C/A C/T A-3') described by Waters *et al.* (1991), we have localized a number of these sequences (Fig. 2.1).

Characterization of Asia-type deletion derivatives: Africa, Rio, and Toronto-type plasmids

Previous molecular analyses have shown that Africa-type gonococcal plasmids are homologous to the Asia-type plasmid, with the exception of a deletion variably reported as ranging from 1.8 to 2.1 kb and reported as being at different locations on the plasmid (Aalen and Gundersen, 1987; Dickgiesser *et al.*, 1982; Yeung *et al.*, 1986). Our laboratory (Aman, 1994) obtained the DNA sequence of 884 bp (Genbank accession number U20375) of pJD5 surrounding the segment deleted from pJD4 in order to ascertain which bases had been deleted. The deletion was 1,826 bp in length and was located approximately 1.9 kb downstream of the *Pst*1 site of pJD4. Additionally, direct repeat DR-30A was absent from pJD5 (Fig. 2.1). The size of pJD5 was estimated to be 5,599 bp. Coupled with sequencing and R/E analysis (Aman, 1994), these data indicate that, relative to pJD4, pJD5 contained no DNA rearrangements. Indeed, the stability observed in gonococcal β-lactamase-producing plasmids has allowed for a PCR-based diagnostic assay to be developed (Dillon *et al.*, 1999).

A DNA sequence of approximately 254 bp of the region surrounding the deletion of the Rio-type (pGJO4717) and several Toronto-type (pJD7, pGC1213, pGC4538, pGC5221,

pGC5228, and pGC5230) plasmids was obtained (Aman, 1994). The deletion in these plasmids, as compared to pJD4, was 2,270 bp. The DNA sequence surrounding the deletion of the five Toronto and Rio plasmids was identical, indicating that, despite widely varying geographic origins, the sequences surrounding the deletions are stable with no variability for the deletion site. The DR-30 is probably implicated in the Rio/Toronto deletion and the size of DR-30B is 95 bp shorter than that in pJD4. Assuming that no other deletions are present on these plasmids, such as the suggested 100 bp deletion on the *Bam*HI-*Hin*dIII fragment of the Rio-type plasmid (Van Embden *et al.*, 1985), the size of all Rio and Toronto-type plasmids is estimated to be 5,154 bp. Although several discrepancies were noted when pJD4 was aligned with an Africa-type plasmid (pFA7) (Sanchez-Pescador *et al.*, 1988), the accuracy of our reported sequence has been confirmed by comparisons with other partially reported gonococcal β-lactamase plasmids (Chen and Clowes, 1987; Gilbride and Brunton, 1990).

Characterization of insertion variants of gonococcal plasmids: Nîmes and New Zealand-type plasmids

The Nîmes-type plasmid, pGF1, shown by R/E analysis to be a variant of the Africa-type plasmid, carries an insertion on the 2.4 kb *Bam*HI fragment (Dillon and Yeung, 1989; Gouby *et al.*, 1986). We further determined that the insertion was on a 1,836 bp *Pst*I-*Spe*I fragment of pGF1. A portion of the DNA sequence of this fragment was determined and was shown to be identical to two sequences on pJD4 (Aman, 1994). DNA sequence analysis demonstrated that the insertion was 1,199 bp in length and is identical to insertion sequence 5 (IS*5*) (Schoner and Kahn, 1981). This insertion sequence was flanked by the sequence 5'-T T A A -3' which we consider to be the gonococcal target sequence for this element, and is

present as a single copy in pJD4. Others have proposed that the target sequence for IS*5* is 5'-C T/A A G/A-3' (Engler and van Bree, 1981; Schoner and Kahn, 1981). Thus, a more universal sequence target for IS*5* might be 5'-C/T T/A A G/A-3'.

Restriction endonuclease analysis of the New Zealand-type plasmid, pAS84/417, indicated that the plasmid is a variant of pJD4, an Asia-type plasmid, containing a 1.8 kb insertion within the 2.4 kb *Bam*HI fragment (Dillon *et al.*, data not shown). A DNA sequence of 3,885 bp from pAS84/417 was obtained (Dillon *et al.*, unpublished) and our laboratory ascertained that 1,883 bp of pJD4 were duplicated and appear as tandem repeats. Except for a few minor differences (data not shown) the two sequences are identical. DR-2 was present as three copies on pAS84/417 (Fig. 2.1) and the total size of the New Zealand-type plasmid is predicted to be 9,309 bp.

We obtained the DNA sequence surrounding Tn*A* of several gonococcal plasmids. We confirmed the findings of a previous report (Chen and Clowes, 1987) which found that the Tn*A* sequence of Africa type plasmids was one nucleotide shorter than the Asia-type (pFA3) plasmid. The same sequence in an Asia-type plasmid (pJD4) as well as New Zealand (pAS84/417), Rio (pGO4717), and Toronto-type (pJD7) plasmids were shown to contain this extra nucleotide. However, the Tn*2* sequence in Africa-type (pJD5) and Nîmes-type (pGF1) plasmids did not. Otherwise, DNA sequences surrounding Tn*2* in gonococcal plasmids were identical and the sequence at the end of the left inverted repeat of Tn*A* in plasmids was 5'-TATCT-3', the presumed transposition target sequence (Chen and Clowes, 1987).

Table 2.2 Comparison of the open reading frames (ORFs)* of pJD4 with previously reported *in vitro* transcription/translation products.

ORF #	Coordinates	Size of protein (kDa) reported by				Possible Function?
		This study[a]	Yeung and Dillon (1985)	Tenover *et al.* (1985)	Biswas *et al.* (1986a)	
1	4561-5997	56.2	56.5		55	
2	1608-624	38.6	43	43	40[b]	RepA[c]
3	3498-2479	39.8	41	41	41	RepB[d]
4	6886-318	31.5	31	30	30	*bla*[e]
5	7264-320[f]	17.4	17.5		18	
6	4392-4096	11.6	16.5	14/15/16[g]	14	Mob[h]
7	7081-7425[i] or 6371-6700	12.8 or 12.3	12.8			
8	4093-3836	10.5	10.8			
9	6461-6700	9.04	8.8			
10	2149-1973	6.7	7.8			
11	3886-4038	6.2	6.5			
12	7016-7162[f]	5.4	5.5			

*. Computer analysis revealed many more ORFs than described by literature.
a. ORFs identified by computer analysis.
b. The 40 kDa protein probably corresponds to ORF#2 of pJD4.
c. Rep protein as described by Gilbride and Brunton (1990).
d. Putative second Rep protein (Pagotto and Dillon, manuscript submitted).
e. Beta-lactamase protein (Chen and Clowes, 1987).
f. ORF #5 and #12 are probably truncated forms of the beta-lactamase protein.
g. Only ORF #6 of pJD4 corresponds to 16 kDa protein as described and mapped by Tenover *et al.* (1985). Protein of 14 and 15 kDda may be degraded products.
h. Reported to be involved in mobilization (Tenover *et al.*, 1985).
i. Not clear which of two ORFs identified by computer analysis match the *in vitro* product.

2.4 DISCUSSION

The present report clarifies the sequence relationships between different gonococcal penicillinase plasmids. The structural diversity observed in some gonococcal β-lactamase-producing plasmids is probably due to the presence of several notable repeated sequences, particularly DR-30 and DR-2. Some repeated sequences are involved in DNA rearrangements such as deletions or duplications (Bachellier *et al.*, 1996). In particular, the repeat DR-30 is implicated in the formation of the deletion in Africa-type plasmids and may be involved in the deletion in Toronto-type plasmids. Recombination between plasmid borne repeats, in a *recA*-dependent manner, has been shown to result in the deletion of one of the repeats as well as any intervening sequence (Bi and Liu, 1996), and is implicated in the formation of the Africa-type plasmid. In this case, we suggest that recombination takes place within the DR-30 repeat and results in the loss of one complete repeat. Because the Africa-type plasmid contains additional nucleotides within DR-30B that are not present in DR-30A (Fig. 2.1), we suggest that the crossover between these two repeats might have occurred within the first 29 bp of the repeats. Although repetitive sequences in close proximity to one another (i.e. less than 300 bp) can also undergo *recA*-independent rearrangements which result in deletions (Lovett, *et al.*, 1993), homologous recombination leading to such deletions increasingly depend on the *recA* gene product as the distance between repeats is increased (Lovett *et al.*, 1993). Bi and Liu (1994) estimated that any exchange involving direct repeats greater than 4 kb apart is RecA dependent. Since DR-30A and DR-30B are only 1.8 kb apart, it remains to be determined whether the gonococcal *recA* product is essential.

The deletions observed in the Toronto/Rio plasmids are not readily explained by the

presence of obvious repeats, even though part of DR-30B is deleted from these plasmids. Based on the predicted model and the location of the deletion (Fig. 2.1), our sequence analysis suggests that parts of the IR-16 and IR-22 may be involved in a rare recombinational event. Within these inverted repeats, a small direct repeat may form, allowing a recombination-mediated deletion to occur similar to that described for the formation of the Africa-type plasmids. Interestingly, even though they are geographically and epidemiologically unrelated, the DNA sequences flanking the Rio and Toronto-type plasmid deletions were identical, an unexpected finding if the deletion involved would have been random.

DR-2 on pJD4 probably caused the duplication observed with the New Zealand-type plasmid pAS84/417. A crossover within two repeats can result in the production of three tandem repeats with the duplication of the DNA sequence between the original two repeats (Roth *et al.*, 1996). This is seen with DR-2 in the New Zealand-type plasmid, which carries three copies of DR-2 (versus two in pJD4) with a duplicated sequence between each consecutive DR-2 pair (Fig. 2.1). This specific event appears to be rare since naturally occurring plasmids of the New Zealand type have only been reported once.

The Nîmes-type plasmid (pGF1) has also been observed only once (Gouby *et al.*, 1986). Structurally, pGF1 is identical to the Africa-type (pJD5) plasmid (Fig. 2.1), except for its IS*5* insertion. To date, IS*5* has not been identified among portions of the *N. gonorrhoeae* FA1090 genomic sequences (Roe *et al.*, 1997) nor has it been reported in *E. coli* C600, the strain used as the *E. coli* background in our studies. However, IS*5* has been reported to be on the chromosome of *E. coli* HB101 (Deonier, 1996), the strain that pGF1 was transformed into initially (Gouby *et al.*, 1986). I, therefore, propose that this sequence

may have originated from an *E. coli* host. A similar phenomenon was reported with pRSF0885, whose host is *Haemophilus influenzae* HR-885; variations in size and restriction endonuclease patterns of this plasmid were reported by several groups and were ultimately shown to be caused by the insertion of IS*1*-K, probably acquired when the plasmid was maintained in an *E. coli* strain (Albritton *et al.*, 1994). Therefore, host dependent alterations may occur in gonococcal plasmids. These plasmids have been shown to undergo spontaneous rearrangements (deletions and insertions) in *E. coli* under non-selective growth conditions (Domenico *et al.*, 1990) and in *N. gonorrhoeae* (Sox *et al.*, 1979). Since R/E maps remained consistent, and overlapping or homologous DNA sequences were identical on different plasmids, and an independent group has obtained an identical DNA sequence for an Africa-type plasmid isolated from *N. meningitidis* (Backman *et al.*, 1998), I am confident that plasmid rearrangement was not occurring. Nevertheless, variants may arise in nature which are either unstable or so rare that they have not been detected in epidemiological screening tests.

In the past, our laboratory has explored various aspects of conjugation using the conjugative plasmids of *N. gonorrhoeae* as well as other conjugative plasmids (Yeung 1989; Dillon *et al.*, 1990). Previous work identified certain areas of the Asia-type plasmid to be mobilizable (gonococcal β-lactamase-producing plasmids are non-conjugative but can be mobilized by certain plasmids) (McNicol *et al.*, 1983; Tenover *et al.*, 1985, Dillon *et al.*, 1989; Piffaretti *et al.*, 1988; Gauthier, 1990). Findings from such studies identified the many DNA fragments from pJD4 as being involved in the mobilization of gonococcal Asia-type plasmids by conjugative (i.e. neisserial or enteric origin) plasmids. The *oriT* sequences identified were based on the reported consensus sequence previously described (Waters *et*

al., 1991) and fall within the fragments identified by conjugation experiments as being able to be mobilized (Fig. 2.1).

Previous work indicated that different conjugative plasmids recognized different DNA sequences involved in mobilization (i.e. where the proteins interact) as well as different *oriT* sequences, depending on the presence or absence of a Mob protein (McNicol *et al.*, 1983; Tenover *et al.*, 1985, Dillon *et al.*, 1989; Piffaretti *et al.*, 1988; Gauthier, 1990). For example, Gauthier (1990) determined that the *Pvu*II/*Ava*I fragment of pJD4 was essential for pJD4 to be mobilized by the IncP plasmid pUB307. Although this fragment lies within the 1.9 kb *Hinf*I fragment reported by Tenover *et al.* (1985), it does not contain the putative Mob protein (ORF#6). Thus, only *cis*-acting sequences were required for mobilization of pJD4 by pUB307 to occur.

Different groups have proposed that the gonococcal β-lactamase-producing plasmids could have originated from a *H. parainfluenzae* host through a conjugational event or from a *H. ducreyi* host by transformation (Brunton *et al.*, 1983; Dillon and Yeung, 1989; Roberts, 1989). One of the hypotheses proposed that the gonococcal plasmids were derived from *H. ducreyi* as two separate events, one involving the Asia plasmid progenitor and the other involving the Africa plasmid progenitor (Roberts, 1989). Structural analysis of these plasmids predicts that, because of the several large repeats present, various deletion or insertion derivatives of the plasmid in the non-Tn*A* coding region are possible and that this can be expected to occur in many bacterial species. This event could include subsequent DNA rearrangements, probably by recombination, affecting DR-30A and DR-30B to give rise to the Africa plasmid, or DR-2 to produce the New Zealand-type plasmid. Thus, two transformation events would not be required for the formation of the smaller Africa-type or

other derivatives of the Asia-type plasmid in *N. gonorrhoeae*. The Toronto and Rio-type plasmids may well represent further recombinational evolution in the gonococcus as their homologs have not yet been identified in *Haemophilus* spp. This hypothesis may be supported in an early study (Sox *et al.*, 1979) that described a variety of uncharacterized gonococcal transformation-derived plasmids with larger or smaller sizes than the Asia-type plasmid which had initially been used to transform the strain.

The question of when and how the 1 bp truncation of Tn*2* occurred in Africa-type plasmids remains an intriguing puzzle. In all strains of *H. ducreyi*, plasmids related to Asia or Africa-type plasmids in the gonococcus carry a complete Tn*2* sequence. It has been proposed that, should gonococcal plasmids arise from *H. ducreyi*, Tn*2* would be truncated as the DNA entered and established itself in the gonococcus (Dillon and Yeung, 1989; Roberts, 1989). This event appears to be specific, because the truncation of Tn*2* is exactly the same in all gonococcal plasmids studied (Chen and Clowes, 1987; our data) with the only difference observed in Africa-type plasmids. Here, truncation omits a nucleotide whereas in Asia, Toronto, Rio and New Zealand-type plasmids, an extra nucleotide is present. As with other deletions in the gonococcus, this process does not appear to be random since the size of the deleted Tn*2* is maintained across many isolates with no obvious epidemiological relationship. It would be interesting to ascertain whether some Africa-type plasmids isolated from the gonococcus in the mid-1970s may in fact carry the additional nucleotide observed on Asia-type plasmids, thereby supporting the proposal that this minor difference between the different plasmids in the gonococcal penicillinase-producing plasmids may have arisen by a mutation.

In conclusion, the exact nature of the sequence differences between gonococcal β-

lactamase producing plasmids was identified. This study should lead to a more complete analysis of the biology of these medically important plasmids.

CHAPTER 3

MOLECULAR AND GENETIC ANALYSIS OF ORIGINS OF REPLICATION OF β-LACTMASE-PRODUCING PLASMIDS OF *NEISSERIA GONORRHOEAE*

(Pagotto F., and Dillon, J.R. (2000) Molecular and genetic analysis of origins of replication of β-lactamase-producing plasmids of *Neisseria gonorrhoeae*. Submitted to *Journal of Bacteriology*)

3.1 INTRODUCTION

Plasmids of Gram-negative bacteria containing three origins of replication are rare (Crosa, 1980; Inuzuka *et al.*, 1980; Banerjee *et al.*, 1992); to date, only three such naturally-occurring plasmids have been described. The first plasmid discovered, F (94.5 kb), contains remnants of three independent replication regions, RepFIC, RepFIA, and RepFIB (Bergquist *et al.*, 1986; Couturier *et al.*, 1988). A 9 kb mini-F plasmid, containing the complete RepF1A region, includes seven genes encoding proteins involved in the replication and maintenance of the plasmid, including a single replication initiation protein (Rep) and two origins, *oriV* and *oriS* (Couturier *et al.*, 1988). Related plasmids lacking the RepF1A region require RepF1B, a less stable replicon (Lane and Gardner, 1979). The RepF1C replicon was shown to have been rendered nonfunctional on the F plasmid by the natural insertion of the transposon Tn1000 (Saadi *et al.*, 1987, Kornberg and Baker, 1992). This replicon, in members of the F plasmid family, resembles the replication region of the plasmid R1 (Kornberg and Baker, 1992). IncN plasmid, pCU1, contains three origins of replication (*oriB*, *oriS*, and *oriV*), all located on a 2 kb DNA fragment which are driven by a single Rep protein (Rajendra Krishnan and Iyer, 1990, Banerjee *et al.*, 1992). The third, and best characterized plasmid with three origins (α, β, and γ), is the 38 kb plasmid RK6. This plasmid also carries genes for two replication proteins, *pir* (encoding the π protein for oris α and γ) and *bis* (Bis for ori β) (Mukhopadhyay *et al.*, 1986). These oris and genes are located on a 4-kb DNA fragment (Mukhopadhyay *et al.*, 1986; Filutowicz and Rakowski, 1998).

It appears that the penicillinase-producing plasmids of *N. gonorrhoeae* contain different and multiple origins of replication as well. This family of plasmids is genetically

related (Pagotto *et al.*, 2000a), and penicillinase production in these plasmids is mediated by a TEM1-type β-lactamase encoded by the Tn*A* transposon Tn*2* which is truncated and includes 84% of tnp*R*, non-coding sequences, the entire *bla* gene, and the right inverted repeat (IR-R) (Fayet *et al.*, 1982; Chen and Clowes, 1987). Initially, a 3.8 kb *Bam*HI-*Pvu*II fragment of a 7.4 kb Asia-type plasmid was shown to be essential for replication (Johnson, 1985). When this fragment was cloned into ColE1-type plasmids such as pBR322, pMB8, and pBluescript KS^+, these vectors which normally depend on DNA polymerase I (Pol I) could be maintained in *polA*$^-$ hosts, indicating that the replication was independent of *polA* (Dillon and Yeung, 1989). Subsequently, Yeung and Dillon (1988) constructed nested deletions of the Asia-type plasmid (pJD4) and deduced that this plasmid had two replication origin regions designated *a* and *b*. The *b* region was further characterized on plasmid pFA3, an Asia-type plasmid similar to pJD4, and included a putative replication protein (Gilbride and Brunton, 1990). None of the replication regions have been additionally characterized since.

In the present study, I further investigate the properties of the origins of replication regions of the β-lactamase-producing plasmids of *N. gonorrhoeae* (Yeung and Dillon, 1988). Since broad-host range gonococcal plasmids such as pJD4 have been shown to replicate in *N. gonorrhoeae*, *Escherichia coli*, *Salmonella minnesota* and *Haemophilus influenzae* (Guiney and Ito, 1982), these studies focussed on origin(s) usage in *E. coli*. Electron microscopy was used to confirm and locate oris in naturally occurring and *in vitro* deletion derivatives of the Asia-type plasmid, pJD4. The incompatibility of the oris to other enteric-derived incompatibility determinants was ascertained. Structural properties for each ori, as determined by DNA sequence analysis and cloning of putative Rep proteins, was determined

by molecular and genetic approaches.

3.2 MATERIALS AND METHODS

E. coli strains and plasmids.

E. coli strains and various plasmids used in this study are listed in Table 3.1. Bacterial cultures were maintained in Brain Heart Infusion broth (Difco, Detroit, MI) containing 15% glycerol and stored at -70^{0}C. *E. coli* strains were subcultured from frozen cells by growing them overnight on Luria Bertani (LB) broth or agar (Difco) at 37^{0}C containing 100 μg/ml ampicillin, 50 μg/ml chloramphenicol or 50 μg/ml tetracycline, where appropriate. Individual colonies were purified by subculturing for 3 days prior to plasmid DNA isolation. Restriction endonuclease (R/E) analysis was performed as previously described (Dillon *et al.*, 1985) to ensure plasmid integrity before enriching for plasmid replicative molecules or for use in incompatibility studies. Plasmid maps are shown in Figure 3.1 and details are based on the submitted sequences generated previously in our laboratory and submitted to Genbank [accession U20374 (pJD4), U20375 (pJD5)].

Construction of pFP5 and pFP9.

Derivatives of pJD5 and pJD9 lacking Tn*2* were constructed as follows: the chloramphenicol acetyltransferase (CAT) cassette was amplified from pACYC184 using primers AC1 (5'-CTAGCTGCAG**GCCGCTGAA**CTGGTGTCCCTGTTGATA-3') (gonococcal uptake sequence in bold) and AC2 (5'-CGTGCTGCAGTTCTGCCATTCATCC-3'), with each primer including a *PstI* site (underlined). The non-Tn*2* region from pJD5 (3,888 bp) was amplified using the primers FP9, 5'-ATGTCTGCAGGCCGCTCTAACCGCT-3' and FP10, 5'CAGTCTGCAGTCGCCGTTCTGGTTG-3'). Similarly, the 1,923 bp non-Tn*2* region of pJD9 was amplified with primers FP7, 5'-CGCTCTGCAGCAACCGAAGCCGTTA-3' and FP8, 5'-CACTCTGCAGCCGTCGGTACTCTCA-3'). PCR-generated products were

digested with *PstI*, ligated, and then transformed into *E. coli* DH5α grown on LB containing chloramphenicol (Dillon *et al.*, 1985). PCR conditions reflected the thermal melting points of the primers and amplification conditions.

Plasmid enrichment and isolation.

Replicative molecules were isolated as described previously (Banerjee and Iyer, 1995), with the following modifications: (1) cells were chilled in an ice bath for 30 min. prior to plasmid extraction; (2) after centrifugation of the lysate, the supernatant containing plasmid DNA was passed through four layers of gauze to remove bacterial debris; (3) to precipitate the DNA (approximately 10-11 ml), 3.5 ml of 50% w/v PEG 8000 and 2 ml of 5 M NaCl were added. The solution was placed on ice for 60 min., centrifuged at 10 000 x g for 20 min. and then the pellet was resuspended in 2 ml TE buffer (pH 8.0). NaCl (5M) was added to a final concentration of 100 mM, followed by the addition of an equal amount of 10 mM Tris-HCl (pH 8.0)-equilibrated phenol:chloroform:isoamyl alcohol (25:24:1). DNA was precipitated with 2 volumes of ice-cold EtOH, washed with 70% EtOH and dissolved in 200 µl TE buffer (pH 8.0) containing 1 µg/ml RNaseA.

Table 3.1. Bacteria strains and plasmids.

E. coli strains	Plasmid	Genbank number	Description/reference
SR1758			Δ(*gal bio*), *thi-1*, *relA1*, *spoT1*; (Banerjee *et al.*, 1992)
SR1672			Δ*polA*, Δ(*gal bio*), *thi-1*, *relA1*, *spoT1*; (Banerjee *et al.*, 1992)
C600			F^-, *thi*-1, *leu*B6, *lac*Y1, *ton*A21, *sup*E44; (Stratagene)
DH5α			F^-, ϕ80d*lac*ZΔM15, *rec*A1, *end*A1, *gyr*A96, *thi*-1, *hsd*R-17(r_k^-, m_k^+), *sup*E44, *rel*A1, *deo*R, Δ(lacZYA-argF)U169; (Stratagene)
BLR(DE3)			F^-, *ompT*, *hsdSB*($r_B^-m_B^-$), *gal*, *dcmD*(*srl-recA*), *306*::Tn*10* (DE3); Novagen
	pBluescript KS II	X52328	Stratagene
	pORF15		pFP9 with 80% ORF15 deleted; this study
	pBlueORF15		pBluescript KSII containing ORF15 under T7 promoter; this study
	pACYC184	X06403	New England Biolabs
C600-JD4	pJD4	U20374	Asia plasmid; (Dillon and Yeung, 1989)
C600-JD5	pJD5	U20375	Africa plasmid; (Dillon andYeung, 1989)
C600-JD7	pJD7	U20419	Toronto plasmid; (Dillon and Yeung, 1989)
C600-JD9	pJD9	U20420	*In vitro* deletion derivative of pJD4; (Yeung and Dillon, 1988)
C600-FP5	pFP5		Non-Tn*2* region of pJD5 PCR-amplified and ligated to chloramphenicol acetyl-transferase gene; this study
C600-FP9	pFP9		Non-Tn*2* region of pJD9 PCR-amplified and ligated to chloramphenicol acetyl-transferase gene; this study
C600-GF1	pGF1	U20421	Nîmes plasmid; (Gouby *et al.*, 1986)

Table 3.1 (con't). Bacteria strains and plasmids.

E. coli strains	Plasmid	Genbank number	Description/reference
C600-AS84/417	pAS84/417	U20422	New Zealand plasmid; (Brett, 1989)
C600-O4717	pGO4717	U55934	Rio plasmid; (van Embden *et al.*, 1985)
	pULB2401		IncFIIA[a]; (Couturier *et al.*, 1988)
	pULB2428		IncI1[a]; (Couturier *et al.*, 1988)
	pULB2429		IncU[a]; (Couturier *et al.*, 1988)
	pULB2433		IncHI2[a]; (Couturier *et al.*, 1988)
	pULB2436		IncHI1[a]; (Couturier *et al.*, 1988)
	pULB2440		IncFIC[a]; (Couturier *et al.*, 1988)
	pULB2426		IncW[a]; (Couturier *et al.*, 1988)
	pULB2424		IncQ[a]; (Couturier *et al.*, 1988)
	pULB2410		IncY[a]; (Couturier *et al.*, 1988)
	pULB2423		IncL/M[a]; (Couturier *et al.*, 1988)
	pULB2406		IncB/O[a]; (Couturier *et al.*, 1988)
	pULB2432		IncN[a]; (Couturier *et al.*, 1988)
	pULB 2425		IncT[a]; (Couturier *et al.*, 1988)
	pULB 2404		IncFIB[a]; (Couturier *et al.*, 1988)
	pULB2439		IncK[a]; (Couturier *et al.*, 1988)
	pUBL2420		IncP[a]; (Couturier *et al.*, 1988)

a- denotes the incompatibility group the plasmid belongs to.

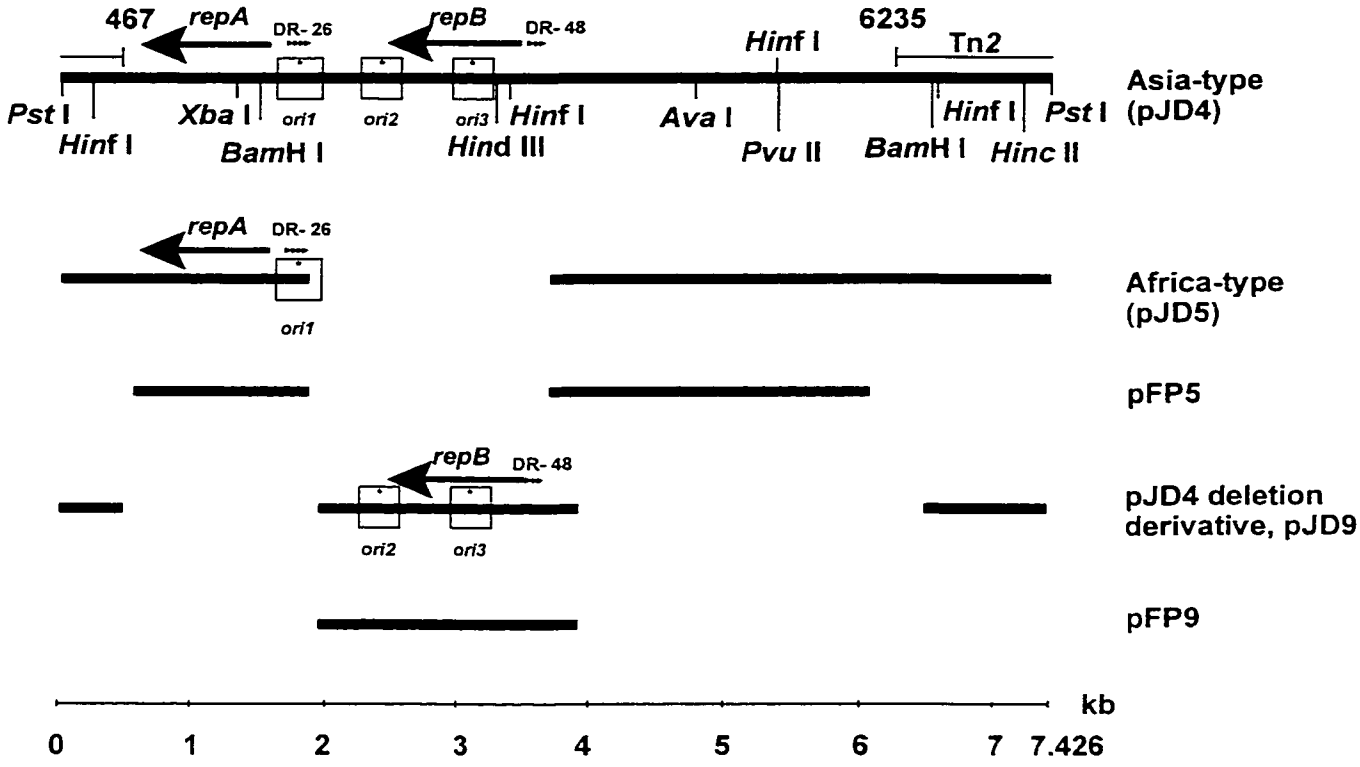

Figure 3.1 Molecular map of plasmids used in this study. Spaces indicate DNA is not present (i.e. missing upon alignment). Replication initiation protein genes are indicated with arrows. the direct repeats by triangles, and origins of replication are depicted as open boxes. The star indicates where DNA replication initiates, based on branch-point analysis (see Figures 3.2-4). Plasmid pFP5 and pFP9 are identical to pJD5 and pJD9, respectively, with the exception that Tn*2* has been replaced by a chloramphenicol acetyltransferase cassette in the FP-series of plasmids.

Electron microscopy and branch-point analysis.

Plasmid DNA, prepared for electron microscopy as described above, was digested with *Pst*I. The *Pst*I site is unique, within Tn*2* and is asymmetrically located with respect to the origins of replication regions; see Fig. 3.1). Approximately 1 μg of linearized plasmid molecules were purified by phenol and chloroform extractions as described above and prepared for EM as described by Ferguson and Davis (1978). Copper grids [300 and 400 mesh, coated with formvar (0.3% in ethylene dichloride)] were used to lift the DNA from the cytochrome-DNA monolayer, followed by staining in uranyl acetate (10 μl of 21.2% uranyl acetate in 0.5 M HCl, made in 90% EtOH, to 10 ml 90% EtOH). There was a 10 min. interval between liftings to allow the molecules to diffuse to the surface, as the hyperphase becomes disturbed following each lift. Four lifts were performed per DNA sample. To enhance the contrast of DNA molecules, the samples were coated with a fine layer of metal particles by rotary shadowing using a Balzers Vacuum Evaporator as previously described (Coggins, 1987). Evaporation was for 30 sec., with the specimen platform rotating at 30-60 rpm, at an angle of 8.5^0, using a 5 cm piece of Pt-Pd wire (80:20 ratio). Micrographs were taken using a Philips EM201 microscope. Replicating molecules were traced and measured for statistical and branch-point analysis as previously described (Coggins, 1987; Wickner and Chattoraj, 1987; Banerjee *et al.*, 1992). All molecules examined under the electron microscope contained only one bubble, as mutliple bubbles were never observed. We chose only those molecules that contained small, replicating bubbles (versus two extended branches) to ascertain the position of the origins. Molecules were measured from the end of each molecule (i.e. *Pst*I site) to the near and far branches of the replication bubble and were

aligned so that the shortest, un-replicated arm was on the left. The relative distance to the nearest (i.e. *Pst*I to beginning of bubble) and farthest (i.e. *Pst*I to end of bubble) branch point are depicted by squares and triangles, respectively (see Figures 3.2, 3.3 and 3.4). The lines were drawn by linear regression analysis of the points obtained, using Sigma Plot version 5.0

Test for DNA polymerase I dependence.

Isogenic *E. coli* strains SR1758 (*polA*$^+$) and SR1672 (*polA*$^-$) (Banerjee *et al.*, 1992) were used to investigate the requirement of various gonococcal plasmids for PolA in replication. Plasmids were transformed into these hosts using electrotransformation (Miller, 1994) or calcium chloride methods (Dillon *et al.*, 1985). Transformants were purified by subculturing them for 3 consecutive days on selective media. Plasmids were isolated and analysed by R/E digestions to screen for possible structural changes (e.g. deletions, insertions, rearrangements).

Incompatibility determination.

Incompatibility tests, which include establishment, maintenance and segregation tests, were performed in *E. coli* DH5α, as described previously (Bergquist, 1987). The establishment test ascertains the ability of a plasmid to establish itself and to replicate in the presence of a resident plasmid. Cells carrying the resident plasmid were electroporated with incoming plasmid DNA and selection was made for one or other (or both) plasmids. The maintenance test determines the ability of two plasmids to co-exist once they have been established within a strain. Cells containing both plasmids were grown overnight in the presence of both antibiotics (approximately stationary phase) before being diluted 10^6-fold into LB broth without antibiotics. Cells were grown in antibiotic-free LB broth for 3 days (10^6-fold

dilution every 12 hours), and subsequently appropriate dilutions of the culture were spread onto LB agar containing antibiotics for the donor, resident, or both plasmids. The segregation test was performed to confirm the existence of weakly compatible plasmids (i.e. in cases where initial transformants isolated carried both plasmids). Colonies carrying both plasmids were grown non-selectively for 48-72 h in LB broth, and cell dilutions were plated on non-selective agar. After 24 hour incubation, colonies were subcultured with sterile toothpicks on LB agar containing antibiotics selecting for the incoming, the resident, or both plasmids. After all experiments (performed 3-5 times), plasmid preparations and R/E analysis on representative transformants were performed to confirm the presence of the plasmid(s), as well as to examine the possibility of rearrangements.

Cloning of RepB (ORF15).

We hypothesized that RepB was equivalent to ORF15 which we initially identified from DNA sequence analysis of pJD4 (Pagotto *et al.*, 2000a). The *repB* gene was PCR-amplified from pJD9 using a Perkin-Elmer 9600 thermal cycler with primers ORF15 (5'-GCGCGAGCTCTGTTTTTTTATTGACC-3') and ORF15COM (5'-GGCGTCTAGAATTTTTCTGTCTCTG-3') (*SacI* site underlined; *XbaI* site double underlined). Amplified DNA (1,101 bp, including 81 bp upstream of start codon) was analyzed using agarose gel electrophoresis followed by ethidium-bromide staining. Gels were photographed using the Gel Print 2000i digital system (Bio Photonics Corp., Ann Arbor, MI). ORF15 amplicons were cleaned using the Qiagen QIAQuick PCR purification kit as described by the manufacturer. The amplicon was digested with *SacI* and *XbaI*, ligated to *SacI*/*XbaI*-digested pBluescript KS+ II and transformed into *E. coli* C600, DH5α, or

BLR(DE3) with selection on ampicillin-containing media. The plasmid content of selected transformants was verified using R/E analysis. The recombinant plasmid selected for further analysis was named pBlueORF15.

Deletion of RepB from pFP9.

Plasmid pFP9 was linearized using the unique *Hin*dIII site found within the 5' end of RepB (1,020 bp, 339 aa, 39, 448 daltons; *Hin*dIII at position 198), and was made blunt-ended using T4 DNA polymerase, creating a frameshift that introduces stop codons throughout ORF15. DNA sequence analysis revealed a truncated form of ORF15 that was 200 bp (67 aa) with a resulting putative molecular weight of 7593 daltons. This mutated plasmid (pORF15) was religated and introduced into *E. coli* BLR(DE3) that contained pBlueORF15 thereby providing ORF15 *in trans* following induction with 1 mM IPTG. Transformants were recovered on media containing both chloramphenicol, ampicillin and IPTG and were analysed of their plasmid content using sequencing and R/E analysis.

***In vitro* transcription/translation reactions.**

The *E. coli* S30 Extract System for Linear DNA Templates (Promega) was used with PCR-amplified DNA from pJD4 using primers FP7/FP8 and FP9/FP10 according to the manufacturer's instruction. [^{35}S]-methionine (15 mCi mL^{-1}) (Amersham Canada) and 1 µl of RNA guard (Pharmacia Biotech Inc.) were also added to each reaction. Products were separated on denaturing 12% polyacrylamide-SDS gels. Gels were dried in a Model 583 Gel Dryer (BIO-RAD) and Kodak X-OMAT™ AR films were exposed overnight to the dried gel and developed. Stephane Bernatchez helped with the *in vitro* transcription/translation experiments. I prepared the DNA for him and he performed the reactions and the protein gel

electrophoresis. I thank him for his help.

DNA sequencing and analysis.

DNA sequencing was performed at the University of Ottawa Biotechnology Research Institute (UOBRI; Ottawa, ON) using an Applied Biosystems 373A DNA sequencer (Alta Biosystems, Mississauga, ON), using the PRISM Ready Reaction DyeDeoxy Terminator Sequencing Kit (Applied Biosystems) in conjunction with the Centri-Sep Spin Columns (Princeton Separations, Adelphia, NJ). Primers were designed using the Primer Designer software package (Scientific and Educational Software, Durham, NC) and were purchased from the UOBRI. Analysis of DNA, RNA and protein sequences were performed using the PC-Gene software (Intelligenetics, Mountain View, CA).

3.3 RESULTS

The gonococcal β-lactamase-producing plasmids have multiple origins of replication

Plasmids pJD4, pJD5 and pJD9 were used to localize the origin(s) of replication in an *E. coli* background using electron microscopy and branch-point analysis (Figures 3.2-4). Branch-point analysis revealed the presence of three clustered, yet spatially distinguishable origins of replication on pJD4, corresponding to initiation coordinates 1867, 2400, and 3100 (based on where the lines meet using regression analysis; see stars on Fig. 3.2). We have named these origins *ori1*, *ori2*, and *ori3*. Molecules corresponding to a complete length expected upon digestion of pJD4 with *Pst*I were used to localize these oris. EM and branch-point analysis now confirms our previous studies, which suggested that pJD4 contained two replication regions, *a* and *b* (Yeung and Dillon, 1988), in that the previously identified *a* region is shown to contain *ori2* and *ori3*, whereas region *b* contains *ori1*.

The Asia-type plasmid pJD4 is 7.4 kb in size, and due to its relatively large size, presented some challenge with respect to the collection of linear molecules of expected length, containing small, replicating bubbles. I was able to separate *ori2* and *ori3* from *ori1* using pJD9, an *in vitro* deletion derivative of pJD4 (Yeung and Dillon, 1988). In pJD9 (Fig. 3.3), the *ori1* and *ori2* mapped to positions 2424 and 3200 (using pJD4 coordinate). Based on the DNA sequence of pJD4 (Pagotto *et al.*, 2000a), these oris correspond to *ori2* and *ori3* of pJD4, respectively (Fig. 3.1).

The naturally occurring Africa-type plasmid, typified by pJD5, was found to have one ori (Fig. 3.4). The origin starts at position 1867 (using pJD4 coordinates) and is the same as *ori1* of pJD4 based on DNA sequencing studies (Pagotto *et al.*, 2000a; Fig. 3.1).

Gonococcal plasmids have at least two incompatibility determinants

There are no established incompatibility groups amongst the gonococcal plasmids. The IncP plasmid pUB307 was shown to mobilize ampicillin resistant gonococcal plasmids from *E. coli* to *N. gonorrhoeae* (Piffaretti *et al.*, 1988), yet no naturally occurring IncP elements has yet to be isolated in the gonococcus. However, plasmids having homology to the IncQ groups, such as RSF1010, have been found in commensal *Neisseria* and *N. meningitidis* (Pintado *et al.*, 1985; Rotger *et al.*, 1986; Facinelli *et al.*, 1987). Therefore, it was of interest to ascertain whether the various origins of replication on pJD4 were incompatible with enteric plasmids (Couturier *et al.*, 1988). Thus, experiments were completed in *E. coli*, the only organism where diverse Inc groups have been established. Plasmids pJD9 (pFP9) and pJD5 (pFP5) were tested for their incompatibility with plasmid constructs containing known incompatibility determinants as described in the Materials and Methods section. These plasmids (pFP5/pFP9) were used because they contain the same oris but different antibiotic resistant markers for selection. All tests revealed that plasmids containing *ori2/ori3* belonged to the IncW group (Table 3.2) whereas *ori1*-containing plasmids belonged to the Inc FII group (Table 3.2).

Incompatibility studies suggested that, since plasmid pJD4 contains all three oris, it should express both Inc phenotypes (W and FII). This was determined to be the case, however, as might be expected, only one was expressed at a time and under different conditions. Plasmid pJD4 was incompatible with the IncW plasmids when incompatibility (i.e. maintenance) tests were performed but expressed IncFII incompatibility, characteristic of *ori1*, in establishment experiments in which pFP5 (*ori1*, IncFII) was resident and pJD4

was the incoming plasmid (data not shown). However, when pFP5 was the incoming plasmid, both plasmids were stably maintained within the cell, indicating that *ori2/3* (IncW) was being used by pJD4. These data indicate that, in *E. coli*, once established pJD4 replicates preferentially from *ori2/3*, as confirmed by the low number of *ori1* bubbles (versus *ori2* and *ori3*) obtained in EM experiments (Fig. 3.2).

A novel Rep protein is required for the replication of pFP9 (ori2 and ori3)

In vitro transcription/translation studies were used to ascertain whether the 1.9 kb DNA fragment with *ori2/ori3* of plasmid pFP9, which excluded the ampicillin resistance determinant, contained genes encoding for proteins that might be involved with replication or maintenance functions. Results from these experiments revealed a single protein of approximately 39 kDa in size (Fig. 3.5, lane 2). Evidence that this protein, named RepB (1,020 bp, 339 aa, 39 449 Da; Pagotto *et al.*, 2000a) might be the Rep protein for *ori2* and *ori3* included the failure to obtain a self-replicating plasmid (pORF15) construct when RepB was truncated by exonuclease digestion (or end-filling) at its unique *Hin*dIII site. However, when RepB was provided *in trans* (on pBlueORF15, Table 3.3) to rescue pORF15 (pFP9 with a truncated *repB*), the plasmid was able to replicate (Table 3.3). When pORF15 was isolated and transformed into a cell not supplying RepB, it was unable to replicate (Table 3.3), indicating a role for RepB in the replication process. Positive controls (pBlueORF15 and pFP9) were able to replicate in the test strain (Table 3.3). Thus, RepB is a novel replication initiation protein for the β-lactamase producing plasmids which functions *in trans*. We have named it RepB to distinguish it from a previously characterized *rep* gene product, RepA (Gilbride and Brunton, 1990).

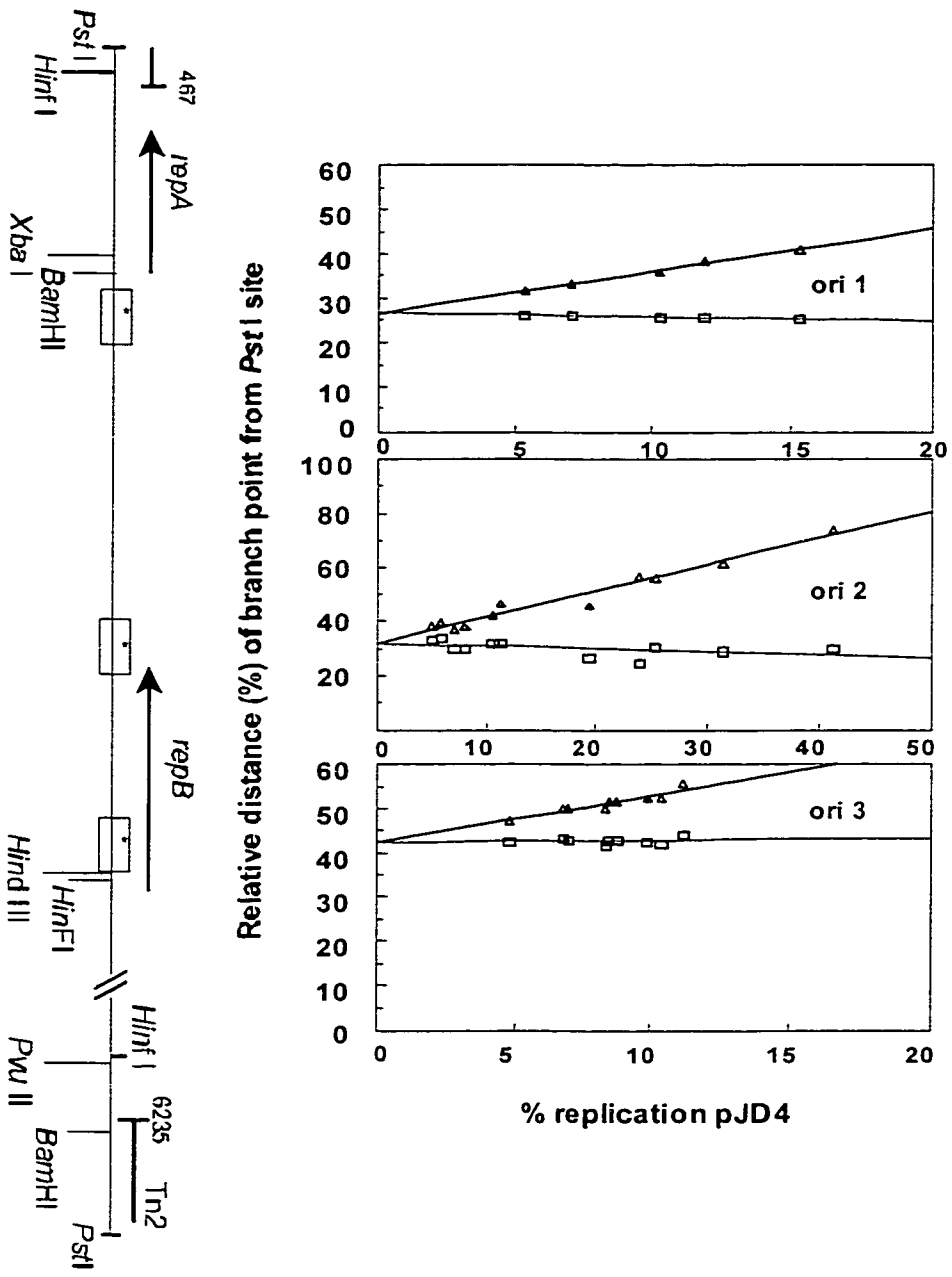

Figure 3.2. Plots of branch-point positions against percent replication of partially replicated plasmid molecules of pJD4 in *E. coli* C600, depicting the location of the replication origins. Plots of branch-point positions against the percent plasmid replication of pJD4 digested with *Pst*I are shown. Measurements of the distances from the *Pst*I site to the branch-points are relative to the linearized molecule digested with the same enzyme and are normalized to 100%. Each molecule has different symbols, representing the relative distances to the nearest (squares) and farthest (triangles) branch-point from the *Pst*I site. The lines were drawn using linear regression analysis. A schematic of the plasmid (compare to Figure 3.1) is shown for orientation purposes. Stars withing the oris (boxes) indicate where the lines meet at the Y-axis, corresponding to the start of DNA replication.

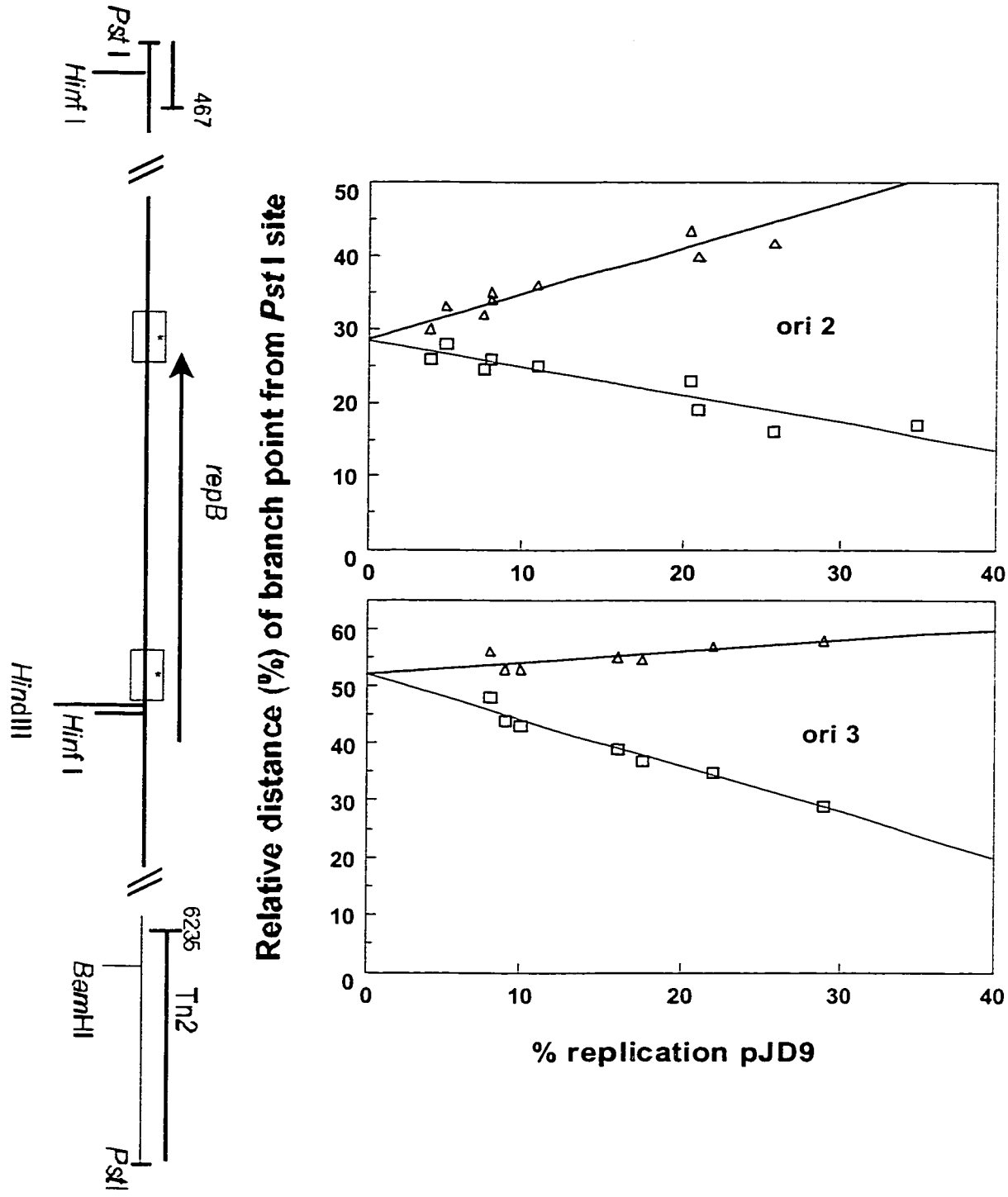

Figure 3.3. Plots of branch-point positions against percent replication of partially replicated plasmid molecules of pJD9 in *E. coli* C600, depicting the location of replication origins. See legend to Figure 3.2 for details.

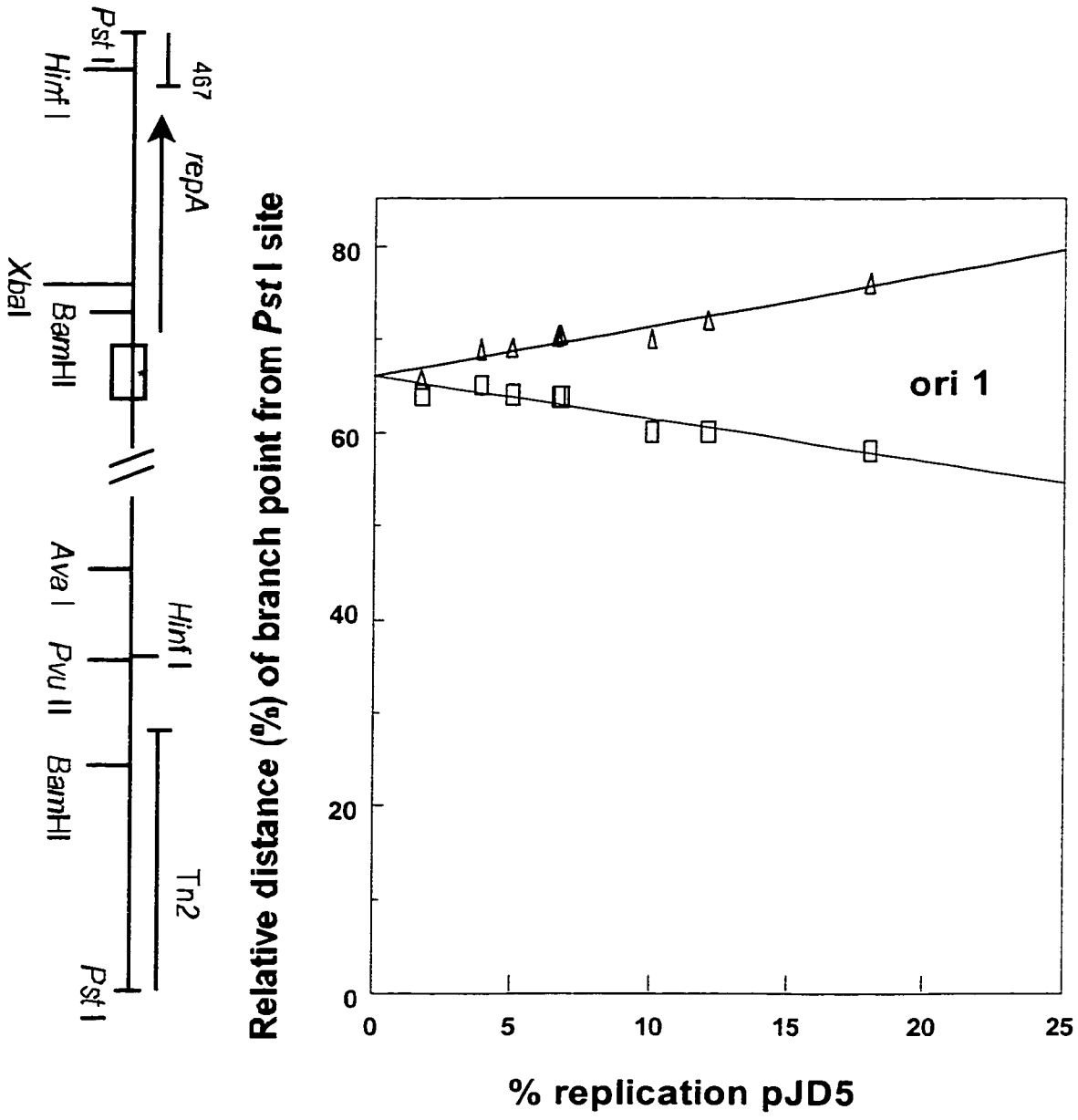

Figure 3.4. Plots of branch-point positions against percent replication of partially replicated plasmid molecules of pJD5 in *E. coli* C600, depicting the location of replication origins. See legend to Figure 3.2 for details.

Table 3.2. Incompatibility results for gonococcal plasmids pJD5/pFP5 (*ori1*) and pJD9/pFP9 (*ori2/ori3*).

Inc group	pJD9/pFP9	pJD5/pFP5	Incompatible?[1]
W	X[2]	X	Yes[3] with pJD9/pFP9
L/M	X	X	No[4]
B/O	X	X	No
H12	X	X	No
F1	X	X	No
Y	X	X	No
N	X	X	No
T	X	X	No
Q	X	X	No
V	X	X	No
FIIA	X	X	Yes with pJD5/pFP5
II	X	X	No
FIB	X	X	No
U	X	X	No

1. Experiments included plasmids being resident as well as incoming.

2. X denotes that plasmid was used.

3. Yes indicates plasmids cannot be maintained in the same cell (i.e. they are incompatible).

4. No indicates that plasmids can be maintained in the same cell.

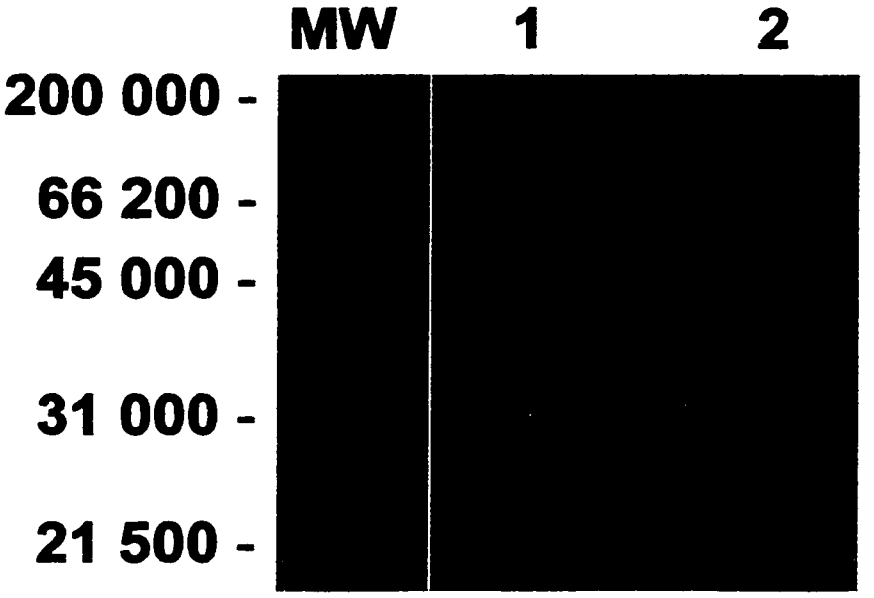

Figure 3.5. *In vitro* transcription/translation. DNA was amplified from pJD5 using primers FP9/FP10 (lane 1) or from pJD9 using primers FP7/FP8 (lane 2). Based on computer analysis, the RepA protein (lane 1) is 38.6 kDa and RepB (lane 2) is 39.4 kDa. MW, molecular weight protein standard.

Table 3.3. The role of RepB using rescue experiments in *E. coli* BLR(DE3).

Plasmid content	Replication of pORF15[1]
pORF15[2]	No
pORF15 + pBlueORF15[3]	Yes
pFP9	Yes
pBlueORF15	Yes

1. Replication was ascertained by isolating plasmid(s) from *E. coli* BLR (DE3) growing in the presence of appropriate antibiotics.

2. 80% of RepB deleted due to end-filling at unique *Hin*dIII site. This plasmid can not replicate autonomously.

3. ORF15 supplied *in trans* was able to rescue and maintain the replication of pORF15.

RepA is necessary for ori1, not required for ori2/3, and is expendable for pJD4 (ori1/2/3)

Other investigators first described a protein (RepA) which was involved with the replication of *ori1* on a fragment of the Asia-type plasmid pFA3, since removal of *repA* abolished replication of a DNA fragment containing *repA* and *ori1* (Gilbride and Brunton, 1990). We confirmed this observation using plasmids pJD4 (*ori1/2/3*) and pJD5 (*ori1*). Functional replication origin regions of plasmids pJD5 (and pJD4) contain *repA*, as identified by *in vitro* transcription/translation experiments and DNA sequence analysis (Pagotto *et al.*, 2000a; Fig. 3.5, lane 1). Inactivation of RepA in plasmid pJD5 by end-filling the unique *Xba*I site near the 5' end of *repA* or the unique *Bam*HI in pFP5 (Fig. 3.1) resulted in a plasmid which could not autonomously replicate (data not shown). To date, we have been unable to rescue a DNA sequence containing the *ori1* region by supplying the RepA protein *in trans*, suggesting that the *repA* gene is required *in cis* for *ori1* to function.

As pJD4 contains both *repA* and *repB*, attempts to knock out either gene were successful only for the *repA* gene (data not shown). That is, all constructs containing an intact *repB* and truncated *repA* gene replicated autonomously whereas no self-replicating plasmids with an intact *repA* and truncated *repB* gene were obtained in an *E. coli* background. Thus, RepA is dispensable for replication of pJD4 in *E. coli*, and is required only for replication of DNA sequences containing only *ori1*.

Gonococcal origins of replication are similar in their structural organization

DNA sequence comparisons between *ori1* and *ori2/ori3* regions on pJD4 and its related plasmids pJD9 (*ori2/3*) or pJD5 (*ori1*) indicated structural similarities between them (Fig. 3.7). Both origins appear to belong to the iteron family, as they both contained iterons (DR-48 for *ori2/3*; DR-26 for *ori1*) and replication initiation proteins (Fig. 3.6). While *repA* is distinct from the actual denaturation site as mapped by EM, *repB* spans all of *ori3* and part of *ori2* (Fig. 3.1). DNA sequence analysis of the promoter regions for *repA* and *repB* revealed that the putative transcription signals (-10 and -35) were located within or adjacent to the direct repeats DR-48 and DR-26 (Fig. 3.6). Interestingly, the two iterons DR-26 and DR-48 were 36% similar (Fig. 3.6C), having an 8 nucleotide conserved sequence motif. Sequence analysis demonstrated that RepA (339 aa,, 39 448 Da) and RepB (328 aa, 38 636 Da) were 61% identical at the nucleotide level and 57% homologous in their amino acid sequence.

Requirements for PolA

Iteron and RNA-regulated plasmids differ in their dependence for DNA pol I (Kornberg and Baker, 1992). Since the gonococcal penicillinase plasmids appeared to have an iteron-based type of organization (Fig. 3.7), the whole family of gonococcal β-lactamase-producing plasmids were tested for their ability to replicate and maintain themselves in an *E. coli polA* deletion mutant (Table 3.4). Interestingly, only plasmid pJD5, and its insertion derivative pGF1 (Nîmes-type) which both carry only *ori1* were unable to grow in a *polA*$^-$ host, lending further support that they belong to the IncFI family.

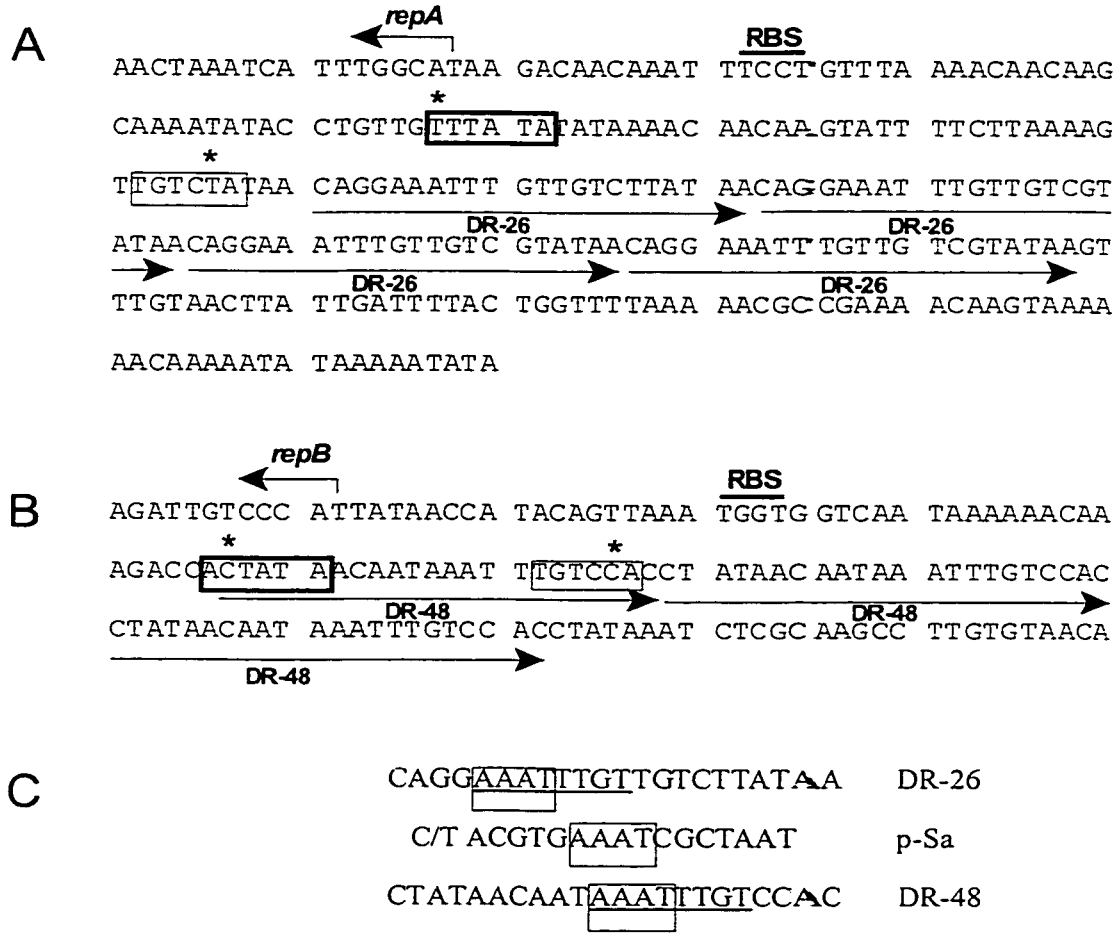

Figure 3.6. Promoter regions for *repA* (A) and *repB* (B). Start sites for Rep proteins, putative ribosome binding sites (RBS), -10 and -35 regions (thick and thin boxes, respectively) and direct repeats (arrows) are indicated. Mismatches to *E. coli* consensus -10 and -35 regions are indicated with astericks. (C) Sequence alignment between DR-26, DR-48 and iterons of plasmid pSa. Underlined sequences indicate identical matches between DR-26 and DR-48 and boxes between DR-26, DR-48 and iterons from pSa.

Table 3.4. Dependence of gonococcal β-lactamase-producing plasmids for DNA polymerase I.

Plasmid	SR1672 (*polA*⁻)	SR1758 (*polA*⁺)
pJD4 (Asia)	+[a]	+
pAS84/417 (New Zealand)	+	+
pJD7 (Toronto)	+	+
pGO4117 (Rio)	+	+
pJD9/pFP9	+	+
pJD5 (Africa)	-[b]	+
pGF1(Nîmes)	-	+
pBluescript KS⁺	-	+
pBlu-*Bam* HI[c]	+	+
pBR322	-	+

a- plasmid was able to maintain itself in the host.
b- plasmid was not able to establish and maintain itself in the host.
c- the large *Bam* HI fragment of pJD4 was inserted into the *Bam* HI site of pBluescript KS.

3.4 DISCUSSION

Plasmids containing three origins of replication, are rare and generally large in size (Crosa, 1980; Inuzuka *et al.*, 1980; Banerjee *et al.*, 1992). Using an *E. coli* background, I was able to show that the gonococcal Asia-type plasmid pJD4 is the smallest described plasmid to date containing three distinct origins of replication (*ori1*, *ori2* and *ori3*). The naturally occurring Africa-plasmid, typified by pJD5, contains a single origin of replication corresponding to *ori1* of pJD4. Current attempts to transform gonococci with plasmids containing *ori2/ori3* have been unsuccessful, indicating that *ori2* and *ori3* may only function in enteric bacteria. The host range of an Asia-type plasmid (*ori1/2/3*) was shown to include gonococci, Enterobacteriaceae, and *Hemophilus influenzae*, but not *Acinetobacter calcoaceticus* or *Pseudomonas aeruginosa* (Guiney and Ito, 1982). However, I have been able to transform *P. aeruginosa* with pFP9 but not pFP5, confirming that *ori1* has a different host range than plasmids containing *ori2/ori3* (Fig. 3.7). Previous work demonstrated that the 3.8 kb *Bam*HI-*Pvu*II fragment was essential for plasmid replication in *E. coli* (Johnson, 1985). This study has shown that this 3.8 kb DNA fragment contains *ori2/ori3* and RepB. Plasmid constructs containing *ori1* and *repA* have been shown to be functional in an *E. coli* background (Gilbride and Brunton, 1990; this study). Recently, plasmids containing *ori1* and *repA* have been shown to be functional in both a gonococcal and *Haemophilus* background (Pagotto *et al.*, 2000b, Chapter 4).

The Africa-type plasmid pJD5, containing *ori1*, demonstrated different replication characteristics than gonococcal plasmids containing *ori2/ori3*. The plasmid R1, used to classify the incompatibility of pJD5/pFP5 in this study, belongs to the IncFII plasmid family.

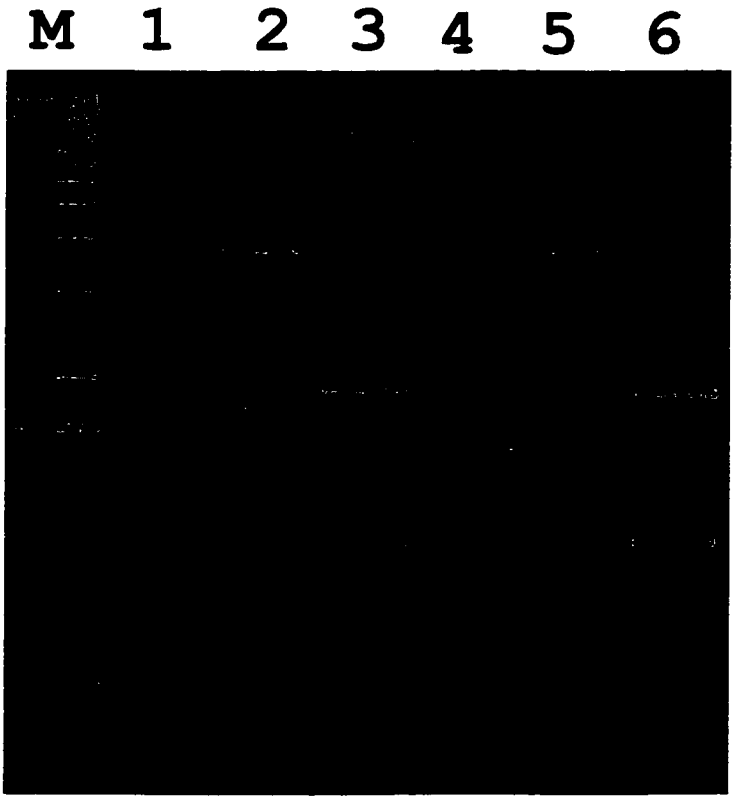

Figure 3.7 Agarose gel electrophoresis of plasmid pFP9 undigested (lanes 1 and 4), digested with *Hin*dIII (lanes 2 and 5) or digested with *Pst*I (lanes 3 and 6) isolated from *Escherichia coli* C600 (lanes 1-3) and *Pseudomonoas aeruginosa* PAO1 (lanes 4-6). M, 1 kb DNA size ladder.

Plasmid R1 does not have iterons for the Rep protein, and is classified as an RNA-regulated plasmid (Helinski *et al.*, 1996). In plasmid R1, translation of the RepA initiation protein is controlled by the antisense molecule *copA* (Light and Molin, 1983; Womble *et al.*, 1984). However, it should be noted that plasmids containing iterons have not been shown to possess antisense molecules associated with replication, copy number or incompatibility properties (Kues and Stahl, 1989; Helinski *et al.*, 1996; del Solar *et al.*, 1998). Thus, the regulation of *repA* in *ori1*-containing gonococcal plasmids and the role of the iterons in the replication process remain to be determined.

Plasmids pJD4 and pJD9/FP9 were shown to be incompatible with the IncW plasmid pUB2426, which contains a 1.5 kb fragment of DNA from the plasmid pSa (Couturier *et al.*, 1988). This 1.5 kb DNA fragment contains *oriV*, three iterons, and a major part of the *repA* gene (Couturier *et al.*, 1988). The RepA protein of pSa is proposed to bind the iterons and thus control its own rate of synthesis (Okumura and Kado, 1992). The iterons were also proposed to be the incompatibility determinant for this plasmid (Okumara and Kado, 1992). Alignment between the consensus sequence of the pSa iterons and DR-48 revealed that they were 41 % identical and contained 4 conserved nucleotides (Fig. 6C). This would suggest that the iterons DR-48 (and those from pSa) would bind to RepB.

Of the iteron-regulated plasmids described to date, only some have experimentally shown definitive binding of the iterons to the Rep protein. Other plasmids have been classified as iteron-regulated based on the genetic organization, and the presence of iterons (Helinski *et al.*, 1996). To attempt to explain the incompatibility observed between pJD4/pJD9 and pUB2426, I propose that iterons on pUB2426 may be interacting with RepB of pJD4/pJD9, and disrupting replication from either *ori2* or *ori3*. At least two possibilities

exist for the role of these iterons. In the first, the iterons from pUB2426 bind and titrate away RepB, thereby reducing replication from *ori2/ori3* (referred to as the titration model). In the second, the iterons from pUB2426 bind to the iterons in *ori2/ori3* containing plasmids and cause a pairing of gonococcal-pUB2426 iterons and shutting off replication through the coupling all plasmid molecules within the cell (referred to as the handcuffing, or coupling model)(Chattoraj, 2000). Which of these situations is occurring is not known at this time, and I have not ruled out a novel mechanism to explain gonococcal plasmid replication. While there are some similarities between the gonococcal iterons DR-48, DR-26 and those of pSa, comparisons of iterons of various plasmids revealed no consensus sequence and showed variation in both number and size (Helinski *et al.*, 1996).

Based on the results obtained in this study, the following model is proposed for replication of β-lactamase producing plasmids of *N. gonorrhoeae*. RepA is essential for *ori1*, and controls its own rate of synthesis by binding to iterons DR-26 which contain its promoter region. Binding of RepA to the iterons would also create a structural distortion in the DNA, allowing for the melting of the two strands, and for the loading of DNA replication proteins at the *ori*. For *ori2* and *ori3*, RepB acts as the replication initiation protein and most likely controls its own rate of synthesis by binding to iterons DR-48. Binding of RepB to the iterons would recruit the replication machinery to either *ori2* or *ori3*, in a similar manner to RepA (*ori1*), and as described for iteron containing plasmids (Kues and Stahl, 1989; Helinski *et al.*, 1996). Exactly how the binding of RepB causes either *ori2* or *ori3* to be used preferentially is unknown. It is most likely that *ori2* and *ori3* mimics the situation described with plasmid R6K, where the gamma origin is rarely used but is required in *cis* for the alpha and beta origin to be used (Kukherjee *et al.*, 1988). We are continuing to investigate the

phenomenon of multiple origins of replication and their differential usage using the gonococcal plasmids as a model system.

In conclusion, this is the first report of a naturally occurring plasmid of *N. gonorrhoeae*, when studied in an enteric background such as *E. coli*, that has three functional origins of replication that also encodes two functionally distinct replication initiation proteins, and belongs to two incompatibility groups. Future work will include studies involving replication of the gonococcal β-lactamase-producing plasmids in some of their other hosts, including *Neisseria*, *Pseudomonas* and *Haemophilus spp.*

CHAPTER 4

STABLE SHUTTLE VECTORS FOR *NEISSERIA GONORRHOEAE, HAEMOPHILUS SPP.* AND OTHER BACTERIA BASED ON A SINGLE ORIGIN OF REPLICATION

(Pagotto, F., Salimnia, H., Totten, P. and Dillon, J.R. (2000) Stable shuttle vectors for *Neisseria gonorrhoeae, Haemophilus* spp. and other bacteria based on a single origin of replication. *Gene* **244:** 13-19.)

4.1 INTRODUCTION

Up until the early 1980's, genetic manipulation of *Neisseria gonorrhoeae* was limited to transformation using naked DNA and naturally competent *N. gonorrhoeae* cells (Stein *et al.*, 1983a; Seifert and So, 1991). In these studies, genes from the gonococcus were isolated by cloning into *Escherichia coli,* manipulated using molecular techniques, then reintroduced into the gonococcus using the natural competency of the gonococcus, which requires the presence of a specific uptake sequence on transforming DNA (Stein *et al.*, 1983a; Silver and Clark, 1995). The DNA, which enters the gonococcus in a linear form, requires the RecA protein and homologous sequences in resident DNA to recombine with the chromosome (Biswas *et al.*, 1986b; Koomey *et al.*, 1987). This technique, used to delete or "knock-out" genes by homologous recombination, was limited because the study of essential genes was not feasible since alteration of these genes caused cell death.

In the last 10 years, a few suicide vectors have been constructed to allow the introduction and subsequent allelic replacement of genes back into the gonococcus. The first of these, pFT180, was constructed by fusing the 4.4 MDa β-lactamase-producing plasmid with the 2.6 MDa cryptic plasmid (Stein *et al.*, 1983a). This vector was large for cloning longer sequences, its DNA sequence was not completely known, and the vector did not contain multiple cloning sites, thereby making cloning of genes more difficult. A derivative of pFP180, pLES92, was created to overcome the limitations of pFT180 (Stein *et al.*, 1983b). pLES92 contained a β-lactamase gene for selection and a *lac* region from pUC9 allowing for the direct selection of hybrid plasmids in appropriate *E. coli* hosts via the disruption of β-galactosidase α complementation (Stein *et al.*, 1983a). There were a few unique restriction sites for cloning. This vector required that the gonococcal uptake sequence

be introduced in order to transform the gonococcus.

In order to study gene regulation as a single copy, not achievable with multi-copy vectors such as pLES92, Silver and Clark (1995) created a translational *lacZ* reporter system to study gene regulation in the gonococcus. The suicide vector, named pLES94, was pUC18-based, and contained a unique *Bam*HI restriction site to allow the cloning of promoters resulting in promoter-*lacZ* gene fusions (Silver and Clark, 1995). The promoter of interest was initially cloned and selected in *E. coli*, using either ampicillin or chloramphenicol selection (Silver and Clark, 1995). Because of the gonococcal *proAB* genes flanking the *lacZ*-promoter fusion, the plasmid would integrate into the gonococcal *proAB* locus via allelic replacement, allowing for the introduction of the promoter-*lacZ* fusion. Once introduced into the gonococcus by transformation, pLES94 allowed for β-galactosidase assays experiments to study gene regulation under varying conditions (Silver and Clark, 1995). Because the origin of replication is enteric in origin, pLES94 is incapable of replicating in the gonococcus as an extrachromosomal element.

In the early 1990's the Tn*Max* vectors were created to allow shuttle mutagenesis (Haas *et al.*, 1993). Using these vectors, based on a series of Tn1721-based mini-transposons, genes were cloned in *E. coli*, subjected to transposon-based mutagenesis, and then reintroduced in the gonococcus or other bacteria as suicide vectors, using chloramphenicol (Tn*Max1*) or erythromycin (Tn*Max2*) resistance (Haas *et al.*, 1993). The Tn*Max* vectors were suitable for targeting of cloned genes, shuttle mutagenesis, and identification of protein export signals as well as rescue of chromosomal markers (Haas *et al.*, 1993). A new collection of Tn*Max* vectors was later developed which included other antibiotic markers such as kanamycin and the M13 forward and reverse sequencing primers

to facilitate sequencing (Kahrs *et al.*, 1995). Various derivatives contained promoterless *trp-lacZ* (Tn*Max11*), *xylE* (Tn*Max10*), *phoA* (Tn*Max6*) or *blaM* (Tn*Max7*, Tn*Max9*) genes, allowing for *in vivo* gene and operon fusions to study gene regulation (Kahrs *et al.*, 1995). Some of the Tn*Max* vectors (pMin) facilitated mutagenesis due to conjugation-related properties, selecting on Tn*Max*-insertions into the cloned, rather than the vector sequences (Kahrs *et al.*, 1995). While these vectors were very useful for targeting and sequencing of genes cloned in *E. coli* and for shuttle mutagenesis of those bacterial species which could not be targeted by direct transposition, it was not possible to work with essential genes as reintroduction would lead to a potentially lethal recombination event.

The most advanced vectors to date are the Hermes shuttle vectors (Kupsch *et al.*, 1996). These vectors are the only vectors where genes can be introduced in the gonococcus as extrachromosomal elements and allow for genetic complementation with cloned genes of both transformable and non-transformable *Neisseria*. The Hermes vectors are composed of a region of the gonococcal p*tetM*25.2 conjugative plasmid that allows plasmid replication and conjugative transfer in *N. gonorrhoeae* (Kupsch *et al.*, 1996). Fused to an origin of replication (p15A) that functions only in *E. coli*, these vectors also contain a selectable marker and a multiple cloning site that is flanked by the integration region of p*tetM*25.2. The Hermes vectors may be introduced by conjugation into both transformable (i.e. piliated) and non-piliated gonococci. A drawback to the Hermes vectors are their size (greater than 7 kb) and the fact that they involve a three step process; 1) cloning of a gene into a Hermes vector and selection in *E. coli*, 2) transformation of the vector into a strain of *N. gonorrhoeae* harbouring a complete p*tetM*25.2 to create a hybrid via gene replacement on the pTET25.2, and 3) conjugative transfer into the recipient gonococcal strain of choice (Kupsch *et al.*,

1996).

Currently, the only vectors available for cloning in *H. ducreyi* are pLS88, or its derivatives, pLSSK/pLSKS (Dixon *et al.*, 1994; Wood *et al.*, 1999). In *H. influenzae*, shuttle vectors containing either one or two oris have been described (Heidecker *et al.*, 1994 and references therein). They are based on the ori from plasmids RSF0885, whose stability in *E. coli* has been questioned (Trieu and McCarthy, 1990), or p15A, shown to be stable in both *E. coli* and *H. influenzae* (Chandler, 1991).

To overcome some of the constraints imposed by these genetic systems, a basic vector, pFP10, capable of stably replicating extrachromosomally in *N. gonorrhoeae* was constructed. This vector contains a single origin of replication which is functional in several genera (*Neisseria, Haemophilus, Escherichia*), is small, and contains a completely characterized DNA sequence. The vector pFP10 was used in cloning experiments to complement a proline-requiring *N. gonorrhoeae* and *E. coli* and to express the green fluorescent protein (GFP) for the first time in both the gonococcus and *Haemophilus ducreyi*.

4.2 MATERIALS AND METHODS

Bacterial strains, plasmids, enzymes and reagents

The bacterial strains and plasmids used in this study are listed in Table 4.1. *N. gonorrhoeae* strains were obtained from our culture collection and *H. ducreyi* strain 35000 and *H. influenzae* strain Rd were maintained in the laboratory of Dr. P. Totten (Department of Medicine, Division of Allergy and Infectious Diseases, University of Washington, Seattle, WA). Strains 35000 and Rd were routinely cultured on charcoal medium [GC-agar base (Difco), 1% GGC (100% GGC is 10% glucose, 1% glutamine, 2.6% cysteine), 1% hemoglobin, 0.2% activated charcoal (Sigma)] at 35^{0}C as previously described (Totten and Stamm, 1994). Strains of *E. coli* were cultured on Luria Bertani (LB) agar (Difco), and incubated at 37°C for 16 hours. Strains of *N. gonorrhoeae* were grown on GC-agar base (GCMB, Difco) supplemented with 1% modified Kellogg's (GCMBK) defined supplement (40 g glucose, 1 g glutamine, 10 ml of 0.5% ferric nitrate solution, 1 ml of 20% cocarboxylase) and were incubated at 35°C for 14-18 hours in a humid atmosphere supplemented with 5% CO_2. The identity of gonococcal strains was confirmed by standard biochemical methods, serological analysis and auxotyping (Dillon *et al.*, 1988). Restriction endonucleases (R/E), T4 DNA ligase, calf intestinal phosphatase and *Taq* polymerase were purchased from Boehringer Mannheim or New England Biolabs and used according to the manufacturer's instruction. Primers were purchased from the University of Ottawa Biotechnology Research Institute.

Table 4.1 Bacterial strains and plasmids used in this study.

Strain/plasmid	Relevant characteristic (source)
E. coli	
HB101	F^-, *thi-1*, *hsdS20*(r_B^-,m_B^-), *supE44*, *recA13*, *ara*-14, *leu*B6, *pro*A2, *lac*Y1, *rps*L20(str^r), *xyl*-5, *mtl*-1 (Gibco)
DH5α	F^-, *end*A1, *hsd*R17 (r_k^-,m_k^+), *sup*E44, *thi*-1, λ^-, *rec*A1, *gyr*A96, *rel*A1, ϕ80d*lac*ZΔM15 (Δ*lac*ZYA-*arg*F (Gibco)
N. gonorrhoeae	
F62	Proline-requiring, streptomycin resistant (J.R. Dillon)
MS11	Wild-type (J.R. Dillon)
CH811	Wild-type (J.R. Dillon)
Haemophilus spp.	
35000	*H. ducreyi* isolated in Manitoba, Canada; laboratory strain (P. Totten)
Rd	*H. influenzae* laboratory strain (P. Totten)
Plasmid	
pEGFP	Green fluorescent protein, Ampicillin resistance (Clontech)
pACYC184	Chloramphenicol resistance (New England Biolabs)
pJD5	β-lactamase plasmid of *N. gonorrhoeae* (Yeung and Dillon, 1988)
pFP10	*ori* from pJD5 + *cat* from pACYC184 (this study)
pFP11	pFP10 + proAB from *N. gonorrhoeae* CH811 (this study)
pFP12	pFP10 + plac-MCS-GFP-MCS from pEGFP (this study)

Transformation and complementation studies

E. coli cells were transformed using the calcium chloride method (Dillon *et al.*, 1985). Briefly, competent cells incubated with 5-100 ng of plasmid DNA on ice for 30 minutes, heat-shocked at 42^{0}C for 2 minutes, and incubated on ice for 5 minutes. Cells were suspended in 1 ml SOC medium (Sambrook *et al.*, 1989), and incubated at 37^{0}C with shaking for 1 hour. Dilutions were plated on LB plates supplemented with 50 μg ml-1 chloramphenicol and incubated overnight at 37^{0}C. *N. gonorrhoeae* strains were transformed by streaking a loopfull of confluent 12-14 hours growth over a 1 cm^2 surface area on GCMBK-agar. DNA (1 μg) was added over the streaked area and the plates were incubated for 6 hours as described above. After 6 hours, the cells were collected using a sterile loop and spread onto GCMBK plates containing 5 μg ml^{-1} chloramphenicol and incubated for 24-48 hours. Transformant colonies were streaked on plates containing 15 μg ml^{-1} chloramphenicol. *E. coli* and *N. gonorrhoeae* transformant colonies were purified by subculturing at least two consecutive times on chloramphenicol-containing medium. Plasmid integrity in both *E. coli* and *N. gonorrhoeae* was subsequently determined by plasmid content analysis and restriction endonuclease analysis as previously described (Dillon *et al.*, 1985). *H. ducreyi* and *H. influenzae* were transformed by electrotransformation and were selected on charcoal medium supplemented with 2 μg ml^{-1} chloramphenicol as described previously (Totten *et al.*, 1995). I would like to thank Dr. P. Totten, who collaborated with us on this project. Dr. Totten transformed *Haemophilus* species with my vector and sent us the strains so I could do the fluorescence microscopy. Media used for selecting *N. gonorrhoeae* F62 or *E.coli* HB101 clones complemented for their proline deficiencies have been described previously (Picard and Dillon, 1989; Sambrook *et al.*,

1989). I acknowledge the help of H. Salimnia, who cloned the gonococcal *proAB* genes into pFP10 and then transformed *N. gonorrhoeae* cells. He also PCR-amplified the multiple cloning site-GFP DNA fragment from pEGFP.

PCR

The origin of replication from pJD5, a naturally occurring, β-lactamase-producing plasmid of *N. gonorrhoeae*, was amplified using primers IT1 (5'-ATGTCTGCAGGCCGTCTGAACCGCTC TAACCGCTT-3') and IT2 (5'-CAGTAAGCTTTCGCCGTTCTGGTG-3') to produce a 3.8 kb amplicon (Fig. 4.1). The chloramphenicol acetyl-transferase cassette (*cat*) was amplified from pACYC184 using primers IR1 (5'-CTAGCTGCAGTGTTGATACCGGGAA-3') and IR2 (5'-CGTGAAGCTTAAGTGCGGTCATCTTCGGTTTCCGT-3')(Fig. 4.1). The uptake sequences from *N. gonorrhoeae* (5'-GCCGTCTGAA-3') (Goodman and Scocca, 1988) and *H. influenzae* (5'-AAGTGCGGT-3') (Fitzmaurice et al., 1984) were added to the primers IT1 and IR2, respectively, as well as the restriction endonuclease sites *Pst*I (single underline; IT1 and IR2) and *Hin*dIII (double-underlined, IT2 and IR1) for cloning purposes. Amplification of the *proAB* genes from *N. gonorrhoeae* CH811 (NR/IB2/plasmid-free) was accomplished using primers HS3 (5'-CCCAAGCTTCGCAGCAGAAATATGGACAC-3') and HS4 (5'-CCCAAGCTTTCGGATTATTCCCTAACCTG-3') containing *Hin*dIII restriction endonuclease sites. PCR amplification was performed in a Perkin-Elmer 9600 Thermocycler (Perkin-Elmer Corp.) with the PCR Core Kit (Boehringer Mannheim) and the following reaction conditions: 1x PCR buffer containing 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 1 μM of each primer and 2.5 U of *Taq* polymerase per 100 μl reaction. Amplified products were purified using the Qiagen PCR purification kit. Primers HS1 (5'-

AATACGCAAACCGCCTCTCC-3') and HS2 (5'-ATCACCGAAACGCGCGAGAC-3') were used to amplify the gene encoding GFP, the flanking multiple cloning sites (MCS), and the promoter region from pEGFP (Clontech). All PCR conditions reflected the thermal melting temperatures of the primers and amplification reactions were adjusted accordingly and are available upon request.

Fluorescence Microscopy

E. coli HB101, *N. gonorrhoeae* F62, and *H. ducreyi* 35000 cells containing pFP12 were used in wet mounts to examine GFP expression. Overnight cultures were observed at 1000X magnification using a Leica DM_RB Microscope or a Zeiss Axioskop (Zeiss X100 oil immersion objective, 100W HBO lamp, standard fluorescein isothiocyanate filter set) and photographed or captured digitally with Image Analysis Software version 5.0 (Northern Eclipse) using a Sony 3 CCD colour video camera (Model DXC-950).

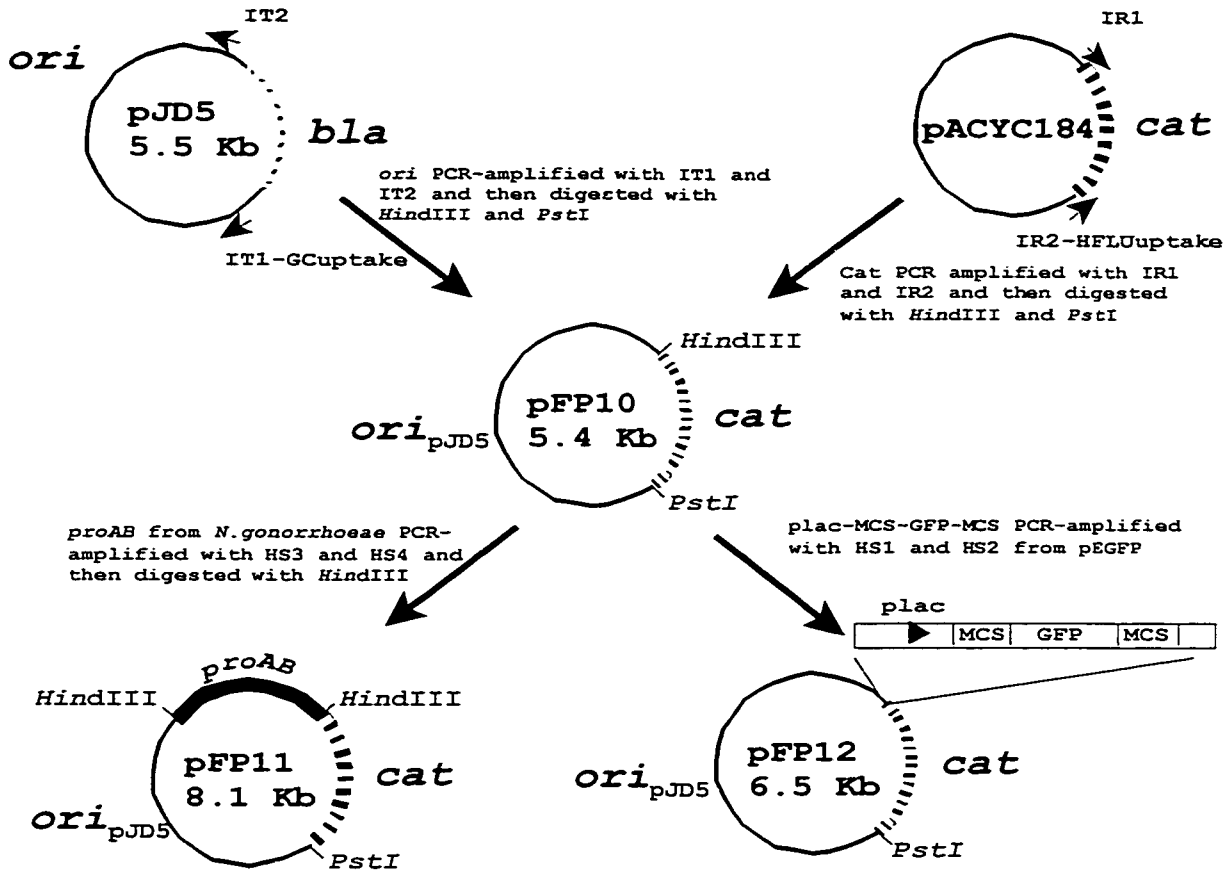

Figure 4.1 Schematic outlining the construction of shuttle vector(s) for *N. gonorrhoeae*. The basic vector, pFP10, contains the origin of replication from pJD5, an Africa-type β-lactamase-producing plasmid from *N. gonorrhoeae*, uptake sequences from *N. gonorrhoeae* (GCuptake) and *Haemophilus influenzae* (HFLUuptake), ligated to the *cat* cassette from pACYC184. The *proAB* genes from *N. gonorrhoeae* CH811 were amplified and cloned into the *Hin*dIII site of pFP10 to generate pFP11. Similarly, the plac-MCS-GFP-MCS DNA fragment was amplified from pEGFP, made blunt-ended, and was cloned into pFP10 (*Hin*dIII digested and blunt-ended), generating pFP12.

4.3 RESULTS AND DISCUSSION

Construction of series of shuttle vectors for *N. gonorrhoeae*

The basic vector, pFPP10, which contains uptake sequences for both *N. gonorrhoeae* and *H. influenzae* and two unique cloning sites was created by amplifying a 3.8 kb fragment of the naturally occurring Africa-type β-lactamase-producing plasmid, pJD5 (coordinates 522-4406, Genbank accession number U20375), which we have ascertained contains a single origin of replication (Yeung and Dillon, 1988; Pagotto *et al.*, 1997, manuscript submitted), and ligating it to a fragment containing the *cat* cassette of pACYC184 (coordinates 3096-370, Genbank accession number X06403)(Fig. 4.1). *N. gonorrhoeae* F62, MS11, and *E. coli* DH5α were successfully transformed with pFP10 at transformation efficiencies of 10^2 (F62, MS11) and 10^7 (DH5α) transformants per μg DNA. This plasmid was stable in both organisms since removal of antibiotic selection (subculturing on non-selective media for 3 days) did not result its loss. No apparent rearrangements, insertions, or deletions were noted with either pFP10 or the resident cryptic plasmid (pJD1 in the case of *N. gonorrhoeae*) as observed by multiple R/E digestions of isolated plasmids (data not shown). This is the first cloning vector functional in both *E. coli* and *N. gonorrhoeae* that uses a single defined origin of replication and which does not require other plasmids for maintenance.

Complementation of *N. gonorrhoeae* F62 and *E. coli* HB101 using pFP11

To demonstrate the ability of pFP10 to genetically complement gonococcal genes *in trans*, pFP11 was constructed by amplifying gonococcal *proAB* genes and cloning them into the *Hind*III site of pFP10 (Fig. 4.1). Transformation and complementation of the proline-requiring strain *N. gonorrhoeae* F62 was done *in trans* (Table 4.2). *N. gonorrhoeae* F62 transformed with or without pFP10 were unable to grow in proline-deficient medium

(Table 4.2), demonstrating the specificity of the insert for complementation. Using pFP11, isolated from the gonococcus, functional, heterologous complementation using *E. coli* HB101, a proline-deficient strain was achieved (Table 4.2).

Expression of green fluorescent protein in *N. gonorrhoeae* and *H. ducreyi*

The use of GFP has never been demonstrated in the gonococcus or in *H. ducreyi*. A plasmid was constructed, pFP12 (Fig. 4.1), from pFP10 by amplifying the *plac*-GFP-MCS fragment (1,126 bp) from pEGFP and cloning it in the *Hind*III site of pFP10 which was made blunt-ended using Klenow enzyme. This inserted sequence also contains several unique restriction enzyme sites that are useful for cloning purposes, both in the 5' MCS (*Sph*I, *Hin*dIII, *Acc*I, *Sal*I, *Sma*I, *Kpn*I) and the 3' MCS (*Not*I, *Stu*I, *Apa*I, *Bsp*120I). Successful transformation of pFP12 into *N. gonorrhoeae* F62 and *H. ducreyi* was achieved and fluorescence was observed (Fig. 4.2). Cells with or without the vector alone (i.e. pFP10) did not demonstrate autofluorescence (Fig. 4.2 c, d). When compared to single *E. coli* cells harbouring pFP12, GFP expression, as measured by fluorescence microscopy, was not as high in single gonococcal cells (data not shown); however, clumps of gonococci were clearly visible and easily observed. Our laboratory has also shown that GFP was functional as a fusion protein with gonococcal *ftsZ* (Salimnia *et al.*, 1999). GFP expression was further confirmed by Western analysis using anti-GFP antibodies and was shown to be insoluble as well as in lower concentrations as compared to *E. coli* (Salimnia *et al.*, 1999). Aside from the fact that gonococcal cells are much smaller than *E. coli* cells, the paler fluorescence in single cells might be explained based on the requirement of GFP for molecular oxygen to form the GFP-fluorophore (Heim *et al.*, 1994; Knapp and Rice, 1995). Since *N. gonorrhoeae* is a microaerophilic organism, it is possible that its intracellular oxygen

concentration is lower than in *E. coli* (i.e. most probably due to its redox potentials) interfering with the proper formation of the GFP-fluorophore. This situation should overcome by using newer generation GFP's which are constantly being modified to increase their fluorescence.

Broad host range of the gonococcal shuttle vectors

Successful transformation of *H. influenzae* Rd and *H. ducreyi* 35000 with pFP10 which was maintained extrachromosomally was shown. Plasmid DNA isolated from these bacteria was re-introduced into *E. coli* HB101 and R/E analysis did not reveal any structural anomalies (deletions, rearrangements). In addition, expression of GFP from pFP12 was also demonstrated in *H. ducreyi* for the first time (Fig 4.2h). Currently, the only vector used for molecular studies in *H. ducreyi* is pLS88, or its derivatives, pLSSK/pLSKS (Dixon *et al.*, 1994; Wood *et al.*, 1999). I have demonstrated that the origin of replication from pFP10 is incompatible with the origin of pLSSK, by using incompatibility experiments (Bergquist, 1987), indicating that the mechanisms underlying the replication of these plasmids are very similar (data not shown). Introduction of the origin from pLSSK in a cell of *E. coli* harbouring the origin from pFP10 caused a dramatic loss in copy number of the gonococcal (i.e. pFP10) origin of replication (data not shown).

Table 4.2 Functional complementation of proline-requiring *Neisseria gonorrhoeae* F62 and *Escherichia coli* HB101 using pFP11.

Strain	Plasmid content	Minimal media with proline	Minimal medium
F62[a]	cryptic	+	-
F62	cryptic + pFP10	+	-
F62	cryptic + pFP11	+	+
HB101	-	+	-
HB101	pFP10	+	-
HB101	pFP11	+	+

a. Wild-type F62; proline requiring.

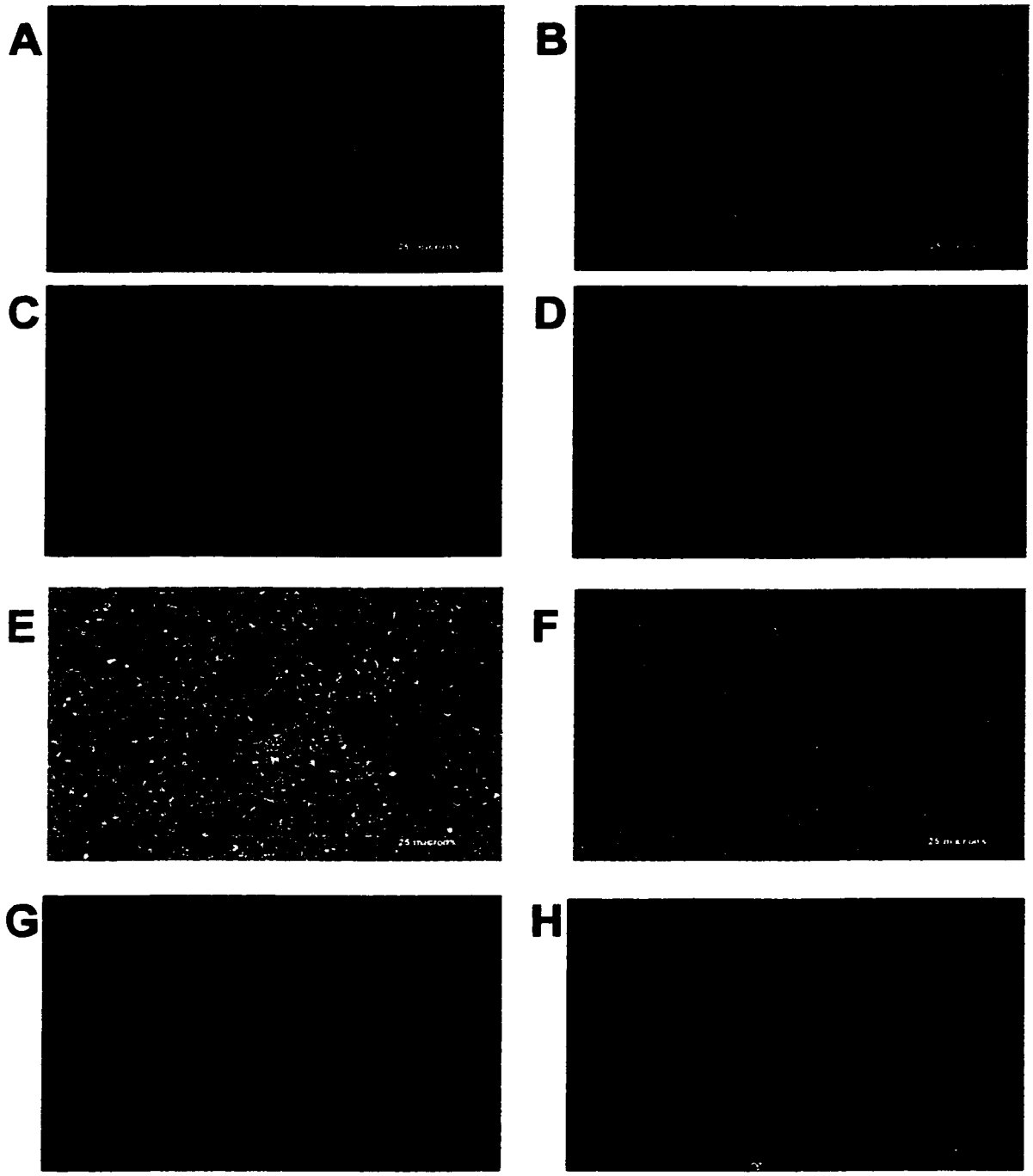

Figure 4.2 Green fluorescent protein expression in *N. gonorrhoeae* F62 and *H. ducreyi* 35000. Depicted are phase-contrast micrographs and fluorescent micrographs of *N. gonorrhoeae* F62 (A, C) and *H. ducreyi* 35000 (B, D), respectively, carrying pFP10, and phase-contrast and fluorescent micrographs of *N. gonorrhoeae* F62 (E, G) and *H. ducreyi* 35000 (F, H) carrying pFP12. Wet mount of cells grown overnight were prepared and photographed using a phase-contrast or fluorescent microscope.

4.4 CONCLUSIONS

A basic, versatile vector (pFP10) has been developed capable of replicating in *E. coli*, *N. gonorrhoeae*, *H. ducreyi*, and *H. influenzae*. Genes cloned in *E. coli* can be easily introduced into these organisms as extrachromosomal elements, allowing complementation of essential genes by supplying them *in trans*. This basic vector is now being further developed to include other selectable markers as well as a multiple cloning site. A genetically defined origin of replication able to replicate in diverse bacteria was used. A single origin is advantageous since the size of the vector is kept small and there is no interference from other origins on the same plasmid that may share similar aspects of DNA replication, thereby decreasing the host range (Couturier et al., 1988). For the first time, the GFP was shown to be functional in *N. gonorrhoeae* and *H. ducreyi*. Thus, GFP may be used as a biological marker in these organisms to study gene expression, regulation, protein localization as well as localization of new bacteria in infection animal models.

CHAPTER 5

THE INTEGRATION HOST FACTOR IS A NECESSARY COMPONENT IN THE REPLICATION OF GONOCOCCAL B-LACTAMASE-PRODUCING PLASMIDS

(Pagotto, F., Szeto, J., Hill, S.A., and Dillon, J.R. (2000) The integration host factor is a necessary component in the replication of gonococcal β-lactamase-producing plasmids. To be submitted to *Gene* in December, 2000)

5.1 INTRODUCTION

Neisseria gonorrhoeae is a Gram-negative diplococcus that is the causative agent of the sexually-transmitted disease, gonorrhea. During the 60's and early 70's, penicillin was the preferred drug of choice for the treatment of uncomplicated gonococcal urethritis. However, penicillin is no longer used to treat such an infection due to the global predominance of isolates which carry penicillinase-producing plasmids (Ison *et al.*, 1998; Moran and Levine, 1995). Previous analysis has indicated that all gonococcal penicillinase-producing plasmids are structurally related, with five distinct groups of β-lactamase-producing plasmids being derived from the prototypic 7.4 kb Asia-type plasmid (pJD4), either through deletions, insertions, or sequence duplications (Brunton *et al.*, 1986a; Pagotto *et al.*, 2000a).

Previous molecular analysis of pJD4 has indicated that this particular plasmid is unusual in that it possesses three origins of replication (Pagotto and Dillon, submitted; see Fig. 3.1). This study also demonstrated that replication of pJD4 required two replication initiation proteins, RepA and RepB, which bound at *ori1* and *ori2/3*, respectively. Furthermore, DNA sequence analysis also revealed the presence of nine putative binding domains for the host supplied Integration Host Factor (IHF) protein (Pagotto *et al.*, 2000a, Fig. 5.1).

In *Escherichia coli*, IHF is a heterodimeric small DNA binding protein composed of α and β subunits encoded by the *himA* and *hip/himD* genes, respectively (Friedman, 1988). IHF recognizes the consensus sequence 5'YAANNNNTTGATW3', where Y= C or T and W = A or T (Craig and Nash, 1984), and when bound to the consensus causes an approximate

180° bend in the DNA (Rice, 1997). In the gonococcus, IHF binds to a similar consensus sequence causing equivalent physical effects (Hill *et al.*, 1997). Moreover, IHF has been implicated in the regulation of several gonococcal genes (e.g. *pilE*) where it serves as a transcriptional cofactor (Hill *et al.*, 1997; Fyfe and Davies, 1998).

In this study, the question of whether IHF played a role in the replication of β-lactamase producing plasmids of *N. gonorrhoeae* due to the identification of the numerous potential IHF binding domains at the replication origin of pJD4 was investigated. Genetic and physical analyses have identified several important IHF binding sites on plasmid pJD4 leading to the proposition that a hierarchy of IHF binding site usage may exist in plasmids that contain more than one IHF binding domain at their replication origins.

5.2 MATERIALS AND METHODS

Bacterial strains and plasmids

The strains of *E. coli* and the plasmids used in this study are listed in Table 5.1. Strain DH5α containing the various plasmid species was grown at 37°C on Luria-Bertani (LB) agar (Difco) or Tryptic Soy Broth (TSB; Difco) supplemented with ampicillin. Strain CJ236 was grown in either 2X yeast tryptone broth (YT; Difco),or, on Tryptic Soy Agar (TSA; Difco) at 37°C. XL1-Blue cells were grown in tryptone yeast phosphate (TYP) [per litre; 16 g Bacto-tryptone, 16 g yeast extract (Difco), 5 g NaCl, 2.5 g K2HPO4] broth with shaking, or on LB or B agar [per litre; 10 g Bacto-tryptone (Difco), 8 g NaCl, 20% glucose, 16 g Agar (Difco] at 37°C. When required, the following antibiotics were added singly or in combination at the following concentration: ampicillin (100μg/mL), chloramphenicol (50μg/mL) and tetracycline (50μg/ml). Plasmids pJD4, pJD5 and pJD9 have been described in detail previously (Yeung and Dillon, 1988; Pagotto *et al.*, 2000a).

Construction of pFP1

Plasmid pFP1 (Fig. 5.1) was constructed by digesting pJD4 with *Bam*HI followed by cloning the 5 kb fragmented into *Bam*HI-digested pBluescript II KS (+) (Stratagene) which had been dephosphorylated with 1 U of calf-intestinal alkaline phosphatase (Pharmacia Biotech) in a 100 μL reaction at 37°C for 1 hour followed by purification using the Qiagen PCR Purification Kit (Qiagen Inc.; Valencia, California). Following transformation of *E. coli* XL1-Blue the recombinant plasmid was verified by restriction digest analysis and DNA sequencing.

Table 5.1. Bacteria and plasmids.

E. coli strains, plasmids, and phages	Genbank number	Relevant characteristics	Source and/or reference
Strain			
DH5α		[*supE*44, Δ*lac*U169 (80 *lacZ* ΔM15), *hsdR*17, *recA*1, *endA*1, *gyrA*96, *thi*-1, *relA*1]	Stratagene
XL1-Blue		[*supE*44, *hsdR*17, *rec*A1, *endA*1, *gyrA*46, *thi*-1, *relA*1, *lac*-1 (F' *proAB*, *lacI*^{q}, *lacZ*ΔM15, Tn10{tet^r}]; (Stratagene)	Stratagene
SR1672		[Δ*polA*, Δ(*gal* bio), *thi*-1, *relA*1, *spoT*1, Kan^r]	Banerjee *et al.*, 1992
CJ236		[*dut*1, *ung*1, *thi*-1, *rel*A1 / pCJ105 (cam^r F')]	Bio-Rad
N99		*E. coli* K12, galK; parental strain	Mendelson *et al.*, 1991
5427		N99, *hupA16*::*kan*, *hupB11*, Δ*himA82*::Tn*10*;	Mendelson *et al.*, 1991
5179		N99, *hupA16*::*kan*, *hupB11*::*cat*, *himD157*::Tn*10*	Mendelson *et al.*, 1991
5477		N99, *hupA16*::*kan*, *hupB11*, Δ*himA82*::Tn*10*	Mendelson *et al.*, 1991
Plasmid			
pJD4	U20374	7.4 kb Asia-type plasmid	Pagotto *et al.*, 2000
pJD5	U20375	5.5 kb Africa-type plasmid,	Pagotto *et al.*, 2000
pJD9	U20420	*In vitro* deletion derivative of pJD4	Yeung and Dillon, 1988
pBluescript KSII (+)	X52328	Ampicillin resistance cloning vector	Stratagene

Table 5.1 Bacteria and plasmids (continued)

E. coli strains, plasmids, and phages	Genbank number	Relevant characteristics	Source and/or reference
pFP1		Large *Bam*HI fragment of pJD4 cloned in pBluescript KSII (+)	This study
pFP1 (Δ3)		pFP1 with IHF binding site 3 removed	This study
pFP1 (Δ4/7)		IHF binding site 4 and 7 removed	This study
pFP1 (Δ4/7D)		13 nucleotides immediately downstream of both IHF binding sites 4 and 7 removed	This study
pFP1 (Δ8/9)		IHF binding sites 8 and 9 removed	This study
pFP1 (Δ4/7/8/9)		IHF binding sites 4, 7, 8 and 9 removed	This study
Phage			
R408		Helper phage used to isolate ssDNA	Strategene

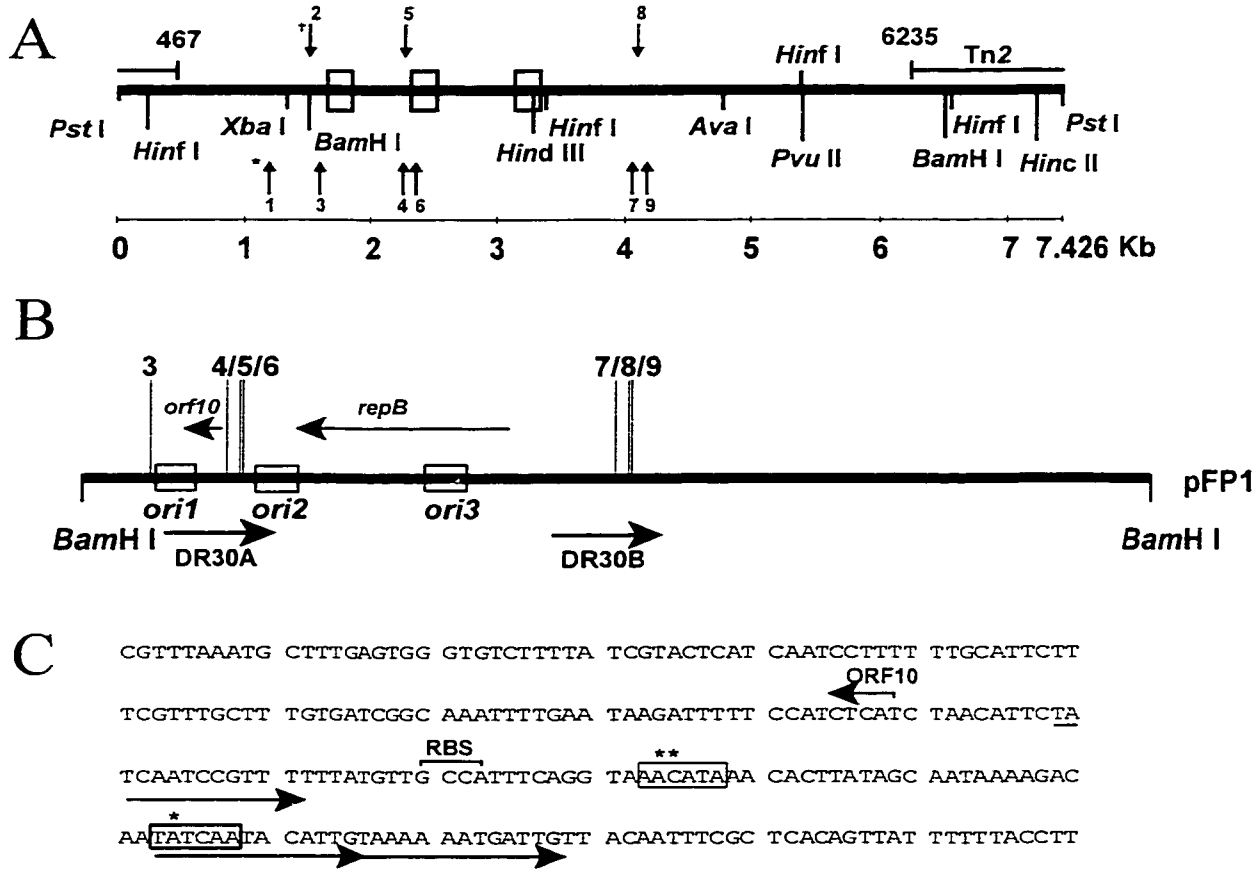

Figure 5.1. Linear map of the gonococcal Asia-type plasmid, pJD4, and its derivative, pFP1. A. Prototype map of pJD4, showing the origins of replication mapped by electron microscopy (boxes), the truncated transposon Tn*2* and the putative integration host factor binding domains (arrows). Star and cross represent perfect matches to the gonococcal and *E. coli* IHF binding domains, respectively. B. The 5 kb *Bam*HI fragment of pJD4 was cloned in the *Bam*HI site of pBluescript to create pFP1. The 507 and 509 bp direct repeats, DR30A, and DR30B, the three oris (boxes) and the genes encoding the replication initiation protein (RepB) and putative open reading frame (Orf10), encoding for a protein of unknown function are illustrated. IHF binding sites 3-9 are shown (vertical lines). C. DNA sequence of the upstream region of ORF10, highlighting the start site of ORF10, putative ribosome binding site (RBS), *E. coli* -10 (thin box) and -35 (thick box) consensus sequences (star indicates mismatches) and IHF binding sites 4, 5, and 6 (arrows).

Transformation of bacteria

Generally, *E. coli* was transformed using the conventional $CaCl_2$ transformation procedure (Dillon *et al.*, 1985).

Preparation of single-stranded pFP1

Helper phage R408 were propagated as described by the manufacturer (Stratagene). Single stranded pFP1 to be used in site-directed mutagenesis experiments was prepared as follows: half of a single overnight colony of *E. coli* CJ236 carrying pFP1 was inoculated into 5 ml of 2X YT containing ampicillin and chloramphenicol and R408 helper phage was added at 10^8 pfu/mL (an approximate multiplicity of infection of 10). The culture was then grown at 37°C with shaking (220 rpm) for about 16 hours. Single-stranded pFP1 was then isolated using the QIAprep M13 kit (Qiagen Inc.; Valencia, California) according to the manufacturer's instructions.

Site-directed mutagenesis of pFP1

Deletions of the IHF binding sites on pFP1 were done using a variation of the Kunkel method (Kunkel *et al.*, 1987). Based on the DNA sequence of pJD4, primers for site-directed mutagenesis were designed and synthesized at the University of Ottawa Biotechnology Research Institute (UOBRI) (Table 5.2). The mutagenic primers were phosphorylated as follows: 100 ng of oligonucleotide, 4 µl of 10X One-Phor-All buffer (Pharmacia Biotech), 4 µl of 10 mM rATP (Promega Corp.; Madison, WI), 9.5 U of polynucleotide kinase (Pharmacia Biotech), and adjusted to 40 µl with ddH_20 and the reactions were incubated at 37°C for 30 mins. Approximately 0.5-1 µg of ss pFP1 was then added to 20 µl of the kinase reaction and incubated 10 min at 65°C, followed by a further incubation for 10 min at room

temperature. Primer extensions were carried out under the following conditions: 4 uL of 10X T4 DNA ligase buffer (Promega Corp.), 2 µl of 2.5 mM dNTPs (Boehringer Mannheim; Laval, Quebec), 4 µl 10 mM rATP (Promega Corp.), 1 µg single-stranded DNA binding protein (Promega Corp.), 1.5 U Klenow enzyme (Promega Corp.), and dH_20 to give a 40 µl reaction. Reactions were incubated at room temperature for 4 hr and the stored at -20°C prior to use. Jason Szeto, a 4th year student in our laboratory, worked, under my co-supervision, on the site-directed mutagenesis and the copy number experiments. I would like to thank him for his dedication and hard work.

Copy number determination

pFP1, and its deletion derivatives, were transformed into *E. coli* strain SR1672 (Dillon *et al.*, 1985). This particular strain was used to ensure that any plasmid replication would occur from cloned pJD4 origins rather than from the ColE1 origin of the pBluescript vector which cannot function in the absence of PolI (Banerjee *et al.*, 1992). Copy number determination was performed as follows: 1 ml overnight cultures of SR1672 containing either pFP1 or its deletion derivatives were subcultured in 24 ml of LB broth supplemented with ampicillin and grown to an optical density (OD_{600}) of approximately 0.5; 2 ml samples were then collected by centrifugation at 10,000 g for 30 sec and the plasmids were isolated using the Quantum Prep Plasmid Miniprep Kit (Bio-Rad; Hercules, California). Colony-forming units per ml (cfu/ml) were then established using 10-fold serial dilutions of the above cultures and the relative copy number was established by comparing equivalent volumes of plasmid DNA on 1% agarose gels. Relative copy number was assessed using the UTHSCSA ImageTool program (University of Texas Health Science Center, San Antonio,

Texas) using the program available at http://ddsdx.uthscsa.edu or ftp://maxrad6.uthscsa.edu. These data were then confirmed using the AlphaEaseTM AlphaImager 1220 (Alpha Innotech Corporation, Washington, DC).

Gel shift analysis

PCR-amplified (forward primer, 5'-GTGGAAATACTGGCCGTT-3'; reverse primer, 5'-TGAAATCAGAATTTTAGCTT-3') or gel purified DNA fragments of pJD4 were end-labelled with [γ-^{32}P]dATP using T4 polynucleotide kinase. The end-labelled DNA fragments were incubated with either purified, gonococcal IHF or purified gonococcal HU preparations (Hill *et al.*, 1997) in a reaction mix consisting of 1 μl BSA (8 μl/ml), 10 μl binding buffer (50 mM Tris [pH 7.4], 1 mM EDTA, 1 mM β-mercaptoethanol, 10% glycerol, 70 mM KCL, 7 mM MgCl, 3 mM CaCl2, with poly(dIdC) added to a final concentration of 50 μg/ml (total reaction of 20 μl). The reactions were incubated at room temperature for 25 min., and terminated by the addition of 1/5 volume of loading buffer (1 mg BSA, 50% glycerol, 0.01 % xylenecyanol). Samples were fractionated on 4.5 % polyacrylamide gels (acrylamide:bis ratios of 29:1) in Tris-borate-EDTA buffer. [Note: The International Pathogenic *Neisseria* Conference is held every two years. At the 1996 meeting in Baltimore, I met with Dr. Stuart Hill, who presented a poster describing the purification of the gonococcal IHF protein. We established a collaboration and I sent him the DNA for him to perform the gel-shift experiments, as he had worked out optimal conditions. I am grateful for his collaborative efforts.]

DNA sequencing

Prior to sequencing, the plasmid DNA was cleaned using the QIAGEN PCR

Purification Kit, and the deletion clones were sequenced at the UOBRI using primers created based on the DNA sequence of pJD4 (Pagotto *et al.*, 2000a). Sequencing was performed using the Applied Biosystems 373A Sequencer (Perkin Elmer; Foster City, CA) to verify that the deletions had occurred.

Table 5.2. Primers used to delete integration host factor binding domains from pFP1.

IHF binding site # (pJD4 coordinates)	Mutagenic primer (5'-3') position (pJD4 coordinates)[1]
3 (1794-1806)	CGTATAAGTTT/GTTACTGGTTTTAAAAACG (1782)
4 (2459-2171); 7 (3988-4000)	CCATCTCATCTAACATTC/ATGTTGCCATTTCA G (2141; 3970)[2]
5 (2223-2235); 6 (2235-2247); 8 (4052-4064; 9 (4065-4076)	GGTAAACATAAACACTTATAGCAATAAAAGA CAA/GTTACAATTCGCTCACAGTTATTTTTTAC C (2189; 4018)[2]

1. / indicates site of IHF binding domain which was removed.

2. The same primer was used for deletion of binding sites 4/7 and 5/6/8/9 since the location of the IHF binding domains were located on two large, direct repeats (Pagotto *et al.*, 2000a).

5.3 RESULTS

The goncoccal β-lactamase-producing plasmids contain multiple IHF binding domains

DNA sequence analysis of pJD4 (Asia-type) indicates the presence of three distinct origins of replication and nine putative binding sites for IHF protein (Pagotto *et al.*, 2000a; Fig. 5.2A; Table 5.3). Comparison between the putative gonococcal IHF binding domains and the *E. coli* consensus sequence indicated considerable degeneracy in the sequences of the proposed IHF binding domains on pJD4, with only site 3 being a perfect match to the consensus (Table 5.3). Consequently, as very few gonococcal IHF binding domains have been studied thus far, it is therefore difficult to come up with a definitive comprehensive consensus gonococcal IHF binding domain.

Gonococcal penicillinase-producing plasmids require IHF for replication in vivo

Based on the above sequence observations, gonococcal plasmids [pJD4 (*ori1/2/3*), pJD5 (*ori1*) and pJD9 (*ori2/3*)] containing the different origins of replication were investigated for their requirements for IHF protein to replicate in *E. coli* (Table 5.4). All plasmids tested were not able to replicate in strains missing one (5179), other (5427) or both (5477) subunits of the IHF protein (Table 5.4). All plasmids replicated in the parental strain (N99), from which IHF insertion mutants were obtained (Mendelson *et al.*, 1991). Collectively, the data presented in Table 5.4 indicate that none of the plasmids could replicate efficiently in the absence of *E. coli* IHF protein, providing further support for a potential role for IHF protein in gonococcal plasmid replication and/or stability.

Table 5.3. Comparison of the IHF sequences found in pJD4 with the *E. coli* and *N. gonorrhoeae* consensus sequences.

Location on pJD4	IHF sequence and position number (mismatches in bold)[1]												
	1	2	3	4	5	6	7	8	9	10	11	12	13
IHF1 (1059-1071)	**A**	A	A	C	A	C	G	T	T	G	A	T	T
IHF2 (1479-1491)	**A**	A	A	T	C	T	T	T	T	G	A	**G**	A
IHF3 (1794-1806)	T	A	A	C	T	T	A	T	T	G	A	T	T
IHF4 (2159-2171)	T	A	**T**	C	A	A	T	**C**	**C**	G	**T**	T	T
IHF5 (2223-2235)	T	A	**T**	C	A	A	T	**A**	**C**	**A**	**T**	T	**G**
IHF6 (2235-2247)	**G**	**T**	A	A	A	A	A	**A**	T	G	A	T	T
IHF7 (3988-4000)	T	A	**T**	C	A	A	T	**C**	**C**	G	**T**	T	T
IHF8 (4052-4064)	T	A	**T**	C	A	A	T	**A**	**C**	**A**	**T**	T	**G**
IHF9 (4065-4076)	**G**	**T**	A	A	A	A	A	**A**	T	G	A	T	T
E. coli consensus	Y[2]	A	A	N[3]	N	N	N	T	T	G	A	T	W[4]
N. gonorrhoeae #1[5]	T	T	A	T	A	T	A	G	A	G	A	T	A
N.gonorrhoeae #2[5]	A	A	A	C	A	A	T	T	A	T	A	T	A
Universal[6]	H[7]	W	A	N	N	N	N	K[8]	W	K	A	T	W

1. Mismatches are with respect to the *E. coli* consensus sequence only (Craig and Nash, 1984).
2. Y = C or T
3. N = A, G, C, or T
4. W = A or T
5. Taken from Hill *et al.* (1997)
6. Relaxed IHF binding domain based on both the *E. coli* and *N. gonorrhoeae* consensus
7. H = C, T, or A
8. K = T or G

Table 5.4. Ability of various gonococcal plasmids to replicate in the presence of functional integration host factor in *E. coli.*

Plasmid	5427 (Δ*himA*)	5179 (Δ*himD*)	5477 (Δ*himA*, Δ*himD*)	N99 (+ control)
pJD4	-	-	–[a]	+[b]
pJD5	-	–	–	+
pJD9	-	-	–	+

a- indicates no growth or transformants obtained
b- indicates growth

Binding of purified gonococcal IHF protein to its domain on plasmid pJD4

As the above data indicate a role for IHF protein in the replication of pJD4, the next step undertaken was an investigation into whether the putative IHF binding domains located within pJD4 could actually bind purified gonococcal IHF protein (Hill *et al.*, 1997) using gel-retardation assays. To facilitate this analysis, three fragments were examined that collectively contained all the putative IHF binding domains of pJD4: i) a 600 bp PCR-amplified DNA sequence that contained IHF binding sites 1 and 2 (Fig. 5.2B, cf lanes 4-6 vs. lane 1); ii) a 1.8 kb gel-purified *Bam*HI-*Hind*III fragment containing IHF binding sites 3-6 (Fig. 5.2C; cf lanes 4-6 vs. lane 1); and, iii) a 1.9 kb *Hinf*I fragment containing IHF binding sites 7-9 (Fig 5.2D; cf lanes 4-6 vs. lane 1). As can be seen in Figs. 5.2B-D, each fragment when bound to purified gonococcal IHF presented a retarded migration profile following electrophoresis through polyacrylamide gels when compared to the unbound fragment. Binding specificity of gonococcal IHF protein to IHF binding sites #'s 1 and 2 is indicated by the competitive binding assay in the presence of non-radiolabelled fragments from pJD4 (Fig. 5.3, lanes 2-6; DNA fragment used is the same as the DNA used in Fig. 5.2B). Interestingly, while HU has not been implicated in plasmid replication, at the highest concentrations of HU protein that were used, a slight shift in the mobility of the DNA could also be observed (most clearly seen in Fig. 5.2C; lanes 2 and 3). As the DNA segments that were used in the gel-shift experiments contained more than one potential IHF binding site, it's possible that cooperative or differential loading of IHF protein onto the multiple sites accounts for the differential binding patterns that are observed as the IHF protein concentration increases (e.g. Fig. 5.2C; lanes 4-6; and perhaps Fig. 5.2D; lanes 4-6).

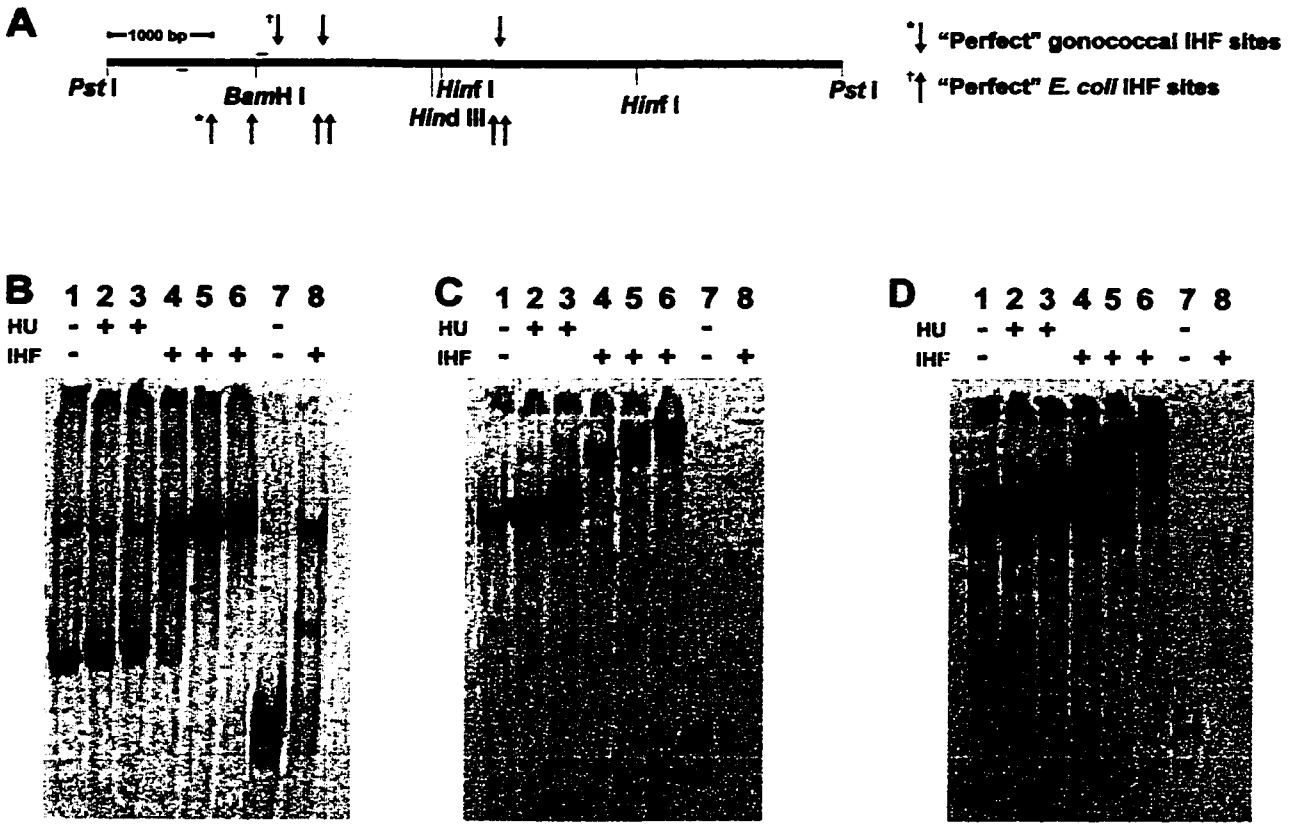

Figure 5.2. Gel-shift analysis of IHF binding to gonococcal origins of replication. (A) Linear map of pJD4, showing IHF sites. (B) PCR product amplified using primers shown in A (horizontal arrows). (C) DNA fragment used was the *Bam*HI/*Hin*dIII fragment. (D) DNA fragment used was *Hin*fI fragment. Lane1, no protein; lanes 2 and 3, HU protein (negative protein control); lane 4-6, increasing amounts of gonococcal IHF (0.5, 1.0, 2.5 μg); lanes 7 and 8, *himA* promoter fragment (positive control). The IHF was able to bind its target sequence, retarding DNA fragment through the gel (lanes 4, 5 and 6).

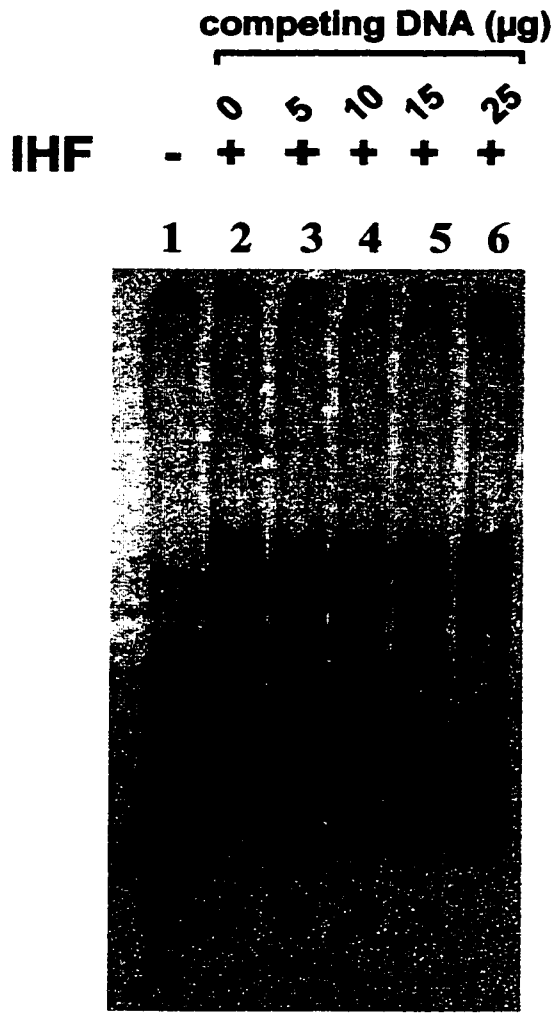

Figure 5.3. Competition assay demonstrating specificity of the gonococcal IHF protein to its binding domains on pJD4. DNA fragment used was the same as in Figure 5.2B. Increasing levels of non-radiolabelled DNA (lanes 2-6) were able to displace the retarded, radiolabelled fragment from the IHF protein back down to the unbound state (lane 1).

Deleting the IHF binding domains and plasmid replication

The previous data demonstrate that purified gonococcal IHF protein binds to DNA segments containing the putative IHF binding domains, and that *E. coli* IHF is required for efficient plasmid replication of the recombinant plasmids. Therefore, in an attempt to delineate which of the putative IHF binding domain(s) is/are important for *in vivo* plasmid replication, deletion derivatives we made where IHF binding domains were selectively removed by site directed mutagenesis and their effect on copy number was assessed (Table 5.5). Because IHF binding sites 4/5/6 and 7/8/9 lie on two direct repeats (Pagotto *et al.*, 2000a), we were unable to generate single IHF knockouts (data not shown). The data presented in Table 5.5 can be summarized as follows: i) removal of binding domains 4/7 or 8/9 caused a slight increase in plasmid copy number; ii) removal of 13 bp immediately downstream of IHF binding sites 4/7 also caused an increase in plasmid copy number; iii) removal of IHF site 3 or sites 4/7/8/9 caused a reduction in copy number; and iv) repeated attempts to obtain constructs with deleted IHF binding sites 5 or 6 (or both) were unsuccessful (data not shown). Because IHF binding sites 1 and 2 lie with the coding region for the required replication initiation protein, RepA (necessary for the replication of *ori1* (17; Pagotto and Dillon, submitted), no attempt was made to modify these sites.

Table 5.5 The effects of removing IHF binding domains on the plasmid copy number of pFP1.

Plasmid (IHF site # deleted)	Relative copy number[1]
pFP1 (wild-type control)	1
pFP1 (Δ3)	0.9
pFP1 (Δ4/7D*)	1.1
pFP1 (Δ4/7)	1.2
pFP1 (Δ8/9)	1.1
pFP1 (Δ4/7/8/9)	0.8

[1] Results of one experiment presented. Repeated experiments yielded similar trends and were verified by two different software packages (see Material and methods).

* 13 nucleotides immediately downstream of IHF sites 4 and 7 were removed.

5.4 DISCUSSION

In studies reported to date, single origins of replication have been used to study the effects the IHF protein has in their replication. However, the role of IHF in the replication of plasmids bearing multiple *oris* is poorly understood. The plasmid R6K, like pJD4, is a plasmid which contains three *oris*, as well as two IHF binding sites (Filutowicz and Rakaowski, 1998). IHF binding studies have only focussed on the γ origin of R6K in isolation (Dellis *et al.*, 1996). Studies have revealed that replication initiation requires IHF binding to only one of two binding sites located in the γ origin (Dellis *et al.*, 1996). *In vitro* studies using the γ origin of plasmid R6K have shown that IHF bound preferentially to the first of two possible IHF binding sites and induced DNA folding (Dellis *et al.*, 1996). Binding of IHF to the second site only occurred if the 106 bp enhancer region involved with stability of the γ origin was absent (Dellis *et al.*, 1992). This suggests that not every IHF binding site found on plasmids possessing multiple IHF binding sites is essential, or that particular IHF sites may be necessary for specific origins. In this study, repeated attempts to recover plasmids with deleted binding sites 5 or 6 were unsuccessful, indicating that these may be required for initiation of replication. Removal of other binding domains causes a slight increase or decrease in replication, reflected by the copy number when compared to unmutated parental plasmid (Table 5.5). In plasmids bearing a single origin of replication, the IHF protein has been proposed to bring together the components of the replication machinery by its bending functions (Stenzel *et al.*, 1987; Bramhill and Kornber, 1988; see Fig. 1.3.4). It is conceivable that the topology of the plasmid molecule within the cell only "reveals" certain domains available for binding the protein.

Studies of IHF binding site dependence in iteron-regulated plasmids have been

conducted with the single *ori* pSC101 and the γ origin of the multiple *ori* R6K. Site-directed mutagenesis was used in both cases to alter key IHF binding sequences to demonstrate their roles in plasmid replication (Stenzel *et al.*, 1987; Dellis *et al.*, 1996). Removal of the IHF binding domain abolished plasmid replication. IHF was required for the gamma but not alpha and beta origins of plasmid R6K (Filutowicz and Appelt, 1988). Plasmid pJD4 is unique because it possesses three origins of replication and nine possible binding sites for IHF protein (Pagotto and Dillon, 1996; Pagotto *et al.*, 2000; Pagotto and Dillon, manuscript submitted). The relationship between the three pJD4 origins and the nine IHF binding sites remains poorly understood. This is in sharp contrast to the much studied pSC101, the plasmid upon which much of our understanding of iteron-regulated replication is based. The plasmid pSC101 carries a single IHF binding site associated with one origin of replication (Stenzel *et al.*, 1987). While my work is based upon *in vivo* genetic experiments in an *E. coli* background, and not in *N. gonorrhoeae* from where pJD4 was originally isolated, it is possible that recognition of essential IHF binding sites differ between the two species. However, based on the fact that the *in vivo* studies demonstrated that *E. coli* IHF can be used in place of gonococcal IHF for the replication of the gonococcal plasmids (Table 5.4), I feel confident that the IHF protein is not an auxilliary but an essential component of plasmid replication in the gonococcus. The role of the IHF protein is probably underestimated in the gonococcus, as gonococcal strains lacking the genes coding for IHF have not yet been produced, indicating its essential nature (Hill, unpublished).

In general, a plasmid may have reduced copy number if a mutation interferes with the normal replication pathway (Kues and Stahl, 1989; Kornberg and Baker, 1992; Helinski *et al.*, 1996). Site-directed mutagenesis altering the three key contact points in the single IHF

binding site of pSC101 has been shown to abolish binding of the protein to DNA as well as inactivate the replication origin (Stenzel *et al.*, 1987). Attempts were made to delete the IHF binding domains using the plasmid pFP1, which carry *ori2/ori3* (Fig. 5.1b). Because IHF binding sites 1 and 2 lie within the gene coding for a replication initiation protein involved with *ori1* (Gilbride and Brunton, 1990; Pagotto and Dillon, submitted), focus was on the remaining binding domains (since altering binding sites 1 and 2 would disrupt the *repA* gene). Deletion of IHF binding site 3 on pFP1 consistently resulted in a decrease in plasmid copy number, when compared to the parental plasmid (Table 5.5). It is possible that removal of this binding site may have caused a significant change in the topology of the DNA, thereby decreasing the initiation of replication from a key origin that is used most frequently in the plasmid (i.e. *ori2* or *ori3*). I believe that *ori1*, while closest to IHF binding site 3 (Figure 5.1b), is not implicated. I have shown that *ori1* of pJD4 functions in the presence of a Rep protein which is not located in the DNA sequence found in pFP1 (*repA* gene located at coordinates 1608-624 on pJD4 (Pagotto and Dillon, manuscript submitted; see Chapter 3). Thus, it is possible that, in pFP1, *ori1* sequences may act as an enhancer element for *ori2* and/or *ori3*. Equally, *ori1* does not function in *E. coli* SR1672 (polA$^-$), so contributions from this origin would only be *in cis*.

The elimination of IHF binding site 3 may have led to reduced initiation efficiency in neighbouring origins, or may have necessitated the recruitment of other IHF site(s) for the initiation of replication at the preferred origin. The binding of IHF to another IHF site (s) (or recruitment of other binding sites via their exposure) may not generate the required bending of DNA at the origin as effectively, thus leading to a decreased frequency of initiation of replication. In addition, the binding of IHF to another IHF binding site (or sites)

may induce alternative DNA topology that alters the loading of the replication machinery. The association of IHF to both IHF binding sites of the γ origin in plasmid R6K has been shown to generate a DNA structure that is different from the structure caused by binding of a single IHF molecule (Dellis *et al.*, 1992). However, it was noted that the binding of the additional IHF protein was only possible if the replication enhancer region was present in the R6K origin (Dellis *et al.*, 1992). It may be possible that different DNA topology can lead to less frequent replication if DnaA and Rep proteins, normally brought together by the bending functions of IHF, are prevented from forming the functional replisome. Plasmid R6K is an example of a plasmid where the frequencies of initiation at each of the three oris, α, β, γ, have been documented. The α origin is used most frequently (48% of the time), followed by a preference for the β (34%) then γ origins (18%) (Crosa, 1980; Inuzuka *et al.*, 1980). Altering IHF binding site 3 in pFP1 resulted in a decreased copy number (Table 5.5). It is possible that replication initiation occurring at either *ori2* or *ori3* be reduced as a direct consequence of the lack of bending functions provided by IHF binding site 3.

The greatest effect on copy number was seen with the plasmid construct pFP1 (Δ4/7/8/9), where IHF binding sites 4, 7, 8, and 9 were removed together (Table 5.5). The decrease in the copy number may partially explain why growth of the strain SR1672 was slower in the presence of ampicillin (data not shown). Since the resistance level to ampicillin responds linearly with gene dosage, bacteria possessing a high copy number plasmid containing an ampicillin resistance gene may be able to grow faster in selective media (Thomas, 1988). Thus, I propose that removal of IHF sites 4/7/8/9 alters either the topology of the DNA molecule, or the accessibility to the remaining IHF binding sites, resulting in a decreased rate of replication initiation.

It has been shown that the copy number of pHS1, a derivative of pSC101, is increased when specific base pair changes occur within the plasmid encoded *rep* gene. The resulting mutant Rep proteins are believed to increase copy number either by binding to the iterons with greater affinity and promoting more efficient replisome formation and/or binding to the promoter region of *rep* with less affinity, thus reducing the autoregulatory effects of Rep (Armstrong *et al.*, 1984). This in turn allows for more Rep protein synthesis that may increase the initiation of replication. IHF binding sites 3-9 are not located within the two *rep* genes (Pagotto *et al.*, 2000). IHF sites 1 and 2 do lie within the *repA* gene encoding for the replication initiation protein described by Gilbride and Brunton (1990). Alterations in either of these sites would affect the protein itself. Only IHF binding site 3 could be deleted individually on pFP1. Sinc10e the remaining IHF binding sites were all found within two direct repeats (Figure 5.1b), such that a deletion of one site would lead to a corresponding deletion of another site in the downstream repeat. There are small open reading frames that lie within the two direct repeats, one of which has been proposed to be involved in mobilization of gonococcal plasmids (Tenover *et al.*, 1985). Thus, it is possible that these small ORFs may play an auxilliary role in the plasmid replication process. Therefore, the effects of a single IHF binding site deletion could only be considered for the ΔIHF 3 clone.

Repeated attempts to remove IHF binding sites 5 and 6 were unsuccessful. These IHF binding sites (including site 4) lie within the promoter region of a small open reading framed, named *orf10* (Fig. 5.1c; Pagotto *et al.*, 2000a). It is possible that removal of binding sites 5 and 6 would affect the transcription and, therefore, levels of ORF10. While the role of ORF10 in the replication of pJD4 has not yet been reported, it can not be ruled out that

ORF10 has some auxilliary role in the replication from either *ori2* or *ori3*. Alternately, independent of the role of ORF10, removal of IHF binding sites 5 and 6 may have altered the topology of the DNA enough to prevent stable replication of the plasmid from either origins.

In this study, high concentrations of the HU protein was able to alter the mobility patterns of gonococcal plasmid DNA (Fig. 5.2C, lanes 2 and 3). HU was considered a sequence-independent DNA-binding protein with a preference for binding bent or kinked DNA (Pontiggia *et al.*, 1993), curved DNA (Shimizu *et al.*, 1995), or DNA containing single-strand breaks or gaps (Castaing *et al.*, 1995); however, it was recently demonstrated that HU was not specific for curved DNA (Azam and Ishihama, 1999). HU has not been implicated in plasmid replication, yet is required at early stages during *in vitro* initiation of replication at *oriC* (Dixon and Kornberg, 1984; Funnell *et al.*, 1986) and, at high levels, has been shown to inhibit the replication of *oriC* plasmids (Ogawa *et al.*, 1985). It still remains unclear if HU is a necessary component of gonococcal plasmid replication, and further studies will be undertaken to investigate this aspect.

In conclusion, this study demonstrated that the gonococcal β-lactamase producing plasmid pJD4, pJD5 and pJD9 require the IHF protein for replication. Deletion of IHF binding sites 3 or 4/7/8/9 in combination cause a decreases the copy number of the plasmid relative to unmutagenized, parental plasmid. Removal of bases downstream from binding sites 4 and 7 caused an increase in copy number. The gonococcal IHF protein was shown to be able to bind IHF sites located on pJD4. Our study is the first of its kind to investigate the role of multiple IHF binding sites in a naturally occurring plasmid containing multiple origins of replication.

CHAPTER 6

CONCLUSIONS AND FUTURE DIRECTIONS

This work primarily focussed on the characterization of mechanism(s) underlying DNA replication, using, as a novel model system, constructed derivatives of two naturally occurring antibiotic resistance plasmid types which have been shown to contain multiple and unique replicons. Prior to attempting an analysis of the replication origins, it was decided to ascertain any structural relationships that may have existed among the gonococcal β-lactamse-producing plasmids (hypothesis #1; chapter 2). The confusion existing in the literature has been resolved with the complete sequence of a prototype Asia plasmid, pJD4 (chapter 2). Equally, analysis of the location of the deletions and/or insertions of related plasmids has allowed for a better understanding of the evolution of these plasmids.

The penicillinase-producing Asia plasmid, pJD4, carried in *Neisseria gonorrhoeae* isolates, was shown to be unusual in that it contains three origins of replication (hypothesis 2; chapter 3). The gonococcal replicon region uniquely carries PolA dependent and independent oris (chapter 3), and has a broad host range different from that of R6K (Kornberg and Baker, 1992; Dillon *et al.*, 1990; see chapter 4), implying that different plasmid and host encoded factors are important for their replication in different hosts. This study also demonstrated, for the first time, that there exists two different replication initiation proteins, RepA and RepB, that are required for origins of replication 1 and 2/3, respectively (hypothesis #3; chapter3). Future experiments will focus on the specific role each of the Rep proteins have on replication oris. Specifically, the Rep proteins will be purified and their ability to proposed iterons will be evaluated using gel-mobility assays and/or footprinting assays. Experiments where a Rep protein will be substituted for the other (as well as iteron substitution) will offer insight into the relationship these initiation proteins have with respect to affecting each other`s function, if any. This is of interest, since obtaining a self-replicating

plasmid (pJD4) containing a truncated *repB* gene was not possible, implying that the *repA* gene product is either not made or is not functional [recall that the reverse was true; a plasmid construct containing a deleted *repA* gene was made, with replication relying on the *repB* gene product, chapter 3).

DNA sequencing analysis revealed several binding sites for proteins such as DnaA and IHF, involved in the replication process of plasmids. Initial genetic experiments with IHF mutant hosts and the presence of multiple IHF boxes in the sequences of the three origins indicate that histone-like proteins are required for the replication of pJD4 and, by association, other gonococcal plasmids (chapter 5). Using purified, gonococcal IHF protein, specificity was shown with DNA fragments containing IHF binding sites in gel-mobility experiments (chapter 5). Ongoing work will address the ability of the gonococcal IHF protein to rescue gonococcal plasmid replication (by supplying it *in trans*) in IHF mutant hosts. Although not directly related to plasmid replication, experiments addressing why a gonococcal IHF mutant was not feasible will be undertaken.

DNA replication is central to all growing organisms, and it is predicted that features of DNA replication are undoubtably shared by non-plasmid replicons and bacteriophages. It is generally accepted that certain molecular components, for example, DNA polymerase, can be intrinsic and common to the replication process in general. The knowledge obtained from these investigations may well be extended to other and seemingly distant replication systems, including bacterial chromosomes and their phages, viral replication and perhaps, replication of eukaryotic chromosomes. Continuation of this work will also allow insight into the host mediated regulation of origin selection and usage in the broad host range plasmid, pJD4. In particular, the role of iterons may now be addressed by purifying the

replication initiation proteins and performing gel-mobility and other DNA binding experiments. It has been shown recently that certain cryptic plasmids of *E. coli* are independent of either antisense RNA and iterons for their copy number control (Burian *et al.*, 1999, del Solar and Espinosa, 2000). In these plasmids, the Rep protein is the only plasmid-encoded element implicated in the initiation and control of plasmid replication (Burian *et al.*, 1999). It will be interesting to see how the gonococcal β-lactamase origins of replication fit in this evolving scenario.

As broad host range plasmids are implicated as vehicles for the spread of newly re-emerged antibiotic resistance genes in bacteria, the development of a new generation of antimicrobial therapies targeted at various components of the replication machinery may also provide new avenues for treatment of microbial infections. An important focus of our laboratory deals with the increase in the emergence of bacterial strains that are resistant to traditionally used antimicrobial drugs. Hence, development of a new generations of antimicrobial therapies targeted at various components of the replication machinery would provide new avenues for treatment of microbial infections. It is now well established that the autonomously replicating DNA plasmids are the main vehicles for the spread of newly emerged antibiotic resistance genes between bacterial cells. In particular, novel drugs or strategies such as the use of antisense RNA (van der Krol *et al.*, 1988; Zhiqiang *et al.*, 1996) may be designed to interfere with the replication of plasmids cohabiting multiple species of bacteria. Proteins such as DnaA, RepA and other smaller factors which are crucial not only for plasmid but also for bacterial replication may therefore represent novel targets for the design of specific antimicrobial interventions. To achieve this goal, it is necessary to understand the mechanisms underlying the initiation of DNA replication of complex

replicons and to identify key proteins and other structural requirements of the replication process and to delineate their role and regulation.

In conclusion, this work represents the first detailed investigation into the mechanism(s) of replication of the β-lactamase-producing plasmids of *N. gonorrhoeae*. In the *Neisseria* field, experts have long searched for molecular tools which would facilitate the study of genes in a gonococcal background. I have been able, through the use of information arising from my work, to construct plasmid vectors which may be used as vectors to put genes back in the gonococcus (hypothesis #5; chapter 4). Perhaps more surprising, is the "scientific buzz" that has been generated with our publication describing the use of the shuttle vector in *Haemophilus* species (see chapter 4). Our laboratory has been busy with plasmid requests since the publication appeared and this should guarantee the "impact" I have made in the laboratory...for a while, at least!

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Yeung, K.-H. (1989) Genetic analysis and molecular characterization of the naturally-occurring penicillinase-producing plasmids in *Neisseria gonorrhoeae.* Ph.D. Thesis. University of Ottawa, Ottawa, Ontario, Canada.

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Zhou, D., Apicella, M.A. (1996) Plasmids with erythromycin resistance and catechol 2,3-dioxygenase or β-galactosidase-encoding gene cassettes for use in *Neisseria* spp. *Gene* **171:** 133-134.

APPENDIX A: PUBLICATIONS ARISING FROM THIS WORK

PUBLICATIONS:

Dillon, J.R., and **Pagotto, F.** 1999. Importance of drug resistance in gonococci: from mechanisms to monitoring. *Current Opinion in Infectious Diseases* **12:** 35-40.

Pagotto, F., Ng, L.-K., Aman, T., Brett, M., Yeung, K.-H., and Dillon, J.R.. 2000. Structural analysis of the family of penicillinase-producing plasmids of *Neisseria gonorrhoeae* based on DNA sequencing. *Plasmid* **43:** 24-34.

Pagotto, F., Saliminia, H., Totten, P., and J.R. Dillon. 2000. Development of stable shuttle vectors based on a unique origin of replication for *Neisseria gonorhoeae, Haemophilus spp.* and other bacteria. *Gene* **244:**13-19.

In preparation/submitted:

Pagotto, F., and J.R. Dillon. 2000. Characteristics of origins of replication from β-lactamase-producing plasmids of *Neisseria gonorrhoeae*. Submitted to *Journal of Bacteriology*

Pagotto, F., Szeto, J., Hill, S.A., and J.R. Dillon. 2000. The role of the integration host factor in the replication of the β-lactamase-producing plasmids of *Neisseria gonorrhoeae*. In preparation. To be submitted to *Gene* in October, 2000.

Pagotto, F., Grigoriev, A., and Dillon, J.R. 2000. Isolation and genetic characterization of the minimal chromosomal origin of replication of *Neisseria gonorrhoeae*. In preparation. To be submitted to *FEMS Microbiology Letters* in December, 2000

PATENT APPLICATIONS

Dillon, J.R., and **Pagotto, F.** Origins of replication and their uses in the development of cloning vectors and other biological products. Submitted April 8, 1999. (Provisional application number 60/128,573 given).

PUBLISHED CONFERENCE ABSTRACTS

Pagotto, F., and J.R. Dillon. 1996. Replication origins of β-lactamase-producing plasmids of *Neisseria gonorrhoeae*. Tenth International Pathogenic Neisseria Conference, Sept. 8-13, Baltimore, Maryland. (Also presented at the Ottawa Life Sciences National Conference & Exhibition, Medical Devices, Agricultural Biotechnology and Technology Transfer Opportunities section, October 29-30, 1996, Ottawa, Ontario).

Pagotto, F.J., Hill, S.A., and Dillon, J.R. 1997. Replication mechanisms of multicomplex gonococcal β-lactamase plasmids. 37th Interscience Conference on Antimicrobial Agents and Chemotherapy, Sept. 28-October 1, Toronto, Ontario.

Salimnia, H, **Pagotto, F.**, and Dillon, J.R. 1998. A shuttle vector for *Neisseria gonorrhoeae*. Eleventh International Pathogenic Neisseria Conference, Nov. 1-6, Nice, France.

Pagotto, F., Hill, S., and Dillon, J.R. 1998. Molecular analysis of gonococcal β-lactamase plasmid replication. Eleventh International Pathogenic Neisseria Conference, Nov. 1-6, Nice, France. Poster was chosen to be discussed at a scientific breakout meeting.

CONFERENCE ORAL PRESENTATIONS:

F. Pagotto. 1996. Presented for Dr. Dillon. Network in the Americas and Caribbean for *Neisseria gonorrhoeae*. 3rd Canadian Conference on International Health: Effectiveness in Health Development. Ottawa, Ontario, Canada, November 10-13.

POSTER COMPETITIONS:

150th Anniversary of the University of Ottawa (1st prize in the Ph.D. poster competition, Department of Biochemistry, Microbiology and Immunology, University of Ottawa, 1998). Poster title: The biology of gonococcal β-lactamase-producing plasmids.

1999 University of Ottawa (Department of Biochemistry, Microbiology and Immunology) annual poster competition (2^{nd} prize in the Ph.D. category). Poster title: Plasmid Biology in *Neisseria gonorrhoeae*.

APPENDIX B: CURRICULUM VITAE

FRANCO JOSEPH PAGOTTO

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Aylmer, Quebec J9J-2P5
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Franco_Pagotto@hc-sc.gc.ca

PERSONAL DATA:

Citizenship: Canadian, Italian
Languages: Italian, English, French
Married, no children

EDUCATION:

1985	High School Diploma D'Arcy McGee High School, Hull Quebec
1987	DEC Health Science Diploma Cegep College, Hull Quebec
1990	B.Sc. (Concentration biology) University of Ottawa, Ottawa, Ontario
1991	B.Sc. (Honours biology/biotechnology option) University of Ottawa, Ottawa, Ontario Thesis title: The acid shock response increases the heat resistance of *Listeria monocytogenes*
1994	M.Sc. (Food Microbiology) Department of Food Science University of Guelph, Guelph, Ontario Thesis title: Phage-mediated detection of mastitis causing *Staphylococcus aureus* and *Escherichia coli* O157:H7 using bioluminescence
1995-present	Ph.D. (Microbiology and Immunology) Department of Biochemistry, Microbiology and Immunology University of Ottawa, Ottawa, Ontario

Thesis title: Genetic and molecular characterization of origins of replication from β-lactamase-producing plasmids of *Neisseria gonorrhoeae*

HONOURS:

University of Ottawa Admission Scholarship (1995-1996)

University of Ottawa Admission Scholarship (1996-1997)

University of Ottawa Travel Award 1996

University of Ottawa Department (Microbiology and Immunology) Travel Award 1997

FCAR (Fonds pour la Formation de Chercheurs et l'Aide à la Recherche) (Ph.D. scholarship) 1997-1998

University of Ottawa Excellence Scholarship 1997-1998

1997 ASM Student Travel Award

Ontario Graduate Scholarship Award (Ph.D) 1998-1999

150th anniversary of the University of Ottawa (1st prize in the Ph.D. poster competition, Department of Biochemistry, Microbiology and Immunology, University of Ottawa, 1998)

1999 University of Ottawa (Department of Biochemistry, Microbiology and Immunology) annual poster competition (2nd prize in the Ph.D. category)

2000 NSERC (Natural Sciences and Engineering Council of Canada) Industrial Research Fellowship Award (to be started by June, 2001)

2000 NSERC (Natural Sciences and Engineering Council of Canada) Visiting Scientist Research Fellowship Award (to be started by June, 2002).

PROFESSIONAL ASSOCIATIONS:

American Society for Microbiology

UNIVERSITY ACTIVITIES:

University Teaching Certificate (University of Guelph, 1991)

Teaching long distance education course (University of Guelph, 1992-1994)

Teaching assistant for food microbiology laboratory course (University of Guelph, 1992-1994)

Judge at Canada-Wide-Science-Fair (1994, Guelph, Ontario)

University of Ottawa Health Sciences Library Advisory Committee (1995-present)

Co-supervision (with Dr. J.R. Dillon) of several 4th year undergraduate students (Department of Biochemistry, Microbiology and Immunology, University of Ottawa, 1996-1999):

Roberto Catana (1996-1997)
Thesis title: Incompatibility and the role of the integration host factor in multicomplex plasmids from *Neisseria gonorrhoeae*

Jason Szeto (1997-1998)
Thesis title: The role of integration host factor binding sites in gonococcal β-lactamase producing plasmid replication

Jean-Placide Rubabaza (1998-1999)
Thesis title: Molecular epidemiology of antibiotic resistance in *Neisseria gonorrhoeae* (Won 1st prize in 4th year poster competition)

PUBLICATIONS:

Farber, J.M., and **F. Pagotto**. 1992. The effect of acid shock on the heat resistance of *Listeria monocytogenes*. *Letters in Applied Microbiology* **15:** 197-201.

Daley, E.F., **F. Pagotto**, and J.M. Farber. 1996. The inhibitory properties of various sponges on *Listeria* spp. *Letters in Applied Microbiology* **20:** 195-198.

Jo-Anne R. Dillon, and **Franco Pagotto**. 1999. Importance of drug resistance in gonococci: from mechanisms to monitoring. *Current Opinion in Infectious Diseases* **12:** 35-40.

F. Pagotto, L.-K. Ng, T. Aman, M. Brett, K.-H. Yeung, and J.R. Dillon. 2000. Structural analysis of the family of penicillinase-producing plasmids of *Neisseria gonorrhoeae* based on DNA sequencing. *Plasmid* **43:** 24-34.

Pagotto, F., Saliminia, H., Totten, P., and J.R. Dillon. 2000. Development of stable shuttle vectors based on a unique origin of replication for *Neisseria gonorhoeae, Haemophilus spp.* and other bacteria. *Gene* **244:** 13-19.

In preparation/submitted:

Pagotto, F., and J.R. Dillon. 2000. Molecular and genetic analysis of origins of replication from β-lactamase-producing plasmids of *Neisseria gonorrhoeae*. Submitted to *Journal of Bacteriology*

Pagotto, F., Szeto, J., Hill, S.A., and J.R. Dillon. 2000. The integration host factor is a necessary component in the replication of the β-lactamase-producing plasmids of *Neisseria gonorrhoeae*. In preparation. To be submitted to *Gene* in October, 2000.

Pagotto, F., and J.R. Dillon. 2000. Isolation and genetic characterization of the minimal chromosomal origin of replication of *Neisseria gonorrhoeae*. In preparation. To be submitted to *FEMS Microbiology Letters* in December, 2000

PATENT APPLICATIONS

Dillon, J.R., and **Pagotto, F.** Origins of replication and their uses in the development of cloning vectors and other biological products. Submitted April 8, 1999. (Provisional application number 60/128,573 given).

PUBLISHED CONFERENCE ABSTRACTS

Pagotto, F., and M.W. Griffiths. 1993. Detection of *Staphylococcus aureus* in dairy cows suffering from mastitis. *Journal of Bioluminescence and Chemiluminescence* **8**: 113.

Pagotto, F.J., and Griffiths, M.W. 1994. Detection of *Staphylococcus aureus* causing bovine mastitis using bioluminescence. *Journal of Dairy Science* **77**: 311.

Pagotto, F.J., and Griffiths, M.W. 1994. Detection of *Staphylococcus aureus* causing bovine mastitis using bioluminescence. *Journal of Animal Science* **72**: 311.

Pagotto, F., and J.R. Dillon. 1996. Replication origins of β-lactamase-producing plasmids of *Neisseria gonorrhoeae*. Tenth International Pathogenic Neisseria Conference, Sept. 8-13, Baltimore, Maryland. (Also presented at the Ottawa Life Sciences National Conference & Exhibition, Medical Devices, Agricultural Biotechnology and Technology Transfer Opportunities section, October 29-30, 1996, Ottawa, Ontario).

Pagotto, F.J., Hill, S.A., and Dillon, J.R. 1997. Replication mechanisms of multicomplex gonococcal β-lactamase plasmids. 37th Interscience Conference on Antimicrobial Agents and Chemotherapy, Sept. 28-October 1, Toronto, Ontario.

Salimnia, H, **Pagotto, F.**, and Dillon, J.R. 1998. A shuttle vector for *Neisseria gonorrhoeae*. Eleventh International Pathogenic Neisseria Conference, Nov. 1-6, Nice, France.

Pagotto, F., Hill, S., and Dillon, J.R. 1998. Molecular analysis of gonococcal β-lactamase plasmid replication. Eleventh International Pathogenic Neisseria Conference, Nov. 1-6, Nice, France.

CONFERENCE ORAL PRESENTATIONS:

F. Pagotto, and M.W. Griffiths. 1994. Phage-mediated detection of *Staphylococcus aureus* causing bovine mastitis by bioluminescence. American Dairy Science Association and American Society of Animal Science Joint Meeting, Animal Health, Pharmacology and Toxicology Division, July 14, University of Minnesota, U.S.A.

F. Pagotto. 1996. Presented for Dr. Dillon. Network in the Americas and Caribbean for *Neisseria gonorrhoeae*. 3rd Canadian Conference on International Health: Effectiveness in Health Development. Ottawa, Ontario, Canada, November 10-13.

POSTER COMPETITIONS:

150th Anniversary of the University of Ottawa (1st prize in the Ph.D. poster competition, Department of Biochemistry, Microbiology and Immunology, University of Ottawa, 1998). Poster title: The biology of gonococcal β-lactamase-producing plasmids.

1999 University of Ottawa (Department of Biochemistry, Microbiology and Immunology) annual poster competition (2nd prize in the Ph.D. category). Poster title: Plasmid Biology in *Neisseria gonorrhoeae*.