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DEVELOPMENT OF A NOVEL SHUTTLE SYSTEM FOR THE TRANSFER OF GONOCOCCAL GENES BETWEEN *ESCHERICHIA COLI* AND *NEISSERIA GONORRHOEAE*

by

Benoit R. Gauthier, B.Sc.

A thesis submitted to the School of Graduate Studies and Research in partial fulfillment of the requirements for the Master of Science degree in Biology

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ABSTRACT

The overall objective of this project was to construct a shuttle vector for the transfer of gonococcal genes between Escherichia coli and Neisseria gonorrhoeae using an in vitro-derived deletion derivative of the 7.2 kb naturally-occurring penicillinase-producing plasmid of N. gonorrhoeae. To improve the frequency of introduction of cloned gonococcal genes in N. gonorrhoeae, uptake sequences presumed to be implicated in gonococcal transformation and modes of introducing plasmid DNA into N. gonorrhoeae were studied. The ability of N. gonorrhoeae strain F62 to selectively acquire a 1.5 kb TaqI fragment derived from the 4.2 kb cryptic plasmid was confirmed by uptake experiments. Sixteen strains of different auxotypes were tested for their ability to acquire the 1.5 kb TaqI fragment. Although all strains were competent for genetic transformation by chromosomal DNA to streptomycin resistance, 3 strains were unable to acquire the 1.5 kb TaqI fragment. No relationship was observed between uptake and auxotype. No correlations were found between the restriction/modification systems of strains that did not acquire the 1.5 kb TaqI fragment and the ones that did. Furthermore, competition experiments performed between chromosomal DNA, the 1.5 kb TaqI fragment and a synthetic oligonucleotide of 37 bp comprising two inverted copies of the uptake sequences flanked by EcoRI sites showed that neither the 1.5 kb fragment nor the oligonucleotide had any effect on the ability of chromosomal DNA to transform a strain that did not recognize the 1.5 kb fragment.

However, the efficiency of transformation of the strain that did acquire the fragment was lowered by the presence of the two competing DNAs. These findings suggest that uptake sequences are not universally recognized in the gonococcus and that N. gonorrhoeae cells possess at least a second transformation system which allows the recognition of heterologous and homologous DNA without the need of uptake sequences.

Conjugation, transformation and electroporation were assessed as possible modes for introducing plasmid DNA into N. gonorrhoeae isolates. Conjugation, using pUB307, an IncP conjugative plasmid, was the most efficient method for introducing plasmid DNA into recipient gonococci. Transformation and electroporation failed to introduce plasmid DNA into N. gonorrhoeae. It was concluded that restriction/modification systems of host strains and non-optimal electroporation conditions were responsible for the failure of introducing plasmid DNA into the gonococcus by transformation and electroporation.

Deletion derivatives of pJD8 (smallest deletion derivative of pJD4) were constructed in order to reduce the size of pJD8 to the smallest mobilizable plasmid. Mobilization of deletion derivatives permitted the refinement of two essential sites required simultaneously for the mobilization of the 7.2 kb penicillinase-producing plasmid of N. gonorrhoeae. These sites were located on

fragments harbouring *mob* and *oriT* functions which were individually identified by others.

Plasmid pGRY4 (5.7 kb), the smallest mobilizable plasmid, was chosen as a basis for the construction of a shuttle vector. The *lacZ* complementation system of *E. coli* and the *xylE* system from *Pseudomonas putida* were investigated as possible positive selection markers for the shuttle vector. The *lacZ* system from the plasmid pGEM-3Z, but not the *xylE* gene, was successfully cloned into the *Bam*HI site of pGRY4 to produce pGCY1. The novel plasmid pGCY1 was mobilized by pUB307 into the gonococcus at a frequency similar to pGRY4. Several problems were encountered when the *proC* gene of *N. gonorrhoeae* was cloned into pGCY1. For example, with the *E. coli* host (χ^{478}), plasmid-free transformants grew on minimal plates without proline indicating that the strain acquired the *proC* gene but the latter was integrated into the chromosome. The strain was later found to be *recA*$^+$ and restriction$^+$ explaining the plasmid-free transformants. Future transformation attempts should be performed in a restriction$^-$ and *recA*$^-$ strain.

RÉSUMÉ

L'objectif principal du projet était de construire un vecteur navette qui permettrait le transfert de gènes gonococciques entre Escherichia coli et Neisseria gonorrhoeae en utilisant un dérivé in vitro du plasmide gonococcique de 7.2 kb qui produit la ß-lactamase. Le rôle des séquences impliquées dans la transformation génétique de N. gonorrhoeae a été étudié afin de déterminer si celles-ci devraient être incorporées au vecteur afin d'augmenter l'efficacité de la transformation. L'habileté de la souche gonococcique F62 d'acquérir de facon sélective un fragment de 1.5 kb généré par la digestion du plasmide de 4.2 kb avec l'enzyme TaqI a été confirmée par des expériences de transformation génétique. Seize souches gonococciques d'auxotypes variés ont été testées pour leur capacité à reconnaître et acquérir le fragment de 1.5 kb contenant une copie de la séquence impliquée dans la transformation sélective de N. gonorrhoeae. Quoique toutes compétentes pour acquérir de l'A.D.N. chromosomal par transformation génétique, 3 des 16 souches ont été incapables de reconnaître et d'acquérir le fragment de 1.5 kb. Aucune relation n'a été observée entre l'auxotype des souches et l'habileté a reconnaître le fragment. Les systèmes de restriction et de modification des 3 souches ont été comparés avec les systèmes de souches qui avaient acquis le fragment de 1.5 kb mais aucune corrélation n'a été décernée entre les systèmes de restriction et la capacité d'acquérir le fragment. Des expériences de compétition entre de l'A.D.N. chromosomal, le

fragment de 1.5 kb et un oligonucléotide synthétique de 37 bases composé de deux copies inversées des séquences de transformation gonococciques ont démontré que l'efficacité de transformation par l'A.D.N. chromosomal de l'une des souches qui n'avait pas acquis le fragment de 1.5 kb n'a pas été affectée par la présence du fragment de 1.5 kb et de l'oligonucléotide de 37 bases. Par ailleurs, l'efficacité de transformation d'une souche qui a acquis le fragment de 1.5 kb a été affectée par la présence compétitive du fragment de 1.5 kb et de l'oligonucléotide de 37 bases. Ces résultats suggèrent que les séquences impliquées dans la transformation génétique du gonocoque ne sont pas reconnues universellement et que N. gonorrhoeae possède au moins un second système de transformation qui serait non spécifique.

La conjugaison, la transformation et l'électroporation ont été étudiées afin de déterminer lequel de ces systèmes possèdait le meilleur rendement pour introduire de l'A.D.N. plasmidique dans N. gonorrhoeae. La conjugaison en utilisant le plasmide de transfer pUB307 (IncP), a été determinée comme étant la plus efficace pour l'introduction de l'ADN plasmidique dans le gonocoque. La transformation et l'électroporation ont été incapables d'introduire de l'A.D.N. dans N. gonorrhoeae. Il a été conclu que les systèmes de restriction des souches et des conditions non optimales pour l'électroporation ont été la cause de l'échec de ces deux modes.

Des dérivés du plasmide pJD8 (le plus petit dérivé du plasmide pJD4) ont été construits *in vitro* afin de créer le plus petit plasmide qui produit la pénicillinase et qui peut être mobilisé dans le gonocoque par pUB307. La mobilisation des différents dérivés de pJD4, pJD5 et de pJD8 nous a permise de raffiner deux sites essentiels pour la mobilisation de pJD4 par pUB307. Ces sites ont été localisés sur deux fragments qui contiennent les fonctions de *mob* et *oriT* impliquées dans la mobilisation du plasmide de 7.2 kb (pJD4).

Le plasmide pGRY4 (5.7 kb), le plus petit plasmide mobilisable par pUB307, a été choisi pour construire le vecteur navette. Le système de complémentation *lacZ* de *E. coli* et le gène *xylE* de *Pseudomonas putida* ont été étudiés comme systèmes de sélection positif pour le vecteur. Le gène *lacZ* et non *xylE* a été cloné dans pGRY4 pour générer le vecteur pGCY1. Le nouveau vecteur navette a été mobilisé à une fréquence similaire a pGRY4.

Certains problèmes ont été encourus lors du clonage du gène gonococcique *proC* dans pGCY1. Par exemple, des transformants de la souche χ^{478} d'*E. coli* ont été obtenus sur milieu minimal déficient en proline indiquant que ceux-ci avaient acquis le gène *proC*. Lors de l'analyse des transformants, aucun plasmide a été retrouvé dans les cellules. Il a été déterminé par la suite que la souche χ^{478} était *recA*$^+$ et restriction$^+$ ce qui expliquerait la présence de transformants sans plasmide. Les expériences à venir sur le clonage

des gènes gonococciques dans pGCY1 devraient être poursuivies dans une souche recA$^-$ et restriction$^-$.

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À MICHÈLE ANDRÉE

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LIST OF ABBREVIATIONS

ATP	adenosine triphosphate
α	alpha
Amp	ampicillin
bp	base pair
ß	beta
bla	beta-lactamase
CFU	colony forming units
Cm	chloramphenicol
cpm	counts per minute
°C	degree celsius
Δ	delta
dATP	deoxyadenosine 5'-triphosphate
dCTP	deoxycytidine 5'-triphosphate
dGTP	deoxyguanine 5'-triphosphate
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	deoxyribonucleotide triphosphate
dTTP	deoxythymidine 5'-triphosphate
dUTP	deoxyuridine 5'-triphosphate
CU	citrulline-uracil-requiring
GCMB	gonococcal medium base
g	gram
hrs	hours
Inc	incompatibility group
IPTG	isopropyl-ß-thiogalactopyranoside
Km	Kanamycin
χ	khi
kb	kilobases
kd	kilodaltons
kv	kilovolts
λ	lambda
L	litre
μF	microfarad
μg	microgram
μL	microlitre
μm	micrometre
mL	millilitre
mM	millimole
min	minute
mob	mob locus
M	mole
Nal	nalidixic acid
ng	nanogram
nm	nanometre
NBT	nitroblue tetrazolium
NR	non-requiring
NP	no plasmid
OD	optical density
oriT	origin of conjugative transfer

oriV	origin of replication
O	ornithine-requiring
OUH	ornithine-uracil-hypoxanthine-requiring
ϕ	phi
r	resistant
rpm	revolutions per minute
RNase	ribonuclease
Rif	rifampicin
SDS	sodium dodecyl sulfate
Sm	streptomycin
Tet	tetracycline
TSA	tryptic soya agar
Tn	transposon
X-Gal	5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside
X-phosphate	5-bromo-4-chloro-3-indolyl phosphate

INTRODUCTION

Neisseria gonorrhoeae, a gram negative coccus, is the causative agent of the human sexually transmitted disease gonorrhoeae. N. gonorrhoeae has a number of properties which contribute to its pathogenicity including the production of an IgA protease (Koomey et al., 1982), pili (Meyer et al., 1982), lipopolysaccharides (Apicella et al., 1981) and outer membrane proteins (Swanson, 1982). In recent years, recombinant DNA methodology has permitted researchers to study these pathogenic elements at the molecular level by investigating the regulation and expression of cloned gonococcal genes in Escherichia coli. One of the major problems of the cloning approach, however, is the re-introduction of cloned DNA into the original N. gonorrhoeae host. Suitable gene transfer methods in N. gonorrhoeae, which allow the study of cloned gonococcal genes under physiological conditions, have not been developed. Several strategies can be employed to achieve this goal, one of them being the construction of a shuttle vector. This study explores this approach and investigates possible modes of plasmid transfer between E. coli and N. gonorrhoeae.

LITERATURE REVIEW

The development of a shuttle system which will allow the transfer of cloned gonococcal genes between E. coli and N. gonorrhoeae requires an understanding of gonococcal genetics in order to determine critical features needed to achieve this goal. The aim of this literature review is to do so by describing: 1) the various naturally-occurring plasmids of N. gonorrhoeae; 2) the genetic exchanges that can occur in the gonococcus; 3) systems which have been already developed for the introduction of cloned genes into N. gonorrhoeae and their disadvantages; 4) the review of restriction/modification systems of the gonococcus; and, 5) the necessity to develop a new and more efficient shuttle system.

I. PLASMIDS OF N. GONORRHOEAE

Plasmids can be classified according to their conjugative properties. Certain plasmids have the ability to self-transfer (conjugative plasmid) by conjugation while others do not (non-conjugative plasmid) (Clark and Warren, 1979). Conjugation is the process whereby DNA is transferred from a donor to a recipient strain by a mechanism involving cell-to-cell contact (Clark and Warren, 1979). This involves the production of proteins which are encoded by the conjugative plasmid (Willetts and Wilkins, 1984). Non-conjugative plasmids do not produce these proteins and are

therefore unable to self-transfer; however, non-conjugative plasmids can be transferred from a donor to a recipient strain by the process of mobilization by conjugative plasmids. In order to be mobilized, regions implicated in this mechanism must be located on the non-conjugative plasmid (Willetts and Wilkins, 1984).

Mobilization can occur by two independent processes, donation and conduction (Clark and Warren, 1979). Donation is the process whereby a non-conjugative plasmid is mobilized by a conjugative plasmid without physical association of the two plasmids. Conduction is the process whereby physical association between the conjugative and non-conjugative plasmids is required in order for transfer to occur (Clark and Warren, 1979). Gonococcal plasmids can be classified according to their conjugative properties.

a) Conjugative plasmids of the gonococcus

The presence of large extrachromosomal DNA lacking any detectable phenotype in N. gonorrhoeae was first reported by Stiffler et al. (1975). This group of novel plasmids had molecular weights of approximately 39.2 kilobase (kb) and shared a common core of 70 to 100% nucleotide sequence similarity, thereby suggesting that they were derived from a common plasmid (Hannah, 1988; Roberts et al., 1979; Sox et al., 1978). Furthermore, the guanine plus cytosine content of these plasmids was determined to be 48 mole %, a percentage similar to gonococcal chromosomal DNA (50%), indicating that the plasmids were probably of gonococcal

origin (Roberts et al., 1979; Sox et al., 1978). The ability of these plasmids to self-transfer to other gonococcal strains was demonstrated shortly after their discovery (Baron et al., 1977; Eisenstein et al., 1977; Kirven and Thornesberry, 1977; Roberts and Falkow, 1977). The host range of the 39.2 kb conjugative plasmid is, however, limited to N. gonorrhoeae and N. cinerea (Flett et al., 1981; Genco et al., 1984; Hannah, 1988; Roberts and Knapp, 1988).

In 1986, N. gonorrhoeae isolates resistant to high levels of tetracycline were isolated and shown to carry a 40.3 kb plasmid (Morse et al., 1986). The 40.3 kb tetracycline-resistance-encoding plasmid was also detected in naturally-occurring isolates of Kingella denitrificans and Eikenella corrodens (Morse et al., 1986). It was initially thought that this novel plasmid was the 39.2 kb conjugative plasmid harboring a tetracycline-resistant determinant tetM, a transposon-borne determinant initially found in the genus Streptococcus (Morse et al., 1986). However, recent studies using restriction endonuclease mapping determined that the 40.3 kb transfer plasmid was different from the 39.2 kb and had most likely evolved in another species (possibly a streptococcus) prior to being transferred to the gonococcus (Gascoyne et al., 1990). In contrast to the limited host range of the 39.2 kb plasmid, the 40.3 kb conjugative plasmid can be transferred from a gonococcal donor strain to a variety of Neisseria species including N. gonorrhoeae, N. meningitidis, N. cinerea, N. flava, N. perflava,

N. sicca, N. subflava, N. flavascens, N. lactamica and N. mucosa (Roberts and Knapp, 1988). To date, these are the only two conjugative plasmids which have been isolated in N. gonorrhoeae strains.

b) Non-conjugative plasmids of the gonococcus.

There are two classes of non-conjugative plasmids in N. gonorrhoeae, penicillinase-producing plasmids and the 4.2 kb cryptic plasmid. In 1976 penicillinase-producing N. gonorrhoeae (PPNG) were isolated in the United Kingdom and in North America for the first time (Ashford et al., 1976; Bowmer, 1976; Percival et al., 1976; Phillips, 1976). Resistance to penicillin was attributed to the production of beta (ß)-lactamase encoded on either a 7.2 kb plasmid or a 5.1 kb plasmid. Isolates from North America which harboured the 7.2 kb plasmid were linked to the Far East (Asian-type) while isolates from the United Kingdom which harboured the 5.1 kb plasmid were linked to West Africa (African-type) (Perine et al., 1977; Roberts et al., 1977). Both plasmids carried roughly 40% of the transposon Tn2 and produced TEM-1 ß-lactamase (Fayet et al., 1982, Roberts et al., 1977). The 7.2 kb and 5.1 kb plasmids were shown to be identical except for a 2.1 kb fragment deleted from the 7.2 kb plasmid (Dickgiesser, 1983; Dickgiesser et al., 1982; Gilbride and Brunton, 1990; Yeung and Dillon, 1985).

Since the initial appearance of the 7.2 kb and 5.1 kb penicillinase-producing plasmids in 1976, several smaller plasmids

have been described: the Toronto-type (4.9 kb), isolated in Canada in 1984 (Yeung et al., 1986); the Rio-type (4.6 kb), epidemiologically linked to Rio de Janeiro (Van Embden et al., 1985); and, the Nimes-type (6.6 kb) isolated in Nimes, France (Gouby et al., 1986). These smaller plasmids were either deletion derivatives of the 7.2 kb plasmid or insertion derivatives of the 5.1 kb plasmid (Yeung and Dillon, 1985; Yeung et al., 1986; Sox et al., 1979).

Gonococcal ß-lactamase-producing plasmids demonstrate a high degree of similarity with a variety of plasmids isolated from Haemophilus spp. (Brunton et al., 1986a; Brunton et al, 1986b; Dillon and Yeung, 1989). Both the 7.2 kb and 5.1 kb plasmids have a G+C content of 41 mole % which is similar to Haemophilus DNA but different from the gonococcal G+C content of 50 mole % (Mayer et al., 1974; Roberts et al., 1977; Sox et al., 1978). Furthermore, the 7.2 kb plasmids of H. parainfluenzae and H. influenzae were shown to be identical to the 7.2 kb plasmid of N. gonorrhoeae (Brunton et al., 1986a; Brunton et al., 1986b). These findings indicate that gonococcal resistance plasmids may have originated from H. parainfluenzae and transferred to N. gonorrhoeae by transformation or conjugation (Brunton et al., 1986a; Brunton et al., 1986b; Dillon and Yeung, 1989).

In addition to the ß-lactamase-producing plasmids, N. gonorrhoeae isolates carry a small 4.2 kb indigenous cryptic

plasmid of unknown function (Mayer et al., 1974, Roberts et al., 1977). Hybridization studies demonstrated that fragments of the cryptic plasmid could integrate into the chromosome of N. gonorrhoeae (Hagblom et al., 1986). The significance of this integration is still unknown. In 1988, Roy et al. isolated a 4.4 kb cryptic plasmid that was identical to the 4.2 kb plasmid except for an insertion of 156 base pairs (bp) in the cryptic plasmid protein B (cppB) gene. The presence of insertions in the cppB suggested a mechanism of modulating gene expression at the transcriptional level by DNA rearrangement (Roy et al., 1988).

The distribution of gonococcal plasmids in N. gonorrhoeae isolates was correlated with their auxotypes (nutritional requirement of a specific isolate) and has been used to classify gonococcal strains ((Dillon et al., 1981; 1986; Dillon and Pauzé, 1981). Non-requiring (NR) and proline-requiring (P^-) isolates often harboured the Asian-type plasmid while the African and Toronto-type plasmids were distributed mostly among non-requiring strains (Dillon et al., 1989). Strains harbouring the conjugative and/or cryptic plasmids of N. gonorrhoeae belong to either the non-requiring or proline-requiring auxotype (Dillon et al., 1989). Proline-citrulline-uracil-requiring (PCU) strains have been characteristically associated with plasmid-free strains in Canada (Dillon et al., 1986; Dillon and Pauzé, 1984).

II. GENETIC EXCHANGE IN THE GONOCOCCUS

The major genetic exchange mechanisms in N. gonorrhoeae are transformation and conjugation (Stein, 1989). Bacteriophages and transposable elements specific to N. gonorrhoeae have not been isolated to date (Goldberg et al., 1978). This section summarizes transformation and conjugation mechanisms in N. gonorrhoeae.

a) Transformation of N. gonorrhoeae

Transformation is defined as the ability of organisms to acquire new genetic material by taking up and incorporating DNA directly from the media (Avery et al., 1944). This process was demonstrated to be the principal natural mechanism mediating transfer of chromosomal genes in N. gonorrhoeae (Sparling, 1966). Initial work suggested that chromosomal genes could be transferred by conjugation by the 39.2kb plasmid since recombinants for a variety of chromosomal markers were observed in prolonged filter matings (Biswas et al., 1976; Roberts and Falkow, 1978). It is now known that these results were obtained not through conjugation but by a DNase-resistant transformation mechanism (Biswas et al., 1985; Steinberg and Goldberg, 1980). Piliated variants of N. gonorrhoeae (colony types 1 and 2) in their log phase were shown to be highly competent for genetic transformation with chromosomal DNA as compared to non-piliated variants (colony type 3 and 4) (Sparling, 1966). However, uptake of penicillinase-producing plasmids by transformation using either piliated or non-piliated variants was

found to be inefficient and usually resulted in deletions of incoming DNA upon transformation unless the recipient contained an homologous plasmid (Biswas et al., 1982; Biswas et al., 1985; Sox et al., 1979). Although certain aspects of gonococcal transformation are well characterized, the mechanism of selective uptake of chromosomal DNA by piliated gonococci is still poorly understood. Initial work performed by Dougherty et al. (1979) demonstrated the preferential uptake by N. gonorrhoeae of gonococcal chromosomal DNA over the chromosomal DNA of other species; both homologous and heterologous DNA interacted with the membrane, but only homologous DNA was taken up into a deoxyribonuclease-resistant form. The DNA was then incorporated into the cell as a linear double-stranded form (Biswas et al., 1986; Biswas and Sparling, 1981).

The selective nature of gonococcal transformation indicated that DNA uptake sequences could be implicated in this process. A similar system is found in Haemophilus influenzae where specific sequences are required for transformation (Danner et al., 1980). In 1982, Graves et al. demonstrated the preferential uptake of a 1.5 kb TaqI fragment and a 3.0 kb MspI fragment, using an 11.5 kb hybrid plasmid composed of the 7.2 kb heterologous ß-lactamase-producing plasmid and the 4.2 kb indigenous plasmid. Both fragments shared a common region of 640 bp entirely derived from the 4.2 kb cryptic plasmid. Competition experiments showed that gonococcal chromosomal DNA but not the 4.2 kb cryptic plasmid DNA hindered the

ability of the 11.5 kb hybrid plasmid to transform N. gonorrhoeae (Graves et al., 1982). The putative uptake sequences of N. gonorrhoeae found on the 640 bp fragment were shown to be different from those of H. influenzae by competition experiments with Haemophilus chromosomal DNA (Graves et al., 1982). Recently, Goodman and Scocca (1988), using chromosomal DNA, have identified the sequence 5'-GCCGTCTGAA-3' as the specific uptake sequence implicated in gonococcal transformation. This sequence was found on the 640 bp fragment of the 4.2 kb cryptic plasmid (Goodman and Scocca, 1988). The sequence was usually found in a dyad symmetry that could form a stable stem-loop structure and function as terminator or attenuator of transcription. However, the dyad symmetry of the uptake sequence was not required for uptake since only one copy was found on the cryptic plasmid (Goodman and Scocca, 1988). In contrast to these observations, Burnstein et al. (1988) found, using a 4.2 kb cryptic plasmid, that in addition to the overlapping 640 bp region of the 1.5 kb TaqI and 3.0 kb MspI fragments, a second region encompassed in a 300 bp DdeI fragment was also acquired by gonococcal cells. A 10-mer (5'GATGCTCTGT-3') common to the 640 bp and 300 bp fragments was identified by DNA sequence analysis; it was different from the uptake sequence described by Goodman and Scocca (1988). This sequence was unable to compete efficiently with chromosomal DNA. They concluded that no specific sequence could account for preferential uptake but that DNA structure could play an important role in gonococcal transformation (Burnstein et al., 1988). Therefore, the role of

uptake sequences in the transformation of N. gonorrhoeae is still uncertain.

The role of piliated variants (colony types 1 and 2) in the transformation of N. gonorrhoeae is poorly understood. Although pili are strongly correlated with competency, several independent groups have demonstrated that pili are not implicated in transformation (Biswas et al., 1989; Mathis and Scocca, 1984). Biswas et al. (1989) created, by chemical mutagenesis, transformation-deficient mutants of piliated N. gonorrhoeae cells, thereby indicating that pili were not implicated in gonococcal transformation. However, another group recently showed, by shuttle mutagenesis, that mutation in the pilus gene of N. gonorrhoeae lowered transformation frequencies by a factor of 10^3 (Seifert et al., 1990). They concluded that pilin expression was therefore required for transformation (Seifert et al., 1990). Variations in membrane proteins between piliated cells and non-piliated cells, other than pilin, indicated that a membrane protein closely linked to or regulated with pilin was probably required for transformation (Klimpel and Clark, 1988).

Transformation of the gonococcus by the penicillinase-producing plasmids of N. gonorrhoeae was shown to be inefficient possibly because these plasmids did not carry a copy of the uptake sequences (Graves et al., 1982). Stein et al. (1983a; 1983b) constructed chimeric plasmids composed of the 7.2kb ß- lactamase-producing

plasmid and 4.2 kb cryptic plasmid, which contained a copy of the putative uptake sequences, and found that, upon transformation, these plasmids underwent deletions and were unstable. Therefore, even in the presence of uptake sequences, the introduction of plasmid DNA by transformation results in unstable deletion derivatives. Burnstein et al. (1988) demonstrated that heterologous DNA such as M13mp19 could be acquired into a DNase resistant state. The stability of the vector in gonococcal cells was however not demonstrated (Burnstein et al., 1988). This indicates that uptake sequences may not always be required for gonococcal transformation.

N. gonorrhoeae and Bacillus subtilis share several characteristics in plasmid and chromosomal DNA transformation. Similar to the gonococcus, plasmid transformation in B. subtilis is less efficient than chromosomal DNA transformation (Dubnau, 1976). However, marker rescue by a homologous plasmid in B. subtilis can increase the efficiency of transformation; a phenomenon also observed in N. gonorrhoeae (de Vos et al., 1981; Biswas et al., 1982). Marker rescue, in both organisms, was attributed to the fact that transforming DNA is cleaved to form a linear molecule before entering the cell. Once inside the cell if it cannot be recirculized by homologous recombination with a resident plasmid (marker rescue) or intergrate into the chromosome it is degraded by restriction endonucleases and lost (de Vos et al., 1981; Biswas et al., 1982). Although both organisms share similarities in transformation, mechanisms of DNA uptake are different. Transformation in B. subtilis is non-specific; both

homologous and heterologous DNA transform B. subtilis and uptake sequences are not required (Dubnau, 1976). The DNA is fragmented and converted to single strand before entering the cell. This processing creates an eclipse which is a time interval where cells are not competent for transformation (Dubnau, 1976). In the gonococcus, the DNA is cleaved once before being processed as a double strand molecule through the membrane. No eclipse has been observed in the gonococcus (Biswas et al., 1985). It appears that organisms such as N. gonorrhoeae, H. influenzae and B. subtilis have developed different but similar strategies to incorporate DNA in order to acquire new functions and survive in a harsh environment.

b) Conjugation in N. gonorrhoeae

The 7.2 kb penicillinase-producing plasmid of N. gonorrhoeae probably originated in H. parainfluenzae and was transmitted to the gonococcus by transformation (Dillon and Yeung, 1989; Sox et al., 1979). However, the spread the 7.2 kb plasmid between gonococcal isolates was attributed to mobilization by the 39.2 kb conjugative plasmid (Eisenstein et al., 1977; Hannah, 1988; Roberts et al, 1977). The 7.2 kb plasmid can be efficiently mobilized by the 39.2 kb conjugative plasmid to species such as N. meningitidis, N. cinerea, N. flava, N. perflava, N. sicca, N. subflava, N. flavascens, and N. mucosa (Genco et al., 1984; Ikeda et al., 1986). The conjugative plasmid also successfully mobilized the 7.2 kb plasmid to strains of H. influenzae and E. coli (Eisenstien et al.,

1977; Flett et al., 1981; Hannah, 1988; Sox et al., 1978). In addition to the 7.2 kb penicillinase-producing plasmid of N. gonorrhoeae, the 5.1 kb plasmid but not the 4.9 kb plasmid was mobilized by the 39.2 kb conjugative plasmid from N. gonorrhoeae to N. cinereae, N. gonorrhoeae and E. coli (Genco et al., 1984; Hannah, 1988). The 7.2 kb plasmid could also be mobilized by several incompatibility group P (IncP) conjugative plasmids and maintained into E. coli, Salmonella minnesota and H. influenzae but not by IncF1, IncFII or IncIα- group plasmids (Guiney and Ito, 1982). Piffaretti et al. (1988) demonstrated the mobilization of the 7.2 kb plasmid from E. coli to N. gonorrhoeae by plasmid pUB307, an IncP conjugative plasmid.

Results of these experiments presumed that mobilization of the 7.2 kb by various conjugative plasmids occurred by donation. Regions on the 7.2 kb plasmid implicated in the conjugative process have been partly characterized by two independent groups using different systems (McNicol et al., 1983; Tenover et al., 1985). Using an H. influenzae background for conjugation studies, McNicol et al. (1983) located a putative origin of transfer (oriT) function, to a 1.9 kb BamHI/HindIII fragment (McNicol et al., 1983). Using a gonococcal background, other workers characterized a 1.9 kb HinfI fragment required for mobilization (Tenover et al., 1985). Tenover et al. (1985) found that this fragment could encode a 16 Kd protein which may be implicated in mobilization of the 7.2 kb plasmid (Tenover et al., 1985).

III. INTRODUCTION OF CLONED GENES INTO N. GONORRHOEAE

Genes from N. gonorrhoeae isolates that have been cloned and characterized have initially been investigated using E. coli systems with few (e.g. recA, protein I, pilin and proAB) being reintroduced into their natural host for further studies (Carbonetti et al., 1988; Koomey et al., 1982; Koomey and Falkow, 1987; Seifert et al., 1990; Stein et al., 1984). Three approaches have been described for the reintroduction of cloned gonococcal genes into N. gonorrhoeae; shuttle vectors, shuttle mutagenesis and transformation followed by homologous recombination with the chromosome.

Only two general purpose cloning vectors have been described for N. gonorrhoeae, pFT180 (Stein et al, 1983b) and pLES2 (Stein et al, 1983a). Plasmid pFT180 is a hybrid plasmid comprising the 7.2 kb penicillinase-producing plasmid and the 4.2 kb naturally-occurring gonococcal cryptic plasmid; pLES2 is a deletion derivative of plasmid FT180 into which the lacZα sequence and the pUC9 multiple cloning sites were incorporated (Stein et al., 1983a). Both of these plasmids, however, have limitations. pFT180 is of limited use because of its large size (11.4 kb), because it contains no multiple cloning sites and lacks a positive selection marker, because it contains sequences involved in recombination and because it is unstable when transformed into N. gonorrhoeae (Stein et al., 1983b). Plasmid pLES2 is also of limited use because it

does not carry the putative uptake sequences found on the cryptic plasmid which are required to efficiently transform N. gonorrhoeae cells (Stein et al., 1983a). Plasmid pLES2, like pFT180, was unstable when transformed into gonococci (V.L. Clark, personal communication). pLES2 was, however, successfully used in cloning genes implicated in the proline biosynthesis pathway of N. gonorrhoeae (Stein et al., 1984). To date, these are the only two shuttle vectors which have been constructed for E. coli and N. gonorrhoeae.

Shuttle mutagenesis was recently applied to study the porin and pilin proteins of N. gonorrhoeae (Carbonetti et al., 1988; Seifert et al., 1990). The basis of this approach is to disrupt a cloned gene in E. coli by using a transposase deficient transposable element harboured on a plasmid. Transposase is supplied to the defective element by a transposase-expressing vector present in the host strain. Cointegrates of the donor molecule which carry the minitransposon and the target molecule are then mobilized and resolved into an E. coli recipient strain. The mutant gene is recovered from the recipient and transformed into the original N. gonorrhoeae host to produce a mutant strain (Seifert et al., 1986). The limitation of this approach is that once re-introduced into the host, the cloned DNA recombines with the chromosome and is therefore not as easily retrievable as plasmid DNA.

Koomey and associates (Koomey et al., 1982; Koomey and Falkow, 1987) developed a system whereby a cloned gene was mutated by introducing the ß-lactamase gene of the 7.2 kb penicillinase-producing plasmid of N. gonorrhoeae into the coding sequence of the gene. The mutated gene was re-introduced into the gonococcus by transformation to create a mutant strain. This approach is limited, however, because the restriction endonuclease map of the gene must be known before it can be mutagenized.

IV. RESTRICTION/MODIFICATION SYSTEMS OF THE GONOCOCCUS

Restriction/modification systems have hindered the re-introduction of cloned gonococcal genes into N. gonorrhoeae (Davies, 1989). Restriction and modification systems are mechanisms by which microorganisms distinguish themselves. Restriction barriers result from the production of restriction endonucleases which bind to and cleave specific DNA sequences (Roberts, 1988). To protect its own DNA from degradation N. gonorrhoeae produces a modification enzyme which binds to and methylates the DNA sequence recognized by a restriction enzyme (Nelson and McClelland, 1987). Thus far, five different restriction endonucleases along with the methylases that recognize the same sequences as the restriction enzymes have been purified from N. gonorrhoeae (Davies, 1989). Isoschizomers of these restriction endonucleases are: HaeII, HaeIII, SacII, HphI and DpnI.

Duff and Davies (1988) have recently revised the nomenclature of gonococcal enzymes so that it reflects that used for other bacterial species (Roberts, 1988). Under the new nomenclature, the prefix S is used for naming methylases instead of M. The prefix R has been retained for naming restriction endonucleases. The suffix for either methylases or restriction enzymes names represents the strain from which they were initially isolated. However, only novel methylases will take the new suffix nomenclature. Characterized methylases will keep their present names. For example, the gonococcal isoschizomer for the restriction endonuclease HaeII was previously named R.NgoII while the methylase for this enzyme was named M.NgoII. Under the new nomenclature the restriction enzyme will now be called R.NgoCI and the methylase S.NgoII.

Davies (1988) suggests that a single gonococcal strain may harbour at least 6 restriction/modification systems. This plethora of restriction endonucleases within one strain represents an efficient restriction barrier difficult for cloned genes to cross. However, Stein et al. (1988) observed that restriction/modification systems of N. gonorrhoeae did not affect the acquisition of foreign DNA by conjugation.

V. OBJECTIVES OF THE PRESENT STUDY

The overall purpose of this project is to develop an efficient shuttle system which would allow the re-introduction of cloned

gonococcal genes into N. gonorrhoeae using a plasmid vector. Although existing shuttle vectors have been successfully used for cloning the gonococcal proAB gene, both pFT180 and pLES2 produce unstable deletion derivatives upon transformation of the gonococcus (personnal communication, V.L. Clark). In order to overcome problems encountered by the shuttle vectors pLES2 and pFT180, and to construct an efficient shuttle system between E. coli and N. gonorrhoeae, uptake sequences implicated in gonococcal transformation and modes of introducing plasmid DNA into the gonococcus were studied.

Controversies regarding the role of uptake sequences have definitely hampered the development of an efficient plasmid/vector permitting the re-introduction of cloned gonococcal genes into N. gonorrhoeae. Thus, one objective of this study is to clarify conflicting reports on the role of uptake sequences in gonococcal transformation and to incorporate these sequences in the shuttle vector if required.

In addition to transformation, conjugation and electroporation were assessed as alternative modes of introducing plasmid DNA into N. gonorrhoeae. Mobilization of the 7.2 kb penicillinase-producing plasmid of N. gonorrhoeae by pUB307, an incP conjugative plasmid, into the gonococcus was recently demonstrated by Piffaretti et al. (1988). Transconjugants were showed to stably maintain plasmids of similar sizes to the 7.2 kb plasmid. Stein et al. (1988) also

demonstrated that, transformed but not mobilized plasmid DNA, was restricted by the gonococcus. Therefore, conjugation might obviate restriction barriers encountered by transformation and permit the introduction of cloned gonococcal genes in N. gonorrhoeae. In recent years, electroporation has become widely used for the introduction of DNA into bacteria. Electroporation of the gonococcus has never been demonstrated. Optimal conditions for the introduction of plasmid DNA into N. gonorrhoeae were determined and the smallest replicating in vitro-derived deletion derivative of the 7.2 kb penicillinase-producing plasmid that could be maintained in both E. coli and N. gonorrhoeae was selected as the basis for the construction of a shuttle vector.

CHAPTER ONE

STUDIES ON THE ROLE OF UPTAKE SEQUENCES IN GONOCOCCAL TRANSFORMATION USING A 4.2 KB CRYPTIC PLASMID: ARE UPTAKE SEQUENCES UNIVERSAL?

INTRODUCTION

The role of uptake sequences in gonococcal transformation is still controversial. Some studies on N. gonorrhoeae transformation suggest that uptake sequences are implicated in this process (Goodman and Scocca, 1988; Graves et al., 1982) while other results indicate that the gonococcus can also acquire DNA without their presence (Burnstein et al., 1988). Most of these studies were accomplished using a limited number of laboratory strains which are not representative of the natural population of N. gonorrhoeae (Graves et al., 1982; Burnstein et al., 1988; Goodman and Scocca, 1988).

The purpose of studies described in this chapter is to investigate the role of uptake sequences in gonococcal transformation with a view to incorporating specific sequences into a shuttle vector. Initial experiments were designed to duplicate the results of Graves et al. (1982). A 4.2 kb cryptic plasmid, instead of a chimeric plasmid, was tested to confirm the preferential uptake of 1.5 kb TaqI fragment containing the 640 bp region implicated in selective uptake. The effect of time on uptake was also investigated as a possible factor influencing preferential uptake. In order to determine whether the 1.5 kb TaqI fragment could be universally recognized by gonococcal strains, thereby bypassing the restriction/modification systems of these strains, sixteen isolates selected among various auxotypes were tested for

their ability to acquire the 1.5 kb TaqI fragment into a DNase-resistant state. The restriction/modification systems of some of these isolates were compared. Competition experiments between chromosomal DNA and competing DNA were performed in order to determine if uptake sequences were universally recognized by N. gonorrhoeae. In addition to the 1.5 kb TaqI fragment, a synthesized oligonucleotide of the uptake sequences was used as a source of competing DNA.

MATERIALS AND METHODS

Bacterial strains and plasmids

Gonococcal strains used in this study are listed in Table 1-1. All strains were from Canadian sources and were submitted by various laboratories to the National Laboratory for Sexually Transmitted Diseases (Laboratory Center for Disease Control, Ottawa, Ontario) for various surveillance studies on N. gonorrhoeae. Auxotypes were confirmed using auxotyping media as described by Hendry and Stewart (1979). Plasmid profiles were verified by the boiling method followed by electrophoresis on 1% agarose gels (Dillon et al., 1985). N. gonorrhoeae strain 2152S, resistant to streptomycin (Sm^r), was constructed by transformation of strain 2152 with chromosomal DNA isolated from a variant strain of F62 (FS62) resistant to streptomycin (Evins and Knapp, 1988). The 4.2 kb cryptic plasmid (pLCDC1) of N. gonorrhoeae was isolated from N. gonorrhoeae strain C1.

Media and growth conditions

Clinical isolates of N. gonorrhoeae were cultured on GC medium base (GCMB; Difco Laboratories, Detroit, Michigan) supplemented with Kellogg's defined supplement as modified by Dillon (1983). Cells were incubated for 18 hours (hrs) in a humid environment at 35° Celsius (°C) in the presence of 5% CO_2.

Table 1-1. Bacterial strains used in uptake experiments.

N. gonorrhoeae strain number	Phenotype[a]	Plasmid content (Kb)
2152	PCU	NP[b]
2253	NR	4.2
2136	NR	4.2
NS-30	OUH	4.2
NS-37	PCUH	NP
NS-63	NR	4.2
NS-506	PUH	4.2
NS-757	NR	4.2
NS-829	P	4.2
NS-888	O	4.2
NS-898	P	4.2
NS-927	OUH	4.2
NS-933	O	4.2
NS-945	NR	4.2
NS-958	CU	4.2
NS-983	P	4.2
FS62	P, Sm^r	4.2
2152S	PCU, Sm^r	NP
C1	NR	pLCDC1; 4.2

a) P: Proline-requiring; O: Ornithine-requiring; C: Citrulline-requiring; U: Uracil-requiring; H: Hypoxanthine-requiring; NR: Non-Requiring. Sm^r: Streptomycin resistant.

b) NP: No Plasmid

N. gonorrhoeae cells were resuspended, when required, in GC base broth prepared by adding to 1 liter of deionized water; 15 grams (g) proteose peptone No. 3 (Difco), 1 g corn starch (BDH Chemicals Ltd., Toronto, Ontario), 4 g potassium phosphate, dibasic (Sigma, St. Louis, Missouri), 1 g potassium phosphate, monobasic (Sigma) and 5 g sodium chloride (BDH). The final pH of the broth was adjusted to 7.2 at 25°C and autoclaved for 15 minutes (min) at 120°C in aliquots of 100 milliliters (mL). When required, streptomycin (Sigma) was added to a final concentration of 500 milligrams/Liter (mg/L)

Selection of N. gonorrhoeae colony types 1 and 2

N. gonorrhoeae colony types 1 and 2 (piliated variants) were initially screened from fresh clinical isolates received at the National Laboratory for Sexually Transmitted Diseases (Kellogg et al., 1963). The piliated colonies were purified from the non-piliated colonies (colony types 3 and 4) each day by selecting colonies of appropriate morphology as viewed through a stereoscopic microscope and subculturing them on GCMB at 35°C in the presence of 5% CO_2 for 18 hrs. Colony types 2 and 3 were used in transformation experiments.

DNA isolation

Chromosomal DNA from N. gonorrhoeae strains FS62 and 2152S was isolated as described by Rodriguez et al. (1983). Gonococcal cells from an overnight culture were harvested from three GCMB plates and

resuspended into 10 mL of TES [50 mM Tris-HCl, pH 8.0 (BDH), 20 mM ethylenediamine tetracetate (EDTA; BDH) and 25% sucrose (BDH)]. Lyzozyme (Sigma) (final concentration of 200 μg/L) and 100 μL of a stock solution of 10 mg/mL of RNase (Boehringer Mannheim Biochemicals, BMC; Dorval, Québec) were added. The preparation was placed on ice for 15 min. One hundred and fifty μL of a 10% solution of sodium dodecyl sulfate (SDS; BDH) was then added and the preparation was set on ice for an additional 5 min followed by the addition of 4 mL of TES. Three phenol (BDH): chloroform (BDH) extractions (1:1 ratio), plus 2 chloroform extractions were performed to remove cell debris and proteins. The supernatants were transferred to clean tubes and the DNA was precipitated by adding 300 μL of 0.5 M ammonium acetate (Sigma) and two volumes of cold 95% ethanol. The fibrous strands of precipitated DNA were spooled out using a glass rod, rinsed in 95% ethanol and resuspended into 400 μL of water.

The 4.2 kb cryptic plasmid, pLCDC1, was isolated from N. gonorrhoeae strain C1 using a large scale alkaline-SDS procedure (Birnboim and Doly, 1979) and purified on a caesium chloride-ethidium bromide density gradient (Maniatis et al., 1982). Cells from N. gonorrhoeae strain C1 were harvested from 10 GCMB plates and resuspended in 2.5 mL of solution I [50 mM glucose (BDH), 10mM EDTA, 0.25 M Tris-HCl pH 8.0, and 2 mg/mL lysozyme (freshly made)]. The cells were placed on ice for 30 min. Five mL of solution II [0.2N NaOH (BDH) 1% SDS] was added and the preparation was set on

ice for another 5 min. A volume of 3.8 mL of solution III [3M sodium acetate (Sigma), pH 4.8] was then added and the lysate was left on ice for 1 hr followed by centrifugation for 10 min at 13,000 rpm in a Sorvall R5C centrifuge (Dupont NEN Research Products, Mississauga, Ontario). The supernatant was ethanol-precipitated by the addition of 2 volumes of cold 95% ethanol and kept at -70°C for 2 hrs. The DNA-containing solution was centrifuged at 12,000 rpm for 10 min in a Sorvall R5C centrifuge. The supernatant was decanted and 9 mL of TE buffer was added to the pellet. After disolving the pellet by vortexing, 8 g of caesium chloride (BDH) and 250 μL of a 10 mg/mL solution of ethidium bromide were added and mixed. The mixture was transferred to quick-seal Beckman ultracentrifuge tubes (Beckman, Toronto, Ontario). Before sealing, the space left between the opening of the tubes and the DNA preparation was filled with paraffin oil. After centrifugation at 55,000 rpm in a Beckman L8-M ultracentrifuge (type TY65 rotor) for 20 hrs at 10°C under vacuum, the DNA bands were visualized under UV light (300 nanometer) (Ultraviolet Product Inc., California). The lower band, corresponding to plasmid DNA, was extracted using an 18 gauge needle and the ethidium bromide was removed using water-saturated isobutanol (Maniatis et al., 1982). Plasmid DNA was precipitated twice in 95% ethanol before being resuspended in 100 μL of water. DNA preparations were stored at 4°C.

DNA concentrations were estimated with a Bausch & Lomb Spectronic 1001 spectrophotometer (Fisher, Ottawa, Ontario) at 260 nm using the relation 1 O.D. unit at A_{260}= 50 ug of DNA/mL.

Restriction endonuclease digestion and gel electrophoresis

The restriction endonuclease TaqI was purchased from Boehringer Mannheim Biochemicals. Reaction mixtures with pLCDC1 were performed in a final volume of 100 μL using a high salt buffer (1/10 of final reaction volume) provided by the manufacturer. Reactions were incubated overnight at 65°C.

Electrophoresis of DNA was carried out in tris-acetate buffer (40 mM Tris base, 20 mM sodium acetate and 1.8 mM EDTA) (Dillon et al., 1985) using 1.0% agarose (Pharmacia, Dorval, Québec) gels in a horizontal submarine apparatus at approximately 15 mA/cm. DNA bands were visualized by UV light (300 nm) after staining the gels with ethidium bromide (stock solution of 10 mg/mL) for 10 min and destaining in water for an additional 10 min. Destained gels were photographed using Polaroid type 52 or type 55 (with negative) films and a Polaroid MP4 camera (Polaroid, Rexdale, Ontario) fitted with a red filter.

Transfer of DNA to solid matrices

DNA fragments were transferred from agarose gels to nylon membranes (Amersham, Toronto, Ontario) according to the method of Southern (1975) as adapted by Dillon et al. (1985). After

electrophoresis, gels were placed in a denaturing solution (0.4 N NaOH and 0.8 M NaCl) for 30 min with gentle agitation to denature the DNA fragments. Gels were then neutralized for 30 min in a neutralization solution (0.5 M Tris-HCl, pH 8.0 and 1.5 M NaCl) with agitation.

The nylon membrane was cut so that it would cover the gel and leave approximately 1 centimeter (cm) on each side. The membrane was equilibrated in 10 X SSC [3 M NaCl and 0.3 M sodium citrate (BDH)] for 30 min. The gel and membrane were mounted on a Southern blot apparatus as described by Dillon et al. (1985) and left overnight at room temperature.

The assembly was taken apart the next morning. The contour of the gel and the wells were traced on the nylon membrane with a fine pencil. The gel was then removed from the membrane, stained, and then visualized under UV light to ensure that DNA transfer was complete. The nylon membrane was gently washed in 2 X SSC to remove any residual agarose, air dried and exposed to UV light for 5 min to fix the DNA irreversably to the membrane.

DNA-DNA Hybridization

Hybridization studies were carried out using both, radioactive and non-radioactive DNA probes.

1. Hybridization using radioactive probes

a) Nick translation

Plasmid pLCDC1 was labelled with [alpha-^{32}P]dATP using the procedure of nick translation developed by Rigby et al. (1977). Purified DNA (1 µg) was resupended in 5 µL of 10 X nick translation buffer (0.5 M Tris-HCl, pH 7.5, 0.1 M $MgSO_4$ and 0.01 M dithiothreitol) and 1 µL of each unlabelled 1 mM dCTP, dGTP and dTTP from a stock solution were added. One hundred pmole of [alpha-^{32}P] (> 3000 Ci/mmol) (ICN Biomedicals Inc., Lisle, Illinois) was incorporated and the solution volume was brought up to 48 µL with sterile water. One µL of DNase I (0.05 µg/mL) and 1 µL of DNA polymerase I (5 units/mL) (BMC) were added and the mixture was incubated at 16°C for 2 hrs. The nick translated DNA was separated from the unincorporated nucleotides by 3 successive ethanol precipitations. The specific activity of probes, determined using a liquid scintillation counter model 1217 Rackbeta (LKB), varied from 1 X 10^6 to 1 X 10^7 cpm/µg of DNA.

b) DNA-DNA hybridization

Hybridization was performed according to a modified procedure of Dillon et al. (1985). Hybridization conditions were carried out at 68°C. The nylon membrane with the DNA was prehybridized for 2 hrs in a sealed plastic bag with 10 mL of prehybridization solution [6 X SSC, 0.5% SDS, 100 ug/mL herring sperm DNA and 5 X Denhardt solution (0.1% Ficoll, 0.1% Polyvinylpyrolidone and 0.1% bovine

serum albumin)]. Ten mL of hybridization solution (prehybridization solution without SDS) containing the radioactive probe was boiled for 5 min to denature the probe before adding it to the prehybridized filter. The hybridization reaction was incubated overnight at 68°C. The following day, the probe was removed and the membrane was washed twice for 15 min each time, in 2 x SSC plus 0.1% SDS at 68°C. The membrane was air dried prior to autoradiography. Autoradiography was performed with intensifying screens at -70°C using Chronex 4 X-Ray films (Dupont NEN Research Products, Mississauga, Ontario). X-ray films were developed after 24 hrs with a Dupont QC1-R/T processor.

2. Hybridization using non-radioactive probes

a) DNA labelling using digoxigenin-labelled dUTP

Plasmid pLCDC1 was labelled according to the recommendations of the manufacturer (BMC). The DNA (1 μg) was first denatured for 10 min in boiling water and quickly chilled on ice. Two μL of hexanucleotide mixture (10 X concentrated hexanucleotide mixture) and 2 μL of dNTP labelling mixture (10 X concentrated mixture pH 6.5; 1mM/L dATP, dCTP, dGTP, 0.65 mM/L dTTP and 0.35 mM/L Dig-dUTP) were then added to the denatured DNA. The volume was made up to 19 μL with sterile water and 1 μL of Klenow enzyme (2 units/μL) (BMC) was added. The reaction mixture was left overnight at 37°C and the next day, labelled DNA was precipitated with 2 μL LiCl (BMC) and 60 μL of cold 95% ethanol at -70°C for 2 hrs. After centrifugation,

two 70% ethanol washes were performed to eliminate unincorporated nucleotides. The sample was dried and resuspended in 50 μL of sterile water.

b) DNA-DNA hybridization

The nylon membrane was prehybridized in hybridization solution (5 X SSC, 0.1% N-lauroylsarcosine; Na-salt, 0.02% SDS and 0.5% blocking reagent provided by BMC) for 2 hrs at 68°C. The solution was then replaced with about 2.5 mL/100 cm^2 filter of hybridization solution containing 10 μL of freshly denatured labelled DNA. According to BMC this amount corresponds to 50 nanograms (ng) of DNA if labelling was optimal. The filter was incubated with the probe at 68°C overnight. The next day, the hybridization solution was removed and the filter was washed twice for 5 min at room temperature in 2 X SSC and 0.1% SDS and twice in 0.1 X SSC and 0.1% SDS for 15 min at 65°C.

c) Immunological detection

The nylon membrane containing the hybridized DNA was briefly rinsed in buffer 1 (100 mM/L Tris-HCl and 150 mM/L NaCl, pH 7.5) and incubated at room temperature in buffer 2 (0.5% blocking reagent in buffer 1) for 30 min with agitation. The filter was rinsed again in buffer 1 and a diluted antibody-conjugate solution (150 mU/mL of polyclonal sheep anti-digoxigenin Fab-fragments conjugated to alkaline phosphatase in buffer 1) was added. The filter was then incubated at room temperature for an additional 30

min with shaking. The solution was poured off and unbound antibody-conjugate was removed by washing the filter twice for 15 min in buffer 1. The membrane was equilibrated with buffer 3 (100 mM/L Tris-HCl, 100 mM/L NaCl and 50 mM/L $MgCl_2$; pH 9.5) and placed in a sealed plastic bag with freshly prepared color solution [45 µL nitroblue tetrazolium (75 mg/mL NBT in 70% dimethylformamide) and 35 µL X-phosphate (50 mg/mL 5-bromo-4-chloro-3-indolyl phosphate, toluidinium salt in dimethylformamide) in 10 mL of buffer 3]. The bag was sealed and placed in the dark until the bands were easily visualized. The color reaction was stopped by rinsing the membrane in buffer 4 (10 mM/L Tris-HCl and 1mM/L EDTA; pH 8.0). The membrane was photographed as described previously.

Gonococcal transformation using chromosomal DNA

Gonococcal transformation using chromosomal DNA was performed as described by Janik and Heym (1976). A loopful of piliated gonococcal cells (colony types 1 and 2) from each of strains FS62, 2152, 2253, 2136, NS-30, NS-37, NS-63, NS-506, NS-757, NS-829, NS-888, NS-898, NS-927, NS-933, NS-945, NS-958 and NS-983 was spread on an area of approximately 4 cm^2 of a GCMB plate with 2.5 µg of total genomic DNA isolated from strain FS62. Plates were incubated for 5 hrs at 35°C with 5% CO_2 prior to the transfer of the cell growth to GCMB plates supplemented with streptomycin (500 mg/mL). Transformants, resistant to streptomycin, were observed after 48 hours of incubation.

Uptake assay using pLCDC1 TaqI fragments

The procedure of Graves et al. (1982) was followed with several modifications. Piliated N. gonorrhoeae cells (colony types 1 or 2) which were transformed by chromosomal DNA, were resuspended into 1 mL of GC broth containing 10 mM $MgCl_2$ to approximately 1 x 10^8 colony forming units/mL (CFU/mL). One μg of TaqI restricted pLCDC1 was added to the suspension and the mixture was incubated at 35°C for up to 1 hr to allow uptake of fragments. DNase I (final concentration of 100 μg/mL) was added and the mixture placed on ice for 20 min in order to degrade any residual DNA left in the broth. The cells were centrifuged and washed twice in cold GC broth before being resuspended in 500 μL of STET buffer (50 mM Tris-HCl, 50 mM EDTA, 5% triton X-100 and 8% sucrose; pH 8.0) for lysis. Lysozyme (final concentration 2 mg/mL) was added and the mixture was left on ice for 20 min. SDS was then added (final concentration 1%) and the lysate was incubated at 65°C for 15 min. After centrifugation, the supernatant was extracted twice with phenol:chloroform (1:1 ratio) and once with chloroform before precipitating the DNA with 5 M ammonium acetate and two volume of 95% ethanol at -70°C. The sample was centrifuged, dried and resuspended into 20 μL of sterile water. Electrophoresis conditions and hybridization with a 4.2 kb probe were performed as described previously.

Restriction endonuclease purification

Restriction endonucleases from crude lysates of strains NS-30, NS-506, NS-933, NS-927, NS-757, 2253 and 2152 were prepared using

the procedure of Stein et al. (1988). Gonococcal cells from an overnight culture were harvested from 10 GCMB plates and resuspended in 8 mL of 10% sucrose (in Tris-HCl; pH 8.0). One mL of 1.0 M NaCl and 2 mg of lysozyme were added to the cell suspension and the mixture was set on ice for 1 hr. One mL of buffer I (200 mM Tris-HCl pH8.0, 100 mM $MgCl_2$, 2 mM EDTA and 76 mM 2-mercaptoethanol) was added and the cells were lysed by sonication with a Braun-Sonic 1510 sonicator at maximum power for 2 min. Cell debris was removed by centrifugation with a Sorvall R5C centrifuge at 15,000 rpm for 15 min. Nucleic acids were precipitated by the addition of polyethyleneimine (Sigma; 1% final concentration). After 45 min on ice, the solution was centrifugated in the Sorvall R5C centrifuge for 15 min at 15,000 rpm and the supernatant decanted to a clean tube. Proteins were precipitated on ice by the addition of ammonium sulfate to a final concentration of 70% (saturation at 0°C for 100 mL equals 43.0g). After 30 min on ice, proteins were collected by centrifugation with the Sorvall R5C centrifuge for 15 min at 15,000 rpm. Proteins were then resuspended in a minimal volume of Buffer II (20 mM Tris-HCl, 1 mM EDTA and 6 mM mercaptoethanol) and were dialyzed against the same buffer for 24 hrs in order to remove any trace of polyethyleneimine. The protein content of the extracts was determined using the Bio-Rad assay (Bio-Rad Laboratories, Toronto, Ontario). Extracts were divided in aliquots of 1 mL and kept in 50% glycerol at -20°C.

The presence of restriction endonuclease activity was determined by digesting lambda DNA with the extracts. Reaction mixtures had final volumes of 20 μL and contained 1 μg of lambda DNA (BMC), 10 mM DTT, the optimal restriction buffer (supplied by BMC) and the cell extract. Reaction mixtures were incubated for up to 1.5 hrs at 37°C before overnight electrophoresis. Digestions were repeated several times in order to confirm patterns produced by the various protein extracts. The patterns produced by the extracts were compared to the pattern of known isoschizomers of gonococcal restriction endonucleases (HaeII, HaeIII and SacII).

DNA competition assays

Chromosomal DNA dose-response curves were first determined for strain NS-30 and NS-888 using a modified protocol of Janik and Heym (1976). Gonococcal cells from strains NS-888 and NS-30 were resuspended into 1 mL of GC broth to a density of 1.5 X 10^8 CFU determined using a 0.5 McFarland nephelometer barium sulfate standard (Paik, 1970). Each suspension was divided into aliquots of 100 μL and an increasing concentration of chromosomal DNA isolated from strain 2152S was added to each sample. The cell-DNA mixtures were individually filtered onto sterile 0.45 μm membrane filters (Millipore Canada Ltd., Mississauga, Ontario) and placed, cell side up on GCMB plates. After 1 hr incubation at 35°C in 5% CO_2, DNase I (100 μg/mL) was added to the filters and the cells were further incubated for 4 hrs. The filters were removed from the plates and vortexed in 1 mL of 0.7% casamino acids. Serial 10-fold dilutions

were prepared and 100 μL plated onto streptomycin-containing GCMB plates. Dilutions were also plated on non-selective media in order to have a total viable cell count for transformation frequencies. Transformation frequencies were calculated as the number of transformants per number of viable cells. Plates were incubated at 35°C supplemented with 5% CO_2 for 48 hrs prior to counting. Transformation frequencies were plotted against increasing concentration of chromosomal DNA and saturation concentrations, represented by the plateau of the curves, were determined. The optimal concentrations for NS-888 and NS-30 were then used in competition experiments with increasing concentrations of either TaqI restricted pLCDC1 fragments or a synthesized 37-mer oligonucleotide comprised of the uptake sequences.

The 37-mer oligonucleotide (5'-GAATTC-GCCGTCTGAA-TATA-TTCAGACGGC-GAATTC-3'), was synthesized by the Ottawa-Carleton Biotechnology Institute (Ottawa, Ontario). The core of the oligonucleotide was composed of two inverted copies of the uptake sequences as found on the gonococcal chromosome (Goodman and Scocca, 1988). Two EcoRI sites were placed at each end of the 37-mer for cloning purposes.

The effects of added species of competing DNA were plotted as a reciprocal plot of T_o/T_x versus C_x/C_o where T_o is the transformation frequency (transformants/total viable cells) in the presence of an optimal concentration of chromosomal DNA; T_x is the

transformation frequency in the presence of competing DNA; C_o is the optimal chromosomal DNA concentration for NS-30 and NS-888 and; C_x is the concentration of competing DNA.

DNA sequence analysis of M13mp19

Burnstein et al. (1988) showed that the replicative form of M13mp19 was acquired into a DNase resistant state by gonococcal cells. In order to determine if M13mp19 carried a copy of the uptake sequences, its sequence (Yanisch-Perron et al., 1985) was compared to the uptake sequences of N. gonorrhoeae. DNA sequence analysis was performed using the University of Wisconsin Genetic Computer Group package (Devereux et al., 1984) provided by the Biomolecular DATA base system of CAN/SND , Canada Institute for Scientific and Technical Information (CISTI).

RESULTS

Confirmation of the preferential uptake of a 1.5kb TaqI fragment derived from pLCDC1

The method described by Graves et al. (1982) was repeated except that only a 4.2 kb cryptic plasmid (pLCDC1) of N. gonorrhoeae was used for transformation in order to confirm the uptake of 1.5 kb TaqI fragment. Restriction endonuclease digestion of pLCDC1 with TaqI produced 6 visible fragments: 1493 bp, 792 bp, 597 bp, 398 bp, 386 bp and 348 bp; Graves et al. (1982) reported two other fragments of 105 bp and 90 bp which were not observed under the conditions described in this study. When these fragments were exposed to FS62 piliated gonococcal cells for 15 min (time recommended by Graves et al.), only the 1.5 kb fragment was incorporated into gonococcal cells (Figures 1-1A and B). However, the possibility that the two smallest TaqI fragments (105 and 90 bp) were taken up can not be excluded since they do not appear on the gel nor on the blot. In order to determine whether uptake of the 1.5 kb fragment was time dependent (i.e., longer incubation periods might be required for other fragments to be internalized), cells and TaqI-digested pLCDC1 fragments were incubated for increasing time intervals. Time had no effect on the preferential uptake of the 1.5 kb TaqI fragment (Figure 1-2A and B). None of the other fragments seen on the gel were observed on the blot indicating that they were not acquired by gonococcal cells. It therefore appears that the 1.5 kb TaqI fragment derived from the cryptic plasmid pLCDC1 is preferentially acquired by N. gonorrhoeae

Figure 1-1A. DNA content of strain FS62 after 15 min incubation with TaqI-restricted pLCDC1 DNA. Lane 1, FS62 without the addition of DNA; lane 2, DNase I treated pLCDCI DNA before being added to cells; lane 3, pLCDC1 DNA restricted with TaqI; lane 4, FS62 cells incubated with the TaqI-restricted pLCDC1. Numbers in the margin indicate molecular sizes (bp) of fragments of TaqI-digested pLCDC1. The unmarked bands are chromosomal DNA (top band) and the 4.2 kb cryptic plasmid (lower band).

Figure 1-1B. Southern blot hybridization of Figure 1-1A with an [alpha-^{32}P]dATP-labelled pLCDC1 probe.

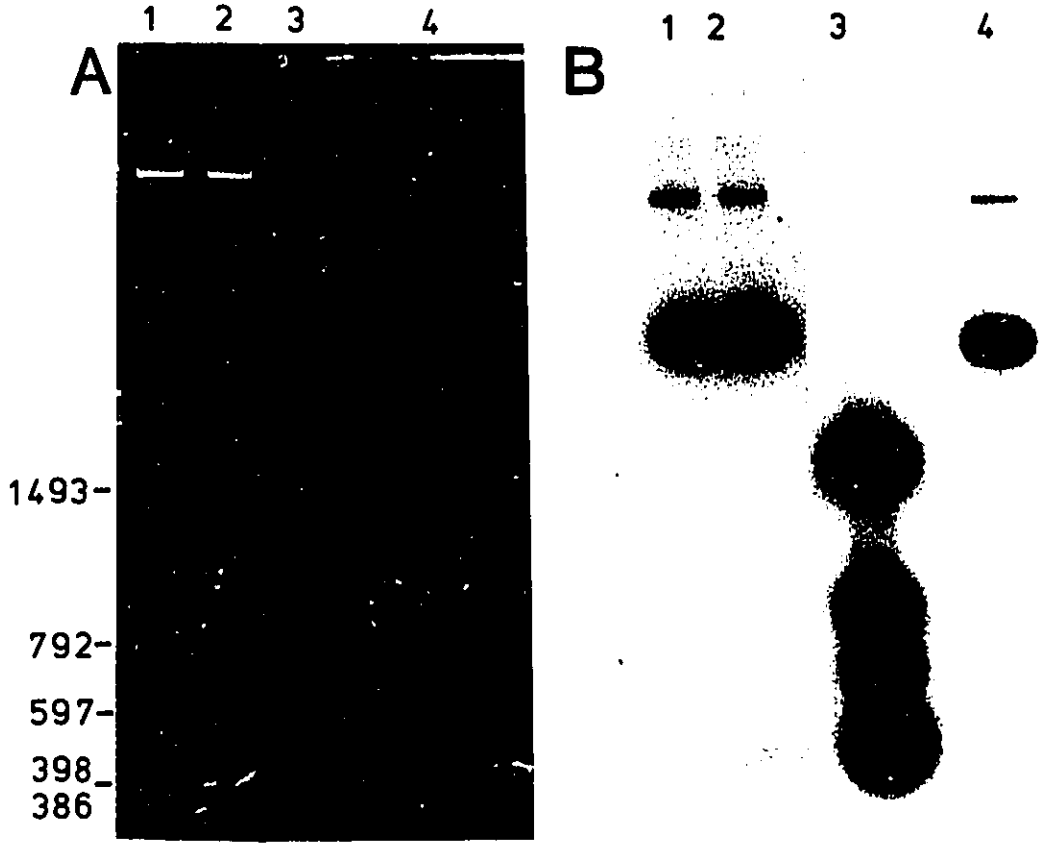
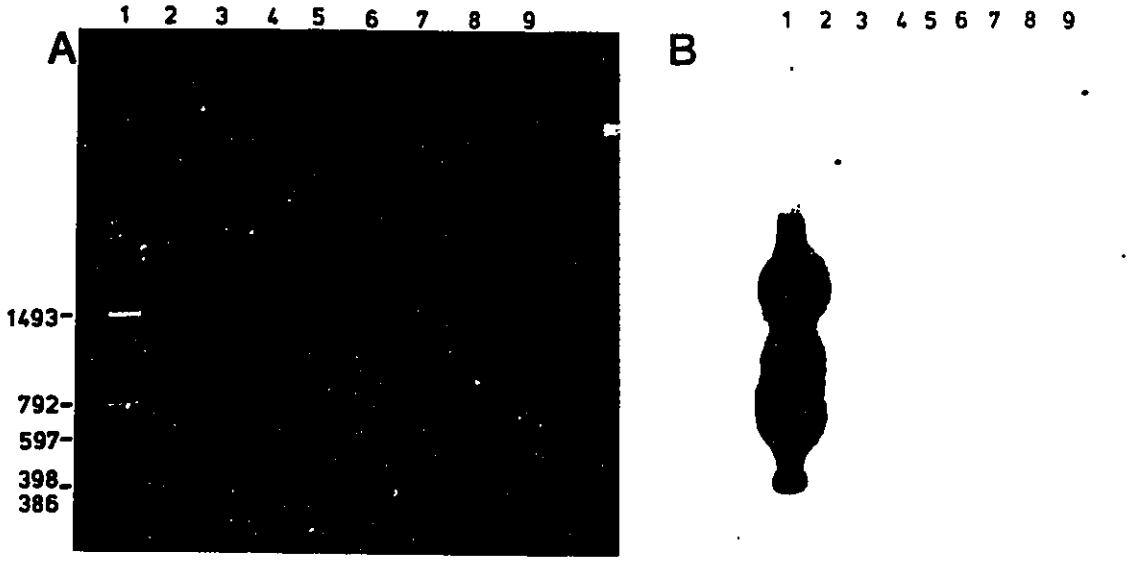

A
1
2
3
4
1493-
792-
597-
398-
386
B
1
2
3
4

Figure 1-2A. DNA content of N. gonorrhoeae strain 2152 cells incubated with TaqI-restricted pLCDC1 for various time intervals. Lane 1, pLCDC1 DNA digested with TaqI; lane 2, 2152 cells without DNA; lane 3, pLCDC1 treated with DNase I prior to the addition of cells. Lanes 4 to 9, 2152 cells incubated with pLCDC1 TaqI fragments for time intervals of 10 (lane 4), 20 (lane 5), 30 (lane 6), 40 (lane 7), 50 (lane 8) and 60 min (lane 9).

Figure 1-2B. Southern blot hybridization of Figure 1-2A with an [alpha-^{32}P]dATP-labelled pLCDC1 probe.


A
1 2 3 4 5 6 7 8 9
1493
792
597
398
386
B
1 2 3 4 5 6 7 8 9

suggesting that putative uptake sequences found on the fragment are required for gonococcal transformation.

Uptake of TaqI-restricted pLCDC1 fragments by gonococcal strains

The ability of different gonococcal strains to preferentially acquire the 1.5 kb TaqI fragment of the cryptic plasmid was tested. Sixteen clinical isolates of N. gonorrhoeae of various auxotypes were examined for their ability to acquire fragments of pLCDC1 generated by TaqI digestion. Strains were initially transformed with chromosomal DNA isolated from strain 2152S to determine if they were competent for genetic transformation. The 16 strains all acquired resistance to streptomycin indicating that they were able to acquire chromosomal DNA by transformation (Table 1-2). Each strain was then transformed with pLCDC1 TaqI fragments (Table 1-2). Three (NS-30, NS-506 and 2253) of 16 strains did not acquire the 1.5 kb TaqI fragment. The auxotypes of these strains were different suggesting that no correlation existed between auxotype and uptake. Results presented in Figures 1-3A and B demonstrate the inability of strains NS30, NS-506 and 2253 to acquire the 1.5 kb TaqI fragment as compared to three strains (2152, NS-933 and NS-927) which acquired the fragment.

Table 1-2. Uptake of the 1.5 kb TaqI fragment of pLCDC1 by gonococcal strains.

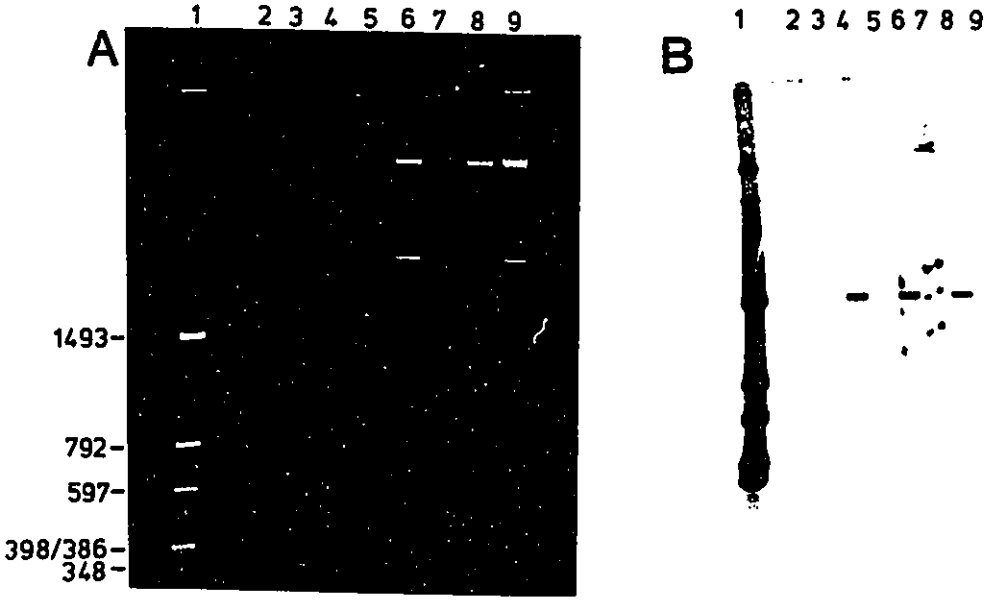
N. gonorrhoeae strain number	Auxotype	Transformation by chromosomal DNA[a]	Uptake of the 1.5kb fragment[b]
2152	PCU	+	3/3
2253	NR	+	0/2
2136	NR	+	2/2
NS-30	OUH	+	0/2
NS-37	PCUH	+	2/2
NS-63	NR	+	2/2
NS-506	PUH	+	0/3
NS-757	NR	+	3/3
NS-829	P	+	2/3
NS-888	O	+	3/3
NS-898	P	+	3/3
NS-927	OUH	+	3/3
NS-933	O	+	3/3
NS-945	NR	+	2/3
NS-958	CU	+	2/3
NS-983	P	+	2/3

a) Transformation was determined on a qualitative basis and is therefore indicated as the ability (+) or inability (-) of strains to express the streptomycin resistance phenotype.

b) The ability of each strain to acquire the 1.5 kb fragment is indicated as the number of positive experiments per total number of experiments.

Figure 1-3A. One % agarose gel of DNA content from gonococcal strains that did or did not acquire the 1.5 kb TaqI fragment. Lane 1, TaqI-digested pLCDC1 DNA; lane 2, 2152 deprived of DNA; lane 3, DNase I-treated pLCDC1 DNA prior to the addition of cells. Lanes 4 to 9 are, respectively, strains 2152, 2253, NS-933, NS-30, NS-927 and NS-506.

Figure 1-3B. Hybridization of Figure 1-3A with a non-radioactive labelled pLCDC1 probe. The 4.2 kb plasmids visualized on Figure 1-3A were removed in order to prevent hybridization with the probe.


A
1 2 3 4 5 6 7 8 9
1493-
792-
597-
398/386-
348-
B
1 2 3 4 5 6 7 8 9

Analysis of the restriction/modification system of N. gonorrhoeae strains NS-30, NS-506 and 2253

In order to determine whether restriction/modification systems were involved in the inability of three strains to acquire the 1.5 kb TaqI fragment, the restriction/modification systems of strains NS-30, 2253 and NS-506 were compared to the systems of four strains (2152, NS-933, NS-927 and NS-757) which acquired the 1.5 kb TaqI fragment. Table 1-3 gives the total protein concentration isolated from each strain along with the optimal restriction conditions used for each assay. Optimal buffers were determined by digesting each reaction mixture with 5 different restriction buffers supplied by Boehringer Mannheim Biochemicals. Results of restriction endonucleases patterns produced for each assay as visualized on a 1% agarose gel are shown in Figure 1-4. Restriction systems of strain 2152 (Figure 1-4, lane 1), NS-933 (lane 3) and NS-927 (lane 4) were similar to the restriction system of strain NS-30 (lane 2), while strains NS-757 (lane 5) and 2253 (lane 7) shared a similar system which was different from the other strains. The restriction system of strain NS-506 (lane 6) was unique compared to other systems. The patterns produced by the various protein extracts were different from the patterns produced by characterized isoschizomers of gonococcal restriction endonucleases [HaeII (lane 8), HaeIII (lane 9), and SacII (lane 10)]. Therefore, the restriction/modification systems of strains NS-30 and 2253 cannot account for the inability of these isolates to acquire the

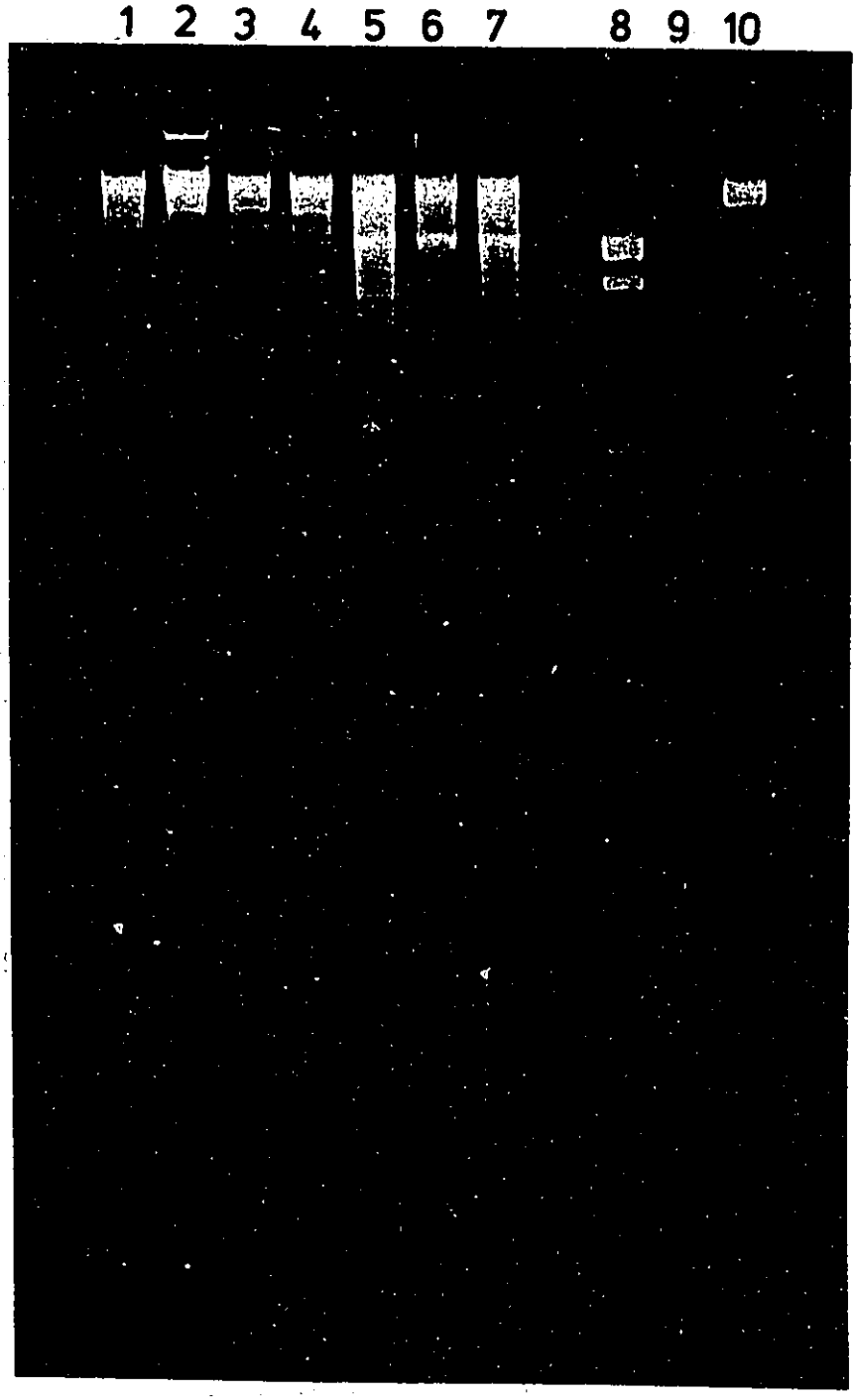
Table 1-3. Total protein concentration isolated from gonococcal strains and optimal endonuclease restriction conditions for the digestion of 1 μg of lambda DNA.

Strain number	Total protein conc. isolated (μg/uL)	Amount of protein required/digestion (μg)	Optimal time (hr)	Optimal buffer[a]
2152	4.7	4.7	1.0	A
NS-30	3.3	6.6	0.5	A
NS-933	3.3	9.9	1.5	A
NS-927	4.7	14.8	1.5	A
NS-757	4.2	8.4	1.0	A
NS-506	9.9	9.9	1.0	M
2253	4.6	9.2	1.5	H

a) 10 X buffer A: 33 mM/L Tris-acetate, 10 mM/L Mg-acetate, 66 mM/L K-acetate and 0.5 mM/L Dithiothreitol.

10 X buffer M: 10 mM/L Tris-HCl, 10 mM/L $MgCl_2$, 50 mM/L NaCl and 1 mM/L Dithioerythritol.
10 X buffer H: 50 mM/L Tris-HCl, 10 mM/L $MgCl_2$, 100 mM NaCl and 1 mM/L Dithioerythritol.

Figure 1-4. Restriction endonuclease patterns of lambda DNA produced by total protein cell extracts isolated from gonococcal strains. Lane 1, 2152; lane 2, NS-30; lane 3, NS-933; lane 4, NS-927; lane 5, NS-757; lane 6, NS-506; lane 7, 2253. Lanes 8 to 10 are isoschizomers HaeII, HaeIII and SacII of N. gonorrhoeae restriction endonucleases.


1 2 3 4 5 6 7 8 9 10

1.5 kb TaqI fragment of pLCDC1 since they share similarities with restriction systems of strains which acquired the fragment.

DNA competition assays

Since NS-30, NS-506 and 2253 were competent for transformation using chromosomal DNA, shared common restriction/modification systems with strains that acquired the 1.5 kb TaqI fragment, and were piliated, suggest that the putative uptake sequences on the 1.5 kb TaqI fragment are not recognized by these strains. To test this hypothesis competition experiments between chromosomal DNA and the 1.5 kb TaqI fragment were performed using strains NS-30 and NS-888. In addition to the 1.5 kb fragment, an oligonucleotide (37-mer) comprised of the uptake sequences reputedly responsible for specific uptake in N. gonorrhoeae was also used in these experiments (Goodman and Scocca, 1988).

In order to determine the DNA concentration at which transformation would be optimal, dose-response curves for chromosomal DNA were established. This concentration would correspond to the amount of DNA required to saturate putative receptors on the cell membrane. Transformation frequencies for increasing chromosomal DNA concentrations are given in Table 1-4A for strain NS-30 and Table 1-4B for strain NS-888. The means for transformation frequencies were then plotted against increasing concentration of chromosomal DNA in order to determine the

Table 1-4. Transformation frequencies of N. gonorrhoeae strains NS-30 and NS-888 with increasing concentration of chromosomal DNA.

A) Strain NS-30

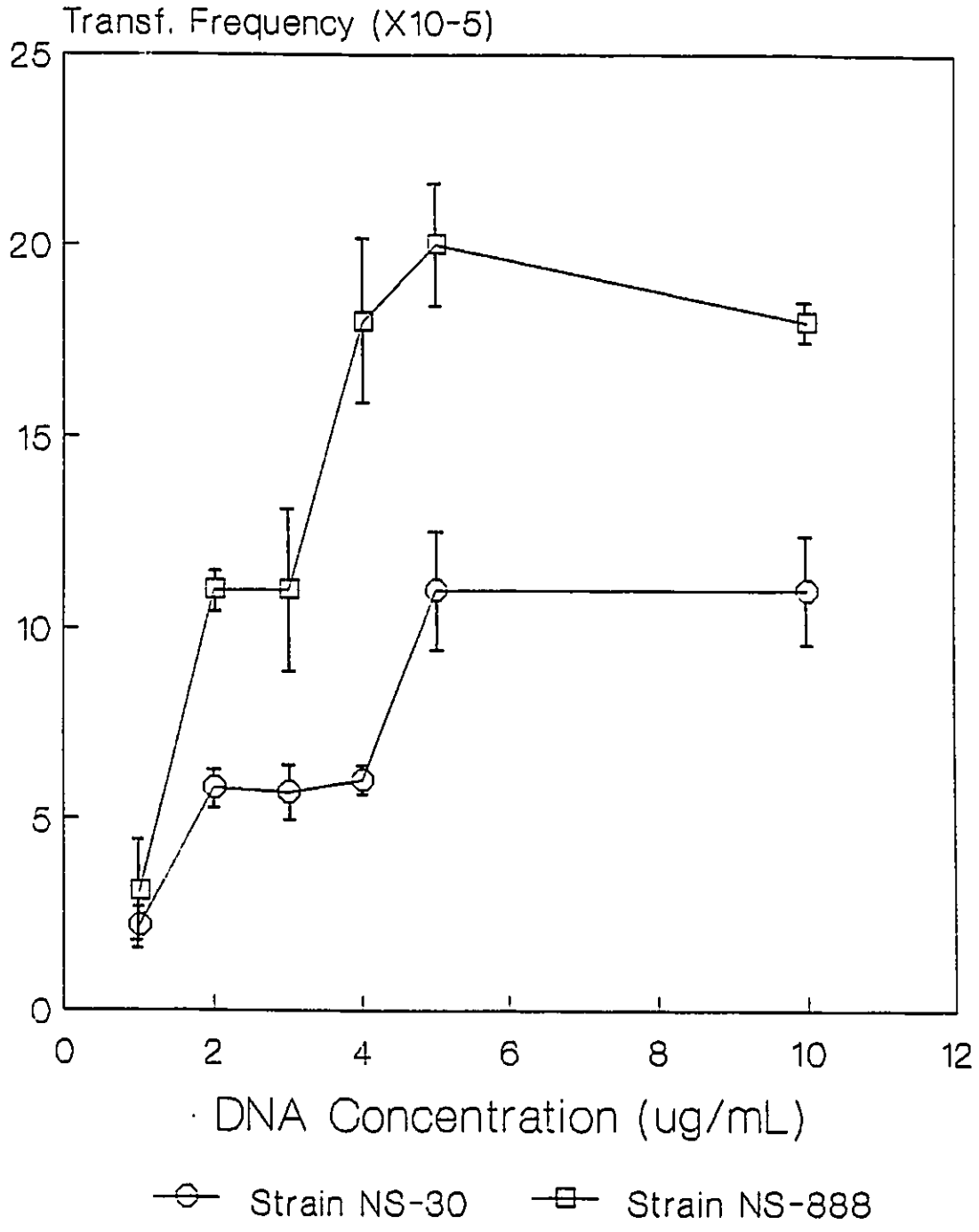
Chromosomal DNA (μg)	Transformation Frequency in expt #[a]: 1	2	3	Mean
1	1.5×10^{-5}	2.4×10^{-5}	2.6×10^{-5}	$(2.2\pm0.6) \times 10^{-5}$
2	6.3×10^{-5}	5.3×10^{-5}	5.7×10^{-5}	$(5.8\pm0.5) \times 10^{-5}$
3	5.1×10^{-5}	6.5×10^{-5}	5.6×10^{-5}	$(5.7\pm0.7) \times 10^{-5}$
4	6.0×10^{-5}	6.3×10^{-5}	5.7×10^{-5}	$(6.0\pm0.3) \times 10^{-5}$
5	8.8×10^{-5}	11×10^{-5}	10×10^{-5}	$(11\pm1.6) \times 10^{-5}$
10	9.4×10^{-5}	12×10^{-5}	12×10^{-5}	$(11\pm1.5) \times 10^{-5}$

B) Strain NS-888

Chromosomal DNA (μg)	Transformation Frequency in expt #: 1	2	3	Mean
1	1.7×10^{-5}	3.3×10^{-5}	4.2×10^{-5}	$(3.1\pm1.3) \times 10^{-5}$
2	11×10^{-5}	10×10^{-5}	11×10^{-5}	$(11\pm0.6) \times 10^{-5}$
3	9.2×10^{-5}	13×10^{-5}	10×10^{-5}	$(11\pm2.0) \times 10^{-5}$
4	17×10^{-5}	18×10^{-5}	20×10^{-5}	$(18\pm1.5) \times 10^{-5}$
5	21×10^{-5}	22×10^{-5}	19×10^{-5}	$(20\pm1.5) \times 10^{-5}$
10	18×10^{-5}	17×10^{-5}	18×10^{-5}	$(18\pm0.6) \times 10^{-5}$

a) Transformation Frequency: Number of transformants per number of viable cells.

Figure 1-5. Dose-response curves using chromosomal DNA for N. gonorrhoeae strains NS-30 and NS-888.


Transf. Frequency (X10-5)
25
20
15
10
5
0
0
2
4
6
8
10
12
DNA Concentration (ug/mL)
Strain NS-30
Strain NS-888

concentration for optimal transformation (Figure 1-5). The concentration for optimal transformation of chromosomal DNA for strains NS-30 and NS-888 was determined to be 5 μg of DNA for1 X 10^8 cells per mL. Therefore, 5 μg of chromosomal DNA was used in competition experiments against increasing concentrations of either the cryptic plasmid or the 37-mer harbouring two copies of the uptake sequences. Results of transformation frequencies are given in Table 1-5A for strain NS-30 and Table 1-5B for strain NS-888. Means for each transformation frequency were then used to determine the ratio T_o/T_x. These ratios were plotted against C_x/C_o as a reciprocal plot (Sisco and Smith, 1979) (Figure 1-6A and B). A positive slope indicates that competition occurred between chromosomal DNA and the competing DNA. A slope of zero indicates that the competing DNA had no effect on the ability of chromosomal DNA to transform gonococcal cells. Results show that both the 1.5 kb TaqI fragment and oligonucleotide had no effect on the ability of chromosomal DNA to transform N. gonorrhoeae strain NS-30. However, the ability of strain NS-888 to be transformed by chromosomal DNA was hindered by the presence of increasing concentration of the oligonucleotide and the 1.5 kb TaqI fragment. It therefore seems that one of the strains that did not acquire the 1.5 kb TaqI fragment (NS-30) did not recognize and acquire the uptake sequences. The strain was, however, able to acquire chromosomal DNA by transformation.

Table 1-5. Competition experiments between 5 μg of chromosomal DNA, the 1.5 kb TaqI fragment and a 37-mer oligonucleotide harbouring copies of the uptake sequences.

A) Strain NS-30
1. 1.5 kb TaqI fragment.

Competing DNA concentration (μg)	Transformation Frequency in exp #: 1	2	3	Mean
0	0.9×10^{-4}	1.0×10^{-4}	1.2×10^{-4}	$(1.0 \pm 0.2) \times 10^{-4}$
2.5	1.0×10^{-4}	2.0×10^{-4}	1.0×10^{-4}	$(1.3 \pm 0.6) \times 10^{-4}$
5.0	1.0×10^{-4}	1.0×10^{-4}	1.0×10^{-4}	1.0×10^{-4}
7.5	1.0×10^{-4}	1.0×10^{-4}	1.0×10^{-4}	1.0×10^{-4}
10.0	1.0×10^{-4}	1.0×10^{-4}	1.0×10^{-4}	1.0×10^{-4}

2. 37-mer oligonucleotide.

Competing DNA concentration (μg)	Transformation Frequency in exp #: 1	2	3	Mean
0	0.8×10^{-4}	1.0×10^{-4}	1.2×10^{-4}	$(1.0 \pm 0.2) \times 10^{-4}$
2.5	1.0×10^{-4}	1.0×10^{-4}	1.0×10^{-4}	1.0×10^{-4}
5.0	1.0×10^{-4}	1.0×10^{-4}	1.0×10^{-4}	1.0×10^{-4}
7.5	0.9×10^{-4}	1.0×10^{-4}	1.0×10^{-4}	$(1.0 \pm 0.1) \times 10^{-4}$
10.0	1.0×10^{-4}	1.0×10^{-4}	1.0×10^{-4}	1.0×10^{-4}

Table 1-5. (cont.)

B) Strain NS-888
1. 1.5 kb TaqI fragment.

Competing DNA concentration (μg)	Transformation Frequency in exp #: 1	2	3	Mean
0	1.0×10^{-4}	1.0×10^{-4}	1.0×10^{-4}	1.0×10^{-4}
2.5	ND[a]	4.5×10^{-4}	5.0×10^{-4}	$(4.8 \pm 0.4) \times 10^{-4}$
5.0	3.7×10^{-4}	4.0×10^{-4}	3.9×10^{-4}	$(3.9 \pm 0.2) \times 10^{-4}$
7.5	ND	2.0×10^{-4}	2.0×10^{-4}	2.0×10^{-4}
10.0	2.0×10^{-4}	2.0×10^{-4}	3.0×10^{-4}	$(2.3 \pm 0.5) \times 10^{-4}$

a) ND; Not determined.

2. 37-mer oligonucleotide.

Competing DNA concentration (μg)	Transformation Frequency in exp #: 1	2	3	Mean
0	1.0×10^{-4}	1.0×10^{-4}	1.0×10^{-4}	1.0×10^{-4}
2.5	4.0×10^{-4}	3.7×10^{-4}	4.5×10^{-4}	$(4.0 \pm 0.4) \times 10^{-4}$
5.0	3.0×10^{-4}	4.0×10^{-4}	4.0×10^{-4}	$(3.7 \pm 0.6) \times 10^{-4}$
7.5	3.0×10^{-4}	3.5×10^{-4}	3.0×10^{-4}	$(3.2 \pm 0.3) \times 10^{-4}$
10.0	1.4×10^{-4}	4.0×10^{-4}	2.0×10^{-4}	$(2.5 \pm 0.5) \times 10^{-4}$

Table 1-6. Ratios of DNA concentrations (C_x/C_o) and transformation frequencies (T_o/T_x) for strain NS-888 and NS-30.

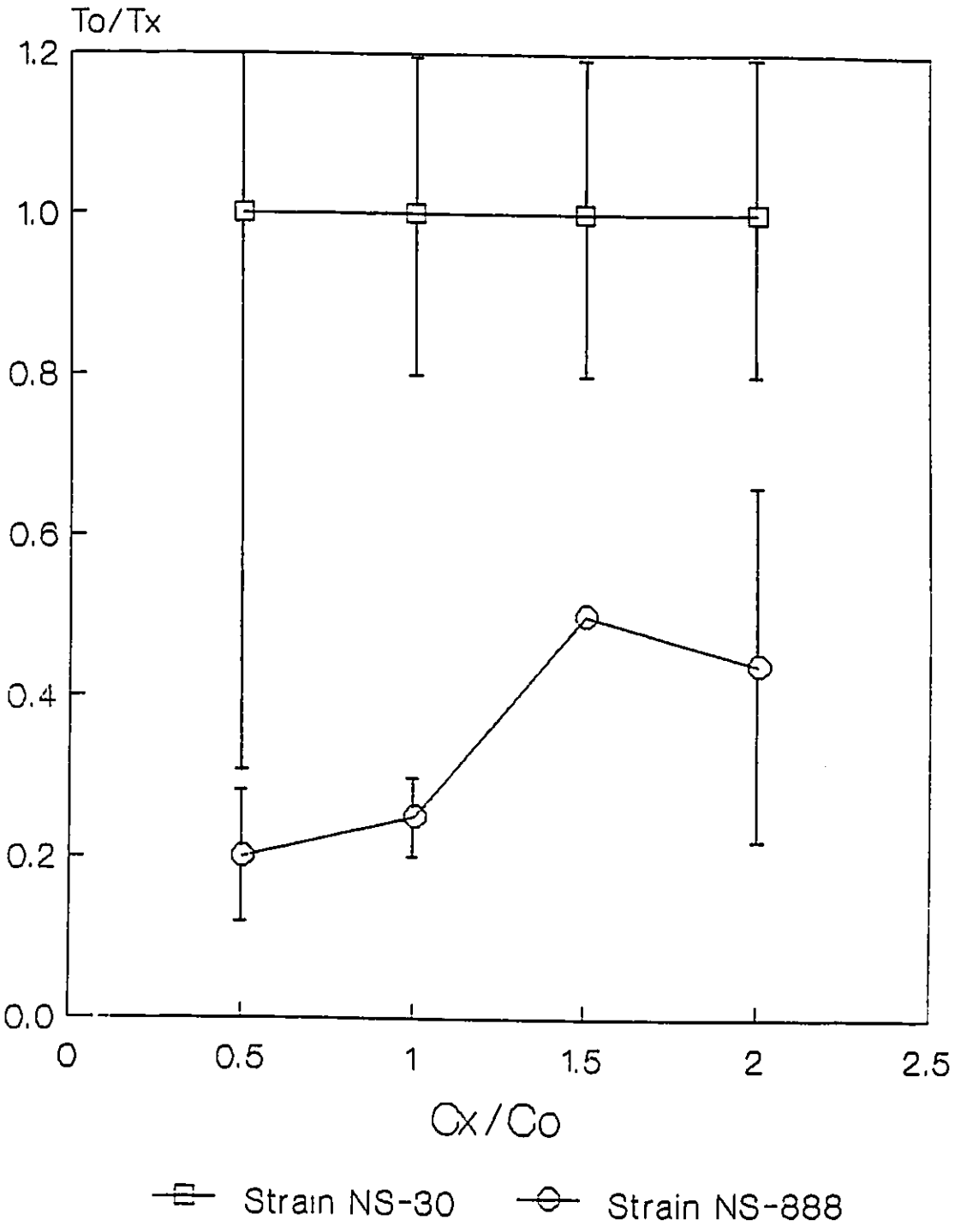
Type of DNA	C_x/C_o	T_o/T_x	
		NS-888	NS-30
1.5 kb TaqI fragment	0.5	0.20±0.08	1.0±0.7
	1.0	0.25±0.05	1.0±0.2
	1.5	0.50	1.0±0.2
	2.0	0.44±0.21	1.0±0.2
37-mer oligonucleotide	0.5	0.25±0.10	1.0±0.2
	1.0	0.27±0.16	1.0±0.2
	1.5	0.30±0.09	1.0±0.3
	2.0	0.40±0.20	1.0±0.2

Figure 1-6. Competitive inhibition of chromosomal DNA by the 1.5 kb TaqI fragment and the 37-mer oligonucleotide containing uptake sequences.

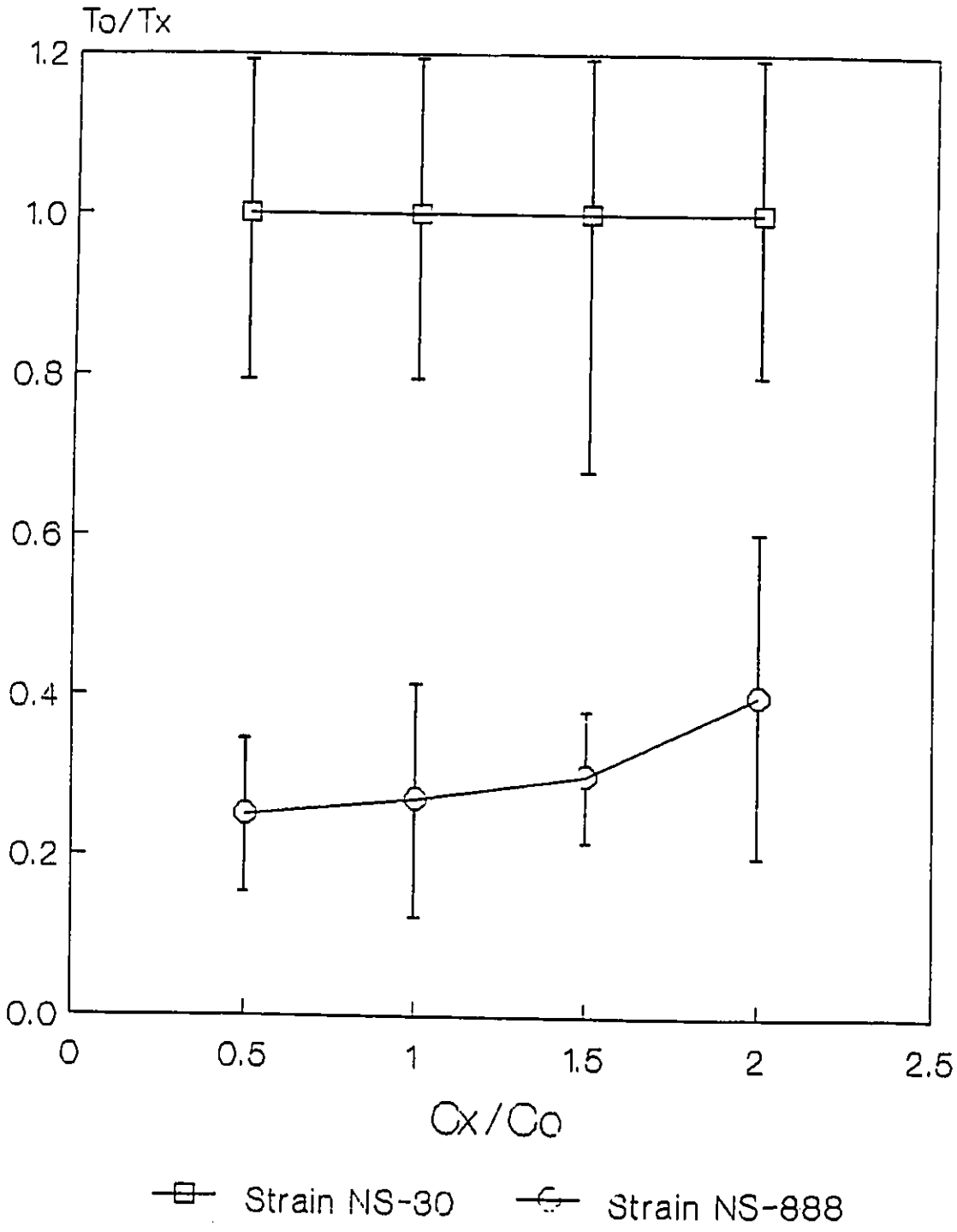
A. 1.5 kb TaqI fragment.

B. Oligonucleotide with uptake sequences.

A


To/Tx
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
0.5
1
1.5
2
2.5
Cx/Co
Strain NS-30
Strain NS-888

B


To/Tx
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
0.5
1
1.5
2
2.5
Cx/Co
Strain NS-30
Strain NS-888

Burnstein et al. (1988) showed, by uptake experiments, that the replicative form of the phage M13mp19 could be acquired by N. gonorrhoeae. A computer DNA similarity search was performed between the phage sequence and the uptake sequences and no similarity greater than 40 % was found between the two sequences. This indicated that M13mp19 transformed the gonococcus without the presence of uptake sequences. These results are similar to what was seen with strains NS-30, NS-757 and 2253.

DISCUSSION

This study confirms the preferential uptake by N. gonorrhoeae strain FS62 of a 1.5 kb TaqI fragment derived from the 4.2 kb cryptic plasmid. The preferential uptake of this fragment was first reported by Graves et al. (1982) using a chimeric plasmid comprising the 7.2 kb and 4.2 kb plasmids. This preferential uptake suggested that the 1.5 kb fragment contained sequences which were recognized by the gonococcus in order to be introduced into the cells by transformation. In fact, Goodman and Scocca (1989) confirmed the presence, on this fragment, of a copy of the uptake sequences (5'-GCCGTCTGAA-3') they initially identified. Burnstein et al. (1988) demonstrated the preferential uptake of an additional 400 bp TaqI fragment from the 4.2 kb plasmid. In their experiment, however, they used a 10 min interval for uptake instead of 15 min. Thus, one may hypothesize that, prolonged intervals of incubation may affect uptake of other fragments. Results from the experiments in this report do not support this hypothesis. With the exception of the 1.5 kb TaqI fragment, none of the fragments visible on the gel were observed in transformed cells for times varying from 0 to 60 minutes. Thus, the ability of the gonococcus to selectively acquire the 1.5 kb TaqI fragment over other fragments cannot be explained by a parameter such as time. Also, time cannot explain the variation seen in uptake between Burnstein et al. (1989), Graves et al. (1982), and this study.

The ability of 16 strains, belonging to different auxotype groups to acquire fragments of the 4.2 kb plasmid restricted with TaqI was tested in order to determine if all gonococcal strains recognized and acquired the 1.5 kb TaqI fragment. Results show that three strains (NS-30, NS-506 and 2253) were unable to acquire the 1.5 kb TaqI fragment or any other fragments from the cryptic plasmid. The remaining 13 strains only acquired the 1.5 kb TaqI fragment confirming our previous results. Although the 3 strains failed to recognize the 1.5 kb fragment, they were competent for transformation using chromosomal DNA. No relationship between auxotype and uptake was observed, as has been noted between plasmid profile and auxotype (Dillon and Pauzé, 1981). Biswas et al. (1989) noticed abnormal colony morphology, such as wrinkled colonies, for mutants which were unable to acquire DNA into a DNase resistant state. Colonies of strains NS-30, NS-506 and 2253 were identical to piliated N. gonorrhoeae cells with no indication of abnormalities.

The restriction assay demonstrated that the inability of N. gonorrhoeae strains NS-30, NS-506 and 2253 to acquire the 1.5 kb TaqI fragment could not be attributed to their restriction/ modification systems. These strains shared similarities with restriction/ modification systems of other strains that acquired the fragment. Patterns produced by the assays using lambda DNA were all different from the patterns produced with known isoschizomers of N. gonorrhoeae (HaeII, HaeIII and SacII). This can be explained by the fact that most gonococcal strains possess at least six

restriction/modification systems some of which are still not characterized (Davies, 1989). Therefore, the pattern produced by one restriction endonuclease will be different than the pattern produced by crude extracts that contain up to six restriction endonucleases. Strain NS-506 (Figure 1-4, lane 6) for example, apparently contained the restriction endonuclease SacII (Figure 1-4, lane 10) but in combination with several uncharacterized restriction endonucleases.

Because three strains were unable to recognize the uptake sequences found on the 1.5 kb TaqI fragment, it appears that uptake sequences for transformation are not universally recognized by naturally-occurring N. gonorrhoeae strains. Furthermore, these results also suggest that transformation may occur by at least two independent mechanisms since the same three strains were competent for genetic transformation using chromosomal DNA. One system would be highly selective and would require the presence of uptake sequences on the incoming DNA while the other system would assimilate foreign DNA.

Several observations support the idea of at least two transformation systems in the gonococcus. If N. gonorrhoeae cells only have a single transformation system requiring uptake sequences which allow discrimination between gonococcal DNA and foreign DNA, why do gonococci retain the ability to produce 6 restriction/modification systems per strain (Davies, 1989)? One possible answer

is that a second transformation system which allows introduction of foreign DNA and requires restriction barriers is also present in the gonococcus. Burnstein et al. (1989) reported that, in addition to the 1.5 kb and 400 bp TaqI fragments of the 4.2 kb plasmid, the replicative form of the phage M13mp19 was also acquired by the gonococcus. No similarities were found between the two DNA sequences suggesting that M13mp19 was acquired by the gonococcus by an alternative transformation process which did not require uptake sequences (this study). Biswas et al. (1989) have recently isolated transformation-deficient mutants by chemical mutagenesis, assigning them to two loci. These were determined on the ability of the strains to be transformed by chromosomal DNA before and after mutagenesis. The results of the present study differ from those of Biswas et al. (1989) in that the 3 naturally-occurring strains that did not recognize the 1.5 kb TaqI fragment were still competent for transformation using chromosomal DNA. Thus, at least two independent transformation systems must be present in the gonococcus and chemical mutagenesis affected only one of the systems. The proposition that at least two transformation processes are present in the gonococcus was strengthened by competition experiments performed using a strain (NS-888) that acquired the 1.5 kb TaqI fragment harbouring a copy of the uptake sequences and one strain (NS-30) that did not. Dose-response curves were first determined in order to calculate the optimal chromosomal DNA concentration for transformation. The concentration found in this study (5 μg of DNA) differed from the concentration (2 μg of DNA)

estimated by Graves et al. (1982). It is, however, interesting to note that both strains have a latent phase in their ability to acquire DNA at concentrations between 2 μg and 4 μg of chromosomal DNA. This could explain why Graves et al. (1982) selected a concentration of 2 μg of DNA, instead of 5 μg, as the optimal concentration. The meaning of this latent phase in both strains is presently unknown. The ability of NS-30 to be transformed by chromosomal DNA was not affected by the presence of either the 1.5 kb fragment or the synthetic oligonucleotide comprising the uptake sequences. This suggested that NS-30 could not recognize uptake sequences but it could be transformed by chromosomal DNA by a different transformation process. Transformation of NS-888 was affected by the two types of competing DNA indicating that this strain has the ability to recognize uptake sequences. This suggests that strains like NS-888 possess at least one transformation process which requires the presence of uptake sequences. It is difficult to assess whether or not strains such as NS-888 possess, in addition to the transformation system which requires uptake sequences, a non-specific transformation system. Similar to NS-30, TaqI fragments of pLCDC1 might have been acquired by strain NS-888 through a non-specific transformation system but these were probably restricted by the cells or recombined with the chromosome and were therefore not detected in the various assays. Finally, Dorward et al. (1989), have recently characterized another transformation mechanism for N. gonorrhoeae. It was found that gonococcal cells could exchange plasmid DNA via membranes vesicles

(blebs) (Dorward et al., 1989). It therefore appears that N. gonorrhoeae has developed several strategies to incorporate homologous and heterologous DNA. This would permit N. gonorrhoeae cells to acquire new gene functions and to propagate them through the gonococcal population.

This study was initially performed in order to determine if the uptake sequences should be taken into consideration for the construction of a shuttle vector. This study demonstrated that 19 % of gonococcal strains were unable to recognize and acquire the uptake sequences. It was also shown by others that even in the presence of uptake sequences plasmid transformation resulted in deletion derivatives (Stein et al., 1983b). Hence, it was concluded that uptake sequences might not render the shuttle vector more efficient in transforming the gonococcus and these were therefore abondoned.

CHAPTER TWO

CONJUGATION, TRANSFORMATION AND ELECTROPORATION OF THE ASIA (7.2 KB), AFRICA (5.2 KB) AND TORONTO (4.9 KB)-TYPE PLASMIDS AND THEIR *IN VITRO*-DERIVED DELETION DERIVATIVES INTO *NEISSERIA GONORRHOEAE*

INTRODUCTION

Few studies have been undertaken which elucidate mechanisms of mobilization of gonococcal non-conjugative plasmids by other plasmids. Fragments with *oriT* and *mob* functions implicated in mobilization of the 7.2 kb plasmid by donation, were individually identified by different researchers (McNicol *et al.*, 1983; Tenover *et al.*, 1985). However, these studies were performed using different host-recipient systems. Although the 7.2 kb penicillinase-producing plasmid of *N. gonorrhoeae* has a broad host range in terms of its mobilization from the gonococcus into other species, experiments demonstrating mobilization by donation of the plasmid from *E. coli* to *N. gonorrhoeae* by pUB307, an *IncP* conjugative plasmid, were reported, only recently (Piffaretti *et al.*, 1988). Piffaretti *et al.* (1988) showed that the 7.2 kb plasmid could be mobilized by pUB307 and stably maintained into the gonococcus.

In addition to pUB307, conjugative plasmids of enteric origin, pR100 (*IncFII*), pR124 (*IncFIV*) and pBG791 (*IncIα*) mobilized the 7.2 kb plasmid between *E. coli* strains (Yeung, 1988). Transconjugants of matings performed with pR100, pBG791 and pR124 harboured plasmids larger than pJD4 (7.2 kb) indicating that the plasmid had been mobilized by conduction (K.-H. Yeung, unpublished data). Cloning of pJD4 *Hind*III/*Bam*HI digests into the vector pACYC184, followed by mobilization of the recombinant plasmids into *E. coli*

by pBG791, resulted in the identification of a 2.4 kb BamHI fragment of pJD4 required for mobilization by conduction (Yeung, 1988). This fragment was different from those identified as carrying oriT and mob functions. Mobilization by conduction of pJD4 into N. gonorrhoeae with pBG791, pR100 and pR124 has never been tested.

Prior to the findings of Piffaretti et al. (1988), the only means of introducing plasmid DNA into N. gonorrhoeae was by transformation (Sparling, 1966). This method often produced unstable deletion derivatives, unless the recipient strain harboured an homologous plasmid which could rescue the incoming plasmid by marker rescue (Biswas et al., 1982; Sox et al., 1979). Stein et al. (1988) showed that plasmid DNA introduced into the gonococcus by conjugation was not subject to restriction barriers. They explained this result based on the fact that restriction endonucleases cannot recognize and, therefore, cannot cleave single stranded DNA required for conjugation. Therefore, conjugation could obviate problems encountered by transformation. Electroporation, a physical process whereby pores are created by applying high-voltage to cells, has become widely used as an alternative mode for introducing DNA into a variety of microorganisms such as E. coli, Campylobacter jejuni, Lactobacillus casei and Streptococcus lactis (Chassy and Flickinger, 1987; Miller et al., 1988; Powell et al., 1988; Taketo, 1988; 1989). Electroporation of N. gonorrhoeae has not yet been reported, but, may offer advantages over

transformation such as obviating the requirement for piliated cells.

In order to evaluate which system was more suitable for introducing plasmid DNA into the gonococcus, the Asia (7.2 kb), Africa (5.2 kb) and Toronto (4.9 kb)-type plasmids of N. gonorrhoeae and their in vitro-derived deletion derivatives (Yeung and Dillon, 1988) were tested for their ability to be introduced into the gonococcus by conjugation, transformation and electroporation. Further studies were undertaken with these plasmids in order to refine the essential oriT and mob regions required for the mobilization of pJD4 by donation. These data were also evaluated for selecting the smallest self-replicating ß-lactamase-producing plasmid which could replicate in both E. coli and N. gonorrhoeae. The ultimate intent was to select this plasmid as the basic entity for constructing an E. coli/N. gonorrhoeae shuttle vector.

MATERIALS AND METHODS

Bacterial strains and plasmids

Bacterial strains and plasmids used in this study are listed in Tables 2-1 and 2-2, respectively. Phenotypes were confirmed by plating on either auxotyping or antibiotic-containing media (Chapter One). N. gonorrhoeae colony types (1, 2, 3 and 4) were maintained as described in Chapter One.

The conjugative plasmids pR100-1, pR124, pBG791 and pUB307 were kindly provided by Drs. S.A. McIntire (University of Texas, Dallas, Texas), V.N. Iyer (University of Carleton, Ottawa, Ontario), G.S. Bezanson (formerly, Laboratory Center for Disease Control, Ottawa, Ontario) and J.-C. Piffaretti (University of Geneva, Geneva, Switzerland). Conjugative plasmids were transferred by conjugation and maintained in E. coli DH1. Non-conjugative plasmids were transformed into strain DH1 harbouring the various conjugative plasmids using the procedure of Miller (1987).

Media and growth conditions

E. coli strains were maintained on Tryptic Soya Agar (TSA, Difco) at 37°C. When required, antibiotics were added at the following concentrations: ampicillin (Amp, Sigma), 100 mg/L; tetracycline (Tet, Sigma), 10 mg/L; rifampicin (Rif, Sigma), 200

Table 2-1. Bacterial strains used for conjugation, transformation and electroporation experiments.

Strains	Phenotype	Source or reference
E. coli		
DH1	F^-, *recA1*, *endA1*, *gyrA96*, *Thi-1*, *hsdR17* (r_k^- m_k^+), *supE44*, λ^-	Maniatis *et al.* (1982)
C600R	Rif^r, F^- *Thi-1*, *leuB6*, *lacY1*, *tonA21*, *supE44*	spont. rifampicin resistant mutant (this lab)
HB101	F^-, *hsdS20* (r_B^- m_B^+), *recA13*, *ara-14*, *proA2*, *lacY1*, *galK2*, *rpsL20*, (Sm^r), *xyl-5*, *mtl-1*, *supE44*, λ^-	Maniatis *et al.* (1982)
N. gonorrhoeae[a]		
2152	PCU	this study
2152S	Sm^r, PCU	this study
NS-757	NR	this study

a) See table 1-1 for abbreviations and strain descriptions.

Table 2-2. Plasmids used for conjugation, transformation and electroporation experiments.

Plasmids	Phenotype	Source or reference
NON-CONJUGATIVE PLASMIDS		
pJD4 (7.2 kb)	Amp^r	Yeung and Dillon (1988)
pJD5 (5.1 kb)	Amp^r	Yeung and Dillon (1988)
pJD6 (3.9 kb)	Amp^r	Yeung and Dillon (1988)
pJD7 (4.9 kb)	Amp^r	Yeung and Dillon (1988)
pJD8 (6.2 kb)	Amp^r	Yeung and Dillon (1988)
pJD9 (3.4 kb)	Amp^r	Yeung and Dillon (1988)
pGRY1 (4.8 kb)	Amp^r	this study
pGRY2 (5.6 kb)	Amp^r	this study
pGRY3 (5.6 kb)	Amp^r	this study
pGRY4 (5.7 kb)	Amp^r	this study
pLES2 (6.2 kb)	Amp^r	Stein *et al.* (1983a)
CONJUGATIVE PLASMIDS (Inc)		
pUB307 (P)	Tet^r, Kan^r	Piffaretti *et al.* (1987)
R124 (FIV)	Tet^r	Iyer V.N.
R100-1 (FII)	Tet^r, Sm^r, Cm^r	McIntire S.A.
pBG791 (Iα)	Sm^r	Benzanson *et al.* (1981)

mg/L; nalidixic acid (Nal, Sigma), 48 mg/L; chloramphenicol (Cm, Sigma), 30 mg/L; kanamycin (Km, Sigma), 30 mg/L; streptomycin (Sm, Sigma), 500 mg/L. Transconjugants from E. coli/N. gonorrhoeae matings were selected using streptomycin/ampicillin-containing media while rifampicin/ampicillin-containing media was used to select transconjugants for matings between E. coli strains.

N. gonorrhoeae strains were grown as described in Chapter One. When required, antibiotics were added at the following concentrations: ampicillin, 10 mg/L; streptomycin, 500 mg/L.

DNA Manipulations and electrophoresis

Restriction endonucleases TaqI, RsaI, DraI, MboII, HinfI, PvuI and AvaII were purchased from either Bethesda Research Laboratories (BRL; Burlington, Ontario) or Promega Biotech (Madison, Wisconsin). Restriction digests were performed using the restriction buffers supplied with the enzymes and the conditions recommended by the manufacturers. DNA ligation with T4 ligase was performed as described by Maniatis et al. (1982). DNA fragments of complementary ends were resuspended in 20 μL of ligation buffer (20 mM Tris-HCl, pH 7.5; 10 mM $MgCl_2$; 0.6 mM ATP; 10 mM dithiothreitol). One μL of T_4 ligase was then added and the mixture was incubated overnight at 16°C. DNA fragments with non-complementary ends were modified to blunt-ends prior to ligation. Fragments to be blunt-ended were resuspended in 18 μL of water, 2 uL of 10 X nick translation buffer and 2 μL of 0.3 mM dNTPs mix. Two μL of Klenow fragment from DNA

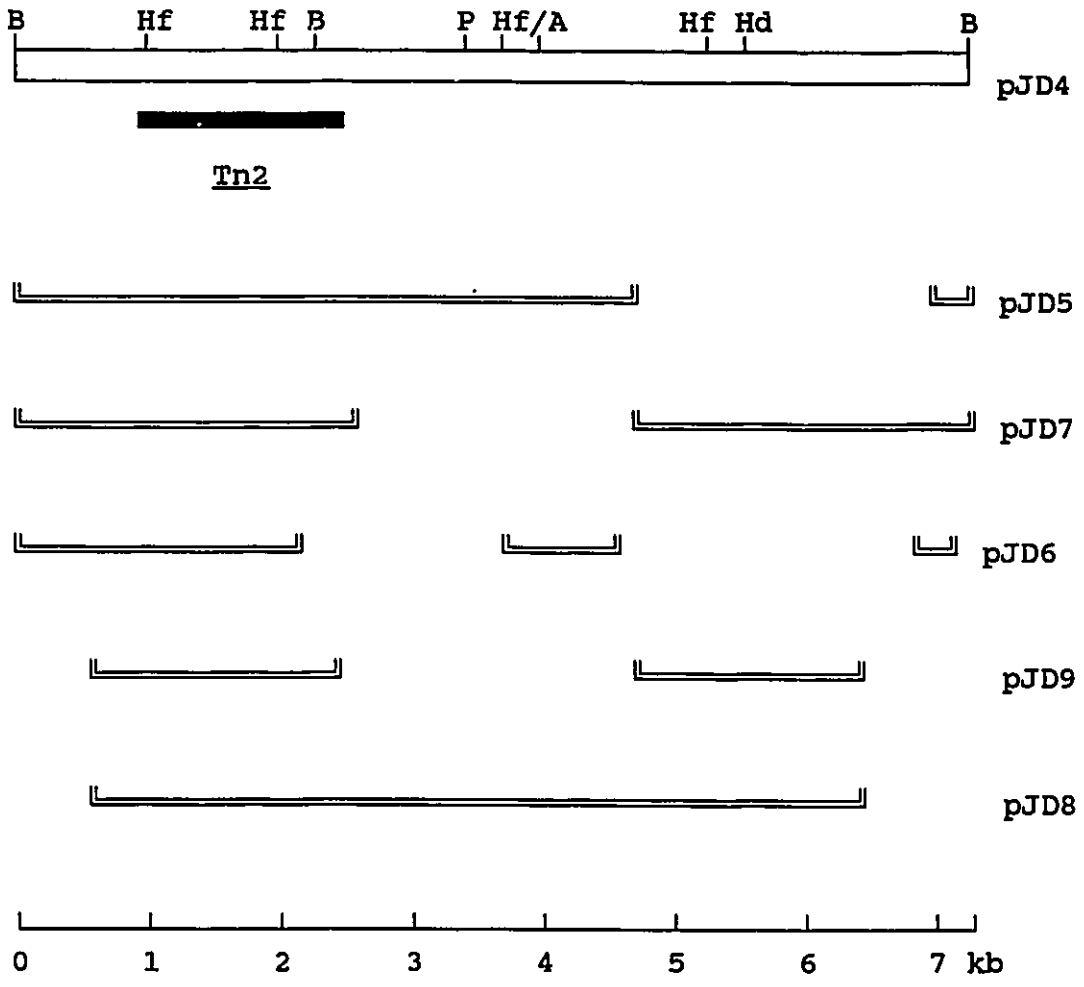
polymerase I (BMC) were added and the reaction mixture was incubated at room temperature for 30 min. The blunt-ended DNA was precipitated with 95% ethanol at -70°C for 2 hrs before ligation.

Electrophoresis of DNA on agarose gels was carried out as described in Chapter One. In addition, polyacrylamide gels (5%) were used to examine restriction endonuclease digests of pGRY3 and pGRY4. Gels were prepared as described by Dillon et al. (1985). Electrophoresis was performed in Tris-borate buffer using the Protean II apparatus (Bio-Rad). Lambda HindIII (BMC) and pBR322 HaeII (BMC) fragments were used as molecular weight standards.

Construction and characterization of in vitro-derived deletion derivatives of pJD4 and pJD5

A series of in vitro-derived deletion derivatives (pJD6, pJD8 and pJD9), generated either from plasmids pJD4 or pJD5 were previously constructed in this laboratory by Bal31 exonuclease digestion (Figure 2-1) (Yeung and Dillon, 1988). Plasmid pJD7 (Toronto-type) is a naturally-occurring deletion derivative of the 7.2 kb plasmid (Yeung and Dillon, 1985). The deletion library was screened again and two new deletion derivatives of pJD4 with

Figure 2-1. Restriction endonucleases map of pJD4, pJD5, pJD7 and their *in vitro*-derived deletion derivatives previously constructed. A: AvaI; B: BamHI; Hd: HindIII; Hf: HinfI; P:PvuII.


B
Hf
Hf
B
P
Hf/A
Hf
Hd
B
pJD4
Tn2
pJD5
pJD7
pJD6
pJD9
pJD8
0
1
2
3
4
5
6
7
kb

smaller molecular weights than pJD8 (6.2kb) were identified and named pGRY3 (5.6 kb) and pGRY4 (5.7 kb) (Results, Figure 2-2). Two addional deletion derivatives of pJD8, pGRY1 and pGRY2, were constructed during this study (Results, Figure 2-2) in order to create the smallest characterized plasmid which could be mobilized by pUB307 and maintained in N. gonorrhoeae.

Mobilization of non-conjugative plasmids

Mobilizations of non-conjugative gonococcal plasmids and their in vitro-deletion derivatives from E. coli DH1 to N. gonorrhoeae 2152S were performed using a filter-mating technique (Guiney and Ito, 1982). E. coli constructs and N. gonorrhoeae 2152S were grown overnight on selective media containing the appropriate antibiotics. Cells were resuspended in 0.7% casamino acids to a concentration of 1.5 X 10^8 colony CFU/mL. Cell concentration was determined using a McFarland barium sulfate standard (Paik, 1970). An equal volume of donor and recipient cells (100 μL) was filtered onto a sterile 0.45 um membrane filter (Millipore Canada Ltd.). The membrane filter was placed, cell-side up, on a GCMB plate and incubated at 35°C with 5% CO_2 for 3 hours. The membranes were resuspended in 1 mL of 0.7% casamino acids and diluted by serial ten-fold dilutions. One hundred μL of each dilution were plated onto GCMB plates supplemented with streptomycin and ampicillin and incubated at 35°C with 5% CO_2 for up to 72 hrs. Transconjugants were tested for penicillinase production using Nitrocefin (Glaxo, Greenford, Middlesex, England) and purified by streaking on

appropriate selective media. Transconjugants were confirmed as N. gonorrhoeae using the Minitek differentiation system (BBL Microbiology Systems, Cockeysville, Maryland). The plasmid content of cells was verified using the alkaline-SDS procedure, as described in Chapter One (Birnboim et al. 1979) followed by agarose gel electrophoresis. This protocol was followed in the mobilization of non-conjugative plasmids by pUB397 from E. coli DH1 to E. coli C600R.

Transformation

Transformation of E. coli was performed as described by Miller (1987). A single colony of E. coli strain DH1 from an overnight culture was grown at 37°C in 50 mL of tryptic soya broth (TSB; Difco) to an absorbance of 0.4-0.5 at 550 nm. The cell broth was centrifuged at 8,000 rpm for 10 min in a Sorvall R5C centrifuge and the pellet resuspended in 25 mL of cold 0.1 M $CaCl_2$ (Sigma). The solution was set on ice for 30 min before being centrifuged again under the same conditions in the Sorvall centrifuge. The supernatant was decanted and the cell paste resuspended in 2 mL of cold 0.1 M $CaCl_2$. Two hundred μL of competent cells were then used for transformation using plasmid DNA isolated from E. coli. One μg of plasmid DNA was mixed with the cells and set on ice for 60 min. Competent cells with DNA were then heated at 42°C for 2 min before adding 800 μL of TSB. Transformed cells were grown for 60 min at 37°C and plated on selective media. Gonococcal transformation using

plasmid JD4 was performed by the protocol of Janik and Heym (1976) as described in Chapter One.

Electroporation

In order to obtain DNA from an isogenic strain, plasmid pJD4 was first mobilized from E. coli DHI to N. gonorrhoeae strain 2152S with the conjugative plasmid pUB307 and isolated from strain 2152S by caesium-chloride ethidium bromide ultracentrifugation (Chapter One). Electroporation of N. gonorrhoeae strains 2152 and NS-757, colony type 2 (competent cells) and type 3 (non-competent cells), with plasmid JD4 was performed using the Gene Pulser™ apparatus (Bio-Rad) according to the manufacturer's instructions. A single colony of an overnight culture from each strain was resuspended into 500 mL of TSB and grown at 37°C to an absorbance of 0.6 at 550 nm. Cells were then centrifuged at 8,000 rpm for 10 min in a Sorvall R5C centrifuge and washed several times in low ionic buffers. After the final wash, the pellet was resuspended in 3 mL of the same buffer. The cell suspension for each strain was divided in aliquots of 50 μL and frozen at -70°C. Electroporation was performed in 0.2 cm electroporation cuvettes containing 40 μL of the cell preparation and 0.5 μg of plasmid DNA. The preparation was set on ice for 10 min prior to electroporation. Electroporation conditions were set at a voltage of 2.5 kV and a capacitance of 25 μF. After the shock, gonococcal cells were either grown in 100 mL of GC broth or plated on GCMB plates and incubated for several hours at 35°C in a humid environment supplemented with 5% CO_2. The

electro-transformants were then plated on ampicillin-containing media and further incubated for up to 72 hrs. E. coli strain DH1 was used as a control. Two parameters were investigated, buffer composition and growth conditions after electroporation.

DNA sequence analysis of one of the origin of replication of pJD4

Seven hundred bp of a 1.6 kb HindIII/BamHI fragment of pJD4, containing one of the origins of replication of the plasmid, was recently sequenced (Yeung and Dillon, 1988). This region also contains the putative oriT of plasmids pGRY3 and pGRY4 which are derived from pJD4. DNA sequence analysis of the 700 bp was performed in order to find novel restriction endonuclease sites which might be used to discriminate any size variation between pGRY3 and pGRY4. The DNA sequence analysis was performed using the University of Wisconsin Genetic Computer Group package (Deveureux et al., 1984) provided by the Biomolecular DATA base system of CAN/SND, Canada Institute for Scientific and Technical Information (CISTI).

RESULTS

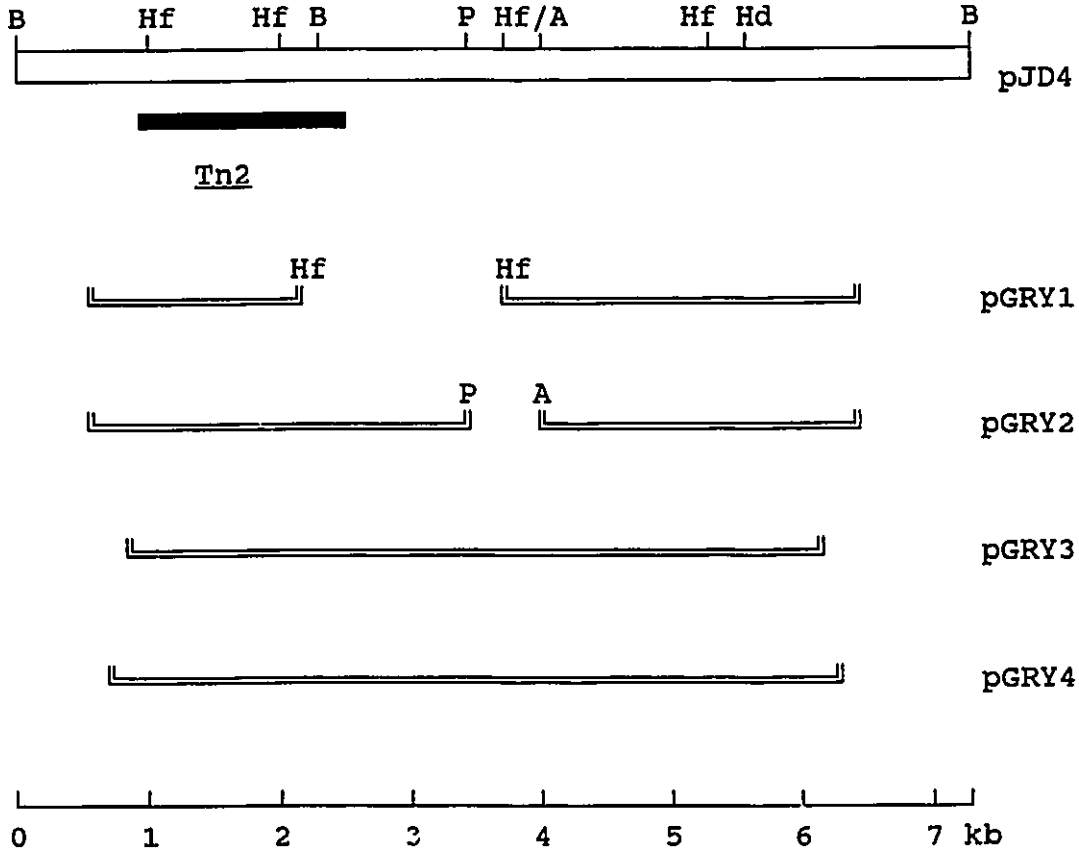
Construction of plasmids pGRY1 and pGRY2

In order to obtain the smallest characterized penicillinase-producing plasmid that could be mobilized and maintained and in N. gonorrhoeae, two novel deletion derivatives of plasmid pJD8 were constructed. Plasmid pGRY1 was constructed by digesting pJD8 with HinfI and PvuII followed by ligation with T_4 ligase (Dillon et al., 1985). The restriction endonuclease PvuII was used to minimize the probability of ligation of a 1.4 kb PvuII-restricted HinfI fragment thereby ensuring the deletion of this fragment (Figure 2-2). Plasmid pGRY2, was constructed by digesting pJD8 with PvuII and AvaI. Digestion of pJD8 with these enzymes generated a 600 bp fragment and a 5.6 kb fragment which were resolved on low melting point agarose (BRL) gel by electrophoresis. The largest fragment (5.6 kb) was isolated from the gel, blunt-ended with klenow enzyme and ligated with T_4 ligase (Dillon et al., 1985) thereby producing the plasmid pGRY2. This plasmid had a 600 bp PvuII/AvaI deletion as compared to the parent plasmid pJD8 (Figure 2-2).

Mobilization of gonococcal resistance plasmids and their deletion derivatives into N. gonorrhoeae by pUB307, pR124, pR100-1 and pBG791

Conjugative plasmids, pR124, pR100-1, pBG791 and pUB307 were tested for their ability to mobilize pJD4, pJD5, pJD7 and their

Figure 2-2. In vitro-derived deletion derivatives of pJD4 characterized and constructed for mobilization experiments. A: AvaI; B: BamHI; Hd: HindIII; Hf: HinfI; P:PvuII. Tn2, truncated transposable element.


B
Hf
Hf
B
P
Hf/A
Hf
Hd
B
pJD4
Tn2
Hf
Hf
pGRY1
P
A
pGRY2
pGRY3
pGRY4
0
1
2
3
4
5
6
7
kb

in vitro-derived deletion derivatives from E. coli DH1 to N. gonorrhoeae strain 2152S (Table 2-3). Plasmid pUB307 mobilized pJD4 but not plasmids pJD5, pJD6, pJD7, pJD9, pGRY1 and pGRY2 into N. gonorrhoeae strain 2152S. Plasmid pUB307 also mobilized pLES2 and deletion derivatives pJD8, pGRY4 and pGRY3 into the gonococcus at frequencies of 1×10^{-6}, 1×10^{-6}, 5.4×10^{-6} and 4.2×10^{-8} respectively. Plasmids pR100-1, pBG791 and pR124 failed to mobilize the transfer of pJD4 between E. coli and N. gonorrhoeae 2152S (Table 2-3).

All plasmid-containing transconjugants were confirmed to be N. gonorrhoeae with the PCU-requiring auxotype. Mobilization of pJD4 into a PCU-requiring strain therefore did not alter the nutritional requirements of its host. Figure 2-3 confirms the presence of these plasmids in gonococcal recipient cells. In all conjugation experiments performed between E. coli and N. gonorrhoeae, pUB307 was never observed in the recipient strain.

Mobilization frequencies of pGRY3 and pGRY4 from E. coli to N. gonorrhoeae varied by 100-fold (Table 2-3) indicating that a vital region affecting mobilization was encompassed by the deletion differentiating these plasmids. Plasmid pGRY3 and pGRY4 were constructed by Bal31 exonuclease digestion and have a 100 bp difference located downstream of the unique HindIII of pJD4 (Figure 2-2). DNA sequence analysis indicated several sites for restriction

Table 2-3. Frequency of mobilization of ampicillin resistance plasmids from E. coli DH1 to N. gonorrhoeae strain 2152S.

Plasmids	Mobilization frequency[a]
pUB307, pJD4	1×10^{-6}
pUB307, pJD5	$< 10^{-8}$
pUB307, pJD6	$< 10^{-8}$
pUB307, pJD7	$< 10^{-8}$
pUB307, pJD8	1×10^{-6}
pUB307, pJD9	$< 10^{-8}$
pUB307, pGRY1	$< 10^{-8}$
pUB307, pGRY2	$< 10^{-8}$
pUB307, pGRY3	4.2×10^{-8}
pUB307, pGRY4	5.6×10^{-6}
pUB307, pLES2	1×10^{-6}
pR100-1, pJD4	$< 10^{-8}$
pBG791, pJD4	$< 10^{-8}$
pR124, pJD4	$< 10^{-8}$

a) Frequencies obtained are the results of at least two independent experiments.

endonucleases TaqI, RsaI, DraI, and MboII within the 700 bp region. Restriction analysis of pGRY3 and pGRY4 using these enzymes did not reveal any size variation of the fragments and their restriction patterns appeared identical (Figure 2-4). The definition of the deletion between these two plasmids based on restriction endonucleases analysis proved inadequate; thus, only DNA sequencing of pGRY3 and pGRY4 will determine the exact location of the deletion distinguishing the two plasmids.

Mobilization of resistance plasmids and in vitro-derived deletion derivatives from E. coli strain DH1 to strain C600R

To determine whether mobilization between E. coli strains proceeded by a similar mechanism as between E. coli and N. gonorrhoeae, conjugation experiments with pUB307 were repeated except that E. coli strain C600R was used as a recipient strain. Plasmid pUB307 mobilized plasmids pJD4, pJD8, pLES2, pGRY3 and pGRY4 into E. coli C600R with similar frequencies as compared to N. gonorrhoeae (Table 2-4). Similarly, plasmids pJD5, pJD6, pJD7, pJD9, pGRY1 and pGRY2 were not mobilized by pUB307 between E. coli hosts. Transconjugants harboured plasmids of similar sizes to the ones used for each mating. Unlike N. gonorrhoeae transconjugants, the conjugative plasmid pUB307 was also transferred into the recipient strain along with the resistance plasmids (Figure 2-5). Mobilization phenotypes of pJD4, pJD5 and their in vitro-derived deletion derivatives with pUB307 between E. coli and N. gonorrhoeae

Figure 2-3. Plasmid content of N. gonorrhoeae transconjugants by agarose gel (1%) DNA-electrophoresis. Lane 1, Lambda HindIII marker; lane 2, E. coli HB101 with pUB307; lane 3, E. coli DH1; lane 4, E. coli DH1 with pUB307; lane 5 to 7, E. coli DH1 construct with pLES2 (lane 5), pJD4 (lane 6) and pJD8 (lane 7); lane 8, N. gonorrhoeae 2152S; lane 9 to 11, gonococcal transconjugants harbouring pLES2 (lane 9), pJD4 (lane 10) and pJD8 (lane 11). Numbers in the margin indicate molecular sizes of fragments produced by lambda HindIII digest (23.1, 9.4, 6.6, 4.4, 2.3 and 2.0 kb).

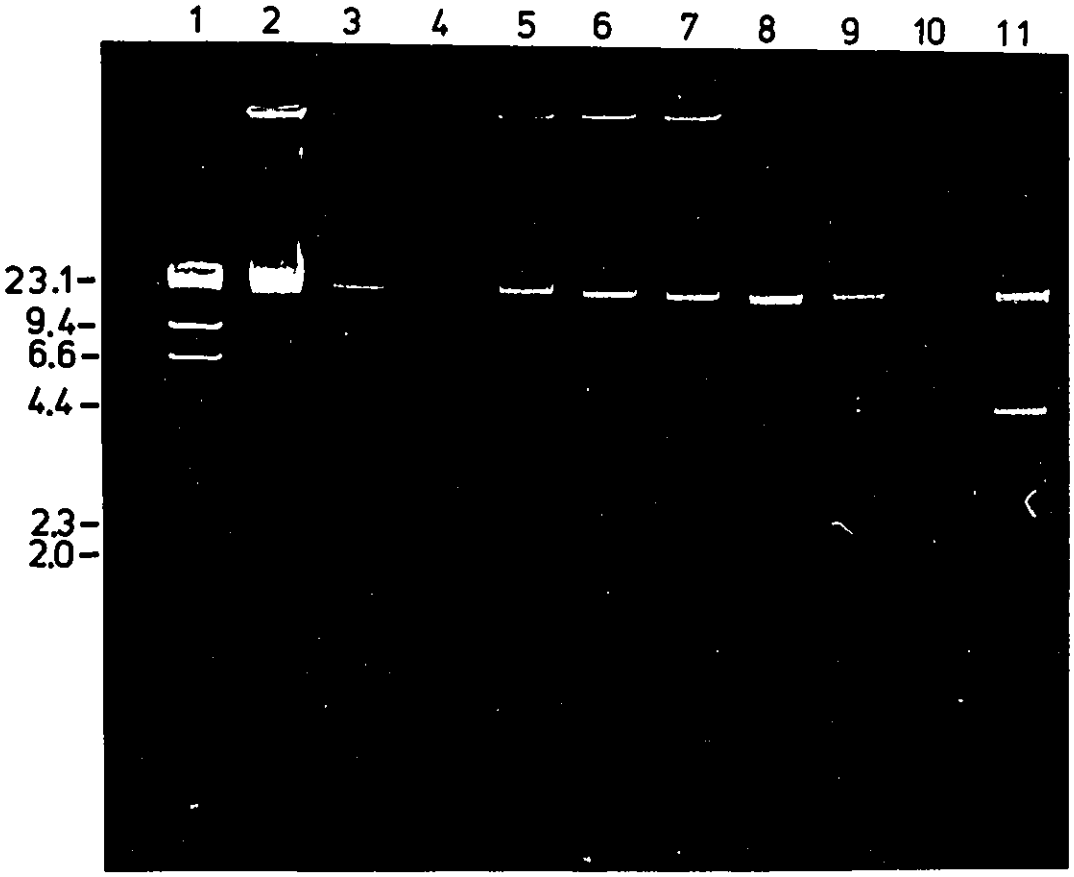

1
2
3
4
5
6
7
8
9
10
11
23.1
9.4
6.6
4.4
2.3
2.0

Figure 2-4. Restriction endonuclease analysis of pGRY3 and pGRY4. Lanes 2 to 5, pGRY3 digested with TaqI (lane 2), RsaI (lane 3), DraI (lane 4) and MboII (lane 5). Lanes 7 to 10, pGRY4 digested with TaqI (lane 7), RsaI (lane 8), DraI (lane 9) and MboII (lane 10). Lanes 1, 6 and 11, marker DNA- HaeII digested pBR322 (1876, 622, 439/430, 370, 227 and 181 bp).

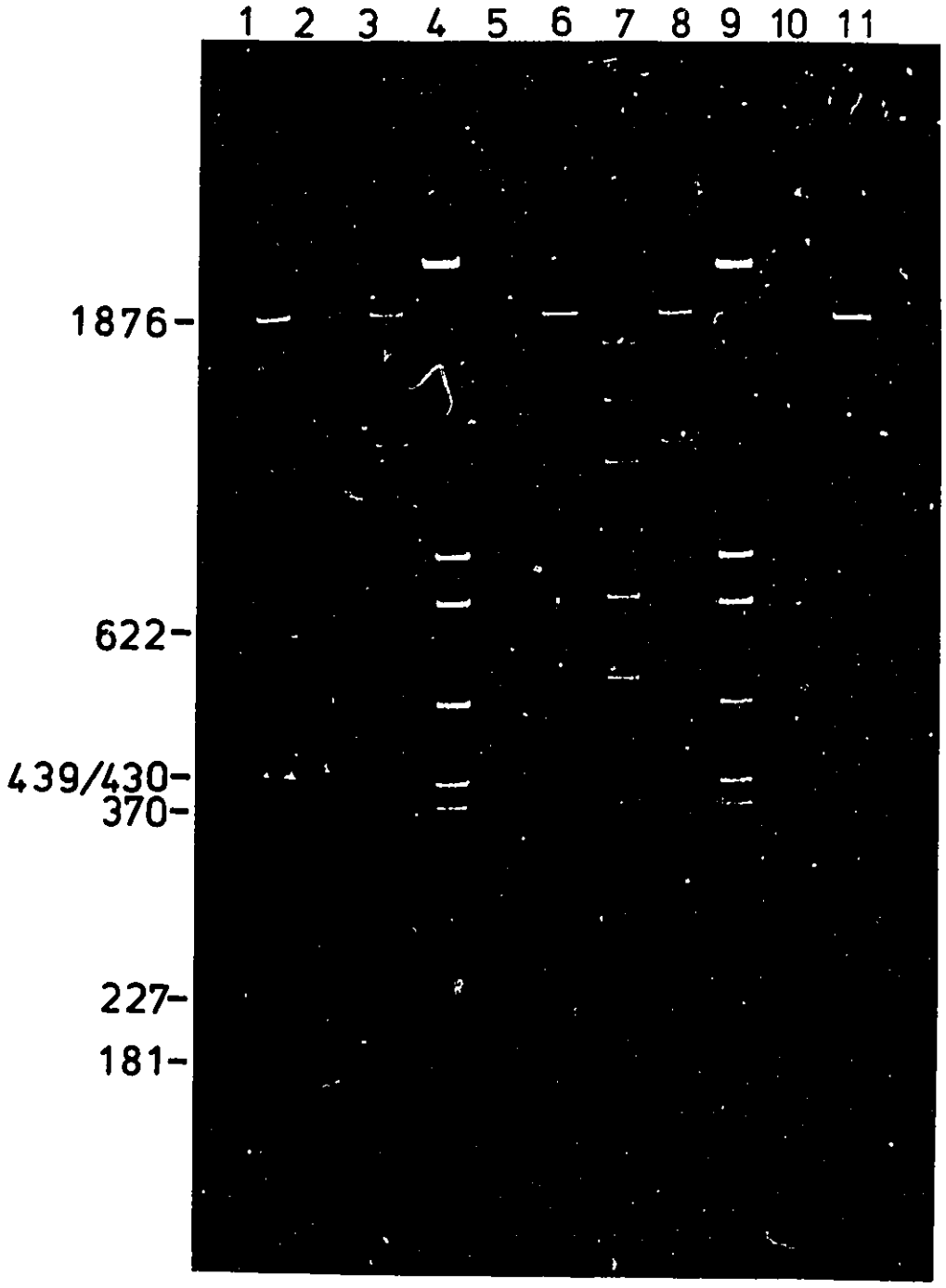

1 2 3 4 5 6 7 8 9 10 11
1876-
622-
439/430-
370-
227-
181-

Table 2-4. Frequency of mobilization of ampicillin resistance plasmids from E. coli DH1 to E. coli C600R using the conjugative plasmid pUB307.

Plasmids	Mobilization frequency[a]
pJD4	3×10^{-6}
pJD5	$< 10^{-8}$
pJD6	$< 10^{-8}$
pJD7	$< 10^{-8}$
pJD8	1×10^{-6}
pJD9	$< 10^{-8}$
pGRY1	$< 10^{-8}$
pGRY2	$< 10^{-8}$
pGRY3	4.2×10^{-8}
pGRY4	5.4×10^{-6}
pLES2	1.2×10^{-6}

a) Frequencies obtained are the result of at least two independent experiments.

Figure 2-5. Plasmid content of an E. coli C600R transconjugant harbouring pJD4. Lane 1, E. coli strain DH1 with pUB307 and pJD4; lane 2, E. coli C600R transconjugant with pUB307 and pJD4. Upper band, pUB307; middle band, chromosomal DNA; lower bands, pJD4 (open circular and covalently close circular).


1 2

and E. coli strains is summarized in Table 2-5. Mobilization of pJD4, pJD5 and their in vitro-derived deletion derivatives into N. gonorrhoeae and E. coli with pUB307 occured through a similar process.

Transformation and electroporation of plasmid DNA into N. gonorrhoeae

Transformation and electroporation methods were investigated as an alternative to conjugation for the mobilization of plasmid DNA into N. gonorrhoeae recipients. Plasmids pJD4, pJD5 and in vitro-derived deletion derivatives were used to transform N. gonorrhoeae strains 2152 and NS-757. No transformants were observed, after numerous attempts of transforming N. gonorrhoeae, with the various plasmids.

Electroporation was also assessed for introducing plasmid DNA into N. gonorrhoeae. Two variables were examined for gonococcal electroporation, buffer composition and growth conditions after electroporation (Table 2-6). Electroporation of N. gonorrhoeae strain 2152 with pJD4 did not produce any electro-transformants. However, electro-transformants were detected with E. coli DH1 indicating that conditions for electroporation of that species were adequate. Massive cell lysis of N. gonorrhoeae, detected by an increase in viscocity in the HEPES/sucrose buffer, was observed after electroporation. Gonococcal cells have the ability to produce

Table 2-5. Mobilization phenotypes of pJD4, pJD5 and their *in vitro*-derived deletion derivatives with the conjugative plasmid pUB307.

Plasmids	Presence of locus on the plasmid		mobilization phenotype	
	mob	*oriT*	*E. coli*	*N. gonorrhoeae*
pJD4	yes	yes	yes	yes
pJD5	yes	no	no	no
pJD7	no	yes	no	no
pJD6	no	no	no	no
pJD8	yes	yes	yes	yes
pJD9	no	yes	no	no
pGRY1	no	yes	no	no
pGRY2	no	yes	no	no
pGRY3	yes	yes	yes	yes
pGRY4	yes	yes	yes	yes

autolysins which hydrolyse the peptidoglycan layer of the cell membrane thereby resulting in cell death (Morse and Bartenstein, 1974). The release of autolysin in the environment creates a cascade affect which results in the lysis of more cells. Expression of autolysins is dependent on high temperature, elevated pH and high concentration of cells, conditions similar to the ones used for electroporation (Young et al., 1980). Magnesium chloride, however, reduces cell death by stabilizing the outer membrane (Young et al., 1980). Therefore, a buffer with a pH of 6.4, containing spermine which inhibits autolysins (Young et al., 1980), glucose, sucrose and supplemented with increasing concentrations of $MgCl_2$ was prepared in order to minimize cell lysis by autolysins (Table 2-6). The presence of cations, however, reduced the time constant (time that takes the current to cross 0.2 cm) which in return lowered the total charge applied to the membranes. The net effect was fewer electro-transformants as observed with E. coli DH1 (Table 2-6). In addition to the new buffer, gonococcal cells were washed in GC broth several times to remove any cell debris caused by electroporation and were then either plated on GCMB plates or incubated in 100 mL of GC broth in order to dilute any autolysin present in the media. The presence of magnesium chloride did stabilize gonococcal cells but did not produce any electro-transformants. Neither colony type 2 nor colony type 3 gonococcal cells affected the ability of N. gonorrhoeae strains 2152S and NS-757 to be electroporated.

Table 2-6. Electroporation[a] of N. gonorrhoeae strains 2152 and NS-757 and E. coli strain DH1 with pJD4.

Buffer[b]	N. gonorrhoeae (T_2/T_3)		E. coli[c]
	2152	NS-757	DH1
HEPES/sucrose	0	0	750
GC	0	0	N.D.[d]
GC/0.5 mM $MgCl_2$	0	0	100
GC/4.0 mM $MgCl_2$	0	0	N.D.

a) The collaboration of F. Picard in some of these experiments is gratefully acknowledged.

b) HEPES/sucrose; 272 mM sucrose, 7 mM sodium phosphate buffer, pH 7.4 and 1 mM $MgCl_2$.
GC; 30 mM glucose, 0.5 mM spermine, 300 mM sucrose and 50 mM HEPES, pH 6.4.

c) Number of transformants/ug of DNA.

d) N.D.; Not determined.

DISCUSSION

Conjugation, transformation and electroporation methods were assessed for their ability to introduce plasmid DNA into N. gonorrhoeae strains. Only the conjugative plasmid pUB307 was able to mobilize pJD4, a 7.2 kb penicillinase-producing plasmid of the gonococcus, from E. coli to N. gonorrhoeae. Conjugative plasmids pR100-1, pR124 and pBG791 were unable to mobilize pJD4 into N. gonorrhoeae. These data thereby confirm observations by Piffaretti et al. (1988); it should be noted that, Piffaretti et al. (1988) obtained a frequency of 1×10^{-4} compared to 1×10^{-6} (present work) in mobilizing the 7.2 kb gonococcal plasmid from E. coli to N. gonorrhoeae. This variation in mobilization frequency may reflect differences in gonococcal recipient strains. All conjugation experiments in this study were carried out with recipient strains of the PCU auxotype, an auxotype associated with the absence of plasmids when isolated naturally. This is the first report describing the mobilization of a resistance element from E. coli to a PCU-requiring strain of N. gonorrhoeae. Others (Hannah, 1988; Mavrommati and Tzelepi, 1989) demonstrated that the 7.2 kb and the 5.1 kb gonococcal penicillinase-producing plasmids could be mobilized from N. gonorrhoeae donor strains to PCU-requiring N. gonorrhoeae recipient strains by the 39.2 kb gonococcal conjugative plasmid. They also observed low mobilization frequencies (1×10^{-5}), suggesting that PCU-requiring strains may be less susceptible

for acquiring plasmid DNA through conjugation (Hannah, 1988; Mavrommati and Tzelepi, 1989).

Mobilization of pJD4, pJD5 and their in vitro-derived deletion derivatives by the conjugative plasmid pUB307 permitted the further characterization of the putative oriT and mob loci required for mobilization of pJD4. Fragments carrying these functions were previously identified by independent groups (McNicol et al., 1983; Tenover et al., 1985). This report shows for the first time the necessity for the simultaneous presence of both regions in order to have mobilization of the 7.2 kb penicillinase-producing plasmid of N. gonorrhoeae. It also appears that both regions are required for the mobilization of pJD4 by pUB307 in either N. gonorrhoeae and E. coli.

Using an E. coli system, Tenover et al. (1985) found that a 16 kd protein encoded on a 1.9 kb HinfI fragment, derived from a 7.2 kb penicillinase-producing plasmid, was required for the mobilization of a chimeric plasmid of the 7.2 kb and 4.2 kb plasmids. This protein was functionally similar to the one isolated from the ColEI plasmid and would be required for the nicking and relaxation complex at the origin of transfer of a 7.2 kb plasmid (Tenover et al., 1985; Warren et al., 1978). Based on mobilization results of deletion-derivatives pJD8, pGRY1 and pGRY2, in this study, a 600 bp PvuII/AvaI fragment of pJD4 was identified as an essential part of the mob region. At the present time it is not

known whether the 600 bp fragment encodes for all or part of the 16 kd protein or whether the fragment contains regulatory elements implicated in the expression of the protein. In vitro transcription and translation experiments would be required to determine which of these possibilities is the correct one.

Using an unclassified H. ducreyi transfer plasmid in a H. influenzae system, McNicol et al. (1983) showed that oriT functions of a 7.2 kb plasmid were located on a 1.9 kb HindIII/BamHI fragment of this plasmid. In the present study, pUB307 (IncP conjugative plasmid) appears to utilize the same origin of transfer as the H. ducreyi transfer plasmid. Plasmids pGRY3 and pGRY4 which vary by a 100 bp upstream of the unique HindIII site of pJD4, had a 100 fold variation in their mobilization frequencies when mobilized with pUB307. The partial loss of mobilization properties of pGRY3 permitted the reduction of the putative oriT from the proposed 1.9 kb BamHI/HindIII to several hundred bases downstream of the unique HindIII site of pJD4. One of the two origins of replication (oriV) of pJD4 was also found within the oriT region (Yeung and Dillon, 1988). The physical proximity of the oriT and OriV have been observed on other plasmids such as pSC101, pRSF1010 and pR6K (Nordheim et al., 1980). The proximity of the two origins does not seem to have any functional relationship for either mobilization or replication but has rather arisen by hitch-hiking during plasmid evolution (Finnegan and Sherratt, 1982; Dillon and Yeung, 1989).

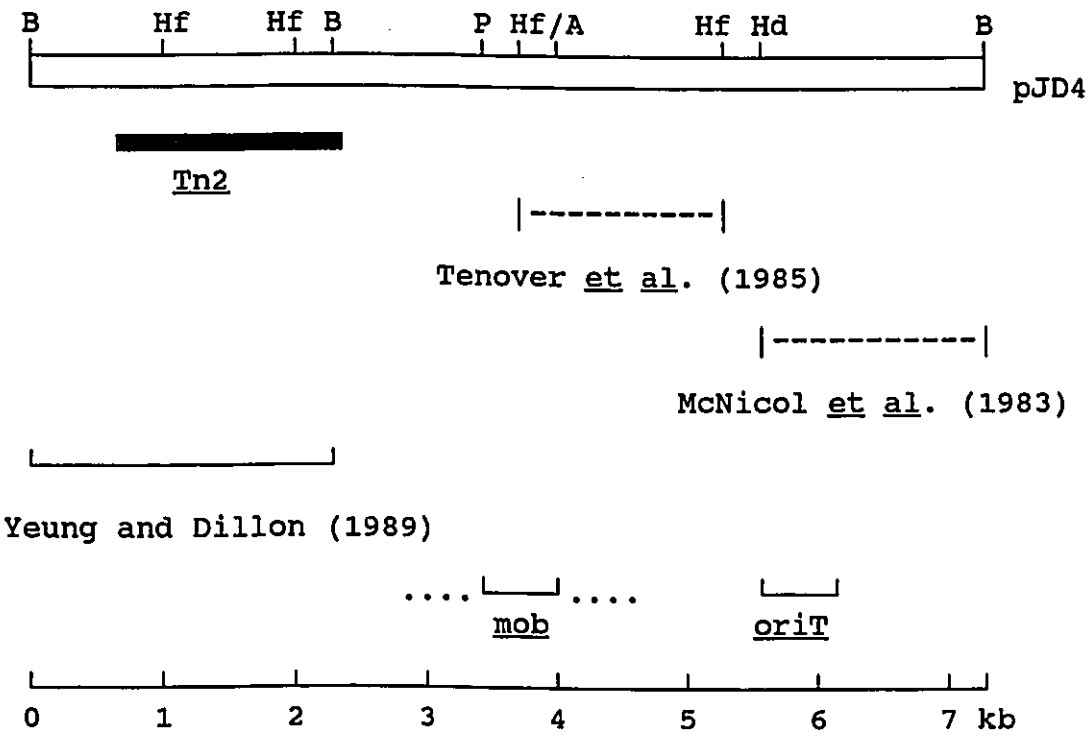
Penicillinase-producing plasmids of N. gonorrhoeae appear to have evolved so that they can be mobilized by several conjugative plasmids in order to propagate in nature. Table 2-7 summarizes what has been achieved, to date, on the mobilization of pJD4 into different host/recipient systems by various conjugative plasmids. The table is accompanied by a map of pJD4 showing critical regions required for the mobilization of this plasmid by either donation or conduction (Figure 2-7). Conjugative plasmids, belonging to different Inc group, use different mob and oriT sites to mobilize non-conjugative plasmids by donation (Willetts and Wilkins, 1984). For example, plasmid pJD5 has lost the origin of transfer (Figure 2-1) but has retained the mob locus. This plasmid was not mobilized by pUB307 (this study) but was mobilized by the endogenous 39.2 kb gonococcal conjugative plasmid from N. gonorrhoeae into both E. coli and the gonococcus (Hannah, 1988; Mavrommati et al., 1989; Roberts et al., 1977). Plasmid pJD7 which has retained the origin of transfer but has lost the mob locus (Figure 2-1) was mobilized by the 40.3 kb tetM gonococcal conjugative plasmid (Sarafian, 1990) but not by pUB307 nor the 39.5 kb plasmid (Table 2-7). These findings indicate that the mob loci, required by pUB307, is also required by the 39.5 kb plasmid of N gonorrhoeae for mobilization. However, the gonococcal conjugative plasmid utilizes a different origin of

Table 2-7. Mobilization phenotype of pJD4 into different host/recipient systems by various conjugative plasmids.

Conjugative plasmids	Region required for mobilization:			Reference
	mob	*oriT*	2.4 kb fragment	
pUB307[a]	yes	yes	no	this study
R100-1[b]	no	no	yes	Yeung (1988)
R124[b]	no	no	yes	Yeung (1988)
pBG791[b]	no	no	yes	Yeung (1988)
pLE2451[c]	yes	no	ND[f]	Tenover *et al.* (1985)
40.3 kb (*tetM*)[d]	no	yes	ND	Sarafian (1990)
pHD147[e]	ND	yes	ND	McNicol (1983)

Mobilization experiments performed between:
a) *E. coli* and *N. gonorrhoeae*
b) *E. coli* strains
c) *N. gonorrhoeae* and *E. coli*
d) *N. gonorrhoeae* strains
e) *H. influenzae* strains.
f) ND; not determined

Figure 2-6. Regions implicated in the mobilization of pJD4 by donation and conduction with conjugative plasmids enumerated in Table 2-7. Fragments harbouring mob and oriT functions were identified in unrelated studies by Tenover et al. (1985) and McNicol et al. (1983) respectively (dash lines). The 2.4 kb BamHI/HindIII region required for mobilization by conduction is also shown (solid line) (Yeung, 1988). The refined mob and oriT loci characterized in this study are depicted by solid lines. Dots extending from each side of the mob locus indicate that the functional mob could span over these regions. Symbols: mob: mob locus; oriT: origin of transfer; Tn2: truncated transposable element. A: AvaI; B: BamHI; Hd: HindIII; Hf: HinfI; P:PvuII.


B
Hf
Hf
B
P
Hf/A
Hf
Hd
B
pJD4
Tn2
Tenover et al. (1985)
McNicol et al. (1983)
Yeung and Dillon (1989)
mob
oriT
0
1
2
3
4
5
6
7 kb

transfer. The mobilization of pJD7 by the 40.3 kb tetM plasmid indicates that this transfer plasmid utilized the same oriT as pUB307 but provided pJD7 a mob protein in trans. In addition to be mobilized by donation, the 7.2 kb penicillinase-producing plasmid of N. gonorrhoeae can also be mobilized by conduction using conjugative plasmids of enteric origin (table 2-7). Therefore, it appears that the 7.2 kb plasmid has evolved and maintained itself within the bacterial world by acquiring independent functions, like a scavenger, in order to adapt to new environments.

It was recently demonstrated that pJD5 could be mobilized from E. coli strain DH1 to strain C600R by the conjugative plasmid pUB307 (K.-H. Yeung, unpublished data). This indicates that an oriT could also be located near the AvaI site of pJD4.

Transformation of gonococcal strains 2152 and NS-757 by plasmid DNA pJD4 was unsuccessful. Studies by Sox et al. (1979) have demonstrated that deletion derivatives of the 7.2 kb plasmid were produced after transformation of an isogenic strain with the 7.2 kb plasmid. The isogenic strain, however, harboured a copy of the 7.2 kb plasmid. They suggested that the transforming DNA which was restricted upon entry into the cell was rescued by the resident 7.2 kb plasmid by marker rescue therefore creating deletion derivatives (Biswas et al., 1982). Marker rescue has also been observed in B. subtilis (de Vos et al., 1981). Hence, marker rescue may explain the occurrence of several deletion derivatives in

nature. In the present study neither N. gonorrhoeae strain 2152 nor strain NS-757 harboured a copy of the 7.2 kb plasmid suggesting that the incoming DNA was restricted by the restriction/modification systems of the strains and degraded.

Although marker rescue could assist in the recovery of a shuttle vector containing cloned genes, this approach could result in a recombinant plasmid with lost or altered cloned genes due to insertion of homologous DNA into the vector. These results led to the conclusion that transformation was not an effective mean for introducing a plasmid vector with a cloned gene into N. gonorrhoeae.

Electroporation of N. gonorrhoeae with plasmid DNA was also unsuccessful. Restriction barriers and non-optimal electroporation conditions probably contributed to the failure to electroporate gonococcal cells. Heat generated by electroporation, high concentration of cells and a basic pH were elements which may have contributed to gonococcal cell lysis by autolysins (Morse and Bartenstein, 1974). A new buffer, with an acidic pH (6.4), containing spermine and magnesium chloride which reduce cell lysis, minimized cell death but did not increase electroporation efficiency. Electroporation was unsuccessful and was, therefore, abandoned as an alternative for introducing plasmid DNA into the gonococcus.

Thus, conjugation using an IncP conjugative plasmid to mobilize pJD4 was determined to be the most successful method of

introducing plasmid DNA into the gonococcus. The smallest mobilizable plasmid, pGRY4 with a molecular weight of 5.7 kb, was chosen to construct the shuttle vector.

CHAPTER THREE

CONSTRUCTION AND TESTING OF THE SHUTTLE VECTOR pGCY1

INTRODUCTION

In order to obtain a functional shuttle vector that can be stably maintained in both E. coli and N. gonorrhoeae, several requirements must fulfilled. The vector must contain one or more selectable markers, contain a positive selection marker harbouring multiple cloning sites, contain no sequences that interfere with the stability of the vector in either organism, be of small size and can be introduced in both E. coli and N. gonorrhoeae by either conjugation, transformation or electroporation.

The most efficient method of introducing plasmid DNA into N. gonorrhoeae was by mobilization of an incoming vector/plasmid by the conjugative plasmid pUB307. The smallest penicillinase-producing plasmid that was mobilized into the gonococcus was pGRY4 (5.7 kb). This plasmid could be stably maintained in both E. coli and N. gonorrhoeae and was therefore selected as the basis for the construction of the shuttle vector. Two approaches were studied for the cloning of a positive selection marker into pGRY4, the lacZ complementation system from E. coli and the xylE gene from Pseudomonas putida.

The lacZ complementation system of E. coli was initially constructed by Messing et al. (1977), who introduced the α subunit of ß-galactosidase into the filamentous phage M13. The ß subunit, harboured on an episome, was complemented by a host strain (Messing

et al., 1977). When transfected with a recombinant phage, the host produced a functional ß-galactosidase which could hydrolyze 5-bromo-4-chloro-indolyl-ß-D-galactoside (X-Gal), supplemented in the media, and release a deep blue 5-bromo-4-chloro-indigo color. In 1981, Messing et al. introduced a multiple cloning site into the α subunit of the M13 construct to produce a versatile cloning vector. The lacZ complementation system has since been used in a variety of cloning vectors and was, therefore, selected as a possible positive selection marker for the shuttle vector. Although not expressed in N. gonorrhoeae, since the gonococcus does not metabolize lactose, this system offers several advantages. The selection of clones would be performed in E. coli, not N. gonorrhoeae, therefore the expression of the system is critical in the enterobacterieceae and not in the gonococcus. Also, the lacZ system possesses a multiple cloning site with a diversity of unique enzymes flanked by two strong promoters which would permit direct sequencing and transcription.

The product of the xylE gene (catechol 2, 3 dioxygenase) which is normally utilized by P. putida in the catabolic pathway of toluene, produces a bright yellow colour when sprayed with cathecol (Franklin et al., 1981). Zukowsky et al. (1983) used this property to construct an expression vector between E.coli and Bacillus subtilis for the identification of DNA fragments that promote gene expression. The assay was found to be rapid, inexpensive, did not require special indicator plates, and offered the advantages of a

genetic indicator test. Catechol 2, 3 dioxygenase is a cytoplasmic protein which is expressed in gram negative microorganisms. This suggests that this protein may be expressed in N. gonorrhoeae. One limitation of this system may be that the requirement for a multiple cloning site could disrupt the gene product.

The novel shuttle vector, pGCY1 constructed with the lacZ complementation system, was tested by cloning gonococcal genes into the multiple cloning site. For this purpose, the proC gene implicated in the proline biosynthesis pathway of N. gonorrhoeae was selected.

MATERIALS AND METHODS

Bacterial strains and plasmids

Bacterial strains and plasmids used in this study are given in Table 3-1. The plasmid pTG402, harbouring the *xylE* gene on a 2.0 kb *Bam*HI/*Xho*I fragment, was kindly provided by D. Speck (Transgène, Strasbourg, France). The plasmid pGFP2, containing the *proC* gene of *N. gonorrhoeae* on a 3.6 kb *Bam*HI fragment, was obtained from F. Picard (Picard and Dillon, 1989). Strains JM101 and JM109 were purchased from Promega Biotech while strain JM83 was provided by N. Bigelow (National Laboratory for Sexually Transmitted Diseases, Ottawa, Ontario). Strain χ^{478} was obtained from the *E. coli* Genetic Stock Center (Yale University, Connecticut).

Media, growth conditions and detection assays

E. coli strains were grown overnight at 37°C. Strains JM101, JM109 were routinely grown on gonococcal auxotyping media lacking proline (Hendry and Stewart, 1979). This was performed in order to ascertain the presence of the F' harbouring the ß subunit of ß-galactosidase. Strains JM83, χ^{478} and HB101 were maintained on TSA plates. Antibiotics, when required, were added at the following concentrations; ampicillin, 100 mg/L; tetracycline, 10 mg/L; kanamycin, 30 mg/L; chloramphenicol, 30 mg/L.

N. gonorrhoeae strain 2152S was grown on GCMB as described

Table 3-1. Bacterial strains and plasmids used in the construction and testing of the shuttle vector pGCY1.

Strain	Relevant phenotype	Source or reference
E. coli		
JM101	*supE*, *thi*, Δ(*lac-proAB*), [F', *traD36*, *proAB*$^+$, *lacI*^{q}*lacZ*ΔM15]	Promega Biotech
JM109	*supE44*, *endA1*, *gyrA96*, *thi*, *relA1*, Δ(*lac-proAB*), *hsdR17*, *recA1*, [F', *traD36*, *proAB*$^+$, *lacI*^{q}*lacZ*ΔM15]	Promega Biotech
JM83	*ara*, Δ(*lac-proAB*), *thi*, *strA*, ϕ80, *lacZ*ΔM15	N. Bigelow
χ^{478}	*proC32*, *leuB6*, *purE42*, *trpE38*, *lysA23*, *metE70*, λ^-	*E. coli* Genetic Stock Center
HB101	F$^-$, *hsdS20* (r_B^-, m_B^-), *recA13*, *ara-14*, *proA2*, *lacY1*, *galK2*, *rpsL20*, (sm^r), *xyl-5*, *mtl-1*, *supE44*, λ^-	Maniatis *et al.* (1982)
N. gonorrhoeae		
2152S	PCU, Sm^r	this study
PLASMIDS		
pGRY4	Amp^r	this study
pGFP2	Amp^r, (*proC* clone)	Picard and Dillon (1989)
pTG402	Amp^r, Cm^r, (*xylE* clone)	Zukowski *et al.* (1983)
pGEM-3Z	Amp^r	Promega Biotech
pUB307	Tet^r, Km^r	Piffaretti *et al.* (1988)

previously (Chapter One). When required, antibiotics were added at the following concentrations: ampicillin, 10 mg/L; streptomycin, 500 mg/L. Transconjugants from E. coli/N. gonorrhoeae matings were selected using streptomycin/ ampicillin-containing media.

Transformants harbouring the recombinant plasmid pGRY4/lacZ (pGCY1) were selected on ampicillin-containing TSA plates supplemented with 20 µL of 50 mg/mL 5-bromo-4-chloro-3-indolyl-ß-D-galactosidase (X-Gal; Promega Biotech) and 100 µL of 0.1 M isopropyl-ß-thiogalactopyranoside (IPTG; Promega Biotech). Colonies expressing the gene turned blue due to the degradation of X-Gal. Strain HB101 recombinants containing pGRY4/xylE were first grown on TSA supplemented with ampicillin and then sprayed with 0.5 M catechol. Recombinants expressing the xylE gene should turn yellow due to the conversion by catechol 2, 3-dioxygenase of cathecol to 2-hydroxymuconic semialdehyde.

Recombinant DNA methodologies

Restriction enzymes BamHI, HaeII, XmaIII, PstI, EcoRI and PvuII were purchased from either Bethesda Research Laboratories or Boehringer Mannheim Biochemicals and used according to the manufacturer's recommendations. Electrophoresis, isolation of DNA fragments from low melting point agarose, preparation of blunt-ended DNA and ligation were performed as described in Chapters One and Two.

The 502 bp HaeII fragment containing the α subunit of the lacZ gene and the 2.0 kb BamHI/XmaIII xylE fragment were extracted from low melting point agarose, after digestion with the appropriate restriction endonucleases, and labelled using the non-radioactive kit from Boehringer Mannheim Biochemicals as described in Chapter One. Transfer of DNA fragments to solid matrices and DNA-DNA hybridizations were also performed as described elsewhere (Chapter One).

Plasmid DNA was transformed into E. coli strains using the method of Miller (1987). Conjugation of pGCY1 between E. coli strain JM83 and N. gonorrhoeae strain 2152S was carried out as outlined in Chapter Two. The alkaline-SDS method of Birnboim and Doly (1979) was used to screen recombinants (Chapter One).

Construction of the shuttle vector pGCY1

1. The lacZ complementation system

The α subunit of the lacZ gene is found on a 502 bp HaeII fragment of the vector pGEM-3Z. In order to retrieve the fragment, plasmid pGEM-3Z was digested with HaeII and resolved on a low melting point agarose gel. The 502 bp was then extracted from the gel.

Plasmid pGRY4 was digested with the restriction endonuclease BamHI at the unique site found on the plasmid (Figure 2-2). This site is found in regions of the plasmid which are not essential for

replication, mobilization or expression of the bla gene (Figure 2-3). pGRY4 and the 502 bp HaeII were rendered blunt-end with Klenow enzyme and ligated together in a molar ratio of 0.5:1.0 (fragment:vector) with T_4 ligase. The mixture was then transformed in either E. coli strain JM101, JM109 or JM83. Transformants were plated on ampicillin-containing media supplemented with X-Gal and IPTG and incubated at 37°C for 24 hrs. Blue colonies were transferred to fresh media.

2. The xylE complementation system

Plasmid vector pTG402 contains the xylE gene on a 2.0 kb BamHI/XhoI fragment derived from the plasmid pWWO (Zukowsky et al., 1983). In order to retrieve the fragment, pTG402 was digested with BamHI and XmaIII which are found at extremities of the gene. The fragments were resolved on low melting point agarose by electrophoresis and the 2.0 kb fragment extracted from the gel.

Plasmid GRY4 was digested with the restriction endonuclease BamHI. The vector and the 2.0 kb BamHI/XmaIII fragment were then blunt-ended and mixed in a molar ratio of 0.5 to 1.0 before being precipitated in 95% ethanol. After ligation, the recombinant molecules were transformed into E. coli HB101 and incubated for 24 hrs at 37°C. Colonies that grew on selective plates were sprayed with 0.5 M catechol.

Cloning of the gonococcal proC gene into pGCY1

Plasmid pGFP2, harbouring the gonococcal proC gene on a 3.6 kb BamHI fragment, was digested with BamHI and the fragments were resolved on a low melting point agarose gel by electrophoresis. The 3.6 kb fragment was isolated from the low melting point agarose and subcloned into the unique BamHI site of the shuttle vector pGCY1. Recombinants were transformed into E. coli strain χ^{478} and plated on proline-deficient plates. Clones containing an active gene should complement the proline deficiency of strain χ^{478}. Recombinants were observed after 72 hrs incubation at 37°C.

RESULTS

Characterization of the shuttle vector pGCY1

1. The lacZ system

When E. coli strain JM83 was transformed with a pGRY4/lacZ ligation mixture, several hundred transformants, which included 5 blue colonies, were obtained. Plasmid profiles revealed that two of the blue colonies were larger than pGRY4. The recombinant plasmids were isolated by caesium chloride-ethidium bromide ultracentrifugation and 0.5 μg of the preparation was digested with PvuII. This restriction endonuclease recognizes two sites within the boundaries of the lacZ gene which will release from the vector a small 379 bp fragment containing part of the gene. Figure 3-1A shows the result of this digestion along with the digestion of pGEM-3Z with PvuII. Both pGRY4/lacZ and pGEM-3Z contained the small 379 bp fragment which was confirmed as the lacZ gene by hybridization (Figure 3-1B). The novel vector was named pGCY1 and is described in Figure 3-2.

The shuttle vector was transformed into E. coli DH1 harbouring the conjugative plasmid pUB307 and mated with N. gonorrhoeae strain 2152S. A mobilization frequency (1.7×10^{-6}) similar to pGRY4 (5.4×10^{-6}) was obtained. The presence of pGCY1 in N. gonorrhoeae was confirmed by lysis of the transconjugants followed by electrophoresis (Figure 3-3).

Figure 3-1A. Characterization of pGRY4/lacZ clones with PvuII. Lane 1, lambda HindIII; lane 2, pGEM-3Z restricted with PvuII; lane 3, pGCY1 (pGRY4/lacZ) restricted with PvuII; lane 4, pGRY4 restricted with PvuII.

Figure 3-1B. Southern blot hybridization of A with a non-radioactive labelled lacZ gene.

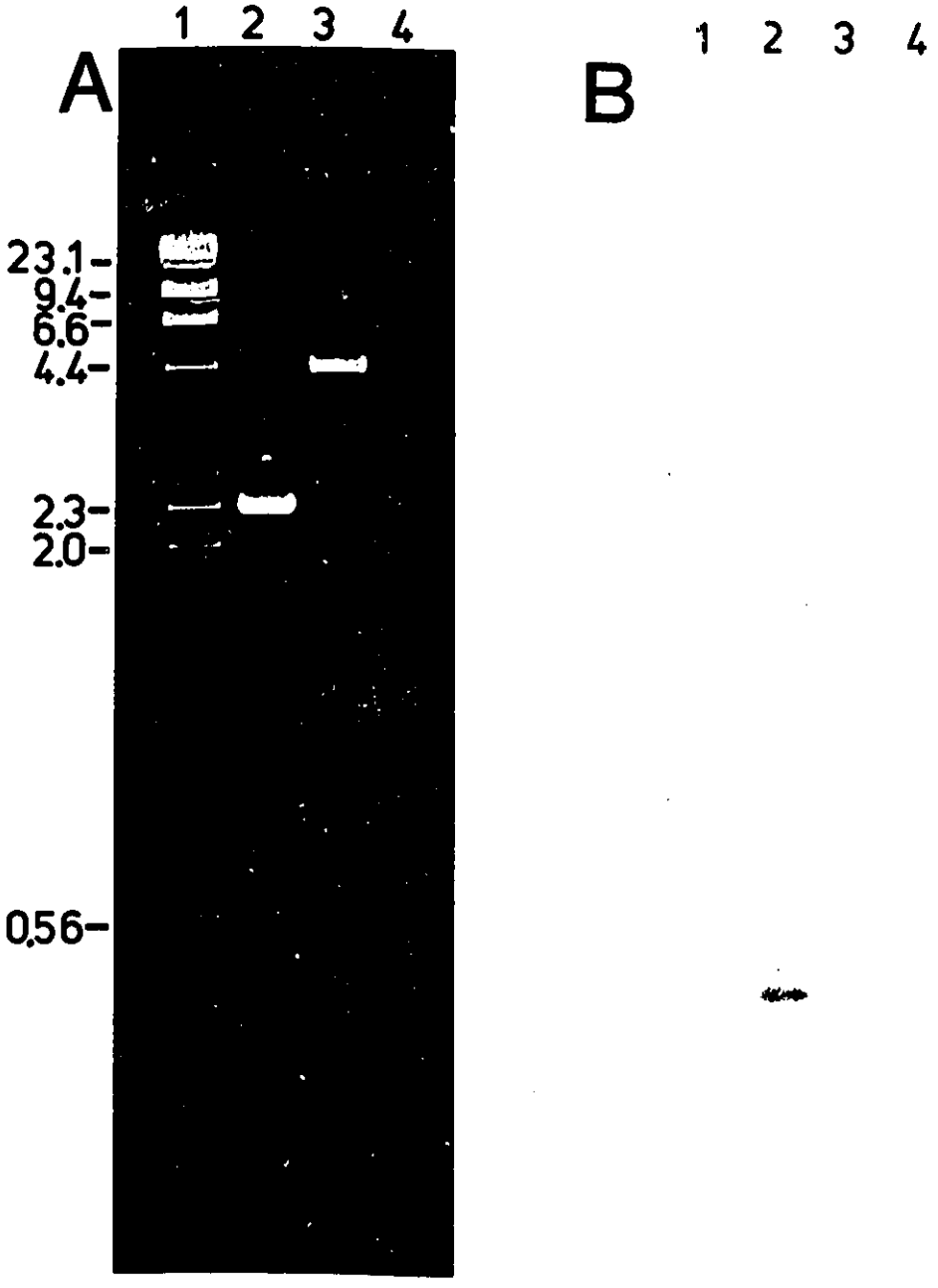

A
1 2 3 4
23.1
9.4
6.6
4.4
2.3
2.0
0.56
B
1 2 3 4

Figure 3-2. Physical map of pGCY1. The putative orientation of the lacZ gene is shown along with the various restriction endonucleases found in the multiple cloning site. mob; mob locus, oriT; origin of transfer.

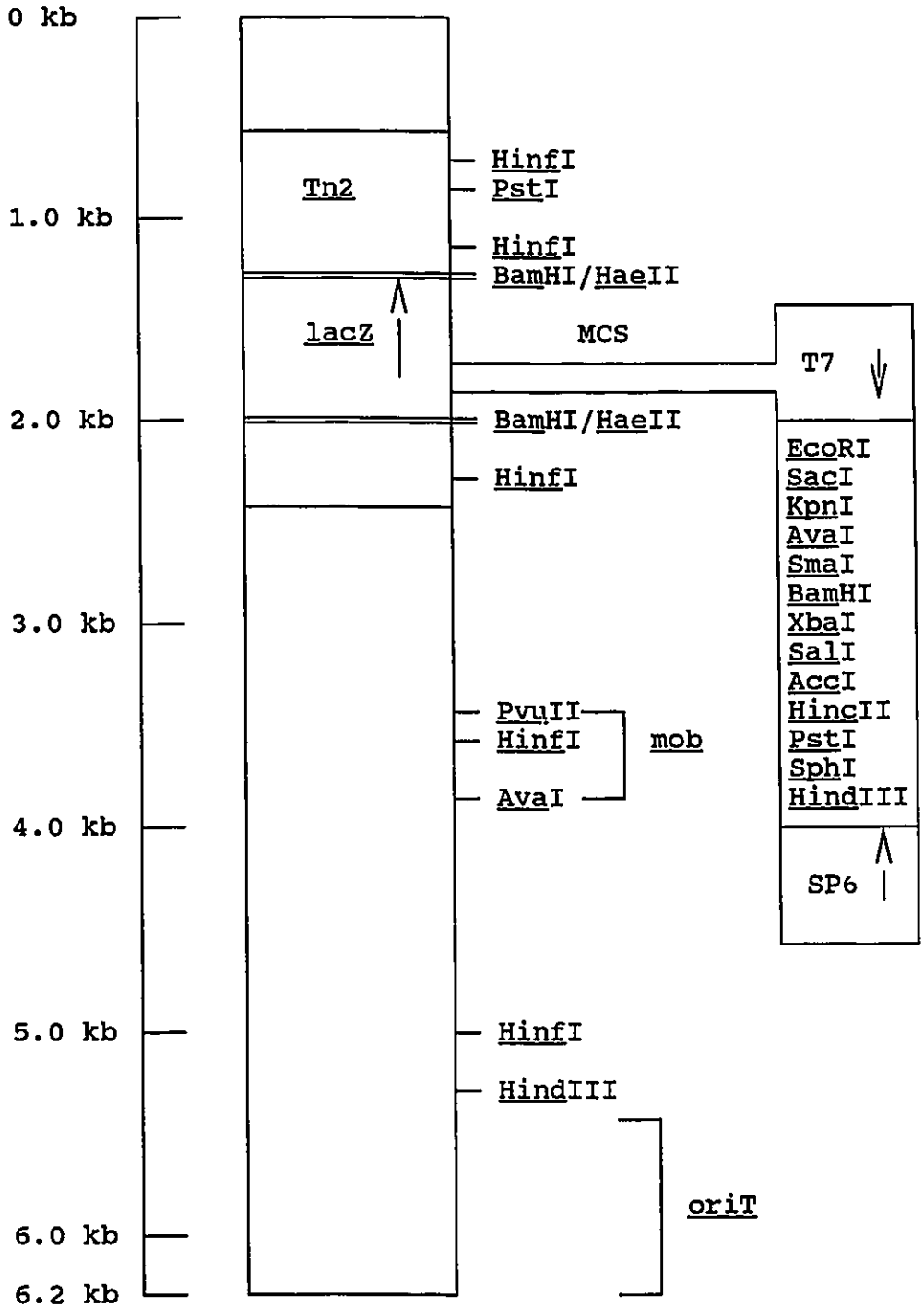
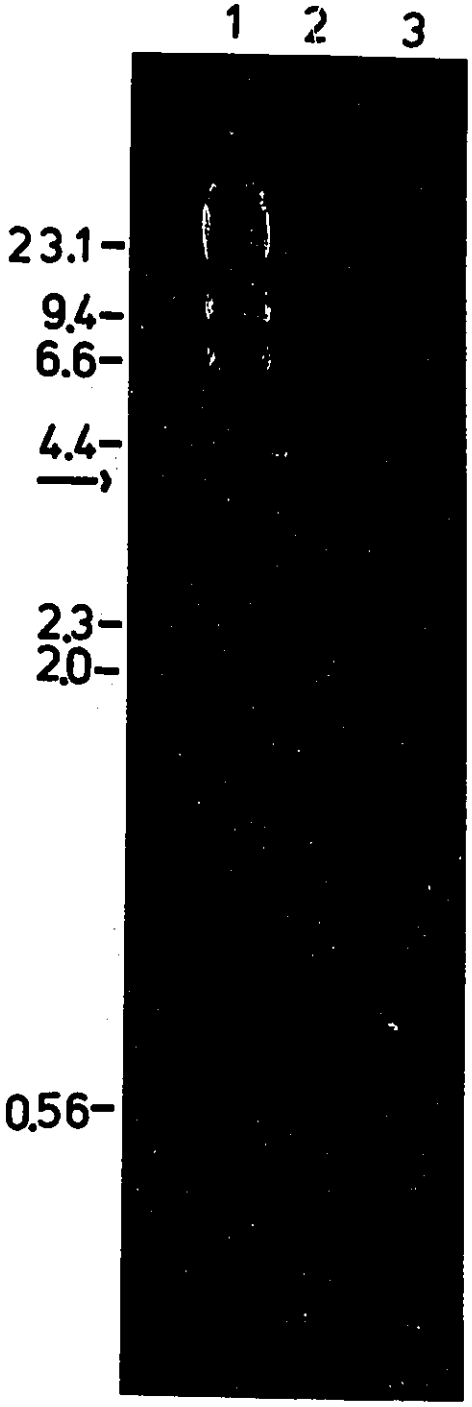

0 kb
1.0 kb
2.0 kb
3.0 kb
4.0 kb
5.0 kb
6.0 kb
6.2 kb
Tn2
lacZ
HinfI
PstI
HinfI
BamHI/HaeII
MCS
T7
BamHI/HaeII
HinfI
EcoRI
SacI
KpnI
AvaI
SmaI
BamHI
XbaI
SalI
AccI
HincII
PstI
SphI
HindIII
SP6
PvuII
HinfI
AvaI
mob
HinfI
HindIII
oriT

Figure 3-3. Plasmid profile of a gonococcal transconjugant harbouring pGCY1. Lane 1, Lambda HindIII; lane 2, DH1/pUB307/ pGCY1; lane 3, 2152S/pGCY1. The arrow points to pJD4 in lanes 2 and 3.


1
2
3
23.1
9.4
6.6
4.4
2.3
2.0
0.56

The orientation of the 502 bp HaeII fragment harbouring the lacZ gene in pGCY1 was putatively determined by double digestion of the plasmid with restriction endonucleases PstI and EcoRI. The two possible orientations of the fragment are depicted on Figure 3-4. Two fragments were generated by the double digestion of pGCY1 with EcoRI and PstI. The larger fragment migrated a distance of 23 mm while the smaller fragment migrated a distance of 48 mm (Figure 3-5). The molecular weights of the fragments were calculated using molecular weight standard curves of lambda HindIII and pBR322 HaeII DNA markers (Table 3-2 and Figure 3-6). The molecular weights of the two fragments were calculated to be 5.0 kb and 1.1 kb. Therefore it appears that orientation A of Figure 3-4 is the correct orientation of the lacZ gene in pGCY1. However, the difference in the size of the fragments between orientation A and B is very small and therefore it is difficult to determine which of the orientation is the correct one. Sequencing of the lacZ/pGRY4 ligated extremities should determine the proper orientation of the gene in pGCY1.

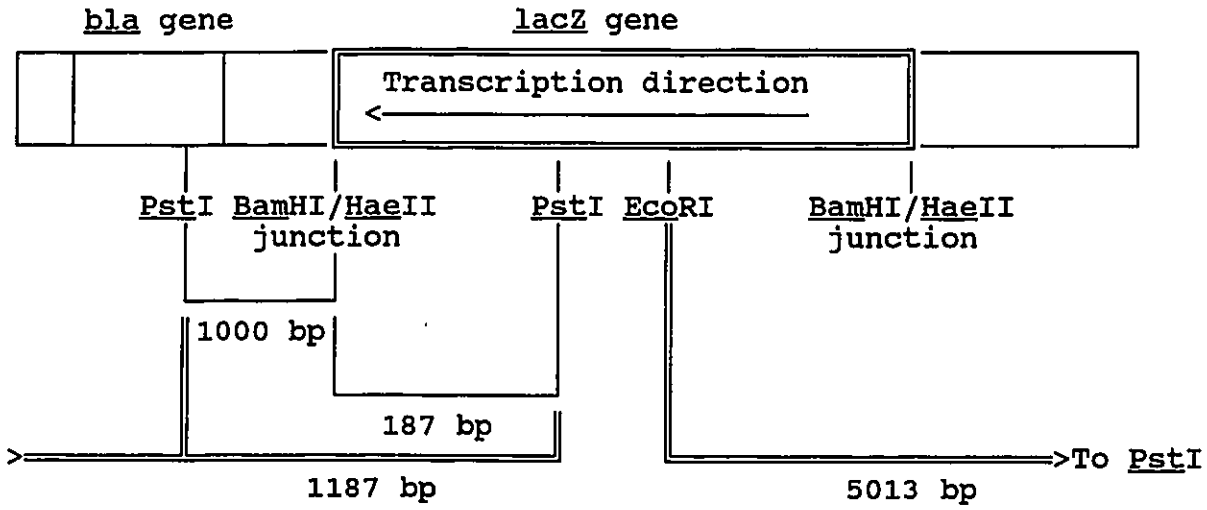
Attempts to transform ligation mixture of pGRY4/lacZ into strains JM109 and JM101 failed to produce transformants. Both strains were, however, transformed by pGEM-3Z indicating that they were competent for genetic transformation. Ligation was confirmed by electrophoresis and transformants harbouring only pGRY4 should have been obtained. This suggested that pGRY4 might

Figure 3-4. The two possible orientations of the lacZ gene in pGCY1.

A) Orientation A should produce two fragments of sizes 1187 bp and 5013 bp.

B) Orientation B should produce two fragments of sizes 1275 bp and 4975 bp. (Figure is not scaled)

A. Orientation A:



B. Orientation B:

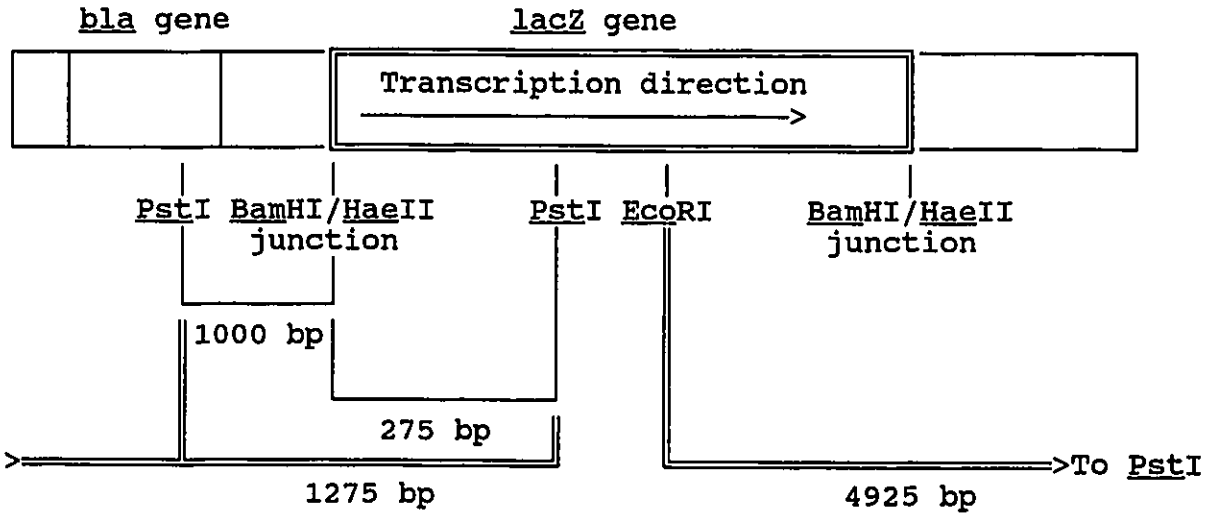


Figure 3-5. Digestion of pGCY1 with EcoRI and PstI. Lane 1, Lambda HindIII; lane 2, pGCY1 restricted with EcoRI and PstI; lane 3, pBR322 HaeII.

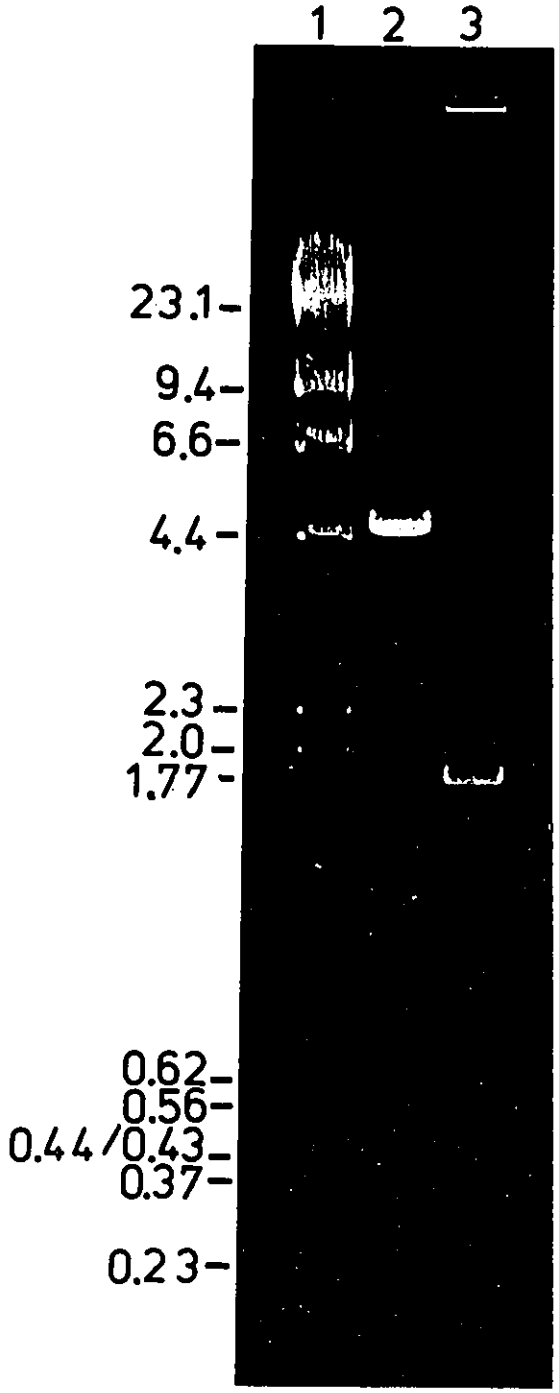
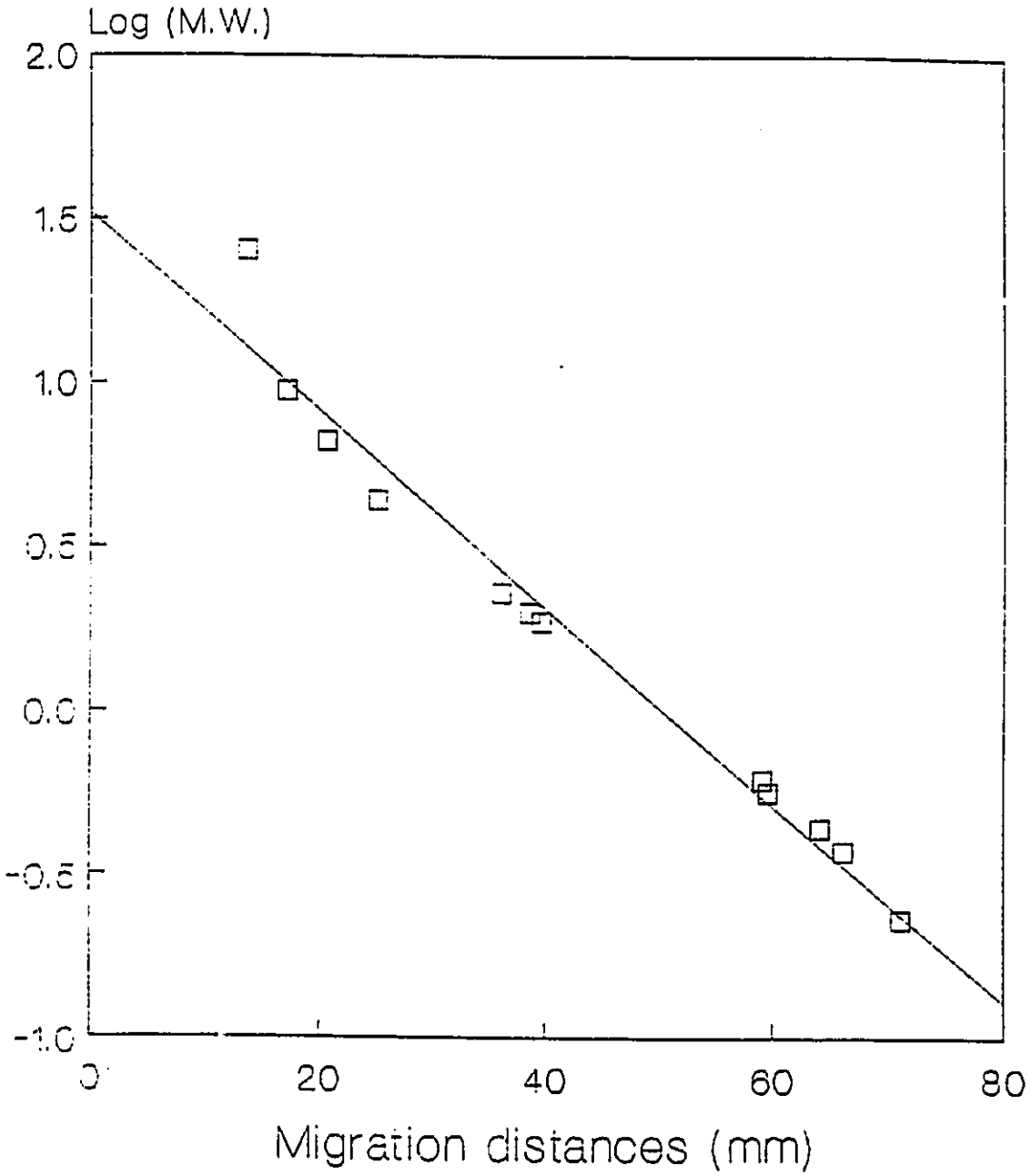

1
2
3
23.1
9.4
6.6
4.4
2.3
2.0
1.77
0.62
0.56
0.44/0.43
0.37
0.23

Table 3-2. Migration distances[a] and molecular weights of lambda HindIII and pBR322 HaeII fragments.

Molecular weight of markers (kb)	Log(M.W.)	Distance migrated by fragments (mm)
Lambda HindIII		
23.1	1.40	13.5
9.4	0.97	17.0
6.6	0.82	20.5
4.4	0.64	25.0
2.3	0.36	36.0
2.0	0.30	38.5
0.56	-0.25	59.5
pBR322 HaeII		
1.876	0.27	39.5
0.622	-0.21	59.0
0.439/0.430	-0.36	64.0
0.370	-0.43	66.0
0.227	-0.64	71.0

a) Refer to Figure 3-5 for migration distances.

Figure 3-6. Standard curves of HindIII-digested lambda and HaeII-digested pBR322 marker DNA. Molecular sizes were in kilobases.


Log (M.W.)
2.0
1.5
1.0
0.5
0.0
-0.5
-1.0
0
20
40
60
80
Migration distances (mm)

not be stable in JM101 and JM109. When JM101 and JM109 were transformed with pGRY4 and pGEM-3Z, transformants were only obtained for plasmid GEM-3Z confirming that pGRY4 was unstable in JM101 and JM109.

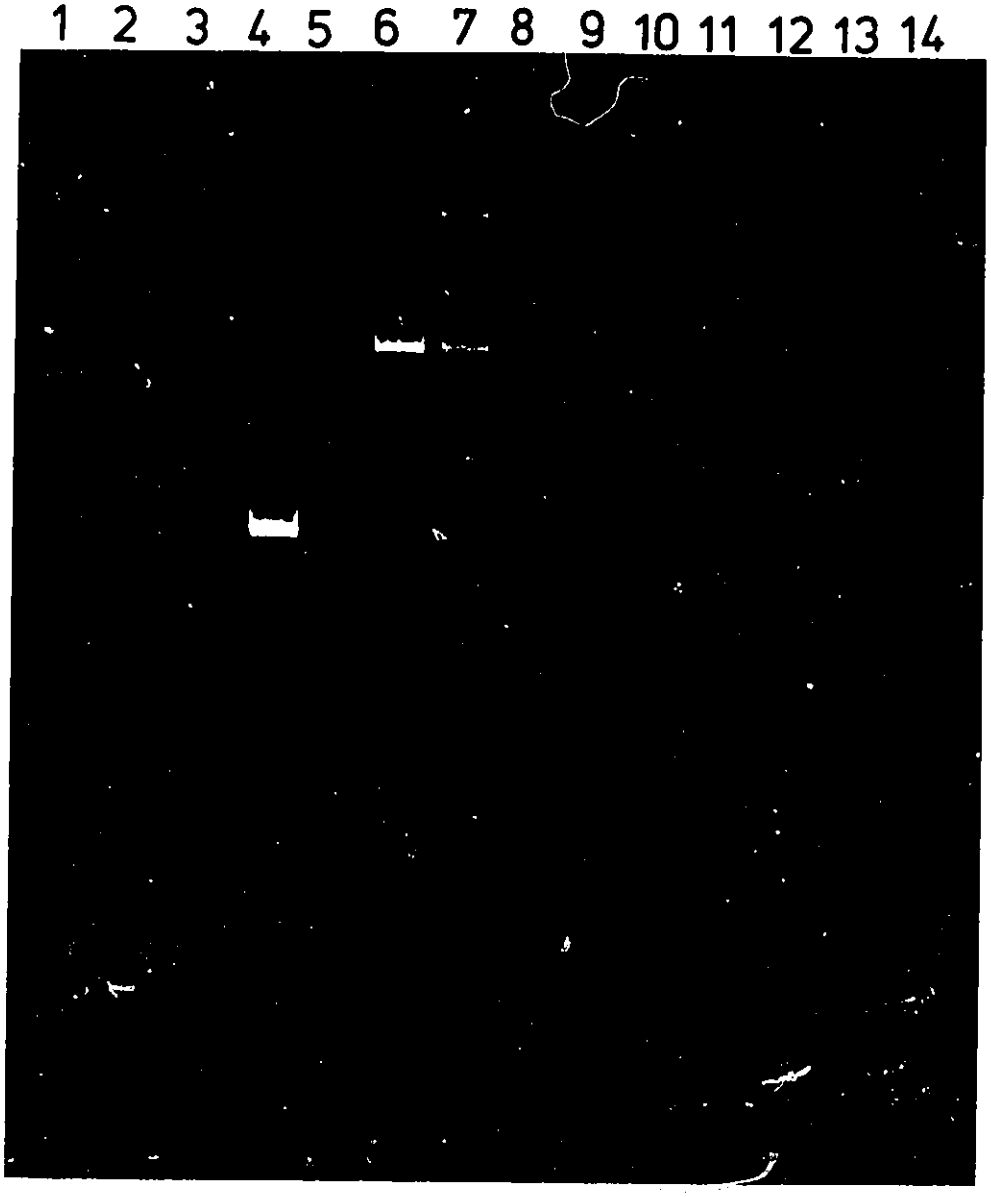
2. The xylE system

Colonies were observed growing on ampicillin selection plates, but these failed to turn yellow after being sprayed with cathecol. Two hundred colonies were lysed in order to determine their plasmid profiles. Figure 3-7 shows the plasmid profiles of 13 transformants, 6 of which contain an unidentified DNA fragment (lanes 4, 6, 7, 9, 11 and 13). Southern hybridization using a xylE probe demonstrated that none of the clones harboured the 2.0 kb fragment of pTG402 (data not shown). These recombinants were not further studied.

Cloning of the gonococcal proC gene into pGCY1

Several unsuccessful attempts were made to clone the proC gene of N. gonorrhoeae into pGCY1. Although transformants were obtained on proline-deficient plates, no plasmid DNA was observed in the clones. These results suggest that the proC gene was integrated into the chromosome of strain χ^{478}. The genotype of the strain is recA$^+$ and restriction$^+$ which strengthens the hypothesis of integration of the gonococcal proC gene into x478 chromosome.

Figure 3-7. Plasmid content of 13 transformants from pGRY4/*xylE* ligation. Lane 1, pGRY4 without an insert. Lanes 2 to 14, pGRY4 with inserts; GRYE-5 (lane 2), GRYE-12 (lane 3), GRYE-18 (lane 4), GRYE-21 (lane 5), GRYE-22 (lane 6), GRYE-25 (lane 7), GRYE-33 (lane 8), GRYE-38 (lane 9), GRYE-39 (lane 10), GRYE-43 (lane 11), GRYE-51 (lane 12), GRYE-52 (lane 13) and GRYE-55 (lane 14).


1 2 3 4 5 6 7 8 9 10 11 12 13 14

DISCUSSION

Two systems were investigated for the cloning of a positive selection marker into pGRY4. Only the lacZ (α subunit) gene was successfully cloned into the plasmid. Although the number of positive clones for the transformation using lacZ recombinants was low, 2 clones were isolated which harboured the lacZ gene. Low melting point agarose interferes with ligation and transformation which would account for low ligation efficiencies. The observation that transformants were obtained with E. coli strain JM83 but not with JM101 and JM109 could be accounted for by the presence of the F' plasmid carried by JM101 and JM109. It is possible that pGRY4 and the F' plasmid are incompatible. This was also observed in M13 cloning experiments where two fragments of 3.2 kb and 2.4 kb, generated by the digestion of pJD4 with HindIII and BamHI, could never be obtained following transformation of the host which contained F'. It was concluded that these fragments contained sequences which were incompatible with the F' plasmid (Gauthier and Dillon, unpublished data). Cloning of the lacZ gene did not appear to alter the mobilization of pGRY4 by the plasmid pUB307 since the mobilization frequency of pGCY1 was similar to pGRY4.

The shuttle vector, pGCY1, offers several advantages over the shuttle vector pLES2. Plasmid pLES2 was derived from a rearranged chimeric plasmid in which critical regions such as replicating and mobilization regions are not characterized (Stein et al., 1983a;

1983b). This plasmid is also very unstable when transformed into the gonococcus (V.L. Clark, personal communication). pGCY1 was constructed from an *in vitro*-derived deletion derivative of pJD4 which is one of the most extensively characterized gonococcal ß-lactamase-producing plasmids. The *lacZ* system which was cloned into pGCY1 also offers a larger number of unique multiple cloning sites than pLES2 (i.e. *Eco*RI, *Sac*I, *Kpn*I, *Sma*I, *Bam*HI, *Xba*I and *Sph*I compared to *Eco*RI, *Sma*I, *Bam*HI and *Sal*I for pLES2). Finally, the multiple cloning site of pGCY1 is flanked by two strong promotors (T7 and SP6) which will permit transcription and sequencing of cloned fragments. This feature is absent in pLES2.

The inability to clone the *xylE* gene into the vector may have either been caused by an over-expression of the cloned gene which resulted in the death of transformants or by the instability of the 2.0 kb fragment cloned in pGRY4. Transformants harbouring larger plasmids were observed, but Southern hybridization of the clones, revealed that the DNA cloned into pGRY4 had no similarity with the 2.0 kb fragment. The absence of *xylE* clones in *E. coli* suggests that transformants harbouring the *xylE* gene did not survive due to over-expression of the gene. In fact, the region of pGRY4 into which the gene was cloned in is surrounded by 5 promotors all active in different organisms (Chen and Clowes, 1987). Two of these, P_a and P_b are promotors used by *E. coli* and *H. influenzae* and are found close to the *Bam*HI site of pGRY4. Therefore, the gene, when cloned into the *Bam*HI site of pGRY4, may have been

constitutively expressed by one of these promotors leading to cell death. The expression of the xylE gene by different promotors has been reported elsewhere, therefore supporting this conclusion (Inouye et al., 1981).

Cloning of a gonococcal gene in pGCY1 has not yet been successful. Attempts to clone N. gonorrhoeae proC gene have failed most likely because of the E. coli host used for selection. E. coli strain x^{478} is a recA$^+$, restriction$^+$ strain derived from E. coli B. Therefore, transforming plasmid DNA, which was purified from an E. coli K-12 derivative may have either been restricted or may have recombined by homologous recombination with the chromosome. The latter would explain why plasmid free colonies grew on media depleted in proline. Therefore, for future cloning, a recA$^-$, restriction$^-$ strain should be selected.

CONCLUSIONS

The overall objective of this study was to construct an efficient shuttle system which would permit the transfer and expression of gonococcal genes in E. coli and N. gonorrhoeae. The shuttle system would consist of a plasmid vector derived from the 7.2 kb penicillinase-producing plasmid of N. gonorrhoeae. The development of an efficient shuttle system required studies on the role of uptake sequences in gonococcal transformation in order to clarify conflicting reports regarding these sequences. As reported by Graves et al. (1982), a 1.5 kb TaqI fragment from the 4.2 kb cryptic plasmid was preferentially acquired by N. gonorrhoeae; however, several strains did not take up the fragment. These strains were transformed by chromosomal DNA and shared common restriction endonucleases with strains that acquired the 1.5 kb TaqI fragment. Competition experiments between chromosomal DNA, the 1.5 kb TaqI fragment and an oligonuclectide comprising uptake sequences suggested that uptake sequences were not universally recognized by gonococcal strains. Thus, it appears that the gonococcus possesses more than one transformation system. This is supported by the present study and previously published data.

Conjugation, transformation and electroporation were assessed as possible modes of introducing plasmid DNA into the gonococcus in order to determine which of these system would be more suitable for the introduction of a plasmid/vector. Conjugation, not transformation or electroporation was found to be suitable for the

introduction of DNA into N. gonorrhoeae. No transformants were observed for transformation of plasmid DNA and this was explained by the restriction/modification systems of strains. Failure to introduce plasmid DNA into N. gonorrhoeae by electroporation was either caused by the restriction/modification systems of strains or inappropriate electroporation conditions.

Mobilization of pJD4, pJD5 and in vitro-derived deletion derivatives by the conjugative plasmid pUB307 permitted the refinement of regions implicated in the mobilization by donation of the 7.2 kb penicillinase-producing plasmid of N. gonorrhoeae. The mob and the oriT loci were shown, for the first time, to be required simultaneously for the mobilization of pJD4 by IncP conjugative plasmids. Plasmid GRY4 (5.7 kb) was found to be the smallest mobilizable plasmid by the conjugative plasmid UB307 and was, therefore, used as the basis for the construction of a shuttle vector.

Results generated from the uptake sequences and mobilization experiments permitted the construction of an efficient shuttle system between E. coli and N. gonorrhoeae. The lacZ gene (α subunit), harboured on a 502 bp HaeII fragment of the vector pGEM-3Z, was successfully cloned into the unique BamHI site of pGRY4 (smallest mobilizable plasmid) to generate the vector pGCY1. Attempts to clone the xylE gene from P. putida failed most likely due to over expression of the gene by one of the promotors of

pGRY4. The shuttle vector pGCY1 offers several advantages over pLES2 such as more unique cloning site and stability in the gonococcus. The vector will be useful in the cloning and characterization of gonococcal genes. Sequencing and unidirectional deletions of cloned fragments will be possible. Finally, pGCY1 will also allow the development of riboprobes for the identification of N. gonorrhoeae isolates.

A shuttle system based on the mobilization of a plasmid vector between E. coli and N. gonorrhoeae was constructed. Plasmid pJD4, from which pGCY1 was derived, can be mobilized into a great number of species (refer to literature review). Therefore, the shuttle system can also be used for species other than E. coli and N. gonorrhoeae.

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