CHARACTERIZATION OF STRUCTURE AND MECHANISM OF MOBILIZATION OF THE AMPICILLIN RESISTANCE PLASMIDS OF *HAEMOPHILUS DUCREYI* AND *NEISSERIA GONORRHOEAE*

A Thesis Presented to the
Department of Medical Microbiology
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of Doctor of Philosophy

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by
Patricia Jean McNicol
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A thesis submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements of the degree of

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This thesis is dedicated to my parents who taught me that one's abilities are limited only by one's fears, and to my husband Rick, who helped me overcome mine.

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Abstract

The plasmids mediating ampicillin resistance in Haemophilus ducreyi and Neisseria gonorrhoeae were characterized with regard to physical structure and maintenance and transfer functions. Restriction endonuclease mapping studies revealed an identity in structure with two exceptions. The plasmids of H. ducreyi contained complete ampicillin transposons, while those of N. gonorrhoeae retained only the portion encoding for TEM-type beta-lactamase. The larger plasmids of H. ducreyi and N. gonorrhoeae contained an insertion element. The plasmids were organized identically with regard to function. Maintenance and transfer regions were located adjacently within common restriction fragments.

While non-conjugative, the plasmids carried a functional OriT site and encoded for the production of OriT site-specific endonuclease(s). The ampicillin resistance plasmids were mobilized efficiently at reproducibly different frequencies by a mobilizing plasmid isolated from H. ducreyi. Hybridization studies detected the presence of homology between the ampicillin resistance and mobilizing plasmids. The frequency of mobilization of the ampicillin resistance plasmids, while independent of the recombination proficiency of the host cell, was correlated with physical structure and with the extent of homology to the mobilizing plasmid.

Since experimental evidence of both this and previous investigations indicated that the ampicillin resistance plasmids have a common origin, most likely in the genus Haemophilus, intergeneric transfer studies were

carried out. Plasmid transfer was effected by conjugation from H. influenzae to N. gonorrhoeae, but at a low frequency and only by the intervention of an intermediate organism.

In vitro coupled transcription-translation of plasmid DNA templates revealed that the ampicillin resistance plasmids encoded several proteins, two of which were also encoded by the mobilizing plasmid. Production of the proteins utilized the entire coding capacity of the ampicillin resistance plasmids. Conversely, only nine percent of the potential coding capacity of the mobilizing plasmid was utilized for the production of proteins. The majority of these proteins was encoded by sequences within the adjacent maintenance and transfer operons.

Introduction.

The functions mediated by plasmid molecules are of particular interest to microbiologists. The ability of plasmids to impart phenotypic traits to the bacterial host cell has far-reaching implications, especially when such traits enhance the pathogenicity of the host cell.

The initial discovery of resistance factors, plasmids mediating resistance to one or more antibiotics, is of significance with regard to the treatment of infectious diseases (Watanabe, 1964). Organisms, previously susceptible to a wide variety of antibiotics, can readily acquire plasmid-mediated resistance. The acquisition of plasmid-mediated ampicillin resistance by Haemophilus influenzae during the early 1970's (Elwell et al., 1975), and subsequently by Neisseria gonorrhoeae in 1976 (Falkow et al., 1976), are excellent examples of the significance of the spread of resistance plasmids to medically important organisms.

The rapid spread of resistance factors is a function of the ability of the plasmid molecules to transfer, either within or between species of bacteria, or to other genera. The transfer of a plasmid can be self-mediated, as is the case with conjugative plasmids. All functions required for transfer are encoded by the molecule itself. However, if a plasmid is non-conjugative, then transfer can be mediated by a mobilizing plasmid co-resident, if only transiently, in the host cell. The non-conjugative plasmid plays either a limited role in transfer, or no role at all.

The mechanism by which a resistance factor is transferred is important in terms of plasmid dissemination. If the plasmid is conjugative, dissemination is a function of the molecule's maintenance requirements and of the host cell's ability to participate in conjugation. If the plasmid is non-conjugative, then dissemination is additionally dependent upon the presence

of a functional mobilizing plasmid.

Dissemination of a plasmid molecule is also dependent upon the ability of the host cell to form a stable mating aggregate (Clark and Warren, 1979). Mating-pair formation is dependent upon many factors, including the presence of specific surface receptors. For this reason, it is not surprising that bacterial cells of different species or genera do not necessarily participate in conjugation. Thus the inability of a host cell to establish a conjugative aggregate acts to limit plasmid dissemination.

Since a plasmid molecule is dependent upon host cell replicative enzymes for maintenance (Bazaral and Helinski, 1970), the plasmid must also carry the appropriate recognition sequences for these enzymes. If the sequences are not recognized by the enzymes of a wide range of potential host cells, then dissemination of the plasmid is limited.

This investigation uses the tools of molecular biology to characterize two unique groups of plasmid molecules. Haemophilus ducreyi, the etiological agent of chancroid, harbours several conjugative and non-conjugative plasmids (Brunton et al., 1979; Albritton et al., 1982; Deneer et al., 1982; Albritton et al ., 1984). Three of these plasmid are of particular interest to this study. Two of the plasmids mediate resistance to ampicillin (Brunton et al., 1979). While non-conjugative, these plasmids can be mobilized by the third plasmid also isolated from H. ducreyi (Deneer et al., 1982). These three plasmids comprise the first group of plasmids under study.

The second group of plasmids under investigation, was isolated from Neisseria gonorrhoeae, the etiological agent of gonorrhea. Two plasmids in this group are non-conjugative and mediate resistance to ampicillin (Perine et al., 1977). The third plasmid in this group is a conjugative plasmid capable of mediating mobilization of the ampicillin resistance plasmids

(Roberts et al., 1979).

The mobilizing plasmid isolated from H. ducreyi is also capable of mediating mobilization of the ampicillin resistance plasmids of N. gonorrhoeae, as well as those of H. ducreyi (Deneer et al., 1982). Brunton et al. (1979) demonstrated that the ampicillin resistance plasmids of H. ducreyi and N. gonorrhoeae are structurally related, yet the extent of relatedness is unknown.

These two groups of plasmids lend themselves to intensive investigation for several reasons. The ampicillin resistance plasmids encode for the production of a beta-lactamase enzyme (Brunton et al., 1979). Production of this enzyme renders the host cell refractory to the effects of ampicillin and related antibiotics, previously the drugs of choice for treatment of chancroid and gonorrhea. Since the ampicillin resistance plasmids of H. ducreyi and N. gonorrhoeae are capable of undergoing mobilization, transfer of these plasmids may result in plasmid-mediated ampicillin resistance in previously sensitive, clinically significant organisms.

This investigation was designed to answer several important questions regarding the structure and function of the ampicillin resistance and mobilizing plasmids.

The extent of the structural relatedness of the ampicillin resistance plasmids was determined, both by restriction mapping, and by determination of the functional organization of the plasmid molecules. Particular attention was paid to identifying the location of transfer and maintenance functions, important functions for plasmid dissemination.

The mechanism of transfer of these plasmids was also of interest. While capable of undergoing mobilization mediated by the mobilizing plasmid of H. ducreyi, it was not known if transfer occurred by an *in trans* or an *in cis* mechanism. Mobilization studies were conducted *in vitro*, to discern

the mechanism by which transfer is occurring.

The influence of physical structure of the ampicillin resistance plasmids upon transfer frequency was unknown. To investigate this question, studies were undertaken to determine if structural differences between the plasmids could be correlated with differences in transfer frequency. Concomittant with this investigation, the extent of homology between the ampicillin resistance plasmids and the mobilizing plasmid was determined.

A structural relationship between the ampicillin resistance plasmids has already been demonstrated (Brunton et al., 1979). The similarity in physical structure may indicate that the plasmids arose in the same genus. Studies were carried out to determine the host range of the ampicillin resistance and mobilizing plasmids with specific emphasis upon transfer between the genera of Haemophilus and Neisseria.

The mobilizing plasmid of H. ducreyi was characterized with regard to structure, by restriction endonuclease mapping and by the identification of the location of transfer and maintenance functions. The extent of homology between the mobilizing plasmids of H. ducreyi and N. gonorrhoeae was determined.

Finally, the number and molecular mass of proteins encoded by these plasmids was determined by the use of in vitro techniques for the transcription and translation of plasmid DNA templates. Particular attention was paid to the identification of those proteins encoded by the transfer and maintenance regions of the plasmid molecules.

The findings of this investigation provide some insight into the functioning of the ampicillin resistance plasmids of H. ducreyi and N. gonorrhoeae, and of the mobilizing plasmid of H. ducreyi in particular. This information may be applicable to plasmid molecules in general.

Literature Review

A. The Plasmid Molecule - Origin and Evolution

1. What is a Plasmid?

Has the gene displaced the cell as an ultimate unit of life? This question was raised by J. Lederberg (1952) in the same communication in which he proposed the generic term plasmid, to describe any extrachromosomal hereditary determinant. The term replaced several others within the literature. Lederberg visualized a plasmid to be an organism living in symbiosis with the host cell. Plasmid replication was independent of chromosome replication. The plasmid was stably maintained by the host cell, yet encoded functions that were non-essential for survival of the cell. Novick (1980) also felt that plasmids were self-sustaining subcellular organisms. He believed that plasmids were independently evolving and more or less on an equal evolutionary footing with viruses. He based his opinion on three distinct characteristics of plasmid molecules. Plasmids were capable of regulating copy number, and thus, unlike the chromosome, were often present in multiple copies within the host cell. Conjugal exchange of plasmids occurred between organisms unable to exchange chromosomal genes. This transfer was dependent upon functions encoded by the plasmid molecules and was independent of functions encoded by the host cell. Finally, conjugal transfer between competing species potentially could enable recipients to survive at the expense of the donor population, a phenomenon not likely to occur if the plasmid molecule was under control of the host cell. In essence, plasmids had evolved the ability to survive even at the expense of the host population.

2. The Isolation and Possible Origin of R-factors.

The significance of the presence of plasmids within bacterial cells was not fully realized until the clinical implications were manifested. Until that point, plasmids had merely been an academic curiosity. The first plasmid encoding multiple resistance to antibiotics was isolated in 1959, after an outbreak of bacillary dysentery in Japan (Watanabe, 1964). The plasmid encoded resistance to four antibiotics, sulfonamide, streptomycin, chloramphenicol and tetracycline, and was capable of transfer between intestinal bacteria, both in vitro and in vivo. By providing a non-essential yet useful function of antibiotic resistance to the host, the plasmid enabled the cell to occupy a particular ecological niche, unaccessible in the absence of the plasmid. Due to the encoded resistance to antibiotics, these plasmids were referred to as resistance factors, or R-factors (Watanabe, 1963). Each R-factor was composed of a portion mediating transfer, the resistance transfer factor (RTF), and a portion carrying resistance genes, the R determinant. The RTF portion of several R-factors shared extensive homology as determined by heteroduplex analysis (Cohen, 1976). Conversely, the R determinant portions were unique. These observations were an indication that each R-factor was composed of regions of separately evolving DNA. Indeed, reversible dissociation of the two portions of the R-factor had been observed. Each portion was capable of existing as an independent replicon co-habiting the host cell (Watanabe, 1971).

3. The Role of Insertion Elements in Plasmid Evolution.

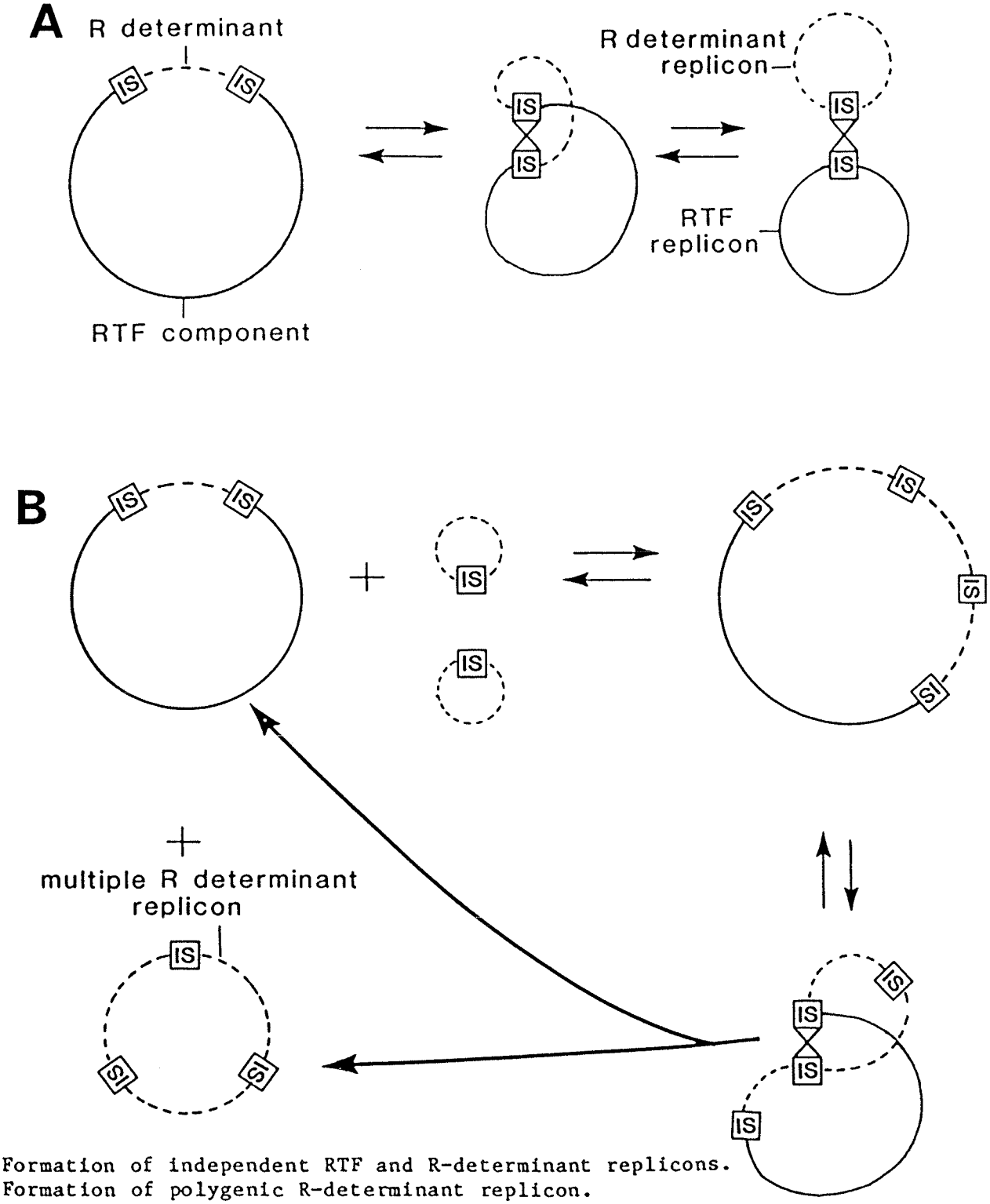
The origin of R-factors was obscure. Watanabe and Fukasawa (1960), hypothesized that multiple drug resistance genes carried on R-factors were acquired in a single step from the chromosome of some unknown bacteria. Since the acquisition of four linked resistance genes was not a probable

occurrence, Falkow et al. (1966) proposed two additional hypotheses. The first envisioned acquisition of resistance genes separately, by a series of recombinational events. The second hypothesis predicted that unique drug resistance determinants were acquired by different R-factors. R-factors encoding multiple resistances were subsequently formed by recombinational events between R-factors co-habiting the same host cell. This second hypothesis proved to be more accurate, when one considered the role of insertional elements in plasmid evolution.

An insertional element (IS element) could be defined as a segment of DNA capable of movement between DNA molecules lacking homology with each other or with the element itself. As IS elements did not require homology for insertion, these elements were capable of joining segments of unrelated DNA. Because of this capability, IS elements have been implicated as a major component in plasmid evolution (Cohen and Shapiro, 1980). IS elements were often found at the boundaries of RTF and R determinants of R-factors. Their presence facilitated the reversible dissociation of the two components into separate replicons as illustrated in Figure 1.

If bounded by IS elements, any gene was capable of transfer by a non-homologous recombination event, completely independent of the recombination system of the host cell. In this way, genes mediating antibiotic resistance could move from one replicon to another. Resistance genes bounded by IS elements are known as transposons (Sherratt, 1982). Several transposons mediating resistance to many different antibiotics have been described (Table 1.). The movement of transposons mediating antibiotic resistance between replicons was a significant step in the evolution of R-factors with multiple R determinants.

Figure 1. Proposed mechanism for reversible dissociation of cointergrate R plasmids at sites of IS elements[1].



A. Formation of independent RTF and R-determinant replicons.
B. Formation of polygenic R-determinant replicon.
1. Modified from Cohen, 1976.

Table 1. Transposons mediating antibiotic resistance[1].

Designation of Tn Elements.

Element	Plasmid Origin	Resistance[2] markers
Tn1	RP4	Ap
Tn2	RSF1010	Ap
Tn3	R1	Ap
Tn4	R1	Ap Sm Su
Tn5	JR67	Km
Tn6	JR72	Km
Tn7	R483	Tp Sm
Tn9	pSM14	Cm
Tn10	R100	Tc

1. From Cohen, 1976.

2. Ap ampicillin; Sm streptomycin; Su sulphonamide; Km kanamycin; Tp trimethoprim; Tc tetracycline.

4. R-factors Pre-date the Antibiotic Era.

R-factors have been isolated from organisms stocked prior to the introduction of antibiotics. Smith (1967) described the presence of R-factors in an Escherichia coli strain first isolated in 1937. Similarily, R-factors were isolated from the gut flora of human populations far removed from civilization (Mare, 1968). Because of these observations, it was obvious that R-factors did not originate as a result of the introduction of antibiotics, however, the rapid spread of R-factors has been facilitated by the often indiscriminate use of antibiotics in animal husbandry and in human and veterinary medicine.

It has been hypothesized that resistance determinants originated as a defence mechanism for organisms producing antibiotics (Davis et al. 1977). Benveniste and Davis (1973) have shown that antibiotic-modifying enzymes of certain Actinomycetes species, natural producers of several antibiotics, were similar to those responsible for plasmid-mediated drug resistance in some enteric bacteria. Thus antibiotic resistance genes were already present in nature prior to their acquisition by clinically significant micro-organisms.

5. Plasmid Replication Function.

Early steps in the evolution of plasmids by necessity involved formation of an origin of vegetative replication site and acquisition of genes specifying the replication function (Cohen, 1976). Excision of specific DNA replication segments from the bacterial chromosome may represent the principle mechanism by which plasmids evolved as independent replicons. Subsequent linkage to genes providing a biological advantage to the host cell would promote proliferation of the plasmid.

While plasmid replication was dependent to a great extent upon host cell enzymes (Bazaral and Helinski, 1970), the molecule maintained two essential features. Each molecule had an origin of vegetative replication (OriV) site as well as a control system regulating the frequency of initiation of replication.

The replication region of a plasmid is a unique sequence of DNA of approximately 450 base pairs in length (Churchward et al., 1983). Within this region are loci encoding functions for plasmid maintenance. The par locus is responsible for partitioning of plasmids between daughter cells. The rep locus, in the case of plasmid pSC101, encodes a protein required for initiation of DNA synthesis. Finally, the inc locus is responsible for incompatability between related plasmids co-existing in a host cell. The incompatability function in plasmid pSC101 and also in ColE1 (Tomizawa and Itoh, 1981) was shown to be due to production of a small RNA molecule. This molecule was a trans-acting repressor of replication, specifically inhibiting the priming of DNA replication. The RNA molecule competitively inhibited the binding of an RNA primer sequence at the OriV site.

The OriV site of plasmid pSC101 is a region rich in A-T base pairs, which facilitates easy separation of strands for initiation of replication. The site also contains three direct repeats of 18 base pairs in length. Churchward et al. (1983) hypothesized that the repeats are recognition sites for proteins involved in DNA replication. Conserved sequences have been found at the OriV site of several plasmids. This was not a surprising observation when one considered that all plasmids were essentially dependent upon host cell enzymes for replication. Therefore, the specific recognition sites for these enzymes had to be present on all plasmid molecules.

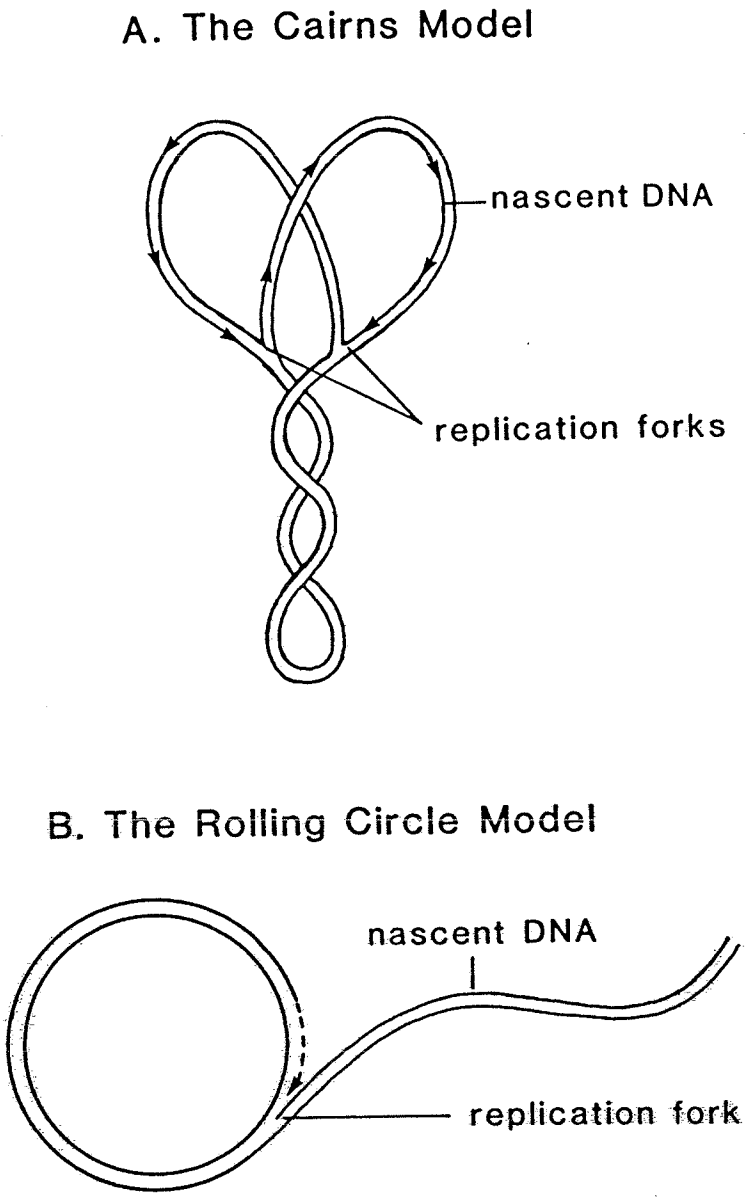
Plasmid molecules have been observed to undergo replication by two distinct mechanisms. The first mechanism, proposed by Cairns (1963), envisioned semi-conservative replication of an open circular plasmid structure. Replication proceeded either uni- or bi-directionally about the plasmid molecule from one or two replication forks respectively. The duplex helical structure was unwound in front of the replication fork as it moved along the molecule (Fig. 2-A).

The second model, proposed by Gilbert and Dressler (1968), was referred to as the rolling circle model. In this model, a round of replication was initiated by nicking of a single strand of the DNA duplex at the OriV site. The free 3' terminus was used as a primer for strand synthesis. Nascent DNA was synthesized forming a molecule of several genomes in length. Thus, the DNA molecule had to be cleaved into genome length fragments, then recircularized, prior to partitioning to daughter cells (Fig. 2-B).

Replication intermediates of several plasmids have been studied by electron microscopy. By measuring the length of replicated and unreplicated arms of a molecule cleaved at a unique site, it was possible to determine both the position of the OriV site and the direction of replication (Lovett et al., 1974). Some plasmids, such as R6K, had more than one OriV site, while ColE1 had only one. Similarly, some plasmids replicated uni-directionally, while for others, replication was bi-directional. The reason for this variance was unknown.

Recent advances in the study of plasmid replication have been made through the use of in vitro replication systems. The groundwork for these studies was laid by the research of Sakakibara and Tomizawa (1974). They determined that exogenous DNA could be replicated in cell extracts supplemented with nucleotides and other co-factors. Using plasmid ColE1 as a

Figure 2. Models for plasmid replication[1].



1. Modified from Broda, 1979.

template they showed that in vitro replication of this plasmid proceeded in a semi-conservative fashion. Products of complete replication were isolated as super-coiled structures, while replicative intermediates were isolated in an open-circular form. They also determined that replication of ColE1 was dependent upon the presence of DNA polymerase I in the cell extract.

Conrad et al. (1979) were able to determine the position of the OriV site and the direction of replication of plasmid RSF1030 using an in vitro replication system. Replication was limited by the addition of 2',3'-dideoxythymidine-5'-triphosphate (ddTTP) to the system, in conjunction with radio-labelled nucleotides. Once ddTTP was incorporated, DNA chain elongation was terminated. Replicative intermediates were isolated and digested with restriction endonucleases. The order in which the fragments were labelled in vitro confirmed the uni-directional mode of replication for this plasmid. In vitro replication gave researchers control over components of the replication system. In this manner, many of the requirements for plasmid replication were determined.

6. Plasmid Transfer Function.

Later stages in the evolution of plasmids most likely included the acquisition of genes to facilitate transfer. The transfer function was of value to the plasmid molecule as transfer represented another mechanism for replication. It was possible for the plasmid to replicate in the sense of vertical transmission to daughter cells upon division of the host cell. However, the ability to transfer, allowed horizontal replication by conjugal passage to other host cells.

Genes responsible for transfer were clustered on the molecule, occupying at least 20 kilobases of the genome (Sherratt, 1982). Mobilizable

non-conjugative plasmids also devoted coding capacity to transfer functions. Approximately 30% of the genome of these plasmids was so occupied.

An essential part of the transfer region was the origin of transfer (OriT) site. This specific DNA sequence was the site of several major biochemical events in plasmid transfer. It was the site for nicking of the DNA duplex as a pre-requisite to transfer. The nicked single strand was conducted to the recipient from this point. The OriT site was also the point for initiation of conjugal DNA synthesis and recircularization in the recipient.

Guiney and Yakobson (1983) identified the OriT site of plasmid RK2 as a 112 base pair sequence. However, for full activity of the site, sequences adjacent to it were required. The OriT site of RK2 contained inverted repeats able to form a cruciform structure which may function in recognition of the site by transfer proteins.

The OriT sites have been sequenced for a few plasmids. The sites in plasmids ColE1 and pBR322 were highly conserved (Clark and Warren, 1979). The sequences for the OriT sites for these two plasmids are presented in Figure 3.

Several plasmids have proteins associated with the OriT site, forming DNA-protein relaxation complexes. Clewell and Helinski (1969) isolated relaxation complexes for plasmid ColE1. They found that agents known to affect protein structure, such as sodium dodecyl sulfate (SDS), induced a change in the structure of the relaxation complex. When the relaxation complex of ColE1 was incubated in the presence of SDS, the covalently closed circular (ccc) form of the complex was altered to an open circular (oc) conformation. Lovett et al. (1974) confirmed these findings and also determined that plasmid ColE2 and R6K could be isolated as DNA-protein relaxation complexes. They noted that treatment with SDS induced a change in the conformation of these plasmids as well.

Figure 3. Nucleotide sequence in the regions of the OriT sites of plasmids ColE1 and pBR322[1].

ColE1

```
GGGGCGCAGC[ 36 intervening ]TGGCTTAACCA
CCCCGCGTCG[ base pairs     ]ACCGAATTGGT
```

pBR322

```
GGGGCGCAGC[ 36 intervening ]TGGCTTAACTA
CCCCGCGTCG[ base pairs     ]ACCGAATTGAT
```

1. Modified from Clark and Warren, 1979.

The protein components associated with the ccc form of the ColE1 relaxation complex were 60,000, 16,000, and 11,000 daltons in mass. However, after treatment with SDS, only the 60,000 dalton protein remained. It was covalently attached to the 5' end of the nicked strand.

A DNA-protein complex has also been isolated for the 4.4 Mdal ampicillin resistance plasmid of Neisseria gonorrhoeae (Guiney and Ito, 1982). However, the OriT site was not localized within the plasmid genome, nor were studies carried out on the bound proteins.

Kupersztoch-Portnoy et al. (1974) proposed two models to describe DNA-protein relaxation complexes. In the first model, ccc DNA was associated at the OriT site with an endonuclease. The endonuclease was activated by agents which denature protein causing a nick in a single DNA strand at the OriT site. In the second model, the DNA pre-existed in a nicked form that was held together by proteins. The first model was probably the more accurate, since Kupersztoch-Portnoy et al. (1974) found that although protein could be dissociated from the relaxation complex by treatment with a high concentration of salt, the DNA remained in a ccc conformation.

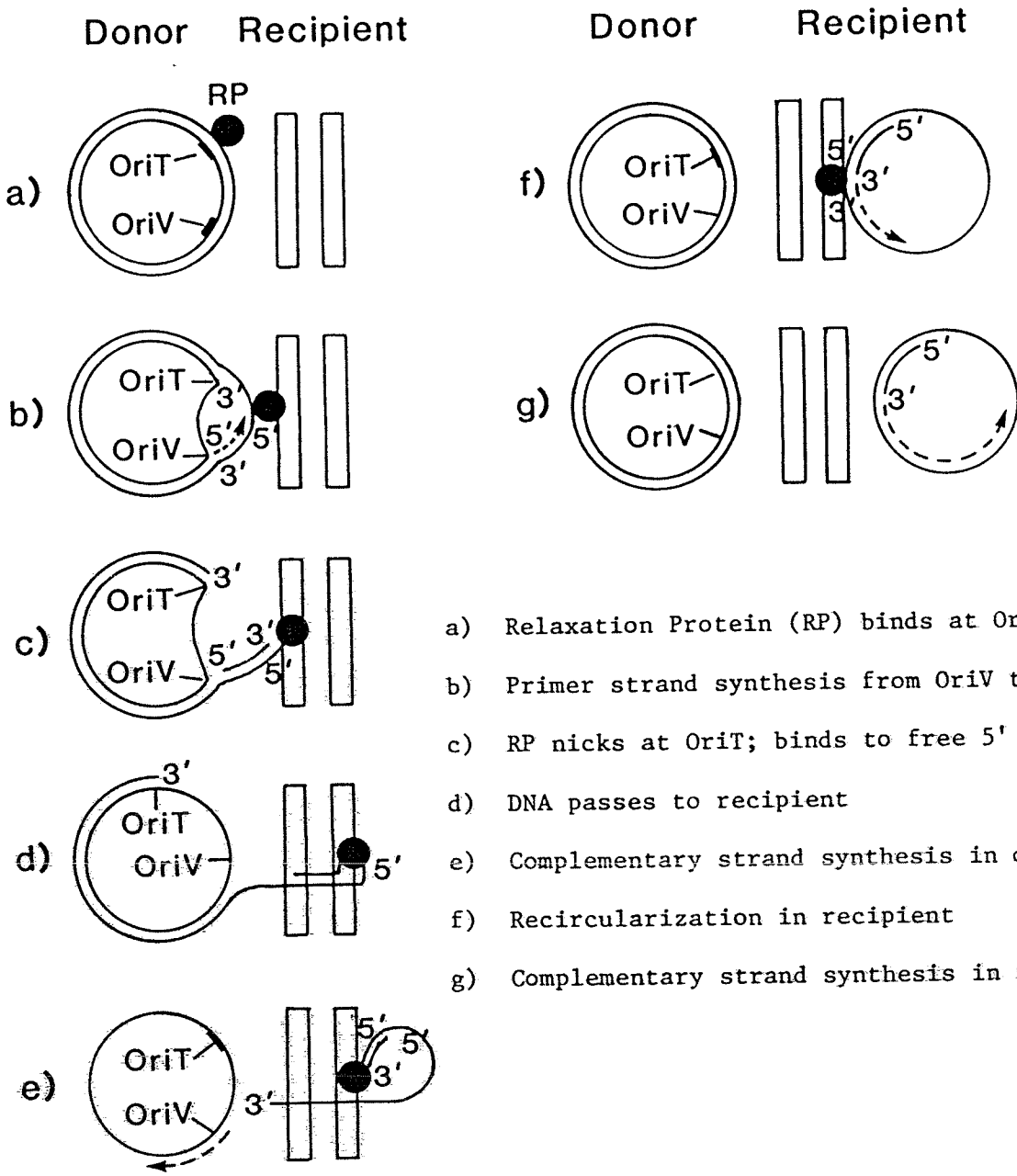
Nordheim et al. (1980) developed a technique for specifically identifying the location of the OriT site within the genomes of plasmids isolated as DNA-protein relaxation complexes. The DNA was specifically nicked at OriT by treatment of the complex with SDS, then a limited nick-labelling procedure was carried out. The nicked DNA was incubated with radio-labelled deoxynucleotides in the presence of DNA polymerase I. Using the free 3' end of the nicked strand as a primer, the enzyme specifically incorporated the deoxynucleotides into the nick site. The labelled plasmid DNA was cut with restriction endonucleases and the fragments were separated by gel electrophoresis. The OriT site was located within the radio-labelled restriction fragment.

Finnigan and Sherratt (1982) noted that the OriT and OriV sites were in close proximity for several plasmids. They speculated that retention of the two sites during plasmid evolution was more likely due to the advantage of conjugal transfer to the spread of a replicon, rather than reflecting a necessary functional relationship between the two sites.

This view was in opposition to a model for conjugal transfer proposed by Nordheim et al (1980). They felt that a functional relationship existed between the OriV and OriT sites (Fig. 4). Plasmid complementary strand synthesis, subsequently taking place in the recipient, was primed in the donor by DNA synthesis from the OriV to the OriT site. Transfer of the primed DNA strand was then effected from the OriT site.

The OriT site of a plasmid was located commonly at the extreme end of a transfer operon encompassing many genes required for DNA transfer. The best characterized transfer operon to date is that of the fertility factor, F. (Willetts and Skurray, 1982). The operon includes at least twenty genes (Fig. 5). The function of many of the genes has been determined. Genes tra A,L,E,K,B,V,W,C,U,F,H,G were required for production of the sex pilus. Surface exclusion, the inability of a host cell containing an F factor to act as a recipient, was mediated by genes tra S and tra T. The product of tra S was a cytoplasmic membrane protein of 18,000 daltons, while tra T encoded production of an outer membrane protein of 25,000 daltons. Plasmid DNA metabolism was controlled by genes tra M,Y,G,D,I,Z. The two genes tra I and tra Z were thought to encode the endonucleases responsible for nicking at the OriT site. Stabilization of mating pair formation was under the control of transfer genes N and G. Finally, the entire operon was under positive control of the tra J gene product. The presence of this product was required for transcription of the other genes. The tra J gene was under negative control of the fertility inhibitor (fin) O and P

Figure 4. Model for a functional relationship between the OriV and OriT sites of a plasmid molecule during conjugal transfer[1].



a) Relaxation Protein (RP) binds at OriT

b) Primer strand synthesis from OriV to OriT

c) RP nicks at OriT; binds to free 5' end

d) DNA passes to recipient

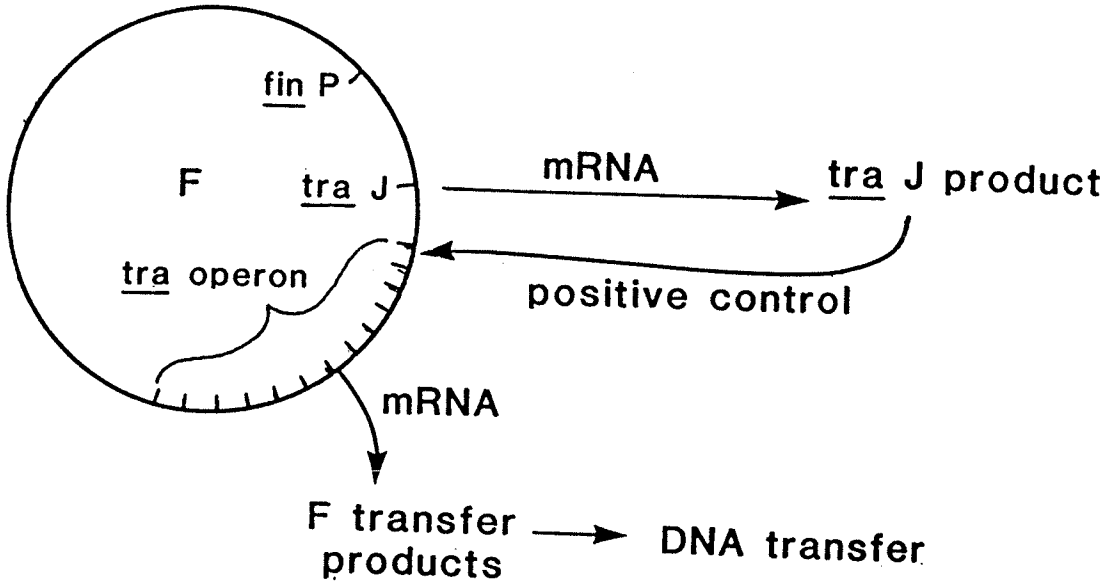
e) Complementary strand synthesis in donor

f) Recircularization in recipient

g) Complementary strand synthesis in recipient

1. Modified from Nordheim et al., 1980.

Figure 5. The transfer operon of fertility factor F.



The tra operon consists of 20 genes. Genes tra A,L,E,K,B,V,W,C,U,F,H,G are required for the production of sex pili. The phenomenon of surface exclusion is mediated by the products of tra S and tra T. Genes tra M,Y,G,D,I,Z are involved in DNA metabolism. The product of the tra J gene is required for transcription of the operon.

genes. The products of these two genes prevented transcription of tra J and thus all plasmid transfer functions.

The F factor was unique amongst conjugative plasmids in that the P gene only, was present within the genome. Therefore, transfer genes were expressed constitutively. However, the fin P gene product of the F factor could interact with the fin O gene product of any other F-like conjugative plasmid co-habiting the host cell. Under these conditions the transfer functions of F were repressed.

B. Transfer of DNA.

DNA can be transferred within and between genera by three different mechanisms. The significance of each mechanism with regard to genetic exchange, is dependent to a large extent, upon the organism.

1. Transformation.

Transformation is defined as the ability of bacteria to acquire a heritable altered phenotype by the acquisition of DNA from an external medium (Deich and Smith, 1980). DNA taken up by this process could be in the form of chromosomal fragments or plasmid DNA. Transformation has been demonstrated in several genera (Spizizen et al., 1966). Some organisms such as Streptococcus pneumoniae, Bacillus subtilus and Neisseria gonorrhoeae were naturally transformable, while Escherichia coli required the presence of specific cations and a heat-shock regime for uptake of exogenous DNA (Cohen et al., 1972). Haemophilus influenzae could be readily transformed with homologous DNA. Uptake of DNA by this organism was dependent upon the presence of a specific 11 base pair recognition sequence within the DNA

molecule (Danner et al., 1982). The sequence, 5'-AAGTGCGGTCA-3', was recognized by a receptor on the cell surface.

Specific stages in the uptake of DNA by Haemophilus were defined by Deich and Smith (1980). The first stage encompassed reversible binding of DNA to the cell surface receptor. In stage two, the cell was committed to uptake of the bound DNA molecule. Duplex DNA was transported into the cell in stage three. Each cell was capable of absorbing 4 to 8 molecules of DNA.

Deich and Hoyer (1982) and Concino and Goodgal (1982) described the DNA receptors on the surface of competent cells of Haemophilus influenzae. Vesicles, generated on the surface during the induction of competence, had uptake sequence-specific binding activity. Loss of competence was accompanied by a release of vesicles into the medium.

Neisseria gonorrhoeae, like Haemophilus, favoured uptake of homologous DNA (Graves et al., 1982). As heterologous DNA failed to compete with uptake of homologous DNA, a specific recognition sequence was speculated to be present in Neisseria DNA. Competence for transformation of Neisseria gonorrhoeae was associated with piliation, although the reason for this was unknown.

The overall importance of transformation as a means of genetic exchange in wild populations is unclear. Due to the natural competence of several different genera, transformation is most likely a viable mechanism of genetic exchange in vivo.

2. Transduction.

In addition to transformation, transfer of DNA between bacterial cells could be mediated by bacteriophage in a process known as transduction (Hayes, 1970). Host cell chromosomal or plasmid DNA could be encapsulated

by phage particles undergoing assembly in an infected cell. Host cells subsequently infected by the particles could acquire, by recombination, gene sequences derived from the chromosome of the former host cell. In the same manner, plasmid molecules could be transferred between host cells by bacteriophage. Jones and Sneath (1970) noted that a large number of R-factors were transferred between enteric organisms in this manner. However, in the genera Haemophilus and Neisseria, transduction did not play a major role in genetic exchange. To date, bacteriophage infecting Neisseria species have never been isolated. While bacteriophage have been identified in the genus Haemophilus (Boling et al., 1973; Samuels and Clarke, 1969), there have been no reports of transduction of either chromosome or plasmid DNA. While transduction was an important means of genetic exhchange in enteric organisms, it was of little value to organisms of the genera Haemophilus and Neisseria.

3. Conjugal Transfer of Plasmid DNA.

While transduction and transformation may have played important roles in the dissemination of plasmids throughout bacterial populations, by far the most efficent system for spread was conjugation.

Plasmid molecules were defined as conjugative if all funtions necessary for transfer were encoded by the plasmid molecule itself (Clark and Warren, 1979). These functions included establishment of mating contact, then transfer and repliconation of plasmid DNA.

The F-factor (fertility factor) has been the best characterized conjugative plasmid to date. The transfer region of this plasmid had been intensely studied (Willetts, 1977). The transfer operon consisted of at least 19 genes, encompassing approximately one-third of the genome (Willetts and Skurray, 1980). Most of the genes were devoted to synthesis

of the F pilus, while the remainder played a regulatory role, or were involved in the DNA transfer process (Clark and Warren, 1979).

Conjugal transfer could be divided into discrete stages (Kingsman and Willett, 1978; Clark and Warren, 1979). Stage one encompassed the formation of a stable mating pair. Mating contact was a collision-dependent process facilitated by both high density of cells and by motility. Evidence has been obtained to suggest that donor cells actively sought out recipients by following a chemotactic gradient (Collins and Broda, 1975).

Mating pair formation was also facilitated by the presence of fimbriae, and in the case of cells harbouring an F factor, by the presence of F pili (Brinton et al., 1964). If mating pair formation was mediated by such sex pili, the pili attached to specific receptors on the surface of the recipient cell (Achtman et al., 1978). Initially, Brinton (1971) speculated that the pilus served as a conduction tube for DNA transfer through the hollow central core. However, more recent data indicated that attachment of the pilus to the recipient triggered pilus retraction, drawing plasmid donor and recipient together (Novotny and Fives-Taylor, 1974).

Stage one of conjugal transfer also included the introduction of a specific nick at the OriT site, then subsequent binding of the 5' and/or 3' ends to a complex at the point of cell-to-cell contact of the mating pair. The nick was introduced in a unique strand at the OriT site by a plasmid-encoded endonuclease (Everett and Willetts, 1980). After nicking, the protein bound covalently to the 5' end of the nicked strand, then acted as a pilot protein provoking exit of DNA from the donor cell.

Kornberg (1974) proposed two hypotheses to describe the junction formed between donor and recipient cells. The first envisioned fusion of the outer membranes with requisite merging of the periplasmic space. The

second hypothesis described a specialized structure for DNA transport which extended between the cytoplasmic membranes of the mating pair, preventing contact between the DNA and the periplasmic spaces.

Stage two in transfer required donor conjugal DNA synthesis, a process quite different from vegetative replication (Bresler et al., 1968). If both the 3' and 5' ends of the transferred strand of DNA were complexed at the cell-to-cell interface, then the transferred strand would enter the recipient in the form of a loop as it was replicated. This stage of transfer is illustrated in Figure 6.

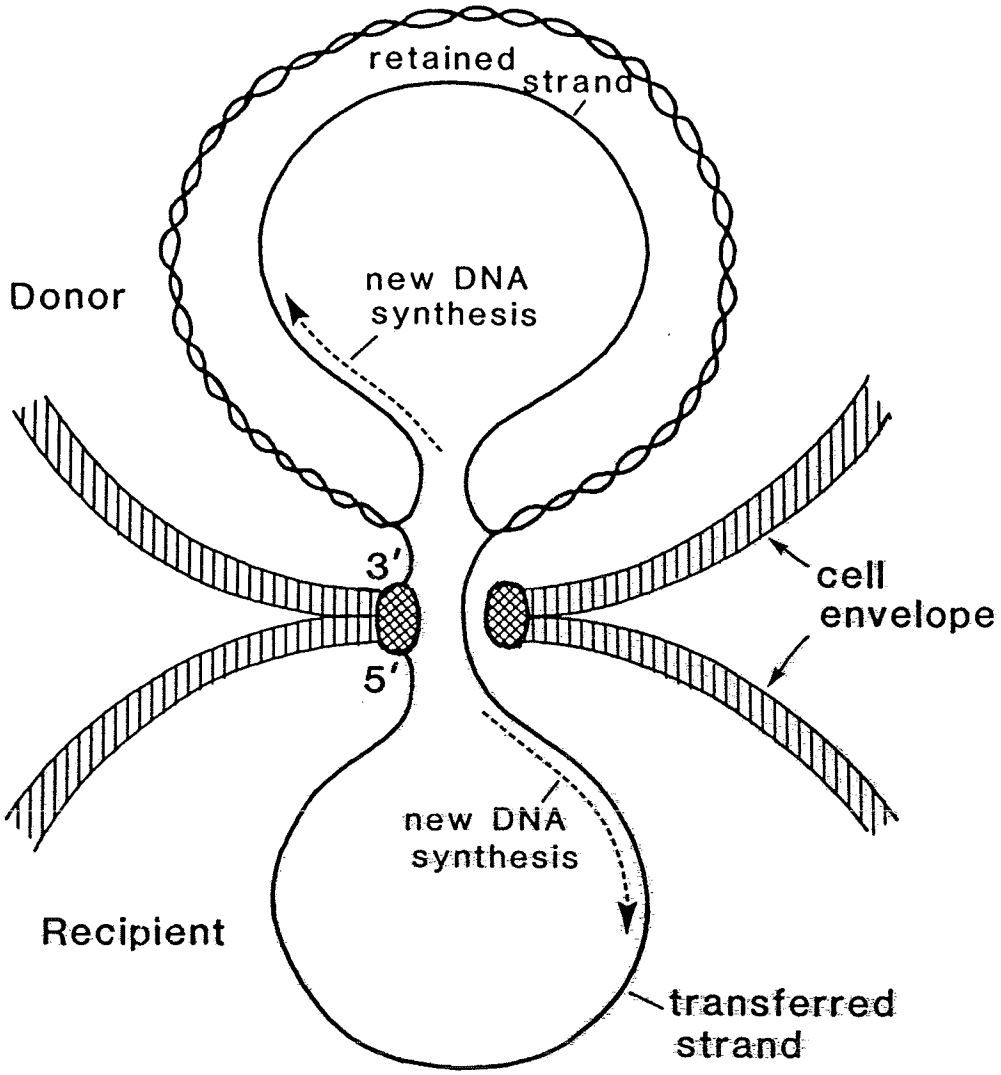
During stage three, the tranferred strand was used as a template for synthesis of a complementary strand. Stages four and five comprised recircularization of DNA strands in both the donor and recipient cells.

4. Mobilization.

Plasmids deficient in transfer function could be transferred by the process of mobilization. Non-conjugative plasmids lacked an OriT site and/or coding sequences for proteins required for self-transfer. A second conjugative plasmid supplied the necessary functions for transfer of the non-conjugative plasmid. These functions were supplied either *in trans* or *in cis*. However, for mobilization to occur, both the conjugative and non-conjugative plasmids had to co-habit, if only transiently, the same host cell. Functions supplied *in trans* were diffusible gene products encoded by the conjugative plasmid. Functions supplied *in cis* were specific gene sequences, such as an OriT site. These functions could only be supplied by the conjugative plasmid by co-integration with the non-conjugative plasmid.

Kilbane and Malamy (1980), while studying mobilization of plasmids by F'*lac*, were able to distinguish four distinct mechanisms by which a non-conjugative plasmid could be mobilized. The first mechanism, Class I

Figure 6. A model for conjugal transfer of plasmid DNA[1].



1. Modified from Willetts, 1981.

mobilization, was mobilization *in trans*. Mobilization was dependent upon the presence of an OriT site in the non-conjugative plasmid. It was from this site that DNA transfer commenced. Mobilization *in trans* was brought about by the interaction of diffusible gene products encoded by the conjugative plasmid with functions encoded by the non-conjugative plasmid. Mobilization *in trans* did not necessitate transfer of the conjugative plasmid to the recipient cell, although transfer of both conjugative and non-conjugative plasmids often occurred.

Mobilization *in trans* was illustrated by the example of F-factor mobilization of the small non-conjugative *E. coli* plasmid ColE1 (Veltkamp and Stuilje, 1981). The F-factor provided functions necessary for mating pair formation, in this case, the F pilus, while ColE1 contributed a functional OriT site and proteins required in the transfer process. Thus the functions supplied *in trans* were gene products encoded by the conjugative plasmid, but required by ColE1 for transfer.

In the absence of an OriT site, mobilization of the non-conjugative plasmid, by necessity, occurred by an *in cis* mechanism. Three mechanisms have been described for mobilization *in cis*. The first mechanism, Class II mobilization, required recombination between the non-conjugative plasmid and the conjugative plasmid and was dependent upon a proficient recombination system in the host cell. The process was *recA* dependent. Subsequent transfer of the co-integrate was initiated from the OriT site of the conjugative plasmid. Both plasmids were transferred to the recipient cell where the co-integrate was resolved into two independent replicons. The second *in cis* mechanism, Class III mobilization, also depended upon co-integration of the two plasmids and transfer from the OriT site of the conjugative plasmid. In this instance, recombination occurred at regions of homology between two plasmids and was independent of the recombination system of the

host cell. Again, both plasmids were transferred to the recipient and were subsequently resolved into independent replicons after transfer. The third mechanism, Class IV mobilization, like Class III, was rec A independent. This mechanism was based upon co-integration at homologous regions created as a result of transposition of a transposable element. Transposition was an 'illegitimate' recombinational event in that segments of DNA with no inherent homology could be joined together. Therefore, the presence of a transposable element on either plasmid could, by the process of transposition, generate homology between the two molecules. Co-integration was an essential step in the transpositional process. Transfer was again effected from the OriT site of the conjugative plasmid. The co-integrate was transferred to the recipient and subsequently resolved into separate replicons.

Willett (1981) examined the conjugative process and made some pertinent observations. Conjugative plasmids were normally 30 kb or greater in size, while smaller non-conjugative plasmids were normally less that 10 kb in size. While non-conjugative, many of these plasmids often carried an OriT site and some essential mobilization genes. Whether conjugative or non-conjugative, approximately one-third of the plasmid was devoted to the transfer process. Obviously, conjugal transfer was of some import, particularily from a survival standpoint. Willett and Skurray (1980) noted that there were few recognition sites for Type II restriction endonucleases within the transfer regions of plasmids, emphasizing the "survivalist" nature of these functions. The lack of restriction sites would optimize conditions for wide dissemination, promoting the spread of plasmids both within and between genera.

Conjugation could be regarded as a successful form of evolution. Essentially a replicative process, conjugal transfer allowed plasmid genes to replicate at a faster rate than the chromosomal genes of the host cell.

C. Recombinant DNA Methodology.

1. The Basic Premise.

Much of the information acquired concerning the nature of the plasmid molecules could be attributed to the use of recombinant DNA technology. The basic premise of this technology was the construction, in vitro, of DNA molecules by joining fragments of DNA from diverse backgrounds. The recombinant molecules were then replicated by introduction into living cells.

Before these procedures became reality, certain basic requirements had to be fulfilled. Mechanisms for cutting and joining DNA from different sources were needed. After recombinant molecules had been constructed, a means of introducing them into living cells for replication had to be found. Once in the cell, techniques for determining the presence of these molecules in vivo were required. It was only through the combined efforts of several researchers that recombinant DNA technology became a reality.

2. Restriction Endonucleases.

Prior to 1970, no method was available for reproducibly cutting duplex DNA molecules into discrete fragments. The existence of enzymes capable of this function was discovered due to the efforts of Lederberg and Meselson (1964). They studied the phenomenon of Lambda bacteriophage restriction by E. coli host cells. When Lambda phage were grown upon E. coli strain C then titred upon E. coli strains C and K, titres were several orders of magnitude lower with strain K. If the phage resulting from infection of strain K were replated upon strain K, titres were normal. The phage were no longer restricted by strain K. Lederberg and Meselson reasoned that the majority of the phage DNA infecting strain K was degraded by restriction

endonucleases. The DNA surviving degradation had been modified by a host enzyme, inhibiting the action of the endonucleases. This was the first indication of the presence of DNA restriction and modification enzymes in a host cell.

Meselson and Yuan (1968) isolated the restriction enzyme from E. coli strain K. The enzyme, a Type I restriction endonuclease, required ATP and S-adenosyl-methionine as co-factors. The enzyme recognized a specific nucleotide sequence, but did not introduce a break in the DNA molecule until it had travelled between 1000 to 1500 base pairs from the recognition site (Rosamond et al., 1979).

Because of the bizarre activity of the Type I endonuclease, it was of little use for cleaving DNA in vitro. However, Kelly and Smith (1970) and Smith and Wilcox (1970) reported the isolation of a Type II restriction endonuclease. This enzyme recognized a specific palindromic sequence and introduced a break within this sequence. By use of these enzymes, the restricted DNA gave rise to discrete fragments of defined length and sequence. The prototype of Type II restriction endonucleases was isolated from Haemophilus influenzae strain Rd. It was called HindI. The recognition sequences for several Type II restriction endonucleases are presented in Table 2.

3. Determination of DNA Fragment Size.

In order to monitor the size of DNA fragments produced, the DNA was radio-labelled then centrifuged through a sucrose gradient. After fractionation of the gradient, the relative sizes of fragments could be determined from their respective position in the gradient. This technique was tedious and did not resolve fragments of similar size. Thorne (1966; 1967) determined that electrophoresis of plasmid DNA through agarose gels would

Table 2. The recognition sequences for some Type II restriction endonucleases.

Enzyme	Recognition Sequence	Source Organism
HindII	(C) (A) 5'G(T) | (G)AC 3' 3'C(A) | (C)TG 5' (G) | (T)	Haemophilus influenzae Rd.
EcoRI	* 5'G | AATTC 3' 3'C | TTAAG 5'	Escherichia coli RY13.
PstI	5'GTGCA | G 3' 3'CACGT | C 5'	Providencia stuartii.
HaeIII	* 5'GG | CC 3' 3'CC | GG 5'	Haemophilus aegyptius.

| site of cleavage.

* modification of base prevents cleavage.

resolve different molecular configurations of the DNA molecules. Aaij and Borst (1972) found that this particular technique could also be used to separate DNA fragments of different molecular weight. They determined that the migration rate of the fragments was inversely proportional to the log of the molecular weight. Fragments could be accurately sized by co-electrophoresing them with molecular weight standards. Staining the gel with an intercalating dye, such as ethidium bromide, rendered the DNA visible under ultra-violet light. Radio-labelling of DNA was no longer necessary.

4. Construction of Restriction Endonuclease Maps.

An initial step in the characterization of a plasmid molecule was the construction of a restriction endonuclease map. After digestion with one or more restriction enzymes, the resulting fragments were ordered to produce a physical map. While several techniques have been devised for mapping, two basic techniques were commonly used (Danna et al., 1973).

The first method was based upon a comparison of fragment size between a partially digested and a completely digested sample of DNA. Partial digestion yielded some fragments comprised of two or more contiguous complete digestion products. By purifying a partial digestion product, incubating it with excess enzyme to complete digestion, then identifying resultant fragments, it was possible to determine which final products were contained in a given partial digestion product. Analysis of several partial digestion products in this way enabled one to determine the order of all fragments.

The second method used for mapping plasmid DNA was digestion of the DNA with multiple enzymes. The cleavage products of one endonuclease were

characterized with respect to size, then digested with a second endonuclease. Analysis of the double-digestion products established the relationship between the cleavage sites of the two enzymes. Both methods are illustrated in Figure 7.

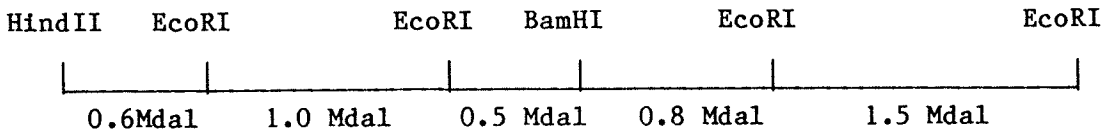
5. Cloning of Restriction Fragments.

The term cloning, with regard to recombinant DNA technology, essentially refers to the selective isolation and replication of a unique DNA molecule. In order for a restriction fragment to be replicated, it had to be joined to a DNA molecule or vector capable of replication in a host cell. Vectors suitable for cloning had to have several desirable properties (Old, R.W., and S.B. Primrose, 1981). A vector with low molecular weight was advantageous for several reasons. The vector was more resistant to damage by shearing than larger molecules. As well, plasmids of low molecular weight were usually present as multiple copies in host cells, facilitating a high gene dosage of the cloned fragment.

Cloning vectors had to confer readily selectable phenoptypic traits upon host cell. Since introduction of DNA molecules into host cells was not a highly efficient process, one had to be able to select from the entire population those cells harbouring the molecules. Selection was facilitated by the presence, within the vector, of at least two genes encoding a selectable phenotypic trait such as antibiotic resistance.

Finally, the vector had to have single sites, preferrably within a gene for scorable phenotype, for a large number of restriction endonucleases. Insertion of a fragment into one of these sites destroyed one of the selectable phenotypic traits imparted by the vector upon the host cell. Thus, one could divide the host cell population into three groups; those cells harbouring intact vector, those cells harbouring vector with an

Figure 7. Restriction endonuclease mapping of plasmid DNA.



A. Mapping by Partial Digestion.
Fragment Molecular Mass (Mdal)

Partial Products	Complete Products
EcoRI	EcoRI
2.3	1.0,1.3
2.1	2.1
4.4	4.4

B. Mapping by Multiple Enzymes.
Fragment Molecular Mass (Mdal)

Complete Digestion Products.

EcoRI	EcoRI + BamHI	EcoRI + BamHI + HindIII
2.1	2.1	1.5,0.6
1.3	0.5,0.8	0.5,0.8
1.0	1.0	1.0
4.4	4.4	4.4

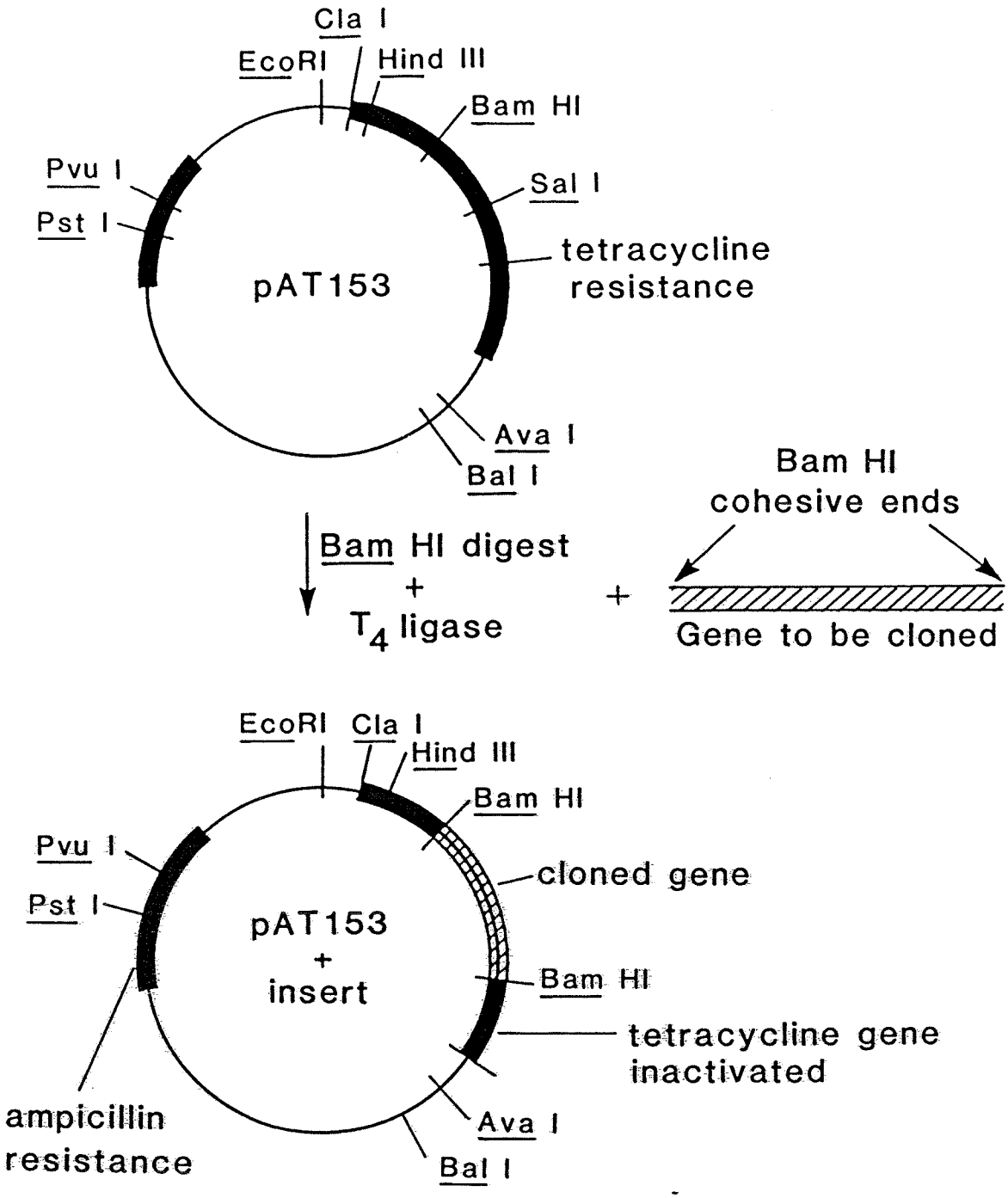
inserted fragment, and those cells with no vector at all.

One of the first steps in cloning was to cut vector DNA and the DNA to be inserted, with the same restriction endonuclease (Fig. 8). The break introduced by many Type II restriction endonucleases produced either 3' or 5' protruding ends. When cut with the same endonuclease, the vector and insert DNA had complementary cohesive ends. By mixing appropriate molar ratios of the two DNA species, recombinant molecules were created. The ends readily associated by hydrogen bonding. However the two or three hydrogen bonds formed between the ends of the fragment and vector molecules were not stable. Covalently bonding of the inserted fragment to the vector was necessary for stability of the DNA molecule.

Both E. coli and bacteriophage T4 produce an enzyme, DNA ligase, with the capability to seal single-stranded nicks between adjacent nucleotides in a duplex chain (Olivera et al., 1968; Gumport and Lehman, 1971). The T4 enzyme had the capability of covalently bonding cohesive as well as blunt-ended fragments.

Bacterial plasmids, encoding resistance to antibiotics were good vectors, particularily those with a high copy number per host cell. While initial cloning experiments were carried out using naturally occurring plasmids as vectors (Chang and Cohen, 1974), improved derivatives were developed. The new vectors combined desirable qualities of several plasmids. One of the most widely used vectors, pAT153, was created in this manner (Twigg and Sherratt, 1980). Plasmid pAT153 encoded resistance to ampicillin and tetracycline (Fig. 8). In addition, insertion of foreign DNA fragments into any of six unique restriction sites resulted in inactivation of one of the resistance genes. Thus a host cell carrying a recombinant plasmid could be selected on the basis of the phenotypic trait imparted by the plasmid.

Figure 8. Insertion of fragments into a cloning vector.



6. Transformation of the Host Cell.

As several useful cloning vectors replicated in E. coli, this bacterium was commonly used as a host cell. The methodology for the introduction of a plasmid molecule into an E. coli host cell was developed by Cohen et al. (1972). E. coli cells were not naturally competent for DNA uptake, but could be made so by a regimen of $CaCl_2$ washes and heat-shock. Cells in the early exponential phase were washed then resuspended in a dilute $CaCl_2$ solution. Recombinant molecules were added to the cell suspension. After approximately one-half hour of incubation, cells were heat-shocked for two minutes at forty-two degrees Celsius. Cells were incubated to allow expression of phenotypic traits encoded by the vector. Selection of cells containing recombinant plasmids was based upon phenotypic traits. If plasmid pAT153 was used as the cloning vector, cells containing the unaltered vector plasmid were resistant to both ampicillin and tetracycline when plated on a medium incorporating the antibiotics. Conversely, cells carrying recombinant plasmids were sensitive to one or the other of the antibiotics, dependent upon the site of insertion of the foreign DNA.

7. In Situ Hybridization.

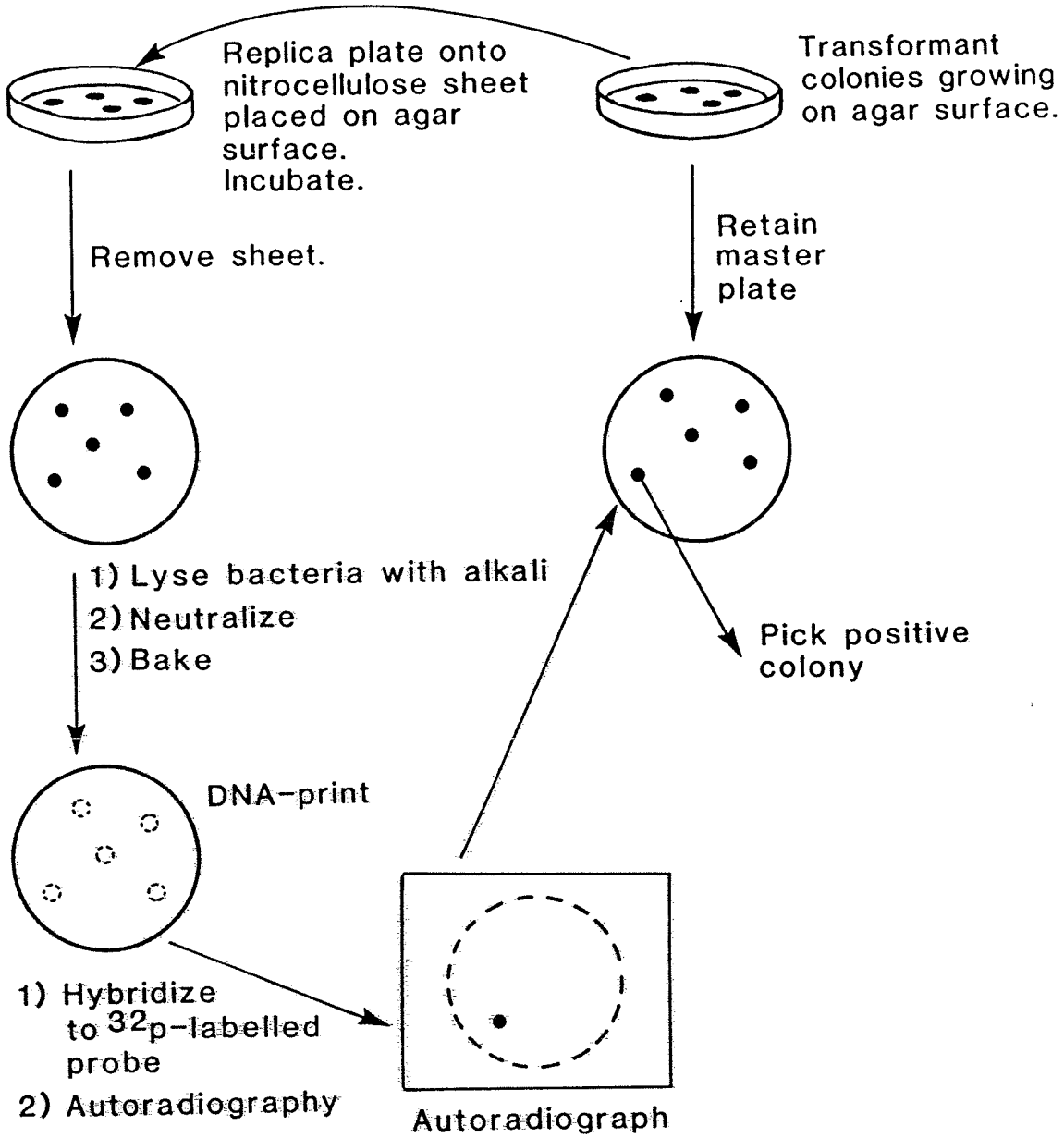
DNA fragments could also be inserted into sites in the vector other than those located within a gene encoding a selectable trait. However, selection of cells containing recombinant plasmids was more complex. Selection was simplified due to techniques developed by Southern (1975).

The Southern blot facilitated detection of homologous sequences in DNA molecules from diverse backgrounds. The technique was based upon the ability of denatured DNA to hybridize, under controlled conditions, to homologous sequences. Grunstein et al. (1975) modified the technique to

allow detection of recombinant plasmids within transformed bacterial cells. Colonies of transformed cells were replica plated onto a nitrocellulose membrane resting on the surface of an agar culture plate. The plate was incubated to allow growth of colonies on the surface of the membrane. The membrane was treated with alkali which lysed the bacterial cells, and denatured DNA extruded with cell contents. The DNA was fixed to the nitrocellulose by baking the membrane at eighty degrees Celsius for four hours. The single-stranded DNA on the filter was hybridized to a radio-labelled single-stranded DNA molecule with sequence homologous to the cloned fragment. If the DNA from the bacterial colony was homologous to the radio-labelled probe molecule, hybridization took place. Autoradiography of the membrane after hybridization revealed the colonies that had DNA sequence homologous to the probe sequence and thus had been transformed by recombinant molecules (Fig. 9).

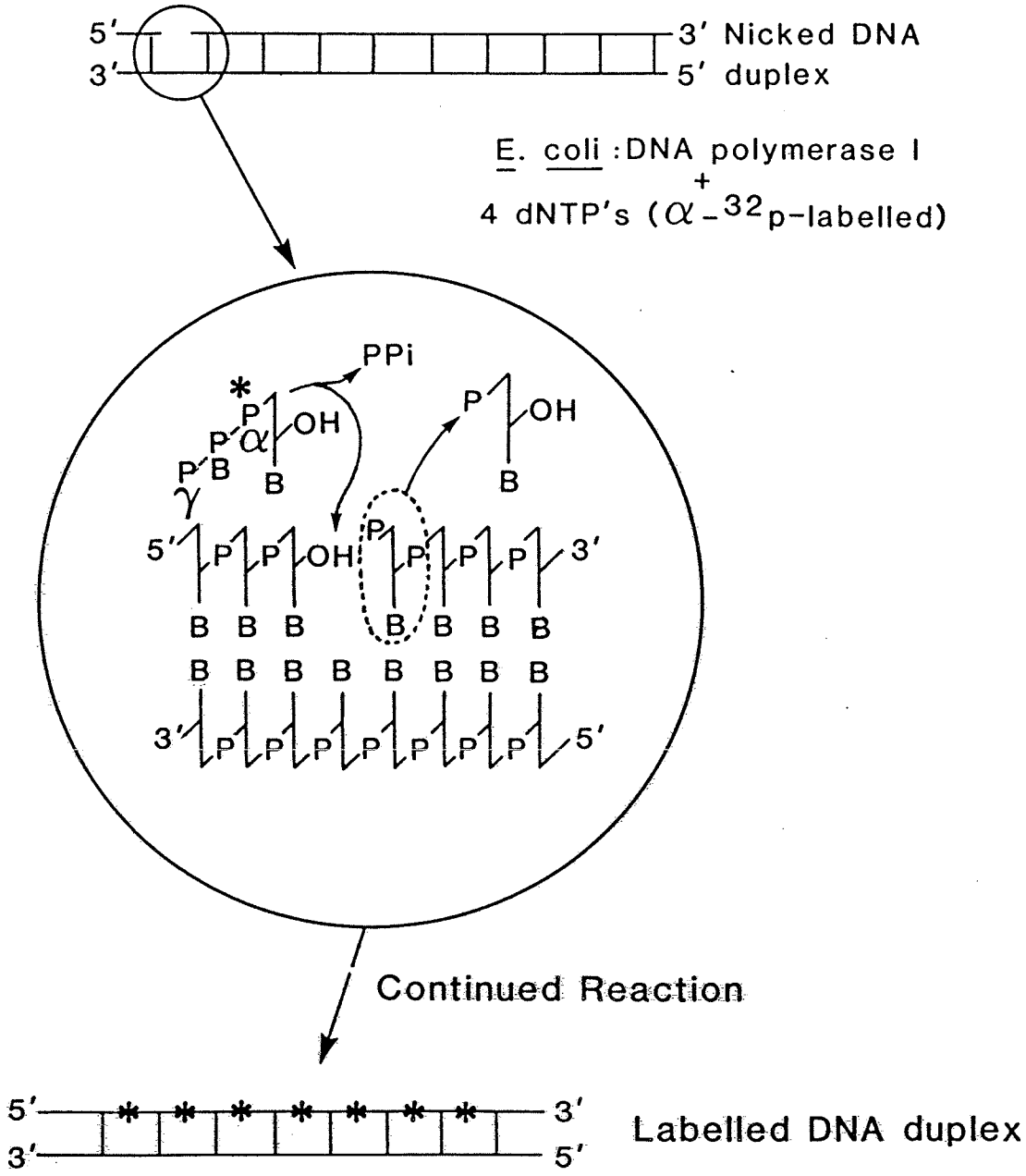
The DNA probe used in hybridization was conveniently radio-labelled by the process of nick-translation (Rigby et al., 1977). This process took advantage of the endonuclease and polymerase activity of E. coli DNA polymerase I. A limited number of nicks were randomly introduced into the DNA molecule by treatment with DNase I (Fig. 10). The free 3'-OH at the nick site acted as a primer for the 5' to 3' polymerase activity of DNA polymerase I. Concomitant hydrolysis of the 5' terminus by the 5' to 3' exonuclease activity of the enzyme, released 5' mononucleotides. The reaction was supplied with the four deoxynucleotides, one with an alpha-^{32}P label. The enzyme progressively incorporated the nucleotides and thus the label into the DNA duplex. Base sequence was unchanged, but the DNA molecule was radio-labelled. After denaturation, the single-stranded molecule could be used as a probe for homologous sequence.

Figure 9. In situ hybridization of DNA[1].



1. Modified from Old and Primrose, 1981.

Figure 10. Nick translation of DNA[1].



1. Modified from Old and Primrose, 1981.

8. Isolation of Plasmid DNA by Dye-Buoyant-Density Gradient Centrifugation.

Large quantities of plasmid DNA were often required for further characterization of molecules. The classical method for isolation of plasmid DNA involved gentle lysis of the host cell, followed by a centrifugation step to remove chromosomal DNA and cell debris. Due to the differential between chromosomal and plasmid DNA, for incorporation of the intercalating dye, ethidium bromide, the two DNA species could be separated by $CsCl_2$-dye-buoyant-density centrifugation (Radloff et al., 1967). The covalently closed circular conformation of the plasmid molecule limited the amount of ethidium bromide that could be bound. However, linear DNA, such as fragmented chromosomal DNA, had no topological constraints and consequently bound more of the dye. This resulted in the chromosomal DNA being of lower density within the gradient than the plasmid DNA. The more dense plasmid band, visualized by ultra-violet light, was selectively collected from the gradient.

D. Analysis of Plasmid-Encoded Proteins.

1. The Minicell System.

Recombinant DNA technology has provided the means for insertion of sequences of "foreign DNA" into plasmids for <u>in vivo</u> production of encoded proteins. However, detection of proteins encoded by the plasmid molecules, against the background of host cell proteins was a difficult task. Many of the inherent problems were eliminated by the use of a minicell system for protein detection.

Minicells were small non-growing anucleate bodies produced by aberrant cell division at the poles of rod-shaped bacteria. While rarely produced by wild-type cells, minicells were continually produced by certain mutants, due to premature activation of division sites at polar regions destined to be located in the central region of the progeny cell.

Several genera have been found to produce minicells, however, E. coli mutants were most commonly used (Alder et al.,1967). Virtually devoid of chromosomal DNA, minicells provided a unique in vitro system for the study of plasmid-encoded proteins. Minicells had functional cell walls, membranes, ribosomes, and energy generating systems capable of maintaining integrity for a long period. The minicells could be separated from larger parental cells by differential centrifugation through sucrose gradients. This step was important, as contamination of a minicell preparation with parental cells would result in the presence of chromosome-encoded proteins, as well as those encoded by a plasmid. Plasmids present in minicell-producing strains were found to segregate into minicells (Inselburg, 1970), however, the exact mechanism for segregation was unknown. Segregation may have been due to residence of the plasmid molecules at the poles of cells, or due to movement to the poles at the time of formation of the minicell septum. Smaller plasmids of less than 10 Mdal were found to segregate more readily into minicells than plasmids of a larger size.

Plasmids directed synthesis of RNA and functional proteins within minicells. Levy (1974) was able to detect functional colicin protein produced within E. coli minicells harbouring plasmid ColE1. Synthesis was carried out in the presence of radio-labelled amino acid so proteins were tagged and could be readily detected. Minicells were lysed and proteins in the lysate were resolved by polyacrylamide gel electrophoresis. The radio-labelled proteins were visualized by fluorography of the gel.

Nothling and Reeve (1980) noted some inherent problems with the minicell system. Isolation of a pure preparation of minicells was time consuming. Backgound noise could be a problem due to translation of residual mRNA from parental cells. Finally, abnormal gene products were sometimes produced due to depletion of amino-acyl tRNA in the minicells.

2. The Maxicell System.

Sancar et al. (1979), described another technique to assist in the characterization of cloned gene products produced by recombinant plasmid molecules. Again, the procedure utilized in vivo incorporation of radiolabelled amino acids into plasmid-encoded proteins, however, synthesis was carried out in E. coli maxicells.

Specific mutant strains of E. coli were highly sensitive to irradiation by ultra-violet light (254nm). Chromosomal DNA was extensively degraded and the cells ceased dividing, yielding large cells. The cells containing plasmids, synthesized plasmid-encoded proteins exclusively. Functioning of the system was dependent upon a large size differential between the plasmid molecules and the chromosome. The smaller the plasmid target, the greater the likelihood that the plasmid remained intact after irradiation. For this reason, this method was not suitable for the study of proteins encoded by large plasmid molecules.

Maxicells were supplied with a source of amino acids including at least one with radio-label. Cells were lysed after a suitable period of incubation, and proteins were again detected by polyacrylamide gel electrophoresis followed by fluorography.

The maxicell sytem was limited by a restriction on plasmid size, and by the presence of residual fragments of chromosome which resulted in background noise.

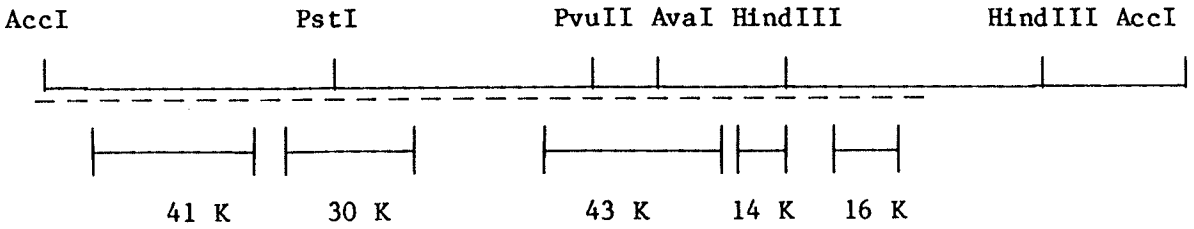
3. Coupled *In Vitro* Transcription-Translation.

A third system was developed for the study of plasmid gene products (Chen and Zubay, 1983). This system was based upon the coupled transcription-translation, *in vitro*, of a DNA template. The method was superior to both the minicell and maxicell systems for several reasons. The system provided quantitative control over components. The DNA template could be a molecule of any size. Linear DNA fragments could be efficiently transcribed and translated as could intact plasmid molecules. This last point was a great advantage. One could compare gene products from an intact plasmid molecule and from the same molecule after restriction at a unique site. A change in the protein profile encoded by the plasmid after restriction indicated that the restriction site was within a gene. In this way, proteins encoded by a plasmid could be correlated with co-ordinates of the restriction endonuclease map. Stein et al. (1983) applied *in vitro* transcription-translation to analysis of the coding capacity of a hybrid gonococcal plasmid (Fig. 11). By using both covalently closed and linear templates, Stein et al. (1983) were able to assign the production of specific proteins to unique regions of the plasmid.

The basic component of the cell-free system was an extract, S-30, prepared from *E. coli*. The extract was prepared from mid-log cells that had been frozen then lysed by the application of pressure. Cell contents were dialysed against a stabilizing buffer, then incubated to allow degradation of pre-existing mRNA. S-30 provided essentially all co-factors and substrates required for RNA and protein synthesis. However, synthesis was improved by supplementation of the reaction mixture with ribonucleotides, tRNA and amino acids. Again, one amino acid was radio-labelled to facilitate detection of nascent protein.

See and Glick (1982) used the coupled transcription-translation system

Figure 11. In vitro transcription-translation analysis of the coding capacity of a hybrid gonococcal plasmid[1].



- - - - Region of hybrid composed of 4.4 Mdal plasmid DNA.

|——| Region encoding for protein.

K $1x10^3$ Daltons.

1. Modified from Stein et al., 1983.

to study expression of cloned DNA sequences. Both covalently closed and linear template were efficiently utilized and background noise was minimal. Expression was somewhat lower with linear template due to degradation by residual endonuclease activity in the cell extract.

Coupled transcription-translation in a cell-free system appeared to be the most accomodating system for analysis of plasmid-encoded proteins. The system eliminated the cell isolation step required for the minicell system. Destruction of templates by ultra-violet irradiation did not pose a problem as it did in the maxicell system. The cell-free system was versatile with regard to template size and conformation and lysis of cells for protein analysis was not necessary. As well, background noise was minimal.

E. Ampicillin Resistance Plasmids of *Haemophilus* species and *Neisseria gonorrhoeae*.

1. Ampicillin Resistance in *Haemophilus*.

Plasmids mediating resistance to ampicillin were first identified in the genus *Haemophilus* in the early 1970's (Elwell et al., 1975). A strain of *H. influenzae* producing beta-lactamase was isolated from a patient with meningitis (Kahn et al., 1974). Similar reports quickly followed (Elwell et al., 1975) indicative of sudden emergence and rapid spread of an R-factor. The enzyme produced by these organisms was a TEM-type beta-lactamase identical to that produced by some members of the *Enterobacteriaceae* (Farrar,Jr., and O'Dell, 1974). Production of this enzyme was encoded by a plasmid-borne ampicillin transposon (TnA) in the enteric organisms and in *Haemophilus* (Elwell et al., 1975). However, the non-TnA portion of the plasmids in *Haemophilus* had a mole % G+C ratio of 40, a value in agreement with the ratio determined for the chromosome of

Haemophilus (De Graff et al., 1976). Thus these plasmids were likely native to the genus.

While the exact origin of the plasmids was unknown, it was speculated that they were created by transposition of TnA from enteric R-factors to indigenous cryptic plasmids of Haemophilus (Laufs et al., 1981). There were two lines of evidence supporting this view. Firstly, phenotypically cryptic plasmids had been isolated from the genus Haemophilus, which could have acted as recipients for the transposition event (De Graaff et al., 1976). Secondly, enteric R-factors were not stable in a background of Haemophilus. Therefore, resistance genes entering the genus Haemophilus by these unstable vectors could be rescued only by insertion into a resident plasmid (Elwell et al., 1975). This speculation did not eliminate the possibility that the plasmids arose in an organism with a G+C molar ratio similar to that of Haemophilus and were subsequently acquired by this genus. The ampicillin resistance plasmids isolated from Haemophilus fell into two size categories; large conjugative plasmids of 30 to 38 Mdal in size containing complete TnA sequences, and small non-conjugative plasmids of approximately 4 Mdal in size containing 40% of TnA (De Graaff et al., 1976; Thorne and Farrar, 1975).

2. Ampicillin Resistance in Neisseria gonorrhoeae.

The clincal significance of the appearance of resistance plasmids in Haemophilus led to speculation over the possible emergence of plasmid-mediated ampicillin resistance in other clinically important orgainsms, particularily Neisseria gonorrhoeae (Falkow et al., 1976). Chromosomally-mediated ampicillin resistance had been increasing in frequency, but these organisms still remained sensitive to therapeutic levels of penicillin (Falkow et al., 1976). Plasmid-mediated penicillin resistant Neisseria

gonorrhoeae strains were initially isolated in 1976. Resistance in these organisms was mediated by beta-lactamase-specifying plasmids of two distinct sizes, that appeared from epidemiological studies, to have arisen from two distinct genetic events. A 3.2 Mdal plasmid had been isolated from outbreaks of gonorrhea in western Africa and England, while a 4.4 Mdal plasmid had been isolated from outbreaks in Asia and in North America (Perine et al., 1977). Previously, two cryptic plasmids of 2.6 and 24.5 Mdal in size, had been isolated from Neisseria gonorrhoeae. These were likely indigenous to Neisseria as the mole% G+C ratio for these plasmids was 50, a value identical to that obtained for the chromosome of Neisseria (Stiffler et al., 1975; Roberts et al., 1979). Conversely, the newly isolated resistance plasmids more closely resembled the plasmids of Haemophilus in mole% G+C ratio and in physical structure as well. This led to speculation that the source of the gonococcal plasmids was the genus Haemophilus (Roberts et al., 1977; Mayer et al., 1974).

The gonococcal plasmids were shown by heteroduplex analysis to contain approximately 40% of TnA, and to share extensive homology with plasmid RSF0885, a 4.1 Mdal ampicillin resistance plasmid isolated from H. parainfluenzae (Roberts et al., 1977). As well, a 4.4 Mdal plasmid isolated from H. influenzae type b, was shown to be 92% homologous in sequence to the 4.4 Mdal plasmid of gonococcus, thus emphasizing the likelihood of a common evolutionary origin (Laufs et al., 1979).

3. Genetic Exchange Between Haemophilus and Neisseria.

The mechanism of transfer of plasmid DNA from Haemophilus to Neisseria remained a mystery. The small ampicillin resistance plasmids isolated from Haemophilus and Neisseria were non-conjugative. Transformation had been the only sexual mechanism identified in Neisseria (Sparling, 1966), but the

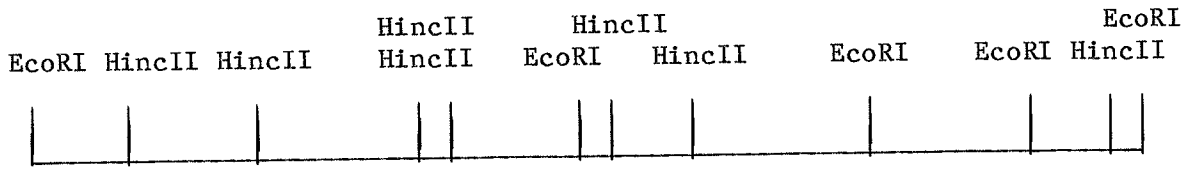
gonococcus was refractory to transformation by DNA isolated from a background of Haemophilus or Escherichia coli. Sparling et al. (1978) were able to demonstrate conjugal transfer of an ampicillin resistance plasmid from the genus Haemophilus to Neisseria gonorrhoeae, although the plasmid was unstable in the gonococcal background. The donor was a strain of H. parainfluenzae harbouring a 4.4 Mdal plasmid. As the ampicillin resistance plasmid was non-conjugative, a co-resident plasmid mediating the transfer was necessary. However such a mobilizing plasmid was not visualized in the donor or in the gonococcal recipient. This caused Sparling et al. (1978) to speculate that the mobilizing plasmid in the donor was phenotypically cryptic and highly unstable in the background of Haemophilus.

4. Mobilization in Neisseria gonorrhoeae.

Eventually, plasmid mobilization within the gonococcus was recorded. The 24.5 Mdal phenotypically cryptic plasmid previously identified in gonococcus (Roberts et al., 1979) was shown to carry mobilization function. To date, little is known with regard to the physical structure of this plasmid. A simple restriction map has been constructed and is presented in Figure 12. Young et al. (1980) noted that insertion of a beta-lactamase gene into the Hinc A fragment destroyed the transfer function of the plasmid. Therefore, this region may contain the transfer operon.

This plasmid mediated the transfer of a 4.4 Mdal ampicillin resistance plasmid from N. gonorrhoeae during intraspecific and intergeneric mating, both in vitro (Roberts et al., 1977; Eisenstein et al., 1977; Kirven and Thornsberry, 1977) and in vivo (Roberts and Falkow, 1979). The plasmid was also capable of mobilizing the 3.2 Mdal ampicillin resistance plasmid of gonococcus, yet was rarely co-resident with this particular plasmid in

Figure 12. Restriction endonuclease map of the 24.5 Mdal mobilizing plasmid of N. gonorrhoeae[1].



1. Modified from Tenover et al., 1980.

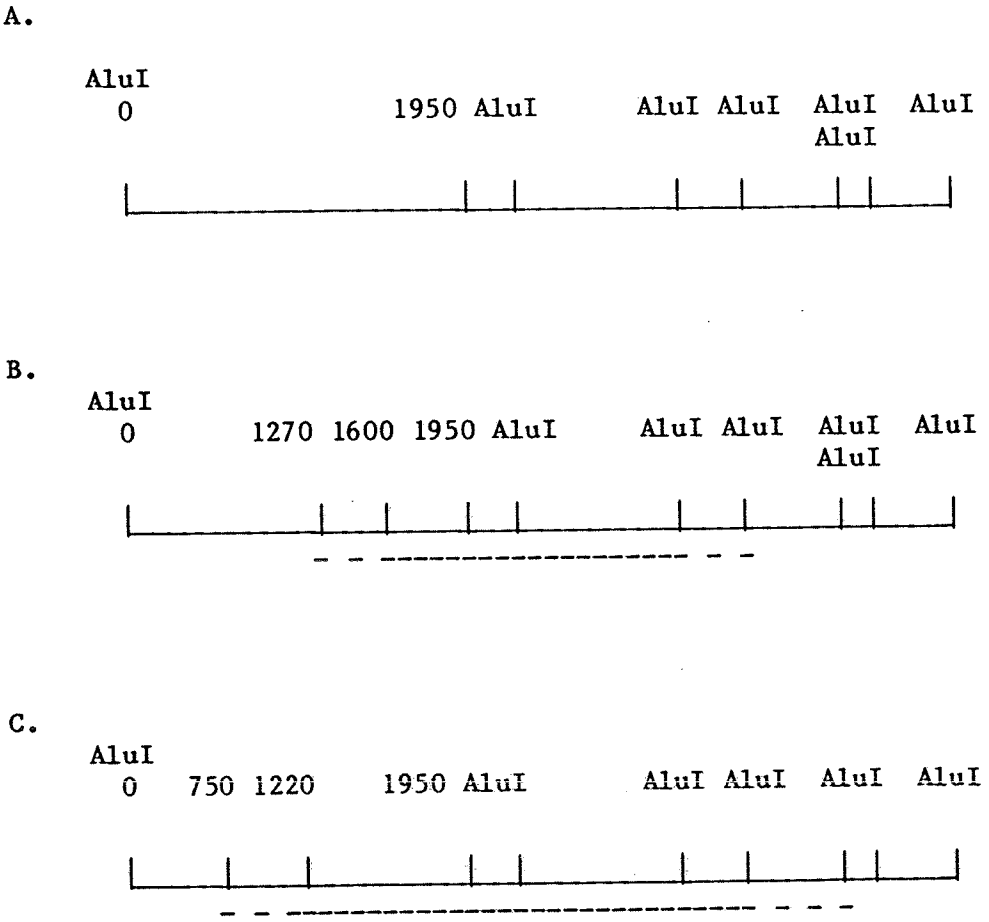
clinical isolates. Conversely, 50% of isolates containing the 4.4 Mdal plasmid contained the 24.5 Mdal mobilizing plasmid (Roberts and Falkow, 1979). Roberts and Falkow (1978) attributed the presence of a 4.4 Mdal plasmid in a number of different strains of gonococcus to conjugal transfer, while spread of a 3.2 Mdal plasmid, normally isolated from identical strains, was attributed to dissemination of a single clone.

5. The Structural Relationship Between the Ampicillin Resistance Plasmids of Neisseria gonorrhoeae.

The 4.4 and 3.2 Mdal plasmids of Neisseria gonorrhoeae had very similar structures. Sox et al. (1979) speculated that the 3.2 Mdal plasmid may have been derived from the 4.4 Mdal plasmid by in vivo transformation of N. gonorrhoeae. By in vitro transformation of N. gonorrhoeae with 4.4 Mdal plasmid DNA, they were able to isolate several derivatives of this plasmid. The most common derivative of the 4.4 Mdal plasmid was a 3.2 Mdal plasmid, identical to the native 3.2 Mdal plasmid. Sox et al. (1979) tested several of these derivatives for their ability to undergo mobilization. While some were still readily mobilized, others were not mobilizable at all (Fig. 13). The authors speculated that the OriT site and/or other gene sequences required for transfer had been lost.

Dissemination of the ampicillin resistance plasmids appeared to take two distinct routes. The 4.4 Mdal plasmid was spread by conjugal transfer due to the presence of a co-resident mobilizing plasmid. The 3.2 Mdal plasmid was derived from the 4.4 Mdal plasmid upon transformation of gonococcus. If the transformed strain carried a mobilizing plasmid, the 3.2 Mdal plasmid could be disseminated by conjugal transfer as well.

Figure 13. Deletion derivatives of the 4.4 Mdal plasmid of N. gonorrhoeae[1].



A. Intact plasmid molecule proficient for mobilization.
B. Deleted plasmid molecule proficient for mobilization.
C. Deleted plasmid molecule in non-mobilizable.

Broken lines indicate deleted regions.

1. Modified from Sox et al., 1979.

6. Plasmid-Mediated Antibiotic Resistance in H. ducreyi.

Plasmid-mediated antibiotic resistance in H. ducreyi has also been observed. Hammond et al. (1978) reported the isolation of an ampicillin resistant strain of H. ducreyi isolated in Winnipeg, Canada, after an outbreak of chancroid. The resistance was mediated by a 5.7 Mdal plasmid with a mole% G+C ratio of 41 (Brunton et al. 1979). This ratio was very close to the value obtained for the chromosome of H. ducreyi and H. influenzae, an indication that the plasmid was most likely indigenous. Maclean et al. (1980) reported that this particular plasmid encoded the production of TEM-type beta-lactamase from a TnA transposon. The 5.7 Mdal plasmid was shown, by heteroduplex analysis, to be closely related to a 4.4 Mdal plasmid mediating ampicillin resistance in Neisseria gonorrhoeae. However, a major difference between the two plasmids was the presence of a complete and functional transposon on the 5.7 Mdal plasmid (Brunton et al., 1981).

Additional ampicillin resistance plasmids have been isolated from H. ducreyi. The molecular mass of these plasmids was epidemiologically correlated with the country from which the strain originated. Plasmids from the Philippines were 7.0 Mdal in size, while those recovered from strains isolated in Mexico and Brazil were 5.7 and 3.2 Mdal in size, respectively (Handsfield et al., 1981).

Resistance of H. ducreyi to antibiotics in addition to ampicillin were noted. Albritton et al. (1982) reported the presence of a 4.9 Mdal plasmid mediating resistance to sulfonamides. In this instance, the mole% G+C ratio of the plasmid was 57, indicating that the plasmid was most likely of enteric origin.

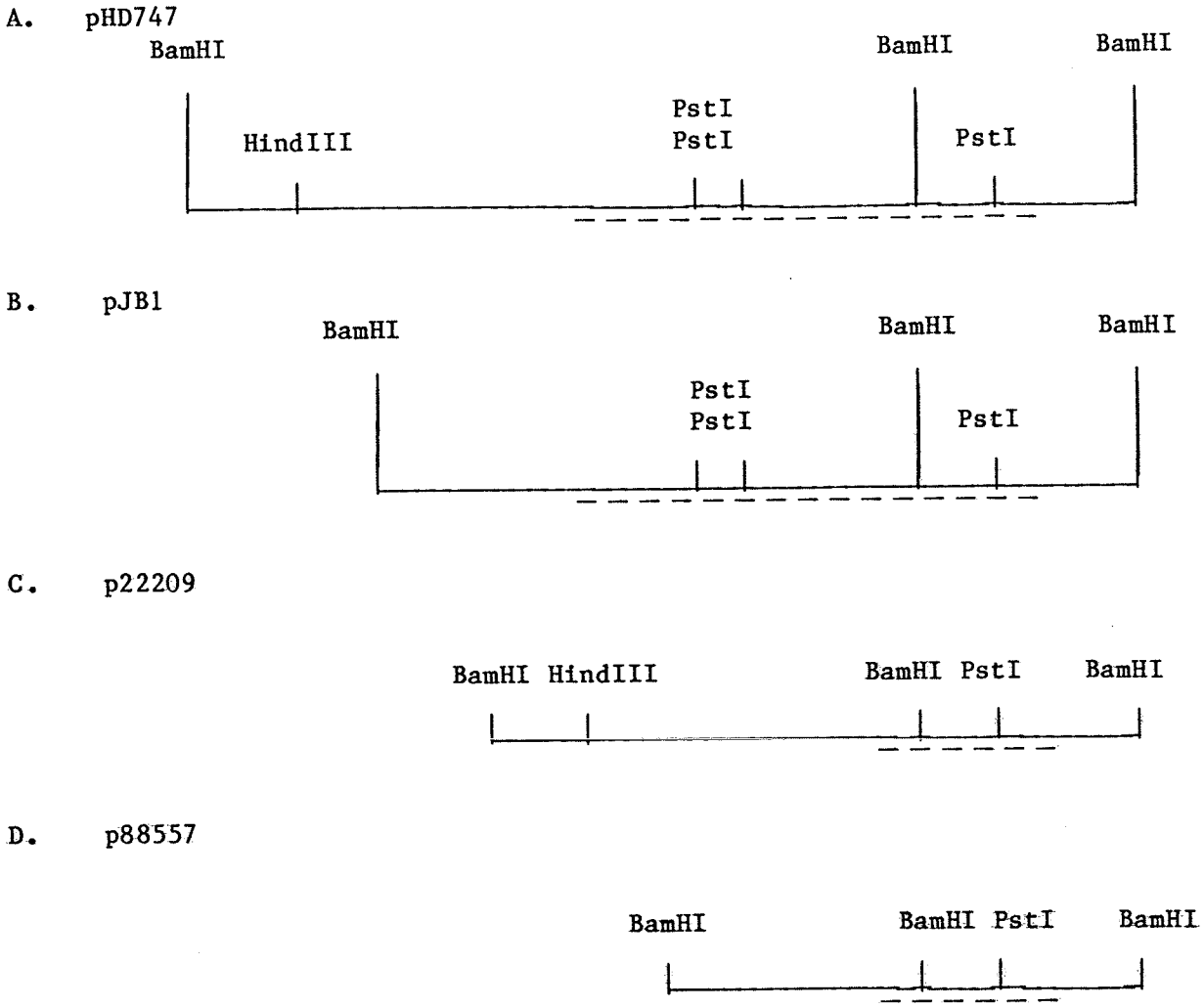
In addition to sulfonamide resistance, Deneer et al. (1982) reported

isolation in Kenya, of a strain of H. ducreyi harbouring a 7.0 Mdal ampicillin resistance plasmid, a 4.9 Mdal sulfonamide resistance plasmid, and a 23.5 Mdal phenotypically cryptic plasmid. The cryptic plasmid mediated transfer of the two smaller plasmids to Haemophilus sp. and E. coli recipients, during in vitro conjugation. This was the first reported incidence of mobilization in the genus Haemophilus, reminiscent of the discovery of a mechanism for conjugation in Neisseria gonorrhoeae (Roberts and Falkow, 1977).

Both the 7.0 and 5.7 Mdal ampicillin resistance plasmids were found by restriction mapping, to have functional Tn2-like transposons (Brunton et al., 1982). By heteroduplex analysis Brunton et al. (1982) determined that the 7.0 Mdal plasmid was completely homologous to the 4.4 Mdal plasmid of N. gonorrhoeae, with the exception of the left-hand portion of the transposon present on the 7.0 Mdal plasmid. Similarily, the 5.7 Mdal plasmid was found to be completely homologous to the 3.2 Mdal plasmid of N. gonorrhoeae (Fig. 14). The authors speculated that the plasmids of N. gonorrhoeae may have been derived from those of H. ducreyi by a transformation event. Deletion of DNA from plasmids after transformation has been previously reported (Sox et al., 1979).

Plasmids mediating resistance to chloramphenicol and tetracycline have also been identified in H. ducreyi (Albritton et al., 1984). While tetracycline therapy was previously effective in the treatment of chancroid, 90% of recent isolates from diverse geographic areas were found to be resistant to the antibiotic. Chloramphenicol and tetracycline resistance were often encoded by the same plasmid. The authors speculated that the presence of plasmid-mediated multiple antiobiotic resistance may become more prevalent in the future.

Figure 14. Restriction endonuclease maps of the ampicillin resistance plasmids of H. ducreyi and N. gonorrhoeae[1].



A. 7.0 Mdal plasmid from H. ducreyi.
B. 5.7 Mdal plasmid from H. ducreyi.
C. 4.4 Mdal plasmid from N. gonorrhoeae.
D. 3.2 Mdal plasmid from N. gonorrhoeae.

_ _ _ _ Position of the ampicillin transposon.

1. Modified from Brunton et al., 1982.

Materials and Methods.

A. Culture Media and Conditions for Growth.

1. Solid Media.

Haemophilus species and *Neisseria gonorrhoeae* were maintained on GC Agar Base (Gibco Laboratories, Madison, Wisconsin) supplemented with 1% bovine haemoglobin (Gibco) and 1% CVA enrichment (Gibco). *N. cinerea* was maintained on Trypticase Soy Agar (Gibco). Cultures were incubated at 35°C in a humid atmosphere of 5% CO_2. *Escherichia coli* strains were maintained on MaConkey Agar without Crystal Violet (Gibco) under growth conditions described above. When appropriate, selective media were prepared with antibiotics at the following concentrations. Ampicillin (Ayerst Laboratories, Montreal, Quebec) was added to a final concentration of 20 ug per ml of medium. Tetracycline (Sigma Chemical Company, St. Louis, Missouri) and nalidixic acid (Sterling-Winthrop Research Institute, Rensseler, New York) were added to a final concentration of 10 ug per ml of medium. Rifampin (Sigma) was added to a final concentration of 25 ug per ml, while streptomycin (Glaxo Corporation, Toronto, Ontario) was added to a final concentration of 1000 ug per ml of medium.

2. Liquid Media.

When grown in liquid culture, *Haemophilus* species were grown in Brain Heart Infusion Broth (BHI) (Gibco). The BHI broth was supplemented with 0.1% nicotinamide adenine dinucleotide (NAD; Sigma) and 0.1% hemin (Sigma) for culture of *Haemophilus* species, but was not supplemented for the culture of *E. coli* strains. When appropriate, antibiotics were included in

the medium at the concentrations previously described. Strains of H. ducreyi, N. gonorrhoeae, and N. cinerea were cultured upon solid medium only.

B. Taxonomic Identification of Bacterial Strains.

All bacterial strains used throughout this study are described in Table 3. The identity of bacterial strains was confirmed by gram reaction, cell and colony morphology, biochemical reactivity, and nutritional requirements. Haemophilus species were identified based upon requirement for hemin (X-factor) and NAD (V-factor). Filter strips impregnated with either factor were obtained from BBL Microbiology Systems, Becton, Dickenson and Co., Cockeyville, Maryland. Strips were placed singly and in combination on plates of GC Base Agar previously inoculated with the test organism in a manner producing confluent growth. Growth on plates with both factors was indicative of H. influenzae. Growth on plates with both factors or with V factor only was indicative of H. parainfluenzae. H. influenzae strain biotypes were biochemically differentiated based upon reaction in API-20E identification strips (Analytab Products, Plainview, New York). Each strip was inoculated with a dense saline suspension of growth from an 18 h plate culture. Biotypes were differentiated on the basis of indole production, urease and ornithine decarboxylase activity. H. ducreyi strains were biochemically inert and were identified on the basis of gram reaction and cell and colony morphology.

N. gonorrhoeae and N. cinerea strains were identified on the basis of gram reaction as well as cell morphology and carbohydrate utilization.

E. coli strains were identified based upon biochemical reaction of API-20E strips.

Table 3. Bacterial strains and plasmids.

Bacterial Strain	Plasmid Complement (x 10^6 daltons)		Phenotype	Source or Reference
H. ducreyi	pHD147	23.5	-	C.I.[1],Kenya.
HD147	pHD747	7.0	Ap[2]	
	pHD447	4.9	Su[3]	
35000	-	-	-	C.I.,Wpg.[4]
35000str	-	-	Str[5]	Spontaneous mutant of 35000.
H. influenzae				
1008	-	-	-	C.I., Wpg.
1008str	-	-	Str	spontaneous
1008rif	-	-	Rif[6]	mutants of 1008.
Rd	-	-	-	(Albritton, 1981)
RdNov	-	-	Nov[7]	transformation of Rd to resistance.
RdNovpHD147	pHD147	23.5	Nov	conjugation of Rd with HD147.
RdpHD747	pHD747	7.0	Ap	(Brunton et al., 1979)
RdpJB1	pJB1	5.7	Ap	(Brunton et al., 1979)
Rdp22209	p22209	4.4	Ap	Transformation of Rd with p22209.
Rdp88557	p88557	3.2	Ap	Transformation of Rd with p88557.
recl-nov	-	-	Nov	Transformation of Rd to *recl*, nov and str.(Notani et al., 1972; Setlow et al. ,1972)
recl-str	-	-	Str	
H. parainfluenzae				
Hpa038	-	-	-	C.I., Wpg.,
Hpa038str	-	-	Str	Spontaneous mutant of Hpa038.
H. aegyptius				
ATCC11116	-	-	Str	American Type Culture Collection.
N. gonorrhoeae				
22209	p22209	4.4	Ap	C.I., Wpg.
	24.5	24.5	-	
	2.6	2.6	-	
88557	p88557	3.2	Ap	C.I., Wpg.
	2.6	2.6	-	

Table 3. Bacterial strains and plasmids. (continued)

Bacterial Strain	Plasmid Complement (x 10^6 daltons)		Phenotype	Source or Reference
N. gonorrhoeae				
NGO29	24.5 2.6	24.5 2.6	- -	C.I., Wpg.
C134E	24.5	24.5	-	C.I., Wpg.
76-061782	24.5 2.6	24.5 2.6	-	Center for Disease Control, Atlanta Georgia
F62	2.6	2.6	-	(Sparling, 1966)
F62rif	2.6	2.6	Rif	spontaneous mutant of F62.
N. cinerea				
NRL32165	-	-	-	C.I., Seattle
E. coli				
C600	-	-	$hsdr^-hsdm^+$ $thr^-leu^-thi^-$	(Appleyard, 1954).
C600str	-	-	$hsdr^-hsdm^+$ $thr^-leu^-thi^-$ Str	spontaneous mutant of C600.
W3110polA	-	-	$polAthi^-$	(DeLucia and Cairns, 1969)
HB101	-	-	pro^-leu^- $thi^-recA13$	(Maniatis, et al., 1982)

1. Clinical Isolate.
2. Ampicillin Resistance.
3. Sulfonamide Resistance.
4. Winnipeg.
5. Streptomycin Resistance.
6. Rifampin Resistance.
7. Novobiocin Resistance.

1. Haemophilus Species.

Haemophilus species are gram-negative coccobacilli. The organisms are facultatively anaerobic and require X (hemin) and/or V (NAD) factors for growth, and reduce nitrate to nitrite.

H. ducreyi is a fastidious organism, requiring X, but not V factor for growth. The organism requires a humid 5% to 10% CO_2 atmosphere with an optimum growth temperature of 33°C to 36°C. Colonies are not easily disrupted and may be pushed intact across the surface of culture medium. Microscopic examination of H. ducreyi reveals a characteristic whorl pattern of cells. H. ducreyi is relatively biochemically inert.

H. influenzae requires both X and V factors for growth. The organism can be divided into serotypes a through f, as dependent upon six serologically distinct capsular polysaccharides. The organism can also be differentiated into biotypes I through VIII, based upon the production of indole and the presence of urease and ornithine decarboxylase enzymes.

H. aegyptius can be differentiated from H. influenzae on the basis of the inability of the former to ferment xylose, but is considered by some investigators to be a biotype of H. influenzae (Kilian, 1976).

H. parainfluenzae is identified on the basis of a requirement for V factor only. The organism can be differentiated into biotypes I through III on the basis of the same biotyping scheme proposed for H. influenzae.

Results of differential tests for the identification of Haemophilus species are presented in Table 4.

2. Neisseria Species.

Neisseria species are gram-negative diplococci. Some of the species have fastidious growth requirements including a requirement for a humid growth environment of 5% to 10% CO_2.

Table 4. Differential tests for identification of Haemophilus and Neisseria species.

Organism	Factor Requirement X	Factor Requirement V	Fermentation of Glucose	Fermentation of Sucrose	Fermentation of Lactose	Production of Indole	Production of Urease	Production of Ornithine Decarboxylase	Nitrate Reduction	Biotype
H. ducreyi										
C147	+	-	-	-	-	-	-	-	+	N/A[1]
35000	+	-	-	-	-	-	-	-	+	N/A
H. influenzae										
1008	+	+	+	-	-	+	+	+	+	I
Rd	+	+	+	-	-	-	+	+	+	IV
recl-nov	+	+	+	-	-	-	+	+	+	IV
recl-str	+	+	+	-	-	-	+	+	+	IV
H. aegyptius										
ATCC11116	+	+	+	-	-	-	+	-	+	N/A
H. parainfluenzae										
Hpa038	-	+	+	+	-	-	+	-	+	III
N. gonorrhoeae										
22209	N/A	N/A	+	-	-	N/A	N/A	N/A	N/A	N/A
88557	N/A	N/A	+	-	-	N/A	N/A	N/A	N/A	N/A
NG029	N/A	N/A	+	-	-	N/A	N/A	N/A	N/A	N/A
C134E	N/A	N/A	+	-	-	N/A	N/A	N/A	N/A	N/A
76-061782	N/A	N/A	+	-	-	N/A	N/A	N/A	N/A	N/A
F62	N/A	N/A	+	-	-	N/A	N/A	N/A	N/A	N/A
N. cinerea										
NRL32165	N/A	N/A	+	+	-	N/A	N/A	N/A	N/A	N/A

1. Not Applicable.

N. gonorrhoeae can be identified on the basis of gram-stain morphology and the ability to utilize glucose. The organism has fastidious growth requirements, and will grow only upon a supplemented medium.

N. cinerea can be differentiated from N. gonorrhoeae by the ability to utilize both sucrose and glucose. As well, the organism is able to grow on unsupplemented medium. Results of differential tests for the identification of Neisseria species are presented in Table 4.

3. Escherichia coli Strains.

Strains of E. coli are gram-negative bacilli. The organisms are faculative anaerobes, and can be differentiated from others members of the Enterobacteriaceae on the basis of several biochemical tests. Results of differential tests for E. coli strains are presented in Table 5.

C. Detection of Beta-lactamase Production.

Bacterial strains growing at ampicillin levels of 20 ug per ml of medium were checked for the production of beta-lactamase. A small amount of growth from a plate culture or a few drops of a broth culture were mixed with a drop of chromogenic cephalosporin solution (Appendix C) (Glaxo Group Research Ltd., Greenford, Middlesex, England) in a well of a microtiter plate. The presence of the enzyme was indicated by a change in the colour of the chromogenic cephalosporin solution from yellow to red within five minutes.

D. Standardization of Cell Culture Suspensions.

It was necessary to accurately correlate broth culture turbidity with

Table 5. Identification of Escherichia coli strains.

Biochemical Test	E. coli strain		
	C600	HB101	W3110polA
Ortho-nitro-phenyl-beta-galactopyranosidase	+	+	+
Arginine Dehydrogenase	-	-	-
Lysine Decarboxylase	+	+	+
Ornithine Decarboxylase	-	-	-
Citrate	-	-	-
Hydrogen Sulfide	-	-	-
Urease	-	-	-
Tryptophan Deaminase	-	-	-
Indole	+	+	+
Voges-Proskauer	-	-	-
Gelatinase	-	-	-
Glucose	+	+	+
Mannose	+	-	+
Inositol	-	-	-
Sorbitol	+	+	+
Rhamnose	+	+	+
Saccharose	-	-	-
Melibiose	+	+	+
Amylose	-	-	-
Arabinose	+	+	+
Oxidase	-	-	-

the number of colony forming units (CFU's) per ml of medium. Turbidity was measured with a Klett-Summerson Photometric Colorimeter (Klett Manufacturing Co., New York, New York) fitted with a number 64 red filter. Serial dilutions of the same cells measured in the colorimeter were plated in duplicate on appropriate medium in order to accurately determine CFU's per ml of medium. In this way, a standard curve was constructed relating Klett units to CFU's per ml of broth suspension for each bacterial species. Standard curves were constructed for E. coli strain C600, H. influenzae strain Rd, and N. gonorrhoeae strain F62. The standard curves are presented in Figure 15.

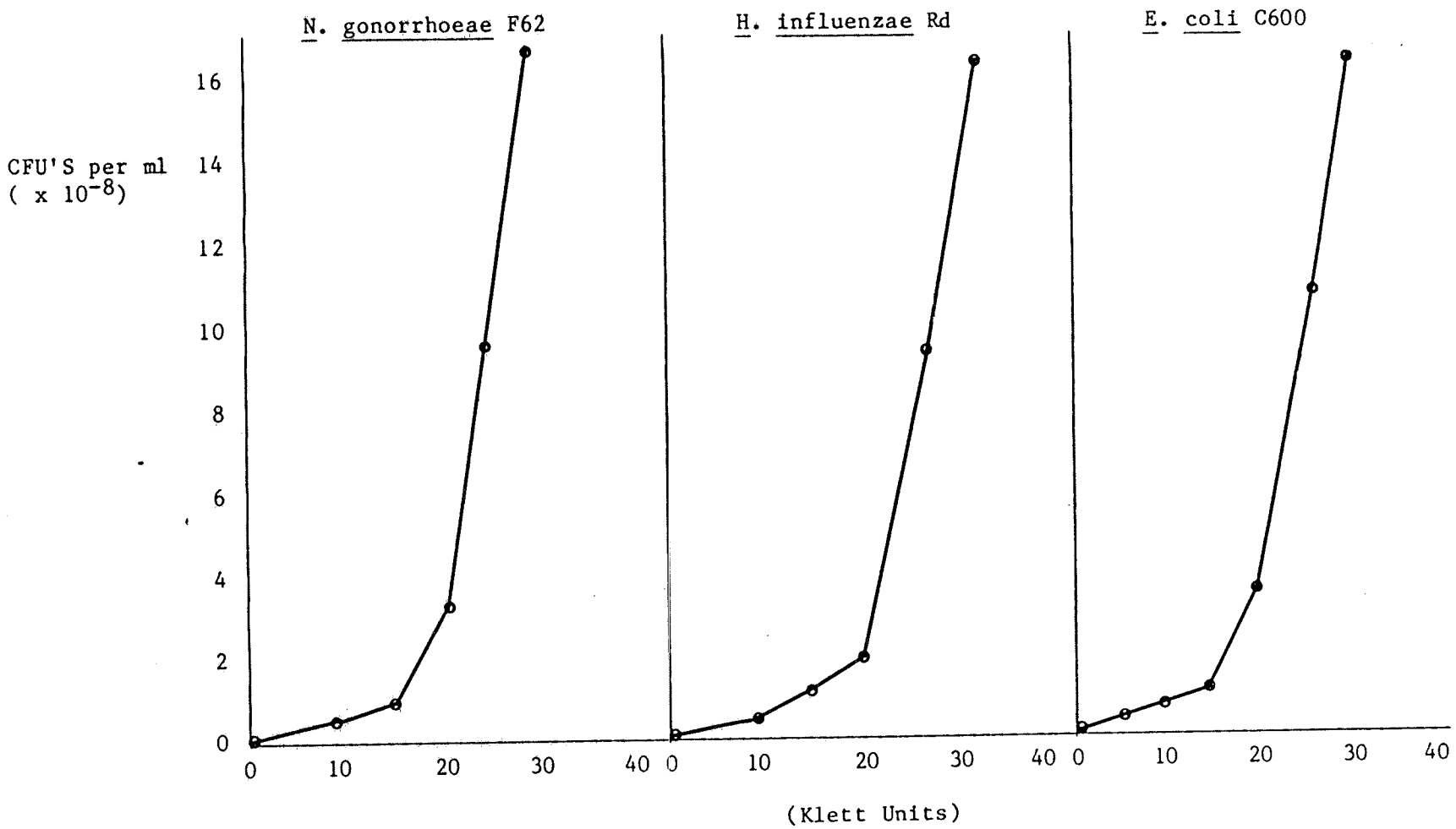
E. Isolation of Plasmid DNA.

1. Rapid Isolation Techniques.

Several rapid methods for detecting the presence of plasmids within host cells were tested. One method gave consistently good results for Haemophilus and Neisseria species, while a second method gave satisfactory results for strains of E. coli. The method used for Haemophilus and Neisseria species was carried out as follows (Klein et al, 1980).

A loopful of cells recovered from an overnight agar culture was suspended in 500 ul of Tris-EDTA-Saline Buffer (TES) (Appendix B) in a 1.5 ml capacity microfuge tube. The suspension was mixed by vortexing. A 100 ul aliquot of freshly prepared lysozyme (Sigma) (10 mg/ml TES) was added and after mixing, the suspension was incubated at 37°C for 0.5 h. A 100 ul aliquot of 10% sodium dodecyl sulfate (SDS) in TES was added, and the tube contents were mixed by inversion. The tube was allowed to stand for 10 min at room temperature. An equal volume of TES-saturated phenol solution was

Figure 15. Standard curves relating Klett units to colony-forming units per ml of cell suspension.



added and mixed by vortex action. The tube was centrifuged for 15 min at 12,800 x g, in a microfuge (Eppendorf model 5412, Sybron Corp., Westbury, New York). The supernate was transferred to a new microfuge tube. The DNA was precipitated with 2 x volume of cold absolute ethanol during a 20 min incubation at -70°C. The precipitated DNA was pelleted by centrifugation for 5 min. The supernate was decanted and the pellet air-dried after inversion of the tube. The dried pellet was resuspended in 50 ul of TES buffer for subsequent electrophoretic analysis.

The Boiling Method of Maniatis et al. (1982) was used for E. coli strains. A 1.5 ml aliquot of a dense overnight broth culture was placed in a microfuge tube. The cells were pelleted by centrifugation for 1 min. in a microfuge. The supernate was removed by aspiration and the cell pellet was resuspended in 350 ul of Triton-Sucrose Lysis Buffer (Appendix B). A 25 ul aliquot of freshly prepared lysozyme (10 mg per ml of 10 mM Tris-HCl, pH 8.0) was added and the tube contents were mixed by vortexing for 3 seconds. The tube was placed in a boiling waterbath for 40 seconds. After centrifugation for 10 min at room temperature, the pellet was discarded and 40 ul of 2.5 M sodium acetate was added to the supernate. The DNA was precipitated by the addition of 2 x volume absolute ethanol and kept for 20 min at -70°C. The DNA was resuspended in 50 ul TES buffer and electrophoretic analysis carried out.

2. Isolation of Purified Plasmid DNA by CsCl-Dye-Buoyant Density Centrifugation.

Highly purified plasmid DNA was isolated from CsCl gradients essentially after the method of Clewell and Helinski (1969). Broth cultures (400 ml) incubated overnight with shaking, were pelleted by centrifugation in an IEC model B-20A centrifuge at 30,000 x g for 20 min. The pellet was

washed by resuspension in 20 ml TES, followed by centrifugation at 30,000 x g for 10 min. The washed pellet was frozen at -20°C, then thawed on ice and resuspended by vortex action in 4 ml of cold Tris-Sucrose Buffer (Appendix B). A 0.5 ml aliquot of freshly prepared lysozyme in 0.25 mM Tris-HCl pH 8.0 (10 mg per ml) was added and incubation continued for an additional 10 min at room temperature. A 1.5 ml aliquot of Triton-lytic Mix (Appendix B) was added and tube contents were gently mixed by inversion. The mixture was kept on ice for 30 min then centrifuged at 120,000 x g at 4°C for 30 min to pellet chromosomal DNA and cell debris. The cleared lysate was enriched in plasmid DNA. Cesium chloride was added to the supernate at a concentration of 0.9 g per ml and dissolved by gentle agitation. A 0.2 ml aliquot of ethidium bromide solution (10 mg per ml of 10 mM Tris-1 mM EDTA buffer, pH 8.0) was added per 7 ml of CsCl solution. The CsCl-EtBr-cell lysate solution was dispensed into polyallomer tubes (No. 326814, Beckman Instruments Inc., Fullerton, California) and centrifuged for 40 h at 18°C at 150,000 x g in a Beckman Model L5-65 ultracentrifuge with a Beckman 50 Ti fixed angle rotor. At the end of the centrifugation period, banded plasmid DNA was visualized under long wave ultra-violet light (Black-Ray Lamp, Model UVL-21, Ultra-Violet Products Inc., San Gabriel, California) and collected by dripping the gradient from the tube base using a Beckman Fraction Recovery System. The fraction containing the plasmid DNA was extracted three times with 1 x volume of CsCl-saturated isopropyl alcohol in order to remove intercalated ethidium bromide. The DNA was dialysed at 4°C against several changes of 10 mM Tris-HCl, pH 8.0. After adjusting salt concentration to 0.3 M with respect to sodium acetate, the DNA was precipitated with 2 x volume absolute ethanol at -20°C overnight, then pelleted by centrifugation at 12,000 x g for 20 min at -10°C. Pelleted DNA was resuspended in 10 mM Tris-HCl, pH 8.0.

F. Determination of DNA Concentration.

The concentration of DNA in a solution was determined spectrophotometrically after the method of Maniatis et al. (1982). Readings were taken at wavelengths of 260 nm and 280 nm using a Unicam SP1800 Ultraviolet Spectrophotometer (Pye Unicam Ltd., Cambridge, England). The reading at 260 nm facilitated calculation of the nucleic acid concentration in a sample by assuming that an optical density (OD) reading of 1.0 corresponded to a concentration of 50 ug DNA per ml for double-stranded molecules. The ratio between the readings at 260 nm and 280 nm provided an estimate of purity of the nucleic acid preparation with regard to protein contamination. Pure preparations of DNA had an OD260/OD280 value of at least 1.8.

G. Gel Electrophoresis.

1. Agarose Gel Electrophoresis.

Agarose gel electrophoresis of plasmid DNA was carried out with a vertical gel electrophoresis system (Model no. 1070-V161, Bethesda Research Laboratories Inc., Rockville, Maryland). Gels were run in Tris-Borate Buffer (Appendix B) at 50 mAmp and 150 Volts for approximately 2.5 h, or until the tracking dye (Appendix C) added to each sample had migrated to within one cm of the bottom of the gel. Gels ranged in concentration from 0.7% to 1.4% agarose (SeaKem Agarose, Mandel Scientific Col, Rockwood, Ontario) in Tris-Borate Buffer (Appendix B). A 40 ul sample containing from 2 to 4 ug of DNA in 30 ul TES Buffer, with 10 ul of tracking dye solution was run per lane. In order to visualize the DNA within the gel matrix, the gel was stained in a 500 ml aqueous solution of ethidium bromide at a concentration of 3 ug per ml. After staining, the gels

were illuminated with a Chromato-Vue Transilluminator (model 0-61, Ultra-Violet Products Inc., San Gabriel, California) and photographed with a Polaroid MP4 camera with Polaroid type 57 high speed film.

2. Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis.

The procedure used for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was essentially that of Laemmli (1970). All reagents are described in Appendix C, while all buffers are described in Appendix B. A vertical gel electrophoresis system (Protean Cell 165-1420, Bio Rad Laboratories, Mississauga, Ontario) coupled to a LKB 2103 power supply (Fisher Scientific Ltd., Winnipeg, Manitoba) was used for protein analysis. Gels varied in concentration from 10% to 15% acrylamide-bis (Appendix C) and were poured to a thickness of 0.75mm. Gels were run at 25 mA constant current in Glycine Buffer (Appendix B) until the tracking dye added to the Loading Buffer (Appendix B) ran off the bottom of the gel, usually within 2 to 3 h. Molecular weight standards (Bio Rad 161-0304) from 10,000 to 100,000 daltons were included on each gel. After electrophoresis, gels were subjected to the following fixing and staining protocol (Fairbank et al., 1971). Gels were fixed overnight in a solution of 25% propanol - 10% glacial acetic acid - 0.05% Coomassie Brilliant Blue. The following day, the gels were placed in a solution of 10% propanol - 10% glacial acetic acid - 0.005% Coomassie Brilliant Blue for 5 to 9 h. Destaining was completed in an aqueous solution of 10% glacial acetic acid.

H. Construction of Plasmid Restriction Endonuclease Maps.

Plasmids were mapped by the use of restriction endonucleases, either

singly, or in combination. The restriction enzymes used throughout this study were obtained from Boerhinger-Mannheim Canada Ltd., Dorval Quebec, and are listed along with their respective recognition sequences in Table 6. For the mapping procedure, a 2 ug quantity of DNA was incubated with the restriction endonucleases at a temperature and in a buffer system recommended by the manufacturer (Appendix B). Digestion was carried out to completion, usually for a minimum of 3 h. Fragments were resolved by agarose gel electrophoresis. Lambda bacteriophage DNA, cleaved with HindIII restriction endonuclease was included on each gel as a standard of molecular weight. Using the distance migrated by the lambda fragments from the origin as the abscissa, and the log molecular weight of the fragments as the ordinate, a standard curve was constructed for each mapping gel (Fig. 16). From this curve, one could determine the molecular weight of fragments of unknown mass co-run with the standards on the gel. Fragments were oriented relative on one another by comparing restriction patterns obtained with single, double and triple enzyme combinations.

I. Cloning of Restriction Endonuclease Fragments.

Vectors used for the cloning of plasmid fragments were derivatives of plasmid pBR322 (Bolivar, 1977). Plasmid pAT153 (Twigg and Sherratt, 1980) encoded resistance to tetracycline and ampicillin, but lacked an origin of transfer site. Plasmid pAT2, the second cloning vector used in this study, was constructed from plasmid pAT153 in the laboratory, in the following manner. Plasmids pAT153 and p22209 were ligated *in vitro* at their unique *Pst* I restriction sites. *E. coli* strain C600 was transformed with recombinant plasmid DNA and selection was made for tetracycline resistance, ampicillin sensitivity of transformants. Screens of transformants revealed

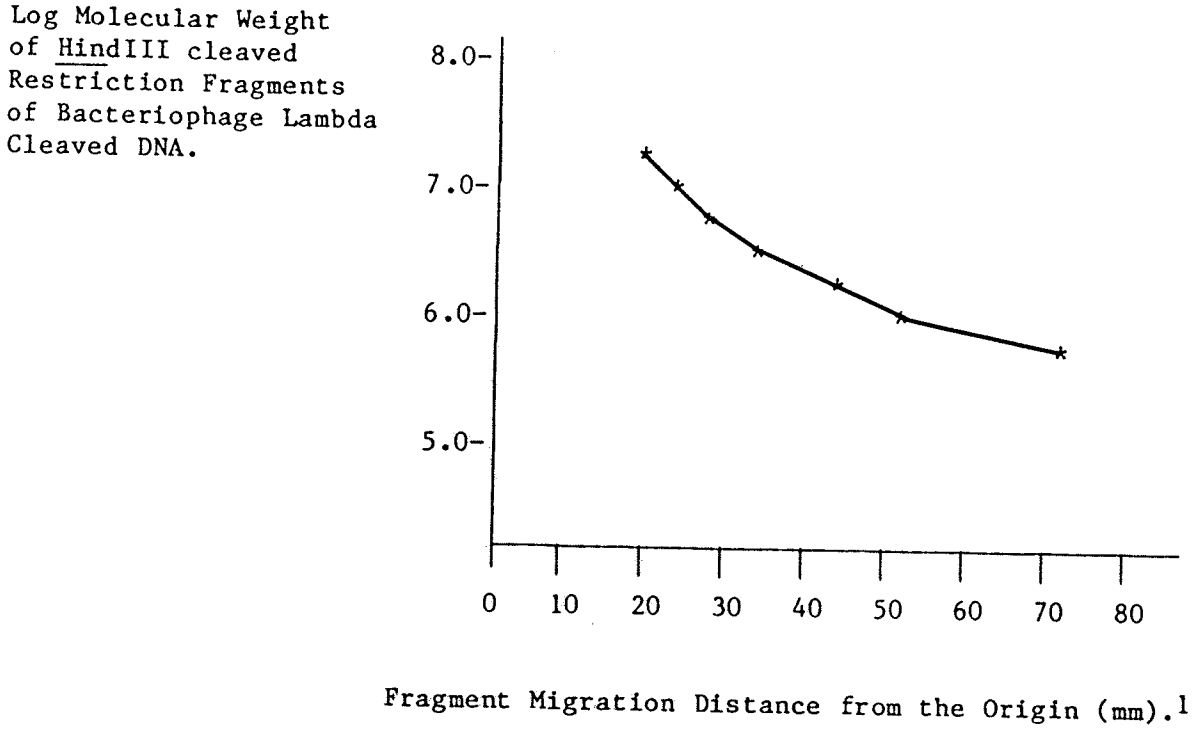
Table 6. The recognition sequences of Type II restriction endonucleases used in the construction of plasmid maps.

Restriction Endonuclease	Recognition Sequence
AvaI	5'G | Py[1]CGPu[2]G 3' 3'C | Pu GCPy C 5'
BamHI	5'G | GATCC 3' 3'C | CTAGG 5'
BglII	5'A | GATCT 3' 3'T | CTAGA 5'
ClaI	5'AT | CGAT 3' 3'TA | GCTA 5'
HaeII	5'PuGCGC | Py 3' 3'PyCGCG | Pu 5'
HindIII	5'A | AGCTT 3' 3'T | TCGAA 5'
PstI	5'CTGCA | G 3' 3'GACGT | C 5'
PvuII	5'CAG | CTG 3' 3'GTC | GAC 5'
SacII	5'CCGC | GG 3' 3'GGCG | CC 5'
XhoI	5'C | TCGAG 3' 3'G | AGCTC 5'
XhoII	5'Pu | GATCPy 3' 3'Py | CTAGPu 5'

\| Point of cleavage within recognition sequence.

1. Pyrimidine.
2. Purine.

Figure 16. Standard curve for the determination of the molecular weight of double-stranded fragments of DNA.



1. Values are dependent upon conditions for electrophoresis.

that many carried a single plasmid smaller in size than either pAT153 or p22209. The plasmid was isolated from one such transformant and mapped by restriction digest. The plasmid was identical to plasmid pAT153 with the exception of the absence of the ampicillin resistance gene. This plasmid, created by spontaneous deletion in vivo, was called pAT2. A diagrammatic representation of the three plasmids is presented in Figure 17.

The quantity of insert to vector DNA used in cloning experiments was in the molar ratio of at least 4:1, favouring isolation of recombinant plasmids as opposed to recircularized vector. The total concentration of DNA within ligation mixtures was 2 pM, with regard to free 5' ends. The ug quantity of insert and vector DNA used in a ligation reaction was calculated based upon the restriction enzyme used. Digestion with different enzymes resulted in different quantities of free 5'end. A sample calculation for deriving these values is given in Table 7.

Vector and insert DNA were digested separately with appropriate concentrations of the same or complementary restriction endonucleases. The reaction mix was extracted once with 1 x volume 10mM Tris-HCl, pH 8.0 saturated phenol. This step was followed by two extractions with 1 x volume 95% chloroform - 5% isoamyl alcohol. Salt concentration was adjusted to 0.3 M with respect to sodium acetate, and the DNA was precipitated with 2 x volume ethanol at -70°C for 20 min. The pellets were dried under vacuum then resuspended, and pooled in a total volume of 20 ul of ligation buffer (Appendix B). Two units of T4 DNA Ligase (Boerhinger-Mannheim) was added. Ligation reactions were carried out overnight at 16°C. The ligation mixture was used directly in the transformation of host cells.

Figure 17. Plasmids pBR322, pAT153, and pAT2.

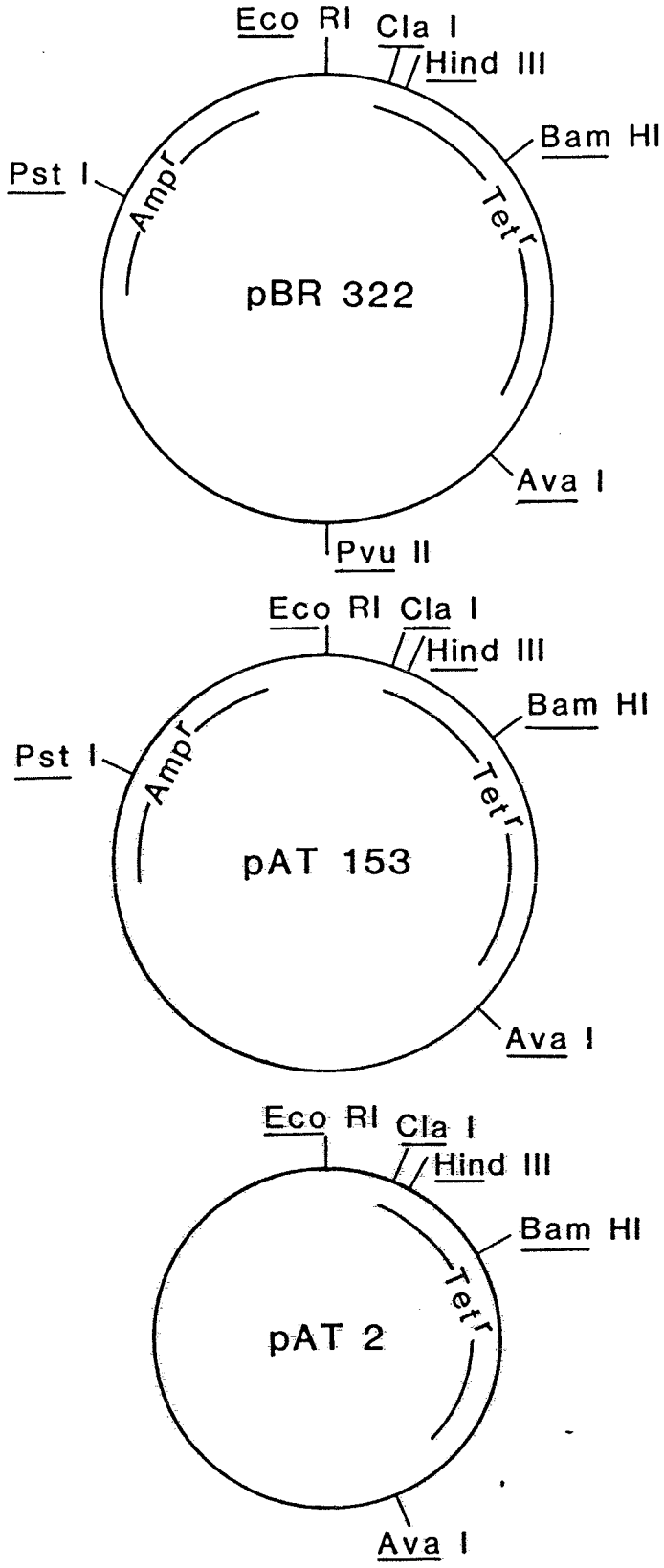


Table 7. Determination of the quantity of vector and fragment DNA required for formation of recombinant plasmids with BamHI restriction endonuclease.

Vector DNA	Fragment DNA
plasmid pAT153 2.6×10^6 daltons	plasmid p22209 4.4×10^6 daltons

1. Calculation for the determination of the number of micrograms of DNA in 1 picomole of molecules.

2.6×10^6 g = 1 Mole	4.4×10^6 g = 1 Mole
2.6 ug = 1 picomole (pM)	4.4 ug = 1 picomole (pM)

2. No. of BamHI sites per plasmid molecule.

pAT153 has 1 site.	p22209 has 2 sites.
1 pM BamHI cleaved pAT153 DNA has 2 pM of 5' ends. 2.6 ug pAT153 = 2 pM 5' ends.	1 pM BamHI cleaved p22209 DNA has 4 pM of 5' ends. 4.4 ug p22209 = 4 pM 5' ends.

3. Ratio of Vector DNA to Insert DNA.

1	:	4
1.3 ug pAT153 = 1 pM 5' ends.		4.4 ug p22209 = 4 pM 5' ends.

4. Total concentration of 5' ends required per reaction is 2 pM.

0.13 ug pAT153 = 0.1 pM 5' ends.	0.44 ug p22209 = 0.4 pM 5' ends.
0.52 ug pAT153 = 0.4 pM 5' ends.	1.76 ug p22209 = 1.6 pM 5' ends.

The ratio of vector DNA to insert DNA is 1:4. The total number of pM of 5' ends in the ligation mixture is 2.

J. Transformation of Bacterial Cells.

The procedure used for the transformation of E. coli strains was a modification of that of Cohen et al. (1972). A 100 ml volume of L Broth (Appendix A) was inoculated with 1 ml of an overnight broth culture. The broth culture was incubated with vigourous shaking at 37°C for 60 to 120 min, until a cell density of 1 x 10^8 per ml had been reached. The culture was chilled on ice for 15 min to stop growth, then the cells were pelleted by centrifugation. The pellet was resuspended in a 50 ml volume of cold 0.1 M $MgCl_2$, then immediately repelleted by centrifugation. This time, the pellet was resuspended in a 5 ml volume of cold 0.1 M $CaCl_2$ and chilled on ice for 20 min prior to being pelleted for a third time. The pellet was resuspended for a final time in 1 ml of cold 0.1 M $CaCl_2$. A 0.2 ml sample of the cell suspension was incubated on ice for 30 min with 50 to 200 ng DNA. Cells were heat shocked for 2 min at 42°C then chilled for an additional 10 min period. The cell suspension was brought to a final volume of 1 ml by the addition of L Broth, and was incubated for 60 min at 37°C. Sample of the culture were plated on medium appropriate for the selection of transformants.

K. In Vitro Conjugation of Bacterial Species.

In order to study the mechanism of plasmid mobilization, in vitro matings were carried out. The procedure utilized either two or three parental strains. Both interspecific and intergeneric matings were carried out.

When matings were carried out with Escherichia coli strains or with Haemophilus species, with the exception of H. ducreyi, cells suspensions were obtained by inoculation of a 10 ml volume of supplemented BHI Broth

with a small amount of growth from an overnight agar culture. The broth culture was incubated with shaking at 37°C until a density of 1 x 10^9 CFU's per ml had been reached. Cultures were then standardized to contain 10^7,10^8 or 10^9 CFU's per ml dependent upon whether the suspended cells were initial or intermediate donors, or final recipients respectively, in the mating aggregate.

When H. ducreyi, N. gonorrhoeae and N. cinerea were used as parental strains, 18 h agar cultures were used as a source of cells. A small amount of the culture was suspended in 10 ml of supplemented BHI Broth and was immediately standardized to contain 10^7, 10^8, or 10^9 CFU's per ml as dependent upon the strains function in the mating aggregate.

The in vitro mating procedure was carried out in the following manner. A 1 ml sample of each parental strain was mixed together by vortexing and collected by filtration onto a 0.45 u polycarbonate filter (Bio-Rad Laboratories, Richmond, California). The filter was placed culture side up on the surface of a chocolate agar plate and incubated overnight to allow plasmid transfer to occur. The next day, the growth on the filter was resuspended in 1 ml BHI Broth and serial dilutions were plated on media to allow selection of donor, recipient and transconjugant cultures. Control plates were included to determine spontaneous mutation rates of parental strains to selective markers. The mobilization frequency of the plasmids was calculated based upon the number of transconjugant colonies per final recipient colony base upon the formula;

$$\text{Mobilization Frequency} = \frac{\text{number of transconjugants}}{\text{number of transconjugants + number of recipients}}$$

The number of transconjugants was added to the number of recipients present at the end of the mating period, to give a value representative of the total number of cells acting as recipients. In general, the number of

transconjugants was so low, relative to the number of recipients, that the addition of transconjugants did not significantly alter the value of the denominator. Mating combinations are presented in Figure 18.

L. Statistical Analysis of Mobilization Frequencies.

Mobilization studies with the ampicillin resistance plasmids of H. ducreyi and N. gonorrhoeae were carried out in triplicate. Mobilization frequencies were calculated for each replicate. A one-way analysis of variance was carried out to determine if the variance in mobilization frequencies within replicates for each plasmid was lower than the variance between replicates for all plasmids. Means derived from the three replicate mobilization frequency values for each plasmid were compared by the Student-Neuman Multiple Range Test.

M. Methodology for the Determination of the Presence of Homology between Plasmids.

The presence of homology between plasmid molecules was determined by the Southern Blot procedure (Southern, 1975). Plasmids to be probed for homologous sequences were digested with several different combinations of enzymes. The resulting fragments were resolved by electrophoresis through a 1% agarose gel. The DNA within the gel matrix was denatured by gently shaking the gel in a solution of 0.4 M NaOH - 0.8 M NaCl for 30 min. After neutralization by gently shaking in a solution of 0.5 M Tris-HCl, pH 7.6 - 1.5 M NaCl for 30 min, the single-stranded DNA was transferred to nitrocellulose paper (Schleicher and Schuell, Keene, New Hampshire) by the blotting

Figure 18. Combinations of parental strains used for *in vitro* conjugation studies.

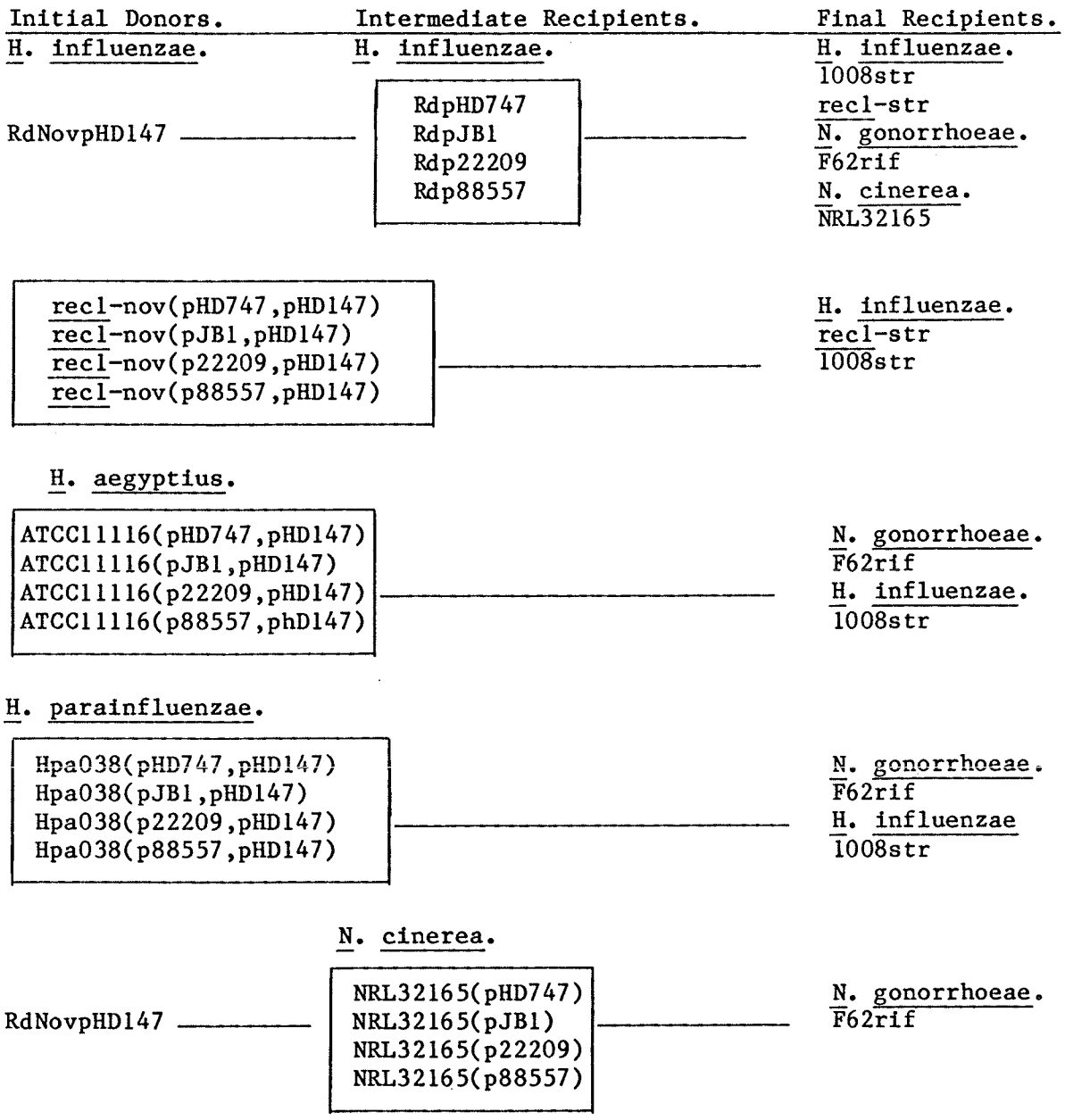


Figure 18. Combinations of parental strains used for *in vitro* conjugation studies. (continued)

Initial Donors.	Intermediate Recipients.	Final Recipients.
N. gonorrhoeae.	*H. influenzae.*	
NGO29	RdpHD747 RdpJB1 Rdp22209 Rdp88557	*N. gonorrhoeae.* F62rif *H. influenzae.* 1008str
76-06178		*N. gonorrhoeae.* F62rif *H. ducreyi.* 35000str
H. ducreyi. HD147		*N. cinerea.* NRL32165 *N. gonorrhoeae.* F62rif

process. After blotting, the nitrocellulose filter was washed in 2 x standard saline citrate (SSC) (Appendix C), then dried for 6 h at 70°C. To prevent non-specific binding of the radio-labelled DNA probe, the nitrocellulose paper was pre-treated in the following manner. The filter was sealed in a plastic bag with 0.2 ml of pre-hybridization mix (Appendix C) per cm^2 of nitrocellulose. The filter was incubated with shaking in a water bath at 68°C for 4 to 18 h. The pre-hybridization mix was removed and the hybridization mix (Appendix C) was added at a volume of 0.1 ml per cm^2 of filter. The hybridization mix also contained 2 x 10^6 disintegrations per min (dpm) of nick-translated denaturated probe DNA per ml. The bag was resealed and hybridization was carried out overnight at 68°C. The following day the filter was washed twice in 2 x SSC - 0.1% SDS at 68°C for 0.5 h each time. The filter was then washed twice in 0.2 x SSC - 0.1% SDS at 68°C for 0.5 h each time. After washing, the filter was air-dried then autoradiography was carried out a -70°C for 4 to 48 h with Dupont Cronex X-ray film and a Dupont Cronex Quanta III intensifying screen.

N. The Nick Translation Procedure.

Plasmid DNA to be used as a probe for homologous sequences, was rendered radio-active by the process of nick translation (Rigby et al., 1977). The procedure was carried out by use of a Nick Translation Kit N.5000 produced by Amersham Co., Oakville, Ontario. Radio-labelled alpha-^{32}P-[dCTP] (3.2 Ci per mole) was obtained from New England Nuclear, Lachine, Quebec. The nick translation procedure was carried out as follows.

A 150 ng quantity of DNA, suspended in 10 mM Tris-HCl, pH 8.0 buffer was placed in a microfuge tube. A 4 ul volume of Solution 1 (Appendix C)

was added, followed by the addition of 50 picomoles of alpha-^{32}P-[dCTP]. A 2 ul aliquot of Solution 2 (Appendix C) was added, then the volume of the reaction mixture was brought to 20 ul by the addition of double-distilled (dd) H_2O. The contents of the microfuge tube were vortexed, then centrifuged briefly. Incubation was carried out for 1.5 h at 14°C. The translation reaction was stopped by the addition of a 40 ul volume of Stop Buffer (Appendix B) followed by heating for 15 min at 68°C. The nick-translated DNA was separated from free nucleotides by Sephadex G-100 (Pharmacia Fine Chemicals, Uppsala, Sweden) column chromatography with TE Buffer (Appendix B) eluent. Excluded radioactivity was pooled and the specific activity (dpm per ug) of the DNA determined by the scintillation counting of a 1 ul sample.

O. An In Vitro Procedure for Locating the Origin of Conjugative Transfer Site of a Plasmid Molecule.

1. Isolation of DNA-Protein Relaxation Complexes.

The location of the origin of transfer (OriT) site within the genome of a plasmid could be determined for some plasmid molecules by the procedure of Nordheim et al (1980). The procedure was based upon the ability of a plasmid molecule to form a DNA-protein relaxation complex. If a plasmid did not form such a complex, then the OriT site could not be located by this procedure.

The procedure was carried out as follows. Duplicate 50 ml BHI Broth cultures of an E. coli or H. influenzae strain, harbouring the plasmid of interest, were grown overnight at 37°C. If the plasmid mediated resistance to ampicillin, the antibiotic was included in the medium at a concentration

of 20 ug per ml. The cultures were pelleted, and the pellets were resuspended and washed twice in a 1 ml volume of cold Tris-sucrose Buffer (Appendix B). A 0.2 ml aliquot of freshly prepared lysozyme (5mg per ml of 0.25 M Tris-HCl, pH 8.0) was added, and the suspensions were incubated for 5 min at room temperature. A 0.4 ml aliquot of 0.25 M EDTA, pH 8.0 was then added and the incubation was continued for an additional 5 min. Cells were lysed by the addition of 1.6 ml of Triton-Lytic Mix (Appendix B) followed by incubation for 15 min on ice. Cell debris and chromosomal DNA were pelleted by centrifugation of the cell lysates at 40,000 x g for 15 min. The cleared lysate of one of the two duplicate cultures was treated for 10 min at 37°C with SDS at a final concentration of 0.25%. This treatment converted plasmid DNA-protein relaxation complexes in the lysate from supercoiled to relaxed open-circular forms. The second culture lysate, treated in the same manner up to this point, was pre-treated by heating at 60°C for 30 min prior to the addition of SDS. The heat treatment prevented the relaxation event from occurring. Any subsequent relaxation of the heat-treated plasmid DNA would be as a result of handling, so the duplicate sample acted as a control for non-specific nicking. Relaxed complexes were purified by passage of the lysate through a Sepharose 4B (Pharmacia) chromatography column (inner diameter 0.7 x 20 cm, flow rate 0.22 ml per min) with TES as the eluent. Fractions of 0.5 ml were collected and fractions two through six were pooled. The pooled fractions were treated with Proteinase K (Beckman) at a final concentration of 1 mg per ml for 15 min at 37 °C. Enzyme action was quenched by extraction with 1 x volume TES-saturated phenol, followed by two extractions with 1 x volume 95% chloroform - 5% iso-amyl alcohol. Salt concentration was adjusted with 0.1 x volume of 3 M sodium acetate. The DNA was precipitated with 2 x volume ethanol at -70°C for 20 min.

2. Labelling of the Relaxation Complex Nick Site.

After in vitro nicking of the DNA-protein relaxation complexes, plasmid DNA was precipitated then dissolved in 10 mM Tris-HCl, pH 8.0. A 1 ug quantity was labelled by a time-limited translation procedure at a temperature that was sub-optimum for the functioning of the Klenow fragment of E. coli DNA polymerase I. The reaction mixture for the labelling procedure contained 1.5 ul of 10X salt solution (0.5 mM Tris-HCl, pH 7.6 , 10 mM Dithiothreitol (Sigma), 75 mM $MgCl_2$, 500 ug per ml Bovine Serum Albumin (Sigma)), 1 ul each of 1.0 mM dATP, dCTP, dTTP (Boerhinger-Mannheim), 6 ul alpha-^{32}P-[dGTP] (3.2 Ci per mole) (New England Nuclear) and 3.5 U E. coli DNA polymerase I - Klenow Fragment (Boerhinger-Mannheim) in a final volume of 25 ul. The reaction was carried out for 10 min at 3°C, then quenched by the addition of 50 ul of stop buffer (10mM Tris-HCl, pH 8.0, 30 mM EDTA, 20 ug plasmid DNA). The sample was heated at 68°C for 15 min. The labelled DNA was purified by passage through a Sephadex G-100 column (Pharmacia). Excluded radioactivity was pooled, extracted with 1 x volume Tris-HCl, pH 8.0-saturated phenol, then with 1 x volume 95% chloroform-5% isoamyl alcohol. DNA was ethanol precipitated as previously described.

3. Identification of the Location of the OriT site.

The precipitated plasmid DNA was resuspended in 20 ul of 10 mM Tris-HCl, pH 8.0 then divided into portions for digestion with several different combinations of restriction enzymes. The resulting fragments were resolved on a vertical 1% agarose gel, stained with an aqueous solution of ethidium bromide (5 ug per ml), then photographed under ultra-violet light. Gels were dried on a dryer (Bio Rad Laboratories, Gel Slab Dryer Model 224) then autoradiography was carried out for 0.5 to 2 h at -70°C as previously

described. The restriction fragment containing the oriT site was specifically labelled.

P. Procedure for Determining the Location of the Origin of Vegetative Replication Site of the Ampicillin Resistance Plasmids of H. ducreyi and N. gonorrhoeae.

1. Cloning of the BamHI restriction fragments of the ampicillin resistance plasmids.

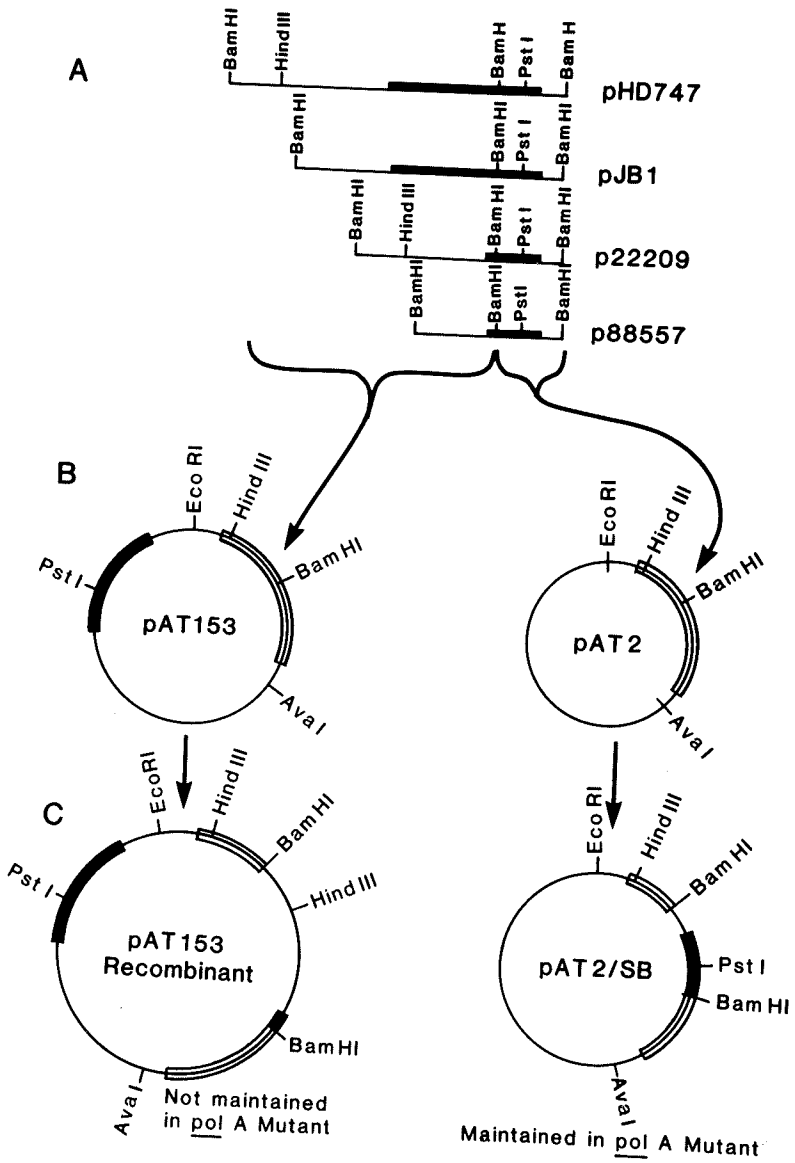
Digestion of the ampicillin resistance plasmid of H. ducreyi and N. gonorrhoeae with BamHI restriction endonuclease resulted in two fragments. The larger fragment was inserted into plasmid pAT153 at the unique BamHI site within the tetracycline gene. Selection of transformants was based upon resistance to ampicillin but sensitivity to tetracycline.

The smaller BamHI fragment contained the gene encoding ampicillin resistance. This fragment was inserted into plasmid pAT2 at the unique Bam HI site within the tetracycline gene. Again, selection of transformants was based upon ampicillin resistance. Transformants were screened for plasmid content. Recombinant plasmids were mapped by restriction endonuclease digestion in order to determine the orientation of the insert. The cloning procedure is illustrated in Figure 19.

2. Determination of the BamHI Fragment Containing the OriV Site.

Both the plasmids pAT153 and pAT2 carry the OriV sequence of plasmid pMB9, a ColE1-like plasmid requiring the presence of DNA polymerase I for maintenance (Kolleck, et al., 1978). Therefore, these plasmids were not maintained in a pol A mutant. Conversely, the ampicillin resistance plasmids of H. ducreyi and N. gonorrhoeae were maintained in a pol A mutant.

Figure 19. Cloning of the BamHI fragments of the ampicillin resistance plasmids of H. ducreyi and N. gonorrhoeae.



A. Restriction endonuclease maps of the ampicillin resistance plasmids.
B. Cloning of the BamHI fragments.
C. Recombinant plasmids.

E. coli strain W3110 pol A was transformed with recombinant plasmid DNA described above. The recombinant plasmid containing the OriV site of the ampicillin resistance plasmids could replicate in the pol A mutant. The location of the OriV site was determined to be within a specific BamHI fragment in this manner.

Q. Direction of In Vitro Replication of the Ampicillin Resistance Plasmids of H. ducreyi and N. gonorrhoeae and Confirmation of the Location of the OriV Site.

1. Introduction.

The direction of replication of several plasmids has been determined by the use of in vitro techniques, specifically replication of plasmid DNA in cell-free extracts (Sakakibara and Tomizawa, 1974; Conrad et al., 1979; Som and Tomizawa, 1982). The methodology used in these studies was applied to the investigation of the direction of replication of the ampicillin resistance plasmids of H. ducreyi and N. gonorrhoeae.

2. Preparation of Cell Extract.

Cell extract was prepared in the following manner. A 750 ml volume of an 18 h L Broth culture of E. coli strain C600 was pelleted by centrifugation. The pellet was resuspended in a 1 ml volume of sucrose-phosphate buffer (10% sucrose - 50 mM KH_2PO_4, pH 7.4). The pellet was frozen at -20°C, then slowly thawed in an ice bath. The total volume of the cell suspension was adjusted to 1.9 ml by the addition of sucrose-phospate buffer. A 75 ul aliquot of 2 M KCl was added in addition to 200 ul of freshly prepared lysozyme solution (4 mg per ml of 50 mM KH_2PO_4) and 60 ul of a 5% solution of Brij-58 (Sigma). The solution was mixed by inversion, then

incubated for 30 min at 0°C. Centrifugation at 26,000 rpm, at 2°C for 20 min followed, in a Beckman L5-65 ultracentrifuge with a Beckman SW 60 rotor. The supernate, collected after the centrifugation step, was the cell-free extract used in subsequent reactions.

3. In Vitro Replication of Plasmid DNA Template in Supplemented Cell-Free Extract.

Plasmid pHD747 DNA template was replicated in supplemented cell-free extract in the presence of alpha-^{32}P-[dCTP]. Two reactions were carried out. In the first reaction (Table 8, column A) plasmid DNA was replicated in the presence of equal molar quanitities of 2',3'-dideoxythymidine (ddTTP), an inhibitor of DNA chain elongation, and 2'-deoxythymidine. In the second reaction (Table 8, column B), ddTTP was ommitted from the reaction mixture. The components of the replication mixtures are presented in Table 8. All components, with the exception of the plasmid DNA, were mixed and allowed to equilibrate for 5 min at 30°C. Template DNA was then added, and after mixing, the reaction mixtures were incubated for 10 min at 30°C. Each reaction mixture was treated with a 25 ul aliquot of deoxyribonuclease-free (DNase-free) ribonuclease (RNase) solution (Sigma) (Appendix C) for 15 min at 37°C, followed by treatment for 15 min at 37°C with Proteinase K (Beckman) in 10 mM Tris-HCl, pH 8.0, at a final concentration of 1 mg per ml. Each reaction mixture was extracted with 1 x volume of TE-saturated phenol. After centrifugation, the aqueous phase was removed, and a 4 ug quantity of plasmid pHD747 DNA was added. The additional plasmid DNA acted as a carrier during Sephadex G-100 column chromotography. TE Buffer, pH 8.0 was used as the eluent. Excluded radioactivity was pooled, extracted once with 1 x volume TE-saturated phenol, then twice with 1 x volume 95% chloroform - 5% isoamyl alcohol. Salt was adjusted to 0.3 M

Table 8. Components of the reaction mixture for in vitro replication of plasmid DNA.

Components	A	B
	(ul)	(ul)
Plasmid DNA (2ug/ul)	0.50	0.50
Cell-free Extract	25.00	25.00
0.1 M $MgCl_2$	5.50	5.50
10 mM ATP	1.80	1.80
10 mM CTP	2.10	2.10
10 mM GTP	1.70	1.70
10 mM UTP	1.80	1.80
10 mM NAD	15.00	15.00
1 M KH_2PO_4	1.90	1.90
2 M KCl	2.50	2.50
5 mM dATP	0.50	0.50
5 mM dGTP	0.35	0.35
5 mM dCTP	0.13	0.13
5 mM dTTP	0.35	0.35
3.2 Ci/mole alpha-^{32}P-[dCTP]	2.50	2.50
1.9 mM ddTTP	1.00	0.00
ddH_2O	12.40	13.40
Total Volume	75.00	75.00

A. Reaction components including ddTTP.
B. Reaction components excluding ddTTP.

with respect sodium acetate, and plasmid DNA was precipitated at -70°C for 20 min with 2 x volume ethanol. The DNA was pelleted by centrifugation, dried under vacuum, then resuspended in an appropriate restriction buffer.

The radio-labelled DNA samples, resulting from the two reactions, were digested with BamHI and HindIII restriction endonucleases. The resulting fragments were resolved by electrophoresis in a 1% agarose gel. The gel was dried and autoradiography was carried out.

4. Confirmation of the Location of the OriV Site by Initiation of Replication with Plasmid DNA Restriction Fragments.

An attempt was made to initiate in vitro replication at the OriV site of plasmid pHD747 using BamHI-HindIII restricted DNA as a template. The contents of duplicate reaction mixtures were as described in Table 8, column B. Incubation was carried out at 30°C for 10 min. The reaction mixtures were treated with DNase-free RNase and Proteinase K as previously described then extracted with TE-saturated phenol and 95% chloroform - 5% isoamyl alcohol. A 4 ug quantity of BamHI-HindIII plasmid DNA was added to each reaction mixture to act as carrier. The labelled DNA in each reaction mixture was freed from contamination with free nucleotides by Sephadex G-100 chromatography. Excluded radioactivity was pooled, adjusted to 0.3 M with respect to sodium acetate, then precipitated with 2 x volume ethanol at -70°c for 20 min. The DNA samples were pelleted by centrifugation, dried under vacuum, then resuspended in 30 ul TE buffer. The samples were run on a 1% agarose gel. The gel was dried, and the restriction digest bands were cut out. After scintillation counting of the bands, activity within each band was determined on the basis of dpm per base pair.

R. Procedure for Determining the Loaction of the OriV and OriT Sites of the Mobilizing Plasmid pHD147 of H. ducreyi.

1. Cloning of the XhoII and ClaI Restriction Fragments of Plasmid pHD147.

As a first step in the analysis of the structural characteristics of plasmid pHD147, a restriction endonuclease map was constructed. Complete cleavage with restriction endonucleases ClaI and XhoII resulted in 3 and 4 fragments respectively. The fragments resulting from digestion with ClaI were cloned into plasmid vector pAT153 at the ClaI site. Insertion into this site inactivated the gene encoding tetracycline resistance, so subsequent selection of transformants was made based upon resistance to ampicillin, but sensitivity to tetracycline.

The XhoII restriction fragments had cohesive ends that were complementary to the cohesive ends of BamHI digested DNA. Therefore, the XhoII restriction fragments of pHD147 were cloned into the BamHI site of pAT153. The tetracycline gene was inactivated by this insertion, so again, selection of transformants was made for resistance to ampicillin, but sensitivity to tetracycline.

E. coli strain C600 transformants were screened for the presence of recombinant plasmids. Plasmids were isolated from transformants by CsCl-dye-buoyant density centrifugation and the presence of insert was confirmed by restriction endonuclease digestion.

2. Replication of pAT153/pHD147 Recombinant Plasmids in a PolA Mutant of Escherichia coli: Identification of the Restriction Fragment Containing the OriV Site.

Plasmid pHD147 was maintained by E. coli strain W3110 polA, while

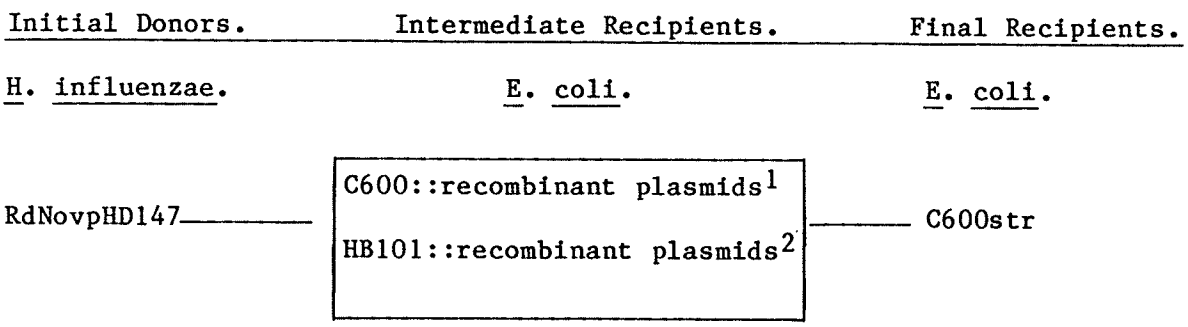
plasmid pAT153 was not. E. coli strain W3110 polA was transformed with the recombinant plasmids described above. Selection of transformants was made for resistance to ampicillin. Transformants were isolated only when the E. coli strain was transformed with specific recombinant plasmids. The pHD147 restriction fragments inserted into these plasmids carried sequences required for maintenance and replication of the recombinant plasmids. Deletion derivatives of these specific plasmids were made by digestion with HindIII restriction endonuclease, then circularization of the larger fragment. In this manner, the maintenance sequences could be specifically located with regard to the restriction endonuclease map of plasmid pHD147.

3. Mobilization of Recombinant Plasmids by Plasmid pHD147 in Recombination Proficient and Deficient Backgrounds: Identification of the Restriction Fragment Containing the OriT Site.

Despite repeated attempts during the course of this study, plasmid pHD147 could not be isolated in vitro as a DNA-protein relaxation complex. For this reason, the procedure of Nordheim et al. (1980) for specifically labelling the nick site of OriT could not be used. However, the location of the OriT site within a specific restriction fragment could be determined by studying the mechanism by which pAT153/pHD147 recombinant plasmids were mobilized by plasmid pHD147.

E. coli strains C600 and HB101 were transformed with recombinant plasmids obtained from the cloning of ClaI and XhoII fragments of pHD147 into pAT153. The host strains were recombination proficient and deficient respectively. Tri-parental matings were carried out as illustrated in Figure 20. Transconjugants were screened for the presence of plasmids. Mobilization of the recombinant plasmids by an in trans or an in cis mechanism was

Figure 20. Tri-parental matings with host strains harbouring pAT153/pHD147 recombinants.



1. C600 is a recombination proficient host cell.
2. HB101 is a recombination deficient host cell.

based upon the following criteria (Kilbane and Malamy, 1980). Mobilization was identified as occurring by an *in trans* mechanism if the following criteria were met.

1. Plasmids present in transconjugants were unaltered in size from those present in parental cells.
2. Co-transfer of plasmid pHD147 to the recipient was not required.
3. Transfer was not dependent upon the recombinational state of the recombinant plasmid host cell.

Mobilization by an *in cis* mechanism was identified by the following criteria.

1. Plasmids present in transconjugants were altered in size.
2. Co-transfer of plasmid pHD147 to recipients was required.
3. Transfer was dependent upon the recombinational state of the recombinant plasmid host cell.

The presence of a functional OriT site was a necessary pre-requisite for mobilization by an *in trans* mechanism (Kilbane and Malamy, 1980). Therefore, those recombinant plasmids mobilized in this manner had to carry a functional OriT site. Since plasmid pAT153 lacked such a site (Twigg and Sherratt, 1979), the site in the recombinant plasmids was provided by the cloned fragments of plasmid pHD147. In this manner, the location of the OriT site of plasmid pHD147 was identified to be within a specific restriction fragment.

S. Analysis of Encoded Proteins by Coupled *In Vitro* Transcription-Translation of Plasmid DNA Templates.

1. Protocol for *In Vitro* Transcription-Translation Reactions.

The proteins encoded by both native and plasmid pAT153-cloned restric-

tion fragments of several plasmids of H. ducreyi and N. gonorrhoeae were determined by the use of a coupled in vitro transcription-translation system. The Prokaryotic DNA-Directed Translation Kit (Code N.380) used in this study was purchased from Amersham. The procedures were carried out as recommended by the supplier.

For each series of reactions, a blank reaction was included. The blank reaction was composed of all reaction components, with the exception of DNA template. As well, a reaction was included containing standard plasmid pAT153 DNA supplied by the manufacturer. All other plasmid DNA template used in these reactions was isolated by CsCl-dye-buoyant density gradient centrifugation and suspended in TE buffer, pH 8.0 at a concentration of 1 ug per ul.

Stock solutions (Table 9) were removed from storage at -70°C and allowed to thaw on ice. A pre-mix solution was prepared for each reaction in a microfuge tube. Each pre-mix solution was composed of three components. The first component was a 7.5 ul aliquot of supplement solution containing sufficient nucleotides for transcription, tRNA for translation, as well as an energy-generating system and inorganic salts. The second component added to the pre-mix solution, was a 3 ul volume of an equimolar solution of amino acids, with the exception of methionine. The third component, a 2 ul aliquot of L-[^{35}S]-methionine (600uCi per mMole, translation grade) was purchased from New England Nuclear.

A 2.5 ug quantity of DNA was added, and the pre-mix solution was brought to a total volume of 12.5 ul with dilution buffer. The contents of each tube were gently mixed and pre-incubated for 5 min at 37°C. To each tube was added a 5 ul volume of the S-30 extract of E. coli supplied by the manufacturer. Tube contents were gently mixed after this addition and incubation at 37°C continued for 60 min. After this period, a 5 ul volume of

Table 9. Stocks solution supplied by Amersham in the Prokaryotic DNA-Directed Translation Kit.

Solution Number	Description
1.	An S-30 extract prepared from E. coli. (Supernatant fraction from a 30,000 x g centrifugation step).
2.	Supplement solution. This contain sufficient nucleotides for transcription, tRNA for translation, an energy-generating system and inorganic salts.
3.	Amino acids minus methionine. An equimolar mixture compatible with the use of L-[^{35}S]-methionine.
4.	Amino acids minus leucine. An equimolar mixture of amino acids compatible with the use of L-[4,5-^{3}H] leucine.
5.	Dilution buffer.
6.	Standard DNA. Plasmid pAT153, circular DNA, 500ug/ml.
7.	Methionine chase solution.
8.	Leucine chase solution.

methionine chase solution was added and incubation was continued for an additional 5 min period. This step facilitated completion of protein chains which may have been terminated prematurely due to a limiting concentration of radioactive methionine. Reactions were terminated by placing reaction tubes into an ice bath.

2. Measurement of L-[^{35}S]-Methionine Incorporation.

A 1 ul aliquot of each reaction mixture was added to separate 500 ul volumes of 1 M NaOH. These solutions were incubated at 37°C for 15 min. This procedure destroyed any RNA within the sample, which could result in background noise. The assay samples were then cooled on ice and then a 3 ml volume of ice-cold 25% trichloro-acetic acid (TCA) solution containing 1 mg per ml casein hydrolysate was added. Proteins within the assay mixtures were precipitated during a 30 min incubation period on ice. The precipitates were collected separately onto 2.5 cm glass fibre discs by filtration, then washed extensively with 5% TCA followed by ethanol. Incorporated activity within the precipitates was determined by scintillation counting.

3. Analysis of Proteins by Polyacrylamide Gel Electrophoresis.

Samples were diluted in a ratio of 1:1 with Loading Buffer (Appendix B), then heated for 5 min at 100°C. Approximately 2 x 10^5 dpm of incorporated radioactivity was loaded onto 12.5% to 15% SDS-polyacrylamide gel with 5% stacking gels. Molecular weight markers were included on the gel. The gel was essentially run, fixed and stained as previously described (See: Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis).

Results.

A. Construction of Restriction Endonuclease Maps for the Ampicillin Resistance Plasmids of H. ducreyi and N. gonorrhoeae.

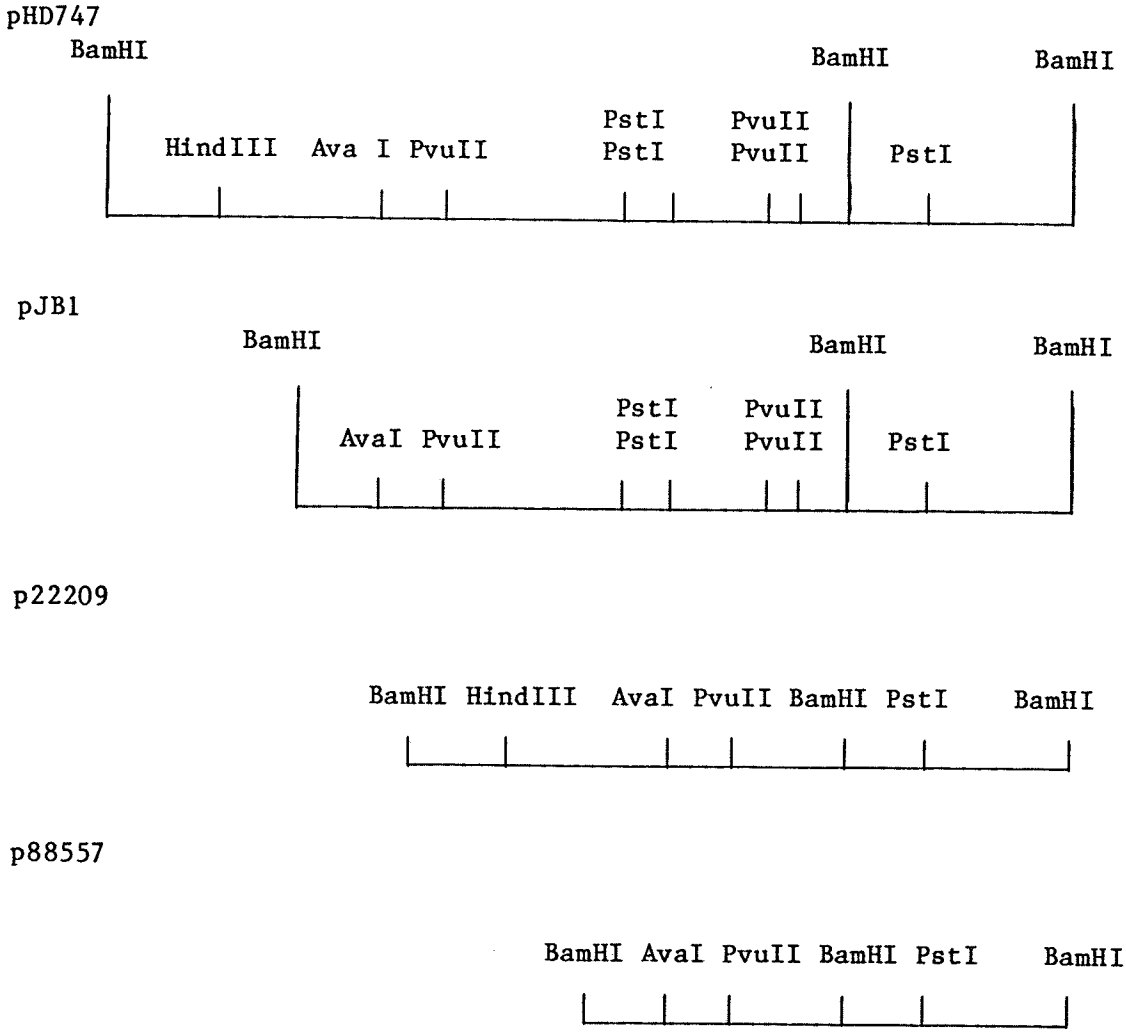
Plasmid DNA, isolated by $CsCl_2$-dye-buoyant density centrifugation was digested with several different restriction endonucleases, both singly and in combination. Fragments from the digestions were resolved by electrophoresis in agarose gels varying in concentration from 0.7% to 1.4%. The molecular mass of the fragments was determined relative to HindIII restriction fragments of known molecular mass from lambda phage DNA, co-run on each gel. The orientation of fragments relative to each other was determined by comparing the molecular mass of fragments from a single digest with the molecular mass of fragments from a double digest. The molecular mass of fragments resulting from restriction endonuclease digestion of the ampicillin resistance plasmids of H. ducreyi and N. gonorrhoeae are presented in Table 10. The restriction endonuclease map constructed for each plasmid from this information is presented in Figure 21.

The ampicillin resistance plasmids of H. ducreyi and N. gonorrhoeae were closely related with regard to structure, with two obvious exceptions. Plasmids pHD747 and pJB1 isolated from H. ducreyi contained complete ampicillin transposons, while plasmids p22209 and p88557, isolated from N. gonorrhoeae contained only partial transposons. In addition, plasmids pHD747 and p22209 contained a 1.3 Mdal region not present in plasmids pJB1 and p88557. This region was identified by the presence of a single HindIII restriction site.

Table 10. Restriction endonuclease fragments resulting from single and double digest of the ampicillin resistance plasmids of H. ducreyi and N. gonorrhoeae.

	Fragment Molecular Mass (x 10^6 daltons)				
Enzyme	BamHI	PstI	PvuII	HindIII	Plasmid
BamHI	1.4, 5.6	0.4, 0.5, 0.9 1.2, 4.0	0.3, 0.35,1.4 2.35, 2.65	1.1, 1.4, 4.5	pHD747
	1.4, 4.3	0.4, 0.5, 0.9 1.2, 2.7	0.3, 0.35,1.2 1.4, 2.35	1.4, 4.3	pJB1
	1.4, 3.0	0.5, 0.9, 3.0	0.8, 1.4, 2.3	1.1, 1.3, 1.9	p22209
	1.4, 1.8	0.5, 0.9, 1.8	0.8, 1.2, 1.4	1.4, 1.8	p88557
PstI	0.4, 0.5, 0.9 1.2, 4.0	0.4, 1.7, 4.9	0.3, 0.4,0.55 0.85, 1.5,3.4	0.4, 1.7, 2.0 2.9	pHD747
	0.4, 0.5, 0.9 1.2, 2.7	0.4, 1.7, 3.6	0.3, 0.4,0.55 0.85,1.5, 2.1	0.4, 1.7, 3.6	pJB1
	0.5, 0.9, 3.0	4.4	1.3, 3.1	2.0, 2.4	p22209
	0.5, 0.9, 1.8	3.2	1.3, 1.9	3.2	p88557
PvuII	0.3, 0.35,1.4 2.35, 2.65	0.3, 0.4,0.55 0.85,1.5, 3.4	0.3,2.35,4.35	0.3, 1.0,2.35 3.35	pHD747
	0.3, 0.35,1.2 1.4, 2.35	0.3, 0.4,0.55 0.85,1.5, 2.1	0.3,2.35, 3.0	0.4, 1.7, 3.6	pJB1
	0.3, 1.4, 2.3	1.3, 3.1	4.4	1.1, 3.4	p22209
	0.8, 1.2, 1.4	1.3, 1.9	3.2	3.2	p88557
HindIII	1.1, 1.4, 4.5	0.4, 1.7, 2.0 2.9	0.3, 1.1,2.35 3.35	7.0	pHD747
	1.1, 4.3	0.4, 1.6, 3.6	0.3,2.35, 3.0	No Site	pJB1
	1.1, 1.4, 1.9	2.0, 2.4	1.1, 3.4	4.4	p22209
	1.4, 1.8	3.2	3.2	No Site	p88557
AvaI	0.3, 0.45,1.4 2.0, 2.8	Not Done	Not Done	0.3, 0.9, 2.8 3.0	pHD747
	0.3,0.45, 0.8 1.4, 2.8	Not Done	Not Done	0.3, 2.6, 2.8	pJB1
	1.0, 2.0, 1.4	Not Done	Not Done	0.8, 3.6	p22209
	0.8, 1.0, 1.4	Not Done	Not Done	3.2	p88557

Figure 21. Restriction endonuclease maps constructed for the ampicillin resistance plasmids of H. ducreyi and N. gonorrhoeae.



B. Mobilization of the Ampicillin Resistance Plasmids of H. ducreyi and N. gonorrhoeae by Plasmid pHD147: Significance of a Recombination Proficient or Deficient Background Upon Mobilization Frequencies.

In order to determine the mechanism of mobilization of plasmids pHD747, pJB1, p22209 and p88557 by plasmid pHD147, in vitro conjugation studies were carried out. Bi- and tri-parental matings were carried out, dependent upon the recombinational proficiency of the host strains. When donor strains were recombination proficient and the final recipient strains were either recombination proficient or deficient, tri-parental matings were conducted. The initial donor strain in the mating aggregates was H. influenzae strain RdNovpHD147, harbouring the mobilizing plasmid pHD147. The intermediate donors, H. influenzae strains RdpHD747, RdpJB1, Rdp22209 and Rdp88557 harboured plasmids pHD747, pJB1, p22209 and p88557 respectively. Thus these strains were resistant to ampicillin. The recombination proficient and deficient final recipients, H. influenzae strains 1008str and DB117recl-str respectively, carried a chromosomally-mediated resistance to streptomycin.

When donor strains were recombination deficient and recipient strains were either recombination proficient or deficient, bi-parental matings were carried out. While the recipients used were H. influenzae strains 1008str or recl-str as previously described, the donor strain was H. influenzae recl-nov, harbouring the mobilizing plasmid pHD147 as well as plasmid pHD747, pJB1, p22209 or p88557. For all conjugation procedures, transconjugants were selected on the basis of resistance to ampicillin and streptomycin. Recipients were selected on the basis of resistance to streptomycin. Mobilization frequencies were calculated based upon the number of transconjugants per final recipient at the end of the mating

period. Each series of mating experiments was repeated in triplicate. The average mobilization frequency calculated for each plasmid is presented in Table 11. All ampicillin resistance plasmids were efficiently mobilized to recipient cells by plasmid pHD147. The mobilization frequencies of the plasmids varied in a reproducible manner. Plasmid pHD747 was mobilized at the highest frequency ($p<0.01$). Plasmid p22209 was mobilized at the second highest frequency ($p<0.01$). The mobilization frequencies of plasmids pJB1 and p88557 were lowest and not significantly different ($p>0.05$). Transconjugants, screened for plasmid content, contained mobilizing plasmid pHD147 in addition to an ampicillin resistance plasmid, as dependent upon the donor strain. Plasmids isolated from transconjugants were unaltered in size from those in parental strains.

C. Intergeneric Transfer of the Ampicillin Resistance Plasmids of H. ducreyi and N. gonorrhoeae.

1. Introduction.

Due to the structural relatedness of the ampicillin resistance plasmids, several in vitro conjugation studies were carried out to determine if transfer from the genus Haemophilus to the genus Neisseria could be effected.

2. Transfer Studies on the Ampicillin Resistance Plasmids from a Background of H. ducreyi or H. influenzae to N. gonorrhoeae and N. cinerea.

Conjugation studies were carried out to determine if transfer of the ampicillin resistance plasmids could be effected to N. gonorrhoeae and

Table 11. Mobilization frequencies obtained in both recombination proficient and deficient backgrounds for the ampicillin resistance plasmids of H. ducreyi and N. gonorrhoeae.

A. Recombination Proficient Host Cells

Plasmid	Mobilization Frequency[1]
pHD747	0.9×10^{-4}
pJB1	0.7×10^{-5}
p22209	0.6×10^{-4}
p88557	0.8×10^{-5}

B. Recombination Proficient Initial Donor and Intermediate Donor Recombination Deficient Final Recipient

Plasmid	Mobilization Frequency
pHD747	2.2×10^{-4}
pJB1	1.5×10^{-5}
p22209	1.0×10^{-4}
p88557	1.5×10^{-5}

C. Recombination Deficient Donor and Recombination Proficient Recipient

Plasmid	Mobilization Frequency
pHD747	1.3×10^{-4}
pJB1	0.5×10^{-5}
p22209	0.7×10^{-4}
p88557	0.6×10^{-5}

D. Recombination Deficient Donor and Recipient

Plasmid	Mobilization Frequency
pHD747	1.0×10^{-4}
pJB1	0.5×10^{-5}
p22209	0.6×10^{-4}
p88557	0.7×10^{-5}

1. Mobilization frequencies calculated on the basis of the number of transconjugants per final recipient. Values are the mean of three replicates.

N. cinerea from a background of H. influenzae or H. ducreyi. When H. influenzae was used as a parental strain, tri-parental matings were carried out with strain RdpHD147 as the initial donor. The intermediate donors were H. influenzae strains RdpHD747, RdpJB1, Rdp22209 and Rdp88557. When H. ducreyi strain HD147, harbouring plasmids pHD147, pHD747 and pHD447 was used as a donor, bi-parental matings were carried out. For all conjugation studies, the recipients were N. gonorrhoeae strain F62rif and N. cinerea strain NRL32165. Gonococcal transconjugants were selected for resistance to ampicillin and rifampin on chocolate agar, however none were isolated. N. cinerea transconjugants, selected for ampicillin resistance on trypticase soy agar, were isolated only when plasmids were transferred from a background of H. influenzae. Ampicillin resistance plasmids within the transconjugants were unaltered in size from those within parental strains. Plasmid pHD147 was transiently maintained in the background of N. cinerea. Results are summarized in Table 12-A and 12-B.

3. Bacterial Species Intermediates in Plasmid Transfer between Haemophilus and Neisseria.

In an attempt to effect plasmid transfer between Haemophilus influenzae and Neisseria gonorrhoeae, H. aegyptius, H. parainfluenzae, and N. cinerea were introduced into the mating aggregrate as donors or intermediate recipients. Bi- or tri-parental conjugation studies were carried out.

In the first series of experiments, H. aegyptius strain ATCC11116, harbouring plasmid pHD147, as well as plasmid pHD747, pJB1, p22209 or p88557 acted as the donor. The recipient was N. gonorrhoeae strain F62rif. Gonococcal transconjugants were selected on the basis of ampicillin and rifampin resistance, however no transconjugants were isolated. As

Table 12. Summary of results of plasmid transfer studies between *Haemophilus* and *Neisseria*.

Initial Donors	Intermediate Recipients	Final Recipients	Transconjugants
A. *H. ducreyi.* HD147	None	*N. cinerea.* NRL32165 *N. gonorrhoeae.* F62rif	None None
B. *H. influenzae.* RdNovpHD147	*H. influenzae.* RdpHD747 RdpJB1 Rdp22209 Rdp88557	*N. gonorrhoeae.* F62rif *N. cinerea.* NRL32165	None Yes all plasmids transferred.
C. *H. aegyptius.* ATCC11116(pHD747,pHD147) ATCC11116(pJB1,pHD147) ATCC11116(p22209,pHD147) ATCC11116(p88557,pHD147)	None	*N. gonorrhoeae.* F62rif *H. influenzae.* 1008str	None Yes all plasmids transferred.
D. *H. parainfluenzae.* Hpa038(pHD747,pHD147) Hpa038(pJB1,pHD147) Hpa038(p22209,pHD147) Hpa038(p88557,pHD147)	None	*N. gonorrhoeae.* F62rif *H. influenzae* 1008str	None Yes all plasmids transferred.
E. *H. influenzae.* RdNovpHD147	*N. cinerea.* NRL32165(pHD747) NRL32165(pJB1) NRL32165(p22209) NRL32165(p88557)	*N. gonorrhoeae.* F62rif	Yes only p22209 transferred.
F. *N. gonorrhoeae* 76-06178	None	*N. gonorrhoeae.* F62rif *H. ducreyi.* 35000str	Yes, 24.5 and 4.4 transferred None
G. *N. gonorrhoeae* NG029	*H. influenzae.* RdpHD747 RdpJB1 Rdp22209 Rdp88557	*N. gonorrhoeae.* F62rif *H. influenzae.* 1008str	None Yes all plasmids transferred.

a positive control for plasmid transfer, H. influenzae strain 1008str was included as a final recipient as well. H. influenzae transconjugants were isolated, indicating that plasmid transfer was occurring. Results are summarized in Table 12-C.

The second series of plasmid transfer experiments used the same recipient strains, however a different donor strain was used. H. parainfluenzae strain Hpa038, harbouring plasmid pHD147, as well as plasmid pHD747, pJB1, p22209 or p88557 acted as the donor. While H. influenzae strain 1008str transconjugants were isolated, as was expected, N. gonorrhoeae strain F62rif transconjugants were not. Thus H. parainfluenzae strain Hpa038 was not an efficient donor for plasmid transfer between Haemophilus and N. gonorrhoeae. Results are summarized in Table 12-D.

Finally, N. cinerea strain NRL32165 was used as an intermediate recipient in a tri-parental mating aggregate. H. influenzae strain RdpHD147 acted as the initial donor and N. gonorrhoeae strain F62rif acted as the final recipient. N. cinerea strain NRL32165 harboured plasmid pHD747, pJB1, p22209 or p88557. A single gonococcal transconjugant, selected on the basis of resistance to both ampicillin and rifampin was isolated. However, only plasmid p22209 was transferred to N. gonorrhoeae. Despite numerous attempts, transfer of the other plasmids was not observed. Results from the three series of transfer experiments are summarized in Table 12-E.

4. Transfer of Plasmids from Neisseria gonorrhoeae to Haemophilus ducreyi.

Plasmid transfer from a background of N. gonorrhoeae to H. ducreyi was also investigated. N. gonorrhoeae strain 76-01678, harbouring a 24.5 Mdal

mobilizing plasmid, a 4.4 Mdal plasmid identical to plasmid p22209, and a 2.6 Mdal cryptic plasmid was used as the donor. H. ducreyi strain 35000str was used as a recipient. Transconjugants were selected for resistance to ampicillin and streptomycin. None was isolated. N. gonorrhoeae strain F62rif was included as a recipient to act as a positive control for plasmid transfer. In this instance, plasmid transfer was effected, and ampicillin and rifampin resistant gonococcal transconjugants were isolated. Results are summarized in Table 12-F.

5. Mobilization of the Ampicillin Resistance Plasmids by the Mobilizing Plasmid of N. gonorrhoeae.

In order to determine if the block to transfer of plasmids between H. influenzae and N. gonorrhoeae was a function of the mobilizing plasmid pHD147 of H. ducreyi, transfer experiments were carried out with transfer mediated by the mobilizing plasmid of N. gonorrhoeae. Tri-parental matings were carried out. The initial donor was N. gonorrhoeae strain NGO29 harbouring a 24.5 Mdal mobilizing plasmid and a 2.6 Mdal cryptic plasmid. The intermediate recipient strains in the mating aggregate were H. influenzae RdpHD747, RdpJB1, Rdp22209 and Rdp88557 harbouring plasmids pHD747, pJB1, p22209 and p88557 respectively. The final recipients were N. gonorrhoeae strain F62rif and H. influenzae strain 1008str. H. influenzae transconjugants, selected for resistance to ampicillin and streptomycin were isolated. N. gonorrhoeae transconjugants, selected for resistance to ampicillin and rifampin were not isolated. The origin of the plasmid effecting mobilization was not the barrier to plasmid transfer between H. influenzae and N. gonorrhoeae. Results are summarized in Table 12-G.

D. Location of the OriT Site of the Ampicillin Resistance Plasmids of H. ducreyi and N. gonorrhoeae.

The ampicillin resistance plasmids of H. ducreyi and N. gonorrhoeae were isolated as DNA-protein relaxation complexes from cleared lysates of both E. coli strain C600 and H. influenzae strain Rd. The complexes were relaxed in vitro by treatment with SDS. The relaxation nick site or OriT site as it is known, was radio-labelled by a limited translation reaction with the Klenow fragment of DNA polymerase I in the presence of dATP, dTTP, dCTP and alpha-^{32}P-[dGTP]. The labelled DNA of each plasmid was isolated separately and cleaved with several different combinations of restriction endonucleases. Resultant restriction fragments were resolved through 1% agarose gels. After drying, autoradiography was carried out. For each plasmid, only one restriction fragment was specifically labelled (Fig. 22). This fragment contained the OriT site. The position of the OriT site, relative to restriction sites on a physical map of each plasmid is illustrated in Figure 23.

The OriT site of plasmids pHD747 and p22209 was located within the 1.1 Mdal BamHI-HindIII restriction fragment. The OriT site of plasmids pJB1 and p88557 was located within the 0.8 Mdal BamHI-AvaI restriction fragment.

E. Location of the OriV Site of the Ampicillin Resistance Plasmids of H.ducreyi and N. gonorrhoeae.

Each of the four ampicillin resistance plasmids had two recognition sites for BamHI restriction endonuclease. Digestion with this enzyme bisected each plasmid into fragments of unequal size. The smaller fragment was common to all four plasmids, and carried the gene mediating

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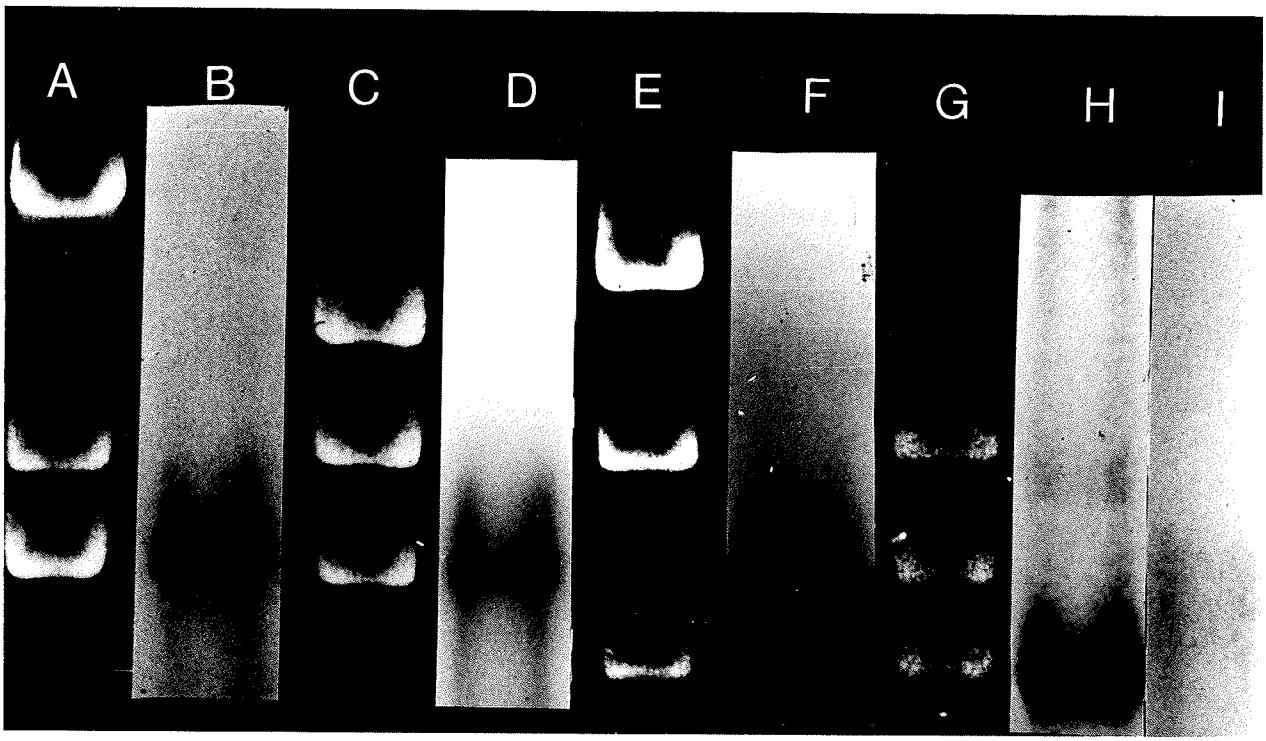
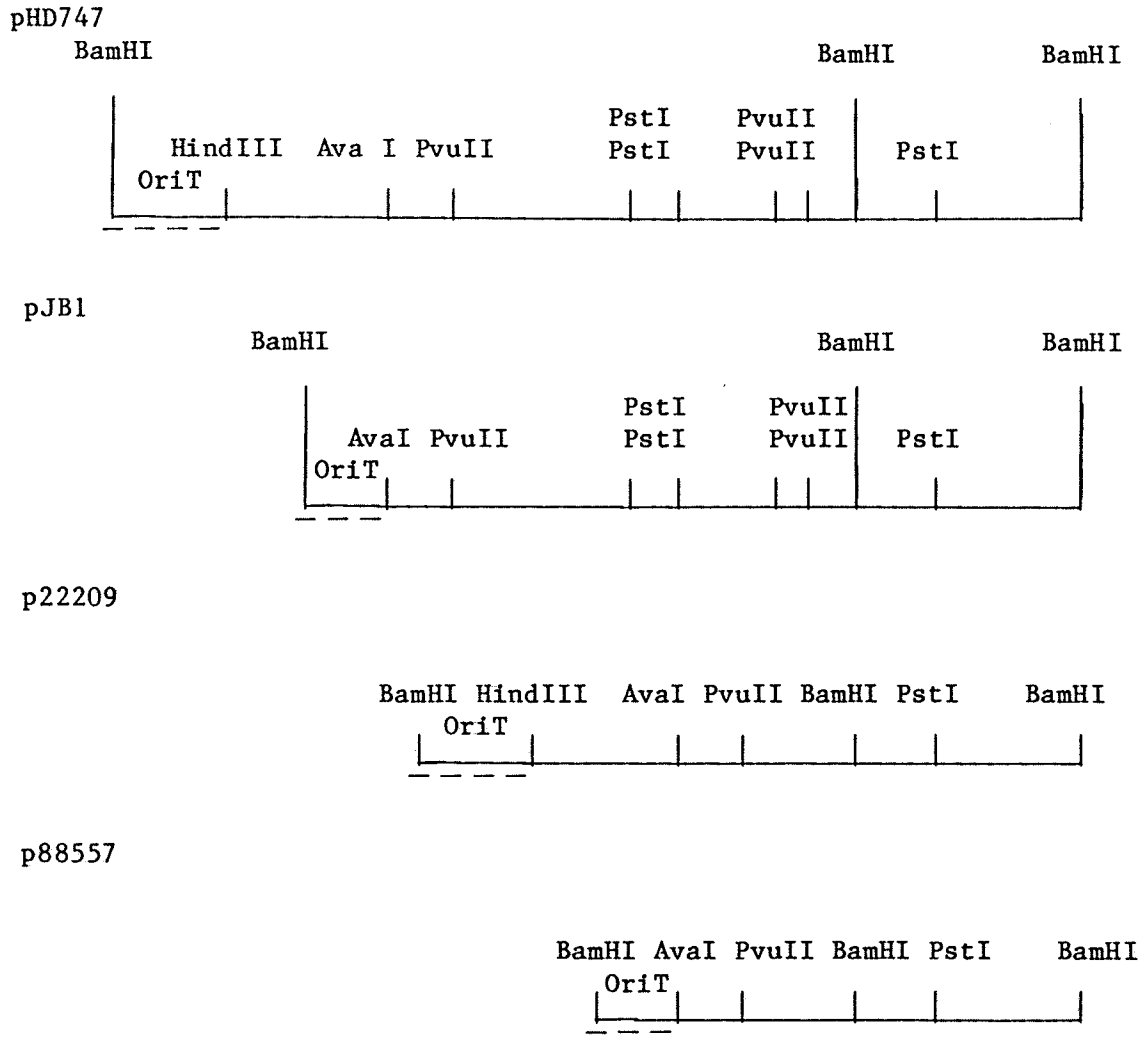
Figure 22. Determination of the position of the OriT sites of the ampicillin resistance plasmids of H. ducreyi and N. gonorrhoeae.



Lane A. BamHI-HindIII restriction fragments of radio-labeled plasmid pHD747 DNA.
Lane B. Autoradiograph of Lane A.
Lane C. BamHI-HindIII restriction fragments of radio-labeled plasmid p22209 DNA.
Lane D. Autoradiograph of Lane C.
Lane E. BamHI-AvaI restriction fragments of radio-labeled plasmid pJB1 DNA.
Lane F. Autoradiograph of Lane E.
Lane G. BamHI-AvaI restriction fragments of radio-labeled plasmid p88557 DNA.
Lane H. Autoradiograph of Lane G.
Lane I. Autoradiograph of radio-labeled plasmid p88557 DNA not treated with SDS. Control for non-specific nicking.

Figure 23. Location of the OriT site of the ampicillin resistance plasmids of H. ducreyi and N. gonorrhoeae.



_ _ _ Location of the OriT site.

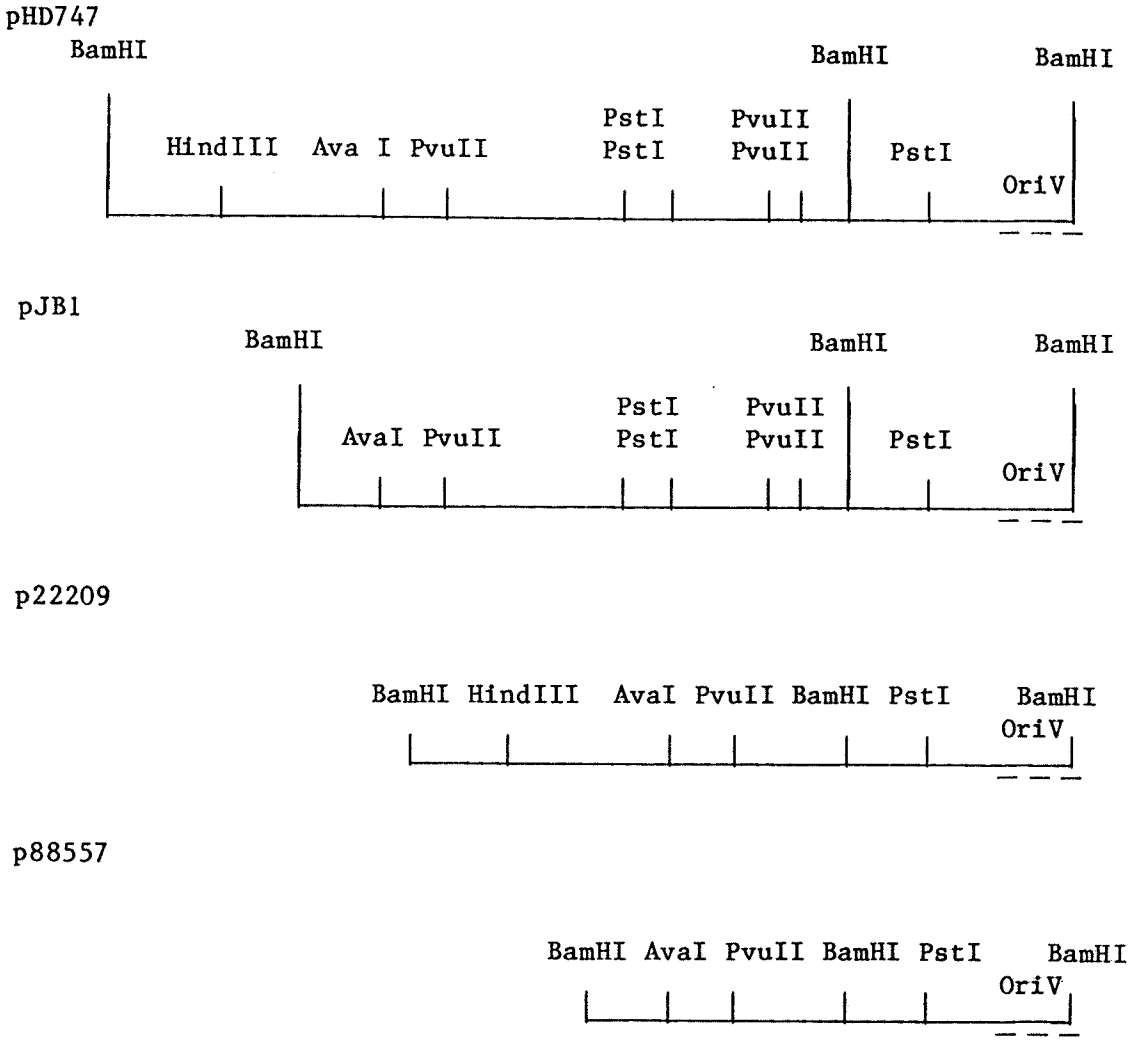
resistance to ampicillin. This fragment was cloned into the BamHI site of plasmid vector pAT2. The larger BamHI fragment, unique to each plasmid, was cloned into the BamHI site of plasmid vector pAT153.

E. coli strain W3110 polA was transformed with recombinant plasmid DNA and transformants were selected for resistance to ampicillin. Since the vector plasmids were not maintained in a polA background, but the ampicillin resistance plasmids of H. ducreyi and N. gonorrhoeae were, only those recombinant plasmids carrying the OriV site of the ampicillin resistance plasmids were maintained. E. coli strain W3110 polA transformants were isolated and screened for plasmid content. Plasmids were isolated and mapped by restriction digest. Only those recombinant plasmids composed of vector pAT2 and the small BamHI fragment common to all the ampicillin resistance plasmids were maintained in the polA mutant. Thus the OriV site of the plasmids was located within this BamHI fragment. While the fragment itself was 1.4 Mdal in size, 0.9 Mdal of the DNA was of ampicillin transposon origin. Thus the OriV site itself was located in a 0.5 Mdal region of the plasmid core between the right terminus of the transposon and the BamHI site (Fig. 24).

F. Direction of Replication of the Ampicillin Resistance Plasmids of H. ducreyi and N. gonorrhoeae.

In vitro replication of plasmid DNA was carried out to determine the direction of replication of the ampicillin resistance plasmids and to confirm the location of the OriV site. Initial studies were carried out to confirm that the ampicillin resistance plasmids could be replicated in vitro in cell-free extracts of E. coli strain C600. When this was confirmed, intensive studies were carried out with plasmid pHD747 as the repre-

Figure 24. Location of the OriV site of the ampicillin resistance plasmids of H. ducreyi and N. gonorrhoeae.



_ _ _ Location of the OriV site.

sentative plasmid of the group. This plasmid was replicated in supplemented cell-free extract in the presence of alpha-^{32}P-[dCTP] under conditions allowing replication to proceed to completion. The labelled DNA was isolated, cleaved with BamHI and HindIII restriction endonucleases and the resulting fragments were resolved by electrophoresis through a 1% agarose gel. After the gel was dried, autoradiography was carried out. As replication proceeded to completion, all restriction fragments were radio-labelled (Fig. 25-A).

In order to limit plasmid replication, the above procedure was repeated, but 2',3'-dideoxythymidine triphosphate (ddTTP) was added in an equal molar concentration to 2'-deoxythymidine triphosphate. Once incorporated, ddTTP prevented DNA chain elongation. When plasmid pHD747 was replicated in vitro under these conditions, replication was terminated a short distance from the OriV site. The plasmid DNA replicated under these conditions was isolated and cleaved with BamHI and HindIII restriction endonucleases. Fragments were resolved by agarose gel electrophoresis. After the gel was dried, autoradiography was carried out.

When replicated in the presence of ddTTP, the 1.4 Mdal BamHI fragment harbouring the OriV site and the 1.1 Mdal BamHI-HindIII fragment adjacent to the OriV site incorporated radio-label (Fig. 25-B). The 4.3 Mdal BamHI-HindIII fragment was unlabelled. The 1.4 Mdal BamHI fragment incorporated less radio-label than did the 1.1 Mdal BamHI-HindIII fragment. This was an indication that replication was proceeding by a uni-directional mode (Fig. 25-C). Bi-directional replication would have resulted in intense labelling of both fragments.

The lower intensity of labelling of the 1.4 Mdal BamHI fragments supported previous data with regard to the location of the OriV site. The site was situated near one end of the fragment. Replication, initiated at

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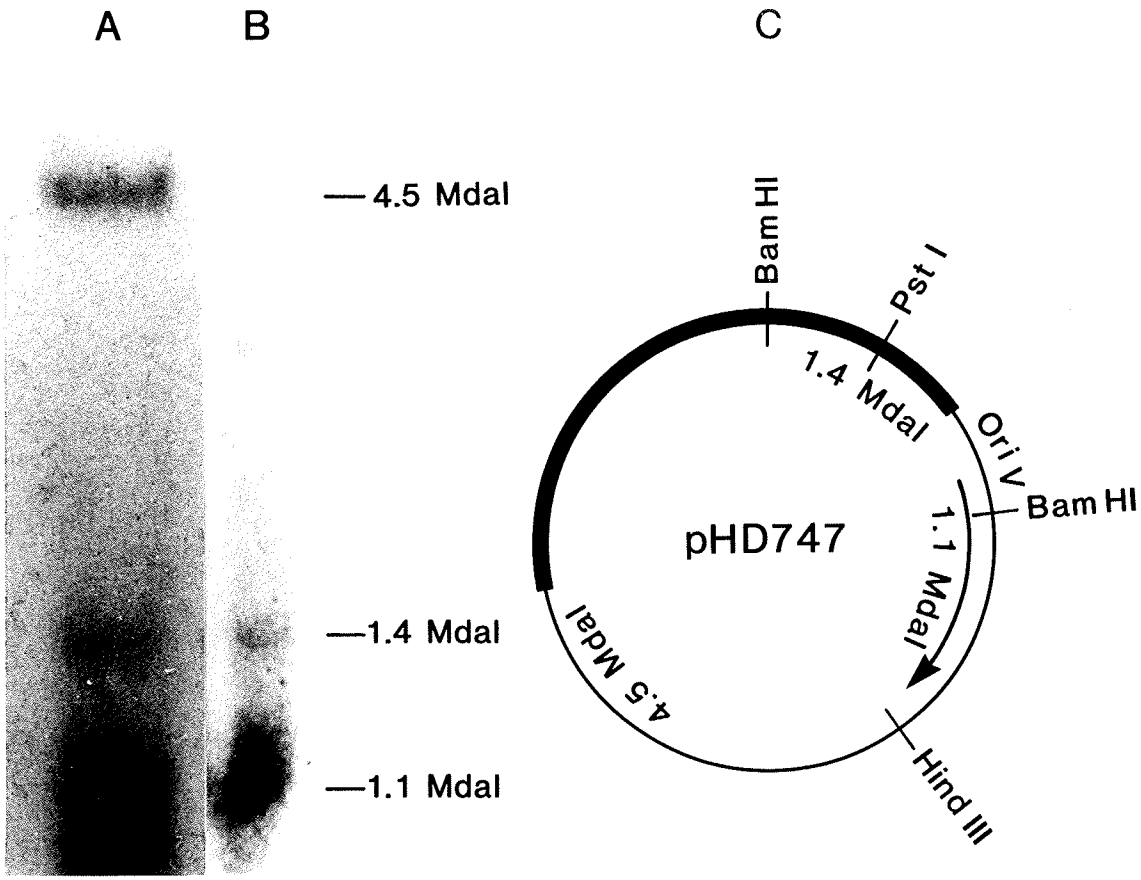
Figure 25. Replication of plasmid pHD747 in vitro.



A. Autoradiograph of BamHI-HindIII restriction fragments of plasmid DNA. Replication proceeded to completion.
B. Autoradiograph of BamHI-HindIII restriction fragments of plasmid DNA. Replication in the presence of ddTTP.
C. Restriction endonuclease map of plasmid pHD747. The location of the origin is indicated by OriV. The direction of replication is indicated by the arrow. The enhanced portion of the plasmid map indicates the position of the ampicillin transposon.

the site, proceeded clockwise with regard to the physical map of the plasmid in Figure 25-C. Thus the majority of radio-label was incorporated into the 1.1 Mdal BamHI-HindIII fragment adjacent to the OriV site. In order for the 1.4 Mdal fragment, harbouring the OriV site to become intensely labelled, the uni-directional replication of the plasmid molecule would have had to go to completion. The presence of ddTTP in the system prevented this. Thus plasmid pHD747 replicated in a uni-directional fashion, from an OriV site situated between the right terminus of TnA and a BamHI recognition site.

To confirm the location of the OriV site, in vitro replication was initiated at the OriV site using pre-restricted plasmid pHD747 DNA as a template. In this instance, the majority of label should be incorporated into the 1.4 Mdal BamHI fragment. It was expected that some label might become incorporated into the remaining fragments due to repair of nicks and the filling in of cohesive ends of the fragments. After replication of DNA, restriction fragments were resolved by gel electrophoresis. After drying the gel, restriction bands were cut from the gel. Label incorporated in each band was determined by counting duplicate samples in a scintillation counter then calculating radio-activity on the basis of counts per minute per base pair. The 1.4 Mdal BamHI fragment containing the OriV site incorporated, on average, 30 cpm per base pair. The 1.1 Mdal and 4.5 Mdal BamHI-HindIII fragments incorporated 5 and 6 cpm per base pair, respectively. These findings support the location of the OriV site within the 1.4 Mdal BamHI restriction fragment.

G. Regions of Homology between the Mobilizing Plasmid pHD147 and the Ampicillin Resistance Plasmids of H. ducreyi and N. gonorrhoeae.

Mobilization by an *in cis* mechanism can be dependent upon the presence of homology between the mobilizing plasmid and the non-conjugative plasmid. For this reason, studies were conducted to determine if mobilizing plasmid pHD147 harboured sequences homologous to those of the ampicillin resistance plasmids pHD747, pJB1, p22209 and p88557.

The four ampicillin resistance plasmids were digested with several different combinations of restriction endonucleases. Fragments were resolved by agarose gel electrophoresis. The DNA within the gel matrix was denatured by treatment with alkali, and after neutralization, was transferred by blotting to nitrocellulose. The transferred DNA was secured to the filter by baking. The single-stranded DNA of the ampicillin resistance plasmids on the filter was hybridized with denatured nick-translated plasmid pHD147 probe DNA, then autoradiography was carried out. The autoradiograph of a BamHI-PvuII digest of the hybridized ampicillin resistance plasmids is presented in Figure 26. By using this autoradiograph, and others resulting from different restriction endonuclease digests, it was possible to determine what regions of the ampicillin resistance plasmids shared homology with the mobilizing plasmid (Fig. 27).

Restriction fragments arising from digestion of the ampicillin transposon portion of the plasmids did not hybridize with the radio-labelled probe. Therefore, the mobilizing plasmid did not share any homology with the ampicillin transposon present on the plasmids. Therefore, the presence of homology within the 1.4 Mdal BamHI fragment was due to sequences within the 0.5 Mdal region of plasmid core. This was also the region shown previously to harbour the OriV site of the ampicillin resistance plasmids. A second region, common to the four ampicillin resistance plasmids also was homologous with pHD147. This region extended from the AvaI site within the plasmid core, rightward to the left terminus of the ampicillin transposon.

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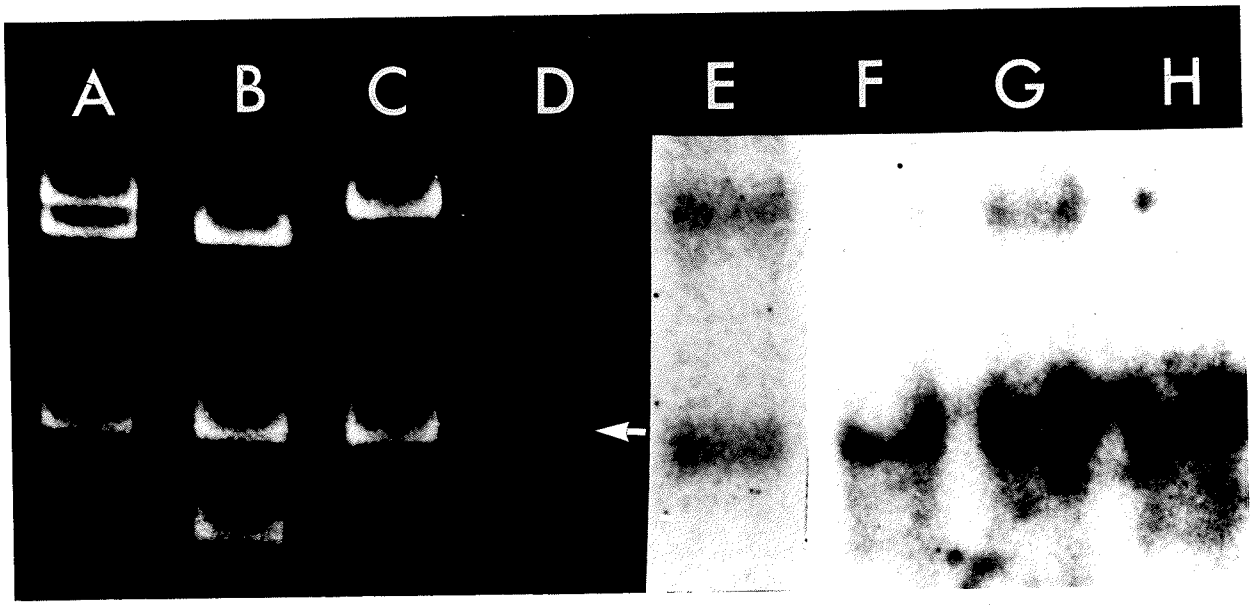
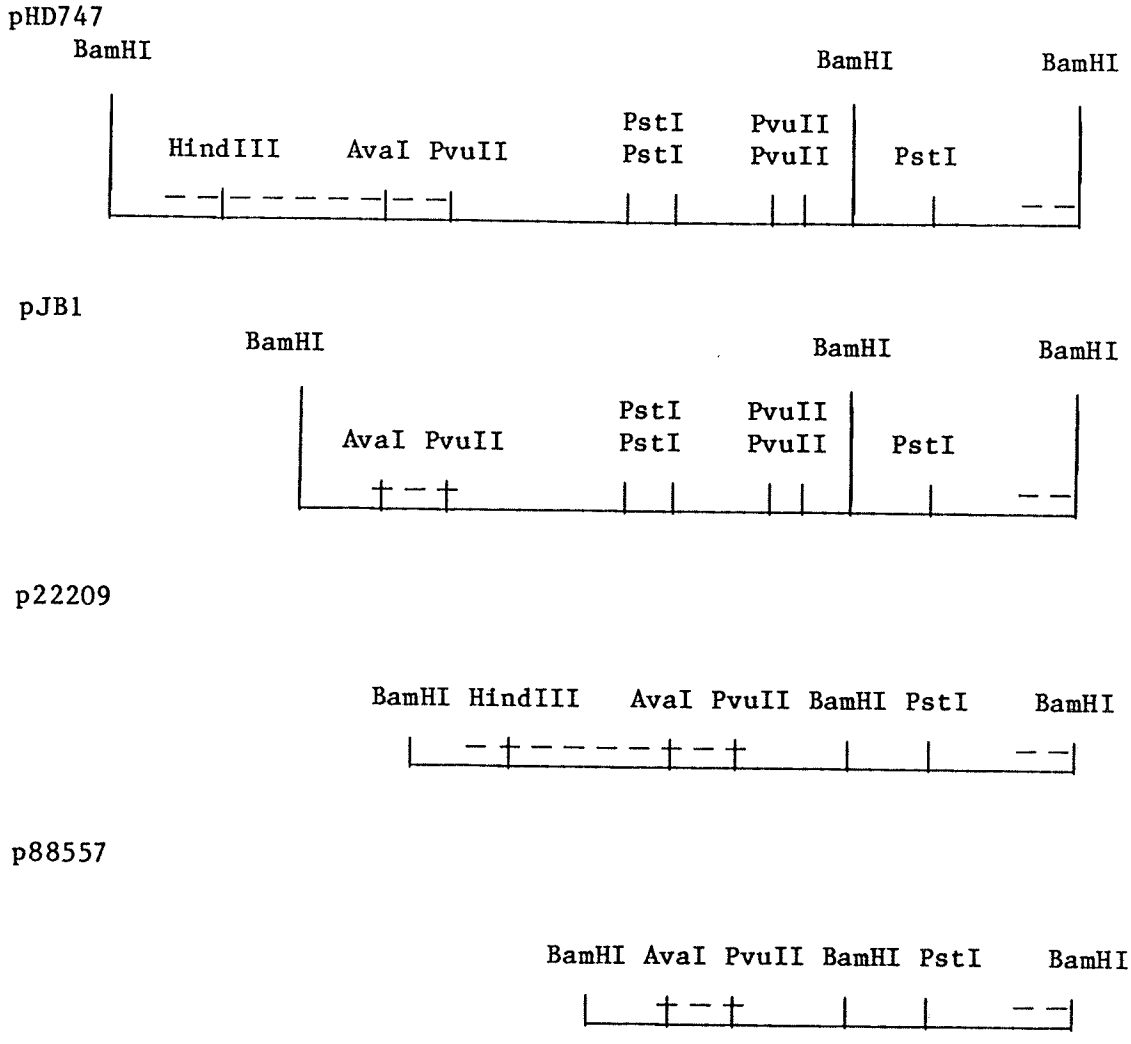
Figure 26. Regions of homology between the ampicillin resistance plasmids of H. ducreyi and N. gonorrhoeae, and the mobilizing plasmid of H. ducreyi.



Lanes A through D. BamHI-PvuII restriction fragments of plasmids pHD747, pJB1, p22209 and p88557 respectively.

Lanes E through H. Autoradiograph of BamHI-PvuII restriction fragments of plasmids pHD747, pJB1, p22209 and p88557 respectively, hybridized to plasmid pHD147 nick-translated DNA.

Figure 27. Location of regions of homology between the ampicillin resistance plasmids of *H. ducreyi* and *N. gonorrhoeae*. and the mobilizing plasmid of *H. ducreyi*.



_ _ _ Location of homology.

These homologous regions were present in pHD747, pJB1, p22209 and p88557.

Plasmids pHD747 and p22209 shared more extensive homology with pHD147. The 1.1 Mdal BamHI-HindIII fragment of these plasmids, in addition to the regions described above, was homologous to the mobilizing plasmid. However, the 0.8 Mdal BamHI-AvaI fragment from pJB1 and p88557 had been previously shown, by heteroduplex analysis, to be homologous with the 1.1 Mdal BamHI-HindIII fragment of plasmids pHD747 and p22209 (Brunton et al., 1979). Yet, this 0.8 Mdal BamHI-AvaI fragment was not homologous to plasmid pHD147. Therefore homology between the 1.1 Mdal BamHI-HindIII fragment of plasmids pHD747 and p22209, and plasmid pHD147 was due to the presence of a portion of the 1.3 Mdal insertion region within this fragment. Thus at least part of this 1.3 Mdal region was homologous to one area of the mobilizing plasmid. While all four ampicillin resistance plasmids shared homology with the mobilizing plasmid, the homologous sequences within plasmids pHD747 and p22209 were more extensive.

H. Restriction Endonuclease Map of the Mobilizing Plasmid pHD147.

As an initial step in the characterization of plasmid pHD147, a restriction endonuclease map was constructed. Digestion was carried out with enzymes used singly and in combination to determine the orientation of restriction fragments. The molecular mass of fragments resulting from these digestions are presented in Table 13. The restriction endonuclease map constructed from these data is presented in Figure 28.

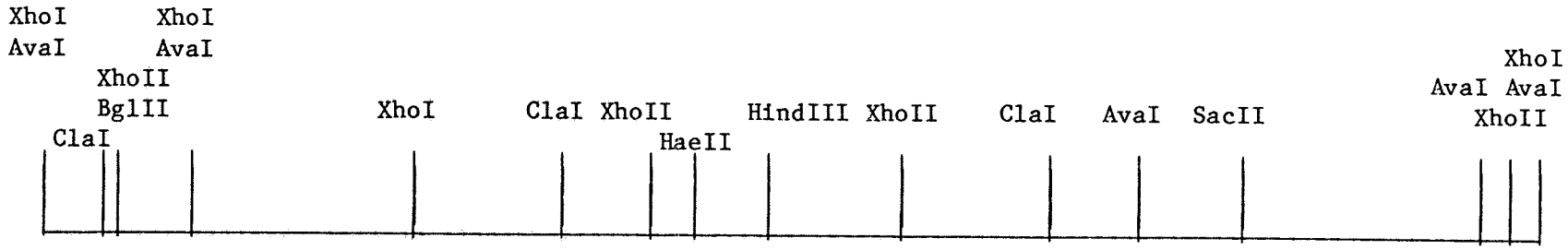
In general, the plasmid did not have an abundance of restriction sites. The plasmid was not cleaved by EcoRI, PstI, PvuII, BamHI, PvuI or SacI restriction endonucleases.

Table 13. Restriction endonuclease fragments resulting from single and double digest of the mobilizing plasmid pHD147 of H. ducreyi

Fragment Molecular Mass (x 10^6 daltons)

Enzyme	XhoI	ClaI	XhoII	BglII	HindIII	SacII	HaeII	AvaI
XhoI	1.8, 4,4, 15,5	0.6,1.2 4.4,2.1 7.5,5.9	0.3,0.7 1.1,2.8 3.2,4.4 9.1	0.7,1.1 4.4, 15.5	1.8, 4.4, 11.0	1.8,4.4 5.4. 10.1	1.8,3.7 4.4, 11.8	0.8,1.8 4.4,5.8 8.9
ClaI	0.6,1.2 4.4,2.1 5.9,7.5	5.9, 7.7, 8.1	0.1,0.9 1.1,2.0 2.8,7.2 7.6	0.1,5.9 7.6,8.1	2.4,3.5 7.6,8.1	2.1,5.9 6.0.7.7	1.6,4.3 7.7,8.1	0.8,0.9 1.8,2.1 4.4,5.8 5.9
XhoII	0.3,0.7 1.1,2.8 3.2,4.4 9.2	0.1,0.9 1.1,2.0 2.8,7.2 7.6	1.0,2.8 8.7,9.2	1.0,2.8 8.7,9.2	1.0,1.3 1.5,8.7 9.2	1.0,2.8 4.1,5.1 8.7	0.5,1.0 2.3,8.7 9.2	0.3,0.7 1.1,2.8 2.9,3.2 4.4,6.3
BglII	0.7,1.1 4.4, 15.5	0.1,5.9 7.6,8.1	1.0,2.8 8.7,9.2	21.7	10.0, 11.7	6.1, 15.6	9.2, 12.5	0.7,0.8 1.1,4.4 5.8,8.9
HindIII	1.8,4.4 4.5, 11.0	2.4,3.5 7.6,8.1	1.0,1.3 1.5,8.7 9.2	10.0, 11.7	21.7	5.6, 16.1	0.8, 20.9	0.8,1.8 4.4,4.4 4.5,5.8
SacII	1.8,4.4 5.4, 10.1	2.1,5.9 6.0,7.7	1.0,2.8 4.1,5.1 8.7	6.1, 15.6	5.6, 16.1	21.7	6.4, 15.3	0.8,1.2 1.8,4.4 4.6,8.9
HaeII	1.8,3.7 4.4, 11.8	1.6,4.3 7.7,8.1	0.5,1.0 2.3,8.7 9.2	9.2, 12.5	0.8, 20.9	6.4, 15.3	21.7	0.8,1.8 3.7,4.4 5.2,5.8
AvaI	0.8,1.8 4.4,5.8 8.9	0.8,0.9 1.8,2.1 4.4,5.8 5.9	0.3,0.7 1.1,2.8 2.9,3.2 4.4,6.3	0.7,0.8 1.1,4.4 5.8,8.9	0.8,1.8 4.4,4.4 4.5,5.8	0.8,1.2 1.8,4.4 4.6,8.9	0.8,1.8 3.7,4.4 5.2,5.8	0.9,1.8 4.4,5.8 8.9

Figure 28. Restriction endonuclease map of the mobilizing plasmid pHD147 isolated from H. ducreyi.



I. The Location, Within Plasmid pHD147, of Sequences Homologous to Regions of the Ampicillin Resistance Plasmids of H. ducreyi and N. gonorrhoeae.

Plasmid pHD147 was digested with several different combinations of restriction endonucleases. The resulting fragments were resolved by agarose gel electrophoresis (Fig. 29-A). Duplicate gels were prepared. The DNA within each gel matrix was denatured by agitating the gels in a solution of alkali. After neutralization, the DNA was transferred by blotting to nitrocellulose. The transferred DNA was secured to the filter by baking. The single-stranded DNA of plasmid pHD147 on one of the filters was hybridized with denatured nick-translated plasmid p22209 probe DNA. Since plasmids p22209 and pHD747 were structurally identical, with the exception of a complete ampicillin transposon within the later, this probe identified the location within pHD147, of sequences homologous to both plasmids p22209 and pHD747. Similarily, the second filter was hybridized with denatured nick-translated plasmid p88557 DNA. This second probe identified the location within pHD147 of sequences homologous to both plasmid p88557 and pJB1. Plasmids p88557 and pJB1 were structurally identical with the exception of a complete ampicillin transposon within pJB1. After hybridization, autoradiography was carried out (Fig. 29-B). From the information provided by the autoradiographs, the location of sequences within pHD147, homologous to the ampicillin resistance plasmids, was determined. Both probes hybridized to identical restriction fragments (Fig. 30).

Sequence homology was present within the region of pHD147 harbouring a unique HindIII restriction site. The homologous region, extended from a unique HaeII restriction site to an XhoII restriction site. This 2.3 Mdal HaeII-XhoII restriction fragment encompassed the 1.3 Mdal insertion region

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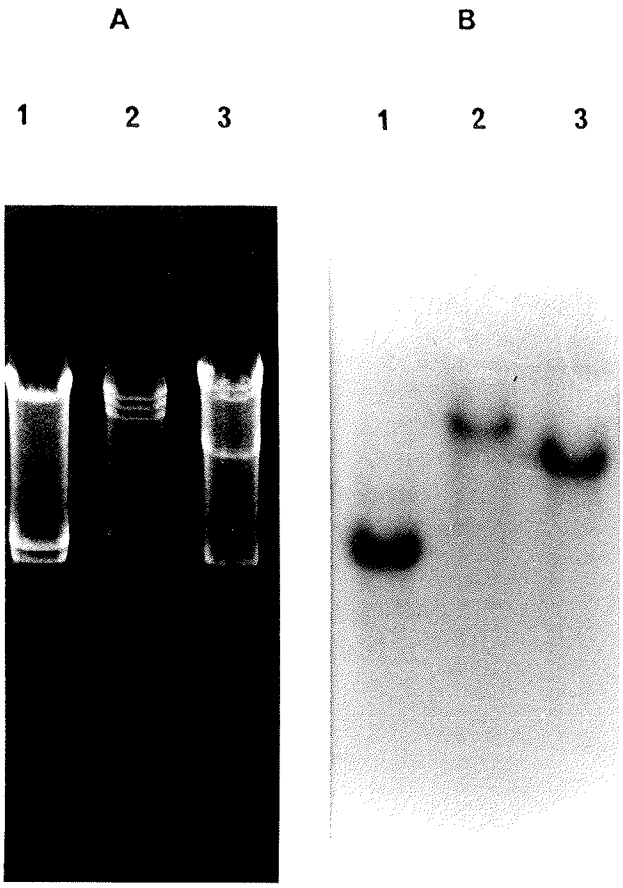
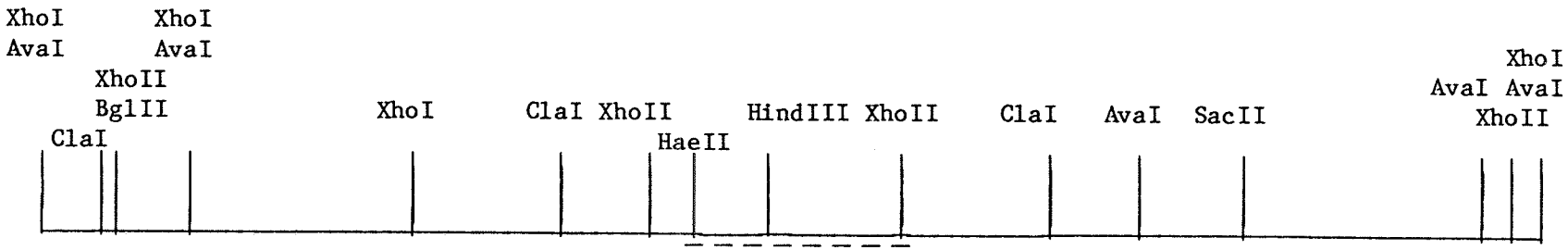
Figure 29. Regions of the mobilizing plasmid pHD147 of H. ducreyi sharing homology with plasmids p22209 and p88557.



Lane A-1. XhoII-HaeII restriction fragments of plasmid pHD147.
Lane A-2. ClaI-SacII restriction fragments of plasmid pHD147.
Lane A-3. ClaI-HaeII restriction fragments of plasmid pHD147.
Lane B-1 through B-3. Autoradiograph of Lanes A-1 through A-3 respectively, after hybridization with nick-translated plasmid p22209 or p88557 DNA.

Figure 30. Region of homology between the ampicillin resistance plasmids of H. ducreyi and N. gonorrhoeae and the mobilizing plasmid of H. ducreyi.



_ _ _ Region of homology.

present within plasmid pHD147, as well as within plasmids pHD747 and p22209.

As previously described, when the ampicillin resistance plasmids of H. ducreyi and N. gonorrhoeae were probed with nick-translated plasmid pHD147 DNA, the region around the OriV site of the ampicillin resistance plasmids demonstrated homology. Within plasmid pHD147, the region homologous to the OriV site region of the ampicillin resistance plasmids was also located within the 2.3 Mdal HaeII to XhoII restriction fragment.

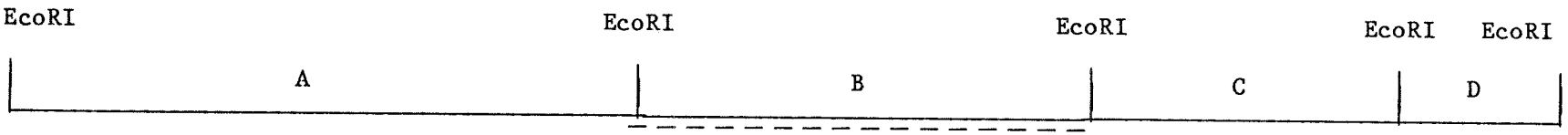
J. The Locations, Within Plasmid pHD147 and the 24.5 Mdal Mobilizing Plasmid of N. gonorrhoeae, of Homologous Sequences.

1. Probing of the 24.5 Mdal Mobilizing Plasmid of N. gonorrhoeae with Nick-Translated Plasmid pHD147 DNA.

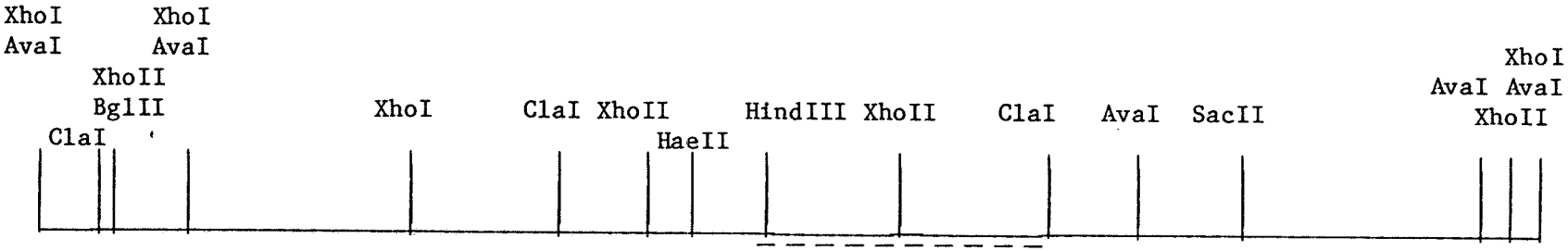
The 24.5 Mdal mobilizing plasmid of N. gonorrhoeae was digested with EcoRI restriction endonuclease. The resulting fragments were resolved by agarose gel electrophoresis, then transferred to nitrocellulose by blotting. The single-stranded 24.5 Mdal plasmid DNA present on the filter was hybridized to denatured nick-translated plasmid pHD147 DNA probe. After hybridization, autoradiography was carried out. Homologous sequences within the two plasmids were present (Fig. 31-A). The EcoRI B restriction fragment of the 24.5 Mdal plasmid harboured sequences that were homologous to those present within plasmid pHD147. The EcoRI B restriction fragment was located within a region of the 24.5 Mdal plasmid determined to function in plasmid transfer (Young et al., 1980).

Figure 31. Location of shared homology within the mobilizing plasmids of H. ducreyi and N. gonorrhoeae.

A. The 24.5 Mdal mobilizing plasmid of N. gonorrhoeae[1].



B. The 21.7 Mdal mobilizing plasmid of H. ducreyi.



1. Modified from Tenover et al. (1980).
- - - Location of Homology.

2. Probing of Mobilizing Plasmid pHD147 of H. ducreyi with Nick-Translated DNA of the 24.5 Mdal Mobilizing Plasmid of N. gonorrhoeae.

Plasmid pHD147 was digested with several different combinations of restriction endonucleases. The resulting fragments were resolved by agarose gel electrophoresis, then transferred by blotting to a nitrocellulose filter. The single-stranded plasmid pHD147 DNA was hybridized to denatured nick-translated DNA of a 24.5 Mdal mobilizing plasmid of N. gonorrhoeae. After hybridization, autoradiography was carried out. The locations of the homologous sequences are presented in Figure 31-B. Homology was present within the 3.5 Mdal HindIII-ClaI restriction fragment of plasmid pHD147. Since this fragment did not harbour the entire 1.3 Mdal insertion region, homology within the HindIII-ClaI restriction fragment was not due to this 1.3 Mdal region. Thus the 1.3 Mdal region found within mobilizing plasmid pHD147, and the ampicillin resistance plasmids pHD747 and p22209 was not present within this 24.5 Mdal mobilizing plasmid of N. gonorrhoeae.

K. Location of the OriV Site of Mobilizing Plasmid pHD147 from H. ducreyi.

1. Cloning of the ClaI and XhoII Restriction Fragments of Plasmid pHD147.

The three fragments resulting from ClaI restriction endonuclease digestion of pHD147 were inserted into the ClaI site of vector plasmid pAT153. Similarily, the four XhoII fragments were inserted into the complementary BamHI site of plasmid pAT153. After an overnight ligation reaction, E. coli strain C600 was transformed with the ligation mixture. Transformants, selected for ampicillin resistance and tetracycline sensitivity, were screened for plasmid content. Recombinant plasmids were

isolated from transformants by $CsCl_2$-dye-buoyant-density centrifugation, then mapped by restriction enzyme digestion.

Plasmid pATB1 was a recombinant plasmid composed of the 2.8 Mdal XhoII fragment of pHD147 inserted into plasmid pAT153. Plasmid pATB5 was a recombinant plasmid composed of the 2.8 Mdal XhoII fragment of pHD147 inserted into plasmid pAT153, but the inserted fragment was in opposite orientation to that of plasmid pATB1. Plasmid pATC5 was a recombinant plasmid composed of the 5.9 ClaI fragment of pHD147 inserted into plasmid pAT153. The ClaI fragment of pHD147 present in this plasmid encompassed the 2.8 Mdal XhoII fragment as well as additional sequences. The recombinant plasmids used in the following studies are presented in Figure 32.

2. Replication of Recombinant Plasmids in the Absence of DNA Polymerase I.

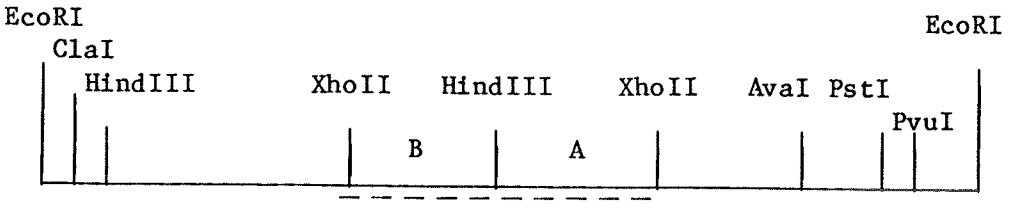
Plasmid pHD147 is maintained by E. coli strain W3110 polA as DNA polymerase I is not required for replication of the molecule. Conversely, plasmid pAT153 is not maintained in this background. Therefore, only those recombinant plasmids carrying the OriV site of plasmid pHD147 will replicate in the E. coli mutant strain. Recombinant plasmid pATB1, pATB5, and pATC5 were found to replicate in the background of E. coli strain W3110 polA. These plasmids carried the OriV site of plasmid pHD147.

3. Location of the OriV Site of Plasmid pHD147.

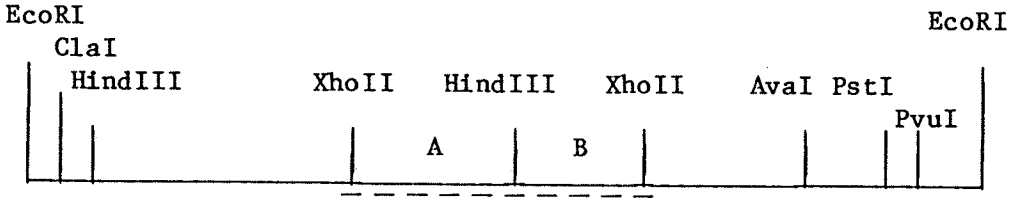
Plasmids pATB1 and pATB5 carried the 2.8 Mdal XhoII fragment of plasmid pHD147 in opposite orientation (Fig. 32). This fragment had a unique site for HindIII restriction endonuclease, as did the plasmid vector pAT153. Recombinant plasmids pATB1 and pATB5 were cut with HindIII restriction endonuclease and the resulting fragments were resolved by agarose

Figure 32. Recombinant plasmid created by insertion of restriction fragments of plasmid pHD147 into vector plasmid pAT153.

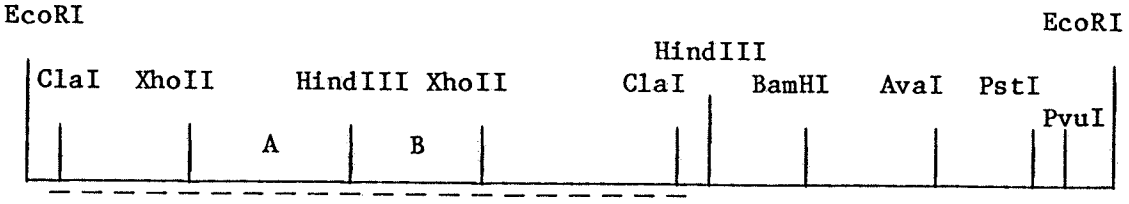
pATB1



pATB5



pATC5



_ _ _ Region derived from plasmid pHD147.

gel electrophoresis. The larger HindIII fragment from each plasmid was recovered from the gel and recircularized by ligation. Maintenance of the deletion derivatives of plasmid pATB1 and pATB5, plasmids pATB1Hind and pATB5Hind respectively (Fig. 33), in a background of E. coli strain W3110 polA, was studied. Only plasmid pATB5Hind was maintained. Therefore the OriV Site of plasmid pHD147 was situated within a 1.3 Mdal region bounded by restriction endonuclease sites XhoII and HindIII (Fig. 33).

L. Location of the OriT Site of Mobilizing Plasmid pHD147.

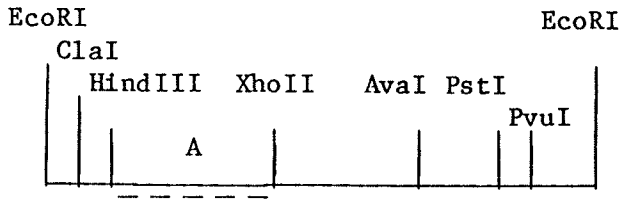
1. Mobilization of pAT153/pHD147 Recombinant Plasmids in Recombination Proficient and Deficient Backgrounds.

Recombinant plasmids derived from insertion of the XhoII and ClaI fragments of plasmid pHD147 into pAT153, were introduced by transformation into E. coli strains C600 and HB101. The E. coli strains were recombination proficient and deficient respectively. Tri-parental matings were carried out. The initial donor, H. influenzae strain RdpHD147 harboured the mobilizing plasmid pHD147. The intermediate recipients, either E. coli strain C600 or strain HB101, harboured one of the recombinant plasmids. The final recipient, E. coli strain C600str, carried chromosomally-mediated resistance to streptomycin. Transconjugants, isolated for resistance to ampicillin and streptomycin, were screened for plasmid content. Results of the mobilization studies are presented in Table 14. Recombinant plasmid pATC5 was the only recombinant plasmid mobilized by an in trans mechanism based upon the following criteria.

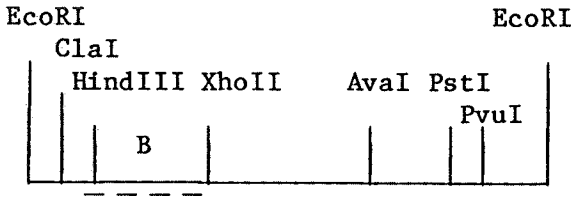
1. Mobilization of pATC5 was independent of the recombinational proficiency state of the host cell.
2. Mobilizing plasmid pHD147 was not visualized in transconjugants.

Figure 33. Deletion derivatives of pAT153/pHD147 recombinant plasmids.

pATB1Hind



pATB5Hind



_ _ _ Region derived from plasmid pHD147.

Table 14. Results of mobilization studies with recombinant plasmids pATB1, pATB5 and pATC5.

Plasmid Mobilized.	Mobilization from a Recombination Proficient Host.	Mobilization from a Recombination Deficient Host.	Plasmid pHD147 Co-transferred.	Plasmid Altered After Transfer
pATB1	+	-	+	+
pATB5	+	-	+	+
pATC5	+	+	-	-

3. Plasmid pATC5, isolated from transconjugants, was identical in size and structure to its counterpart in the parental host cell.

All other recombinant plasmids, including plasmids pATB1 and pATB5, were mobilized by an *in cis* mechanism. Classification of *in cis* mobilization was based upon the following criteria.

1. Mobilization was effected only if the host was recombination proficient.
2. Mobilizing plasmid pHD147 was visualized in transconjugants.
3. Plasmids isolated from transconjugants were altered in size and structure relative to those present in parental host cells.

Mobilization of a non-conjugative plasmid by an *in trans* mechanism required the presence, within the plasmid, of a functional OriT site (Kilbane and Malamy, 1980). Since vector plasmid pAT153 did not have an OriT site (Twigg and Sherratt, 1980), mobilization of a pAT153/pHD147 recombinant plasmid by an *in trans* mechanism was dependent upon such a site being present within the pHD147 restriction fragment inserted into pAT153. Only one recombinant plasmid, pATC5 was mobilized by an *in trans* mechanism. The pHD147 insert within this recombinant plasmid harboured a functional OriT site.

2. Location of the OriT Site.

Plasmid pATC5 carried the 5.9 Mdal ClaI fragment of pHD147 (Fig. 32). This fragment harboured a unique site for HindIII restriction endonuclease, as did the plasmid vector pAT153. Recombinant plasmid pATC5 was cleaved at the two sites with HindIII restriction endonuclease and the resulting fragments were resolved by agarose gel electrophoresis. The larger of the two fragments was recovered from the gel and recircularized by ligation

(Fig. 34). E. coli strain HB101 was transformed with the ligation mixture. A transformant carrying the HindIII deletion derivative of pATC5 was isolated. The derivative plasmid, pATC5Hind, was checked for the ability to undergo mobilization by an in trans mechanism. Mobilization from the recombination deficient host did not occur. When plasmid pATC5Hind was introduced into recombination proficient E. coli strain C600 by transformation, the plasmid could be mobilized, but only by an in cis mechanism. Thus the OriT site had been deleted from plasmid pATC5Hind. From this information, it was possible to determine the location of the OriT site of plasmid pHD147. The site was situated within the 1.1 Mdal region, bordered by ClaI and XhoII restriction endonuclease recognition sites (Fig. 34).

M. Coupled Transcription-Translation of Plasmid DNA Templates In Vitro.

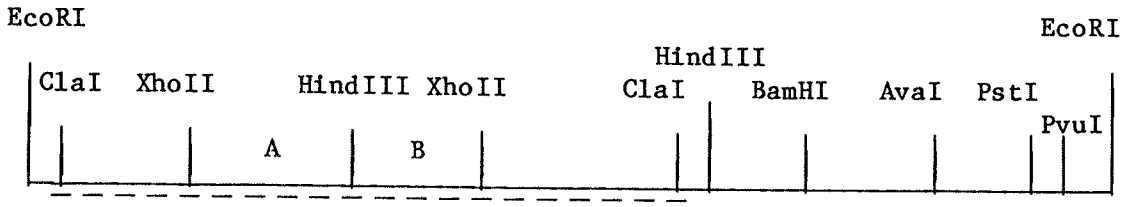
Proteins, produced by coupled transcription-translation of native and recombinant plasmid DNA templates, were compared on the basis of molecular weight by SDS-polyacrylamide gel electrophoresis.

1. Proteins Encoded by the Ampicillin Resistance Plasmids of H. ducreyi and N. gonorrhoeae.

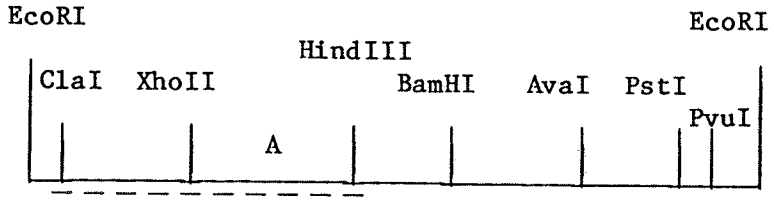
The DNA of plasmids p88557, p22209, pJB1 and pHD747 was used as template for coupled transcription-translation reactions in the presence of L-[^{35}S]-methionine. The resulting proteins were resolved by SDS-polyacrylamide gel electrophoresis. Gels ranged in concentration from 12.5% to 15% acrylamide. After electrophoresis, autoradiography was carried out. An autoradiograph of the gel is presented in Figure 35-A. As an aid in interpretation of the autoradiograph, a line-drawing of the autoradiograph is

Figure 34. Plasmid pATC5Hind, a deletion derivative of recombinant plasmid pATC5.

pATC5



pATC5Hind



_ _ _ Region derived from plasmid pHD147.

NOTICE/AVIS

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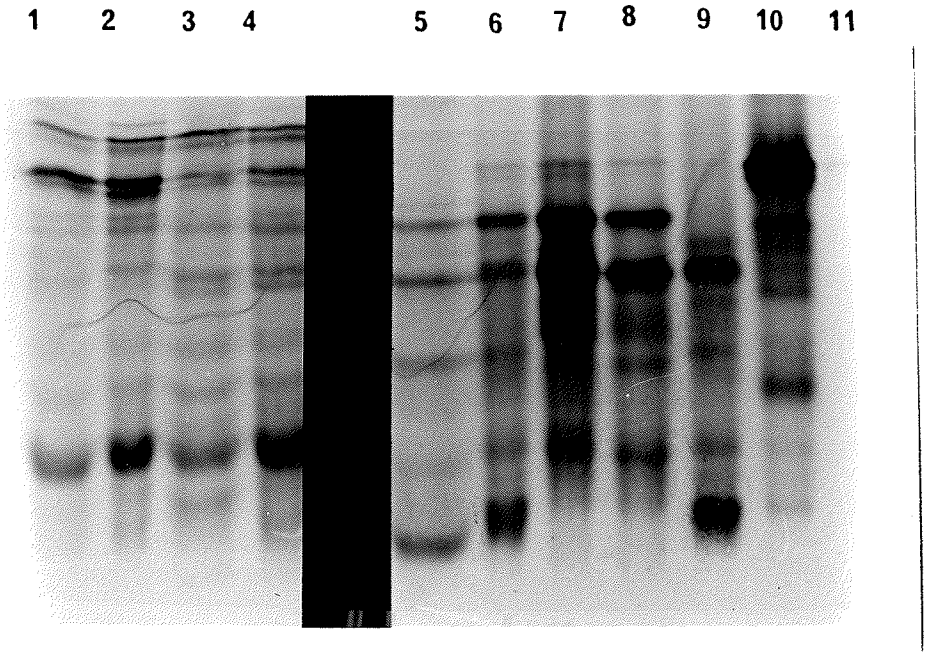
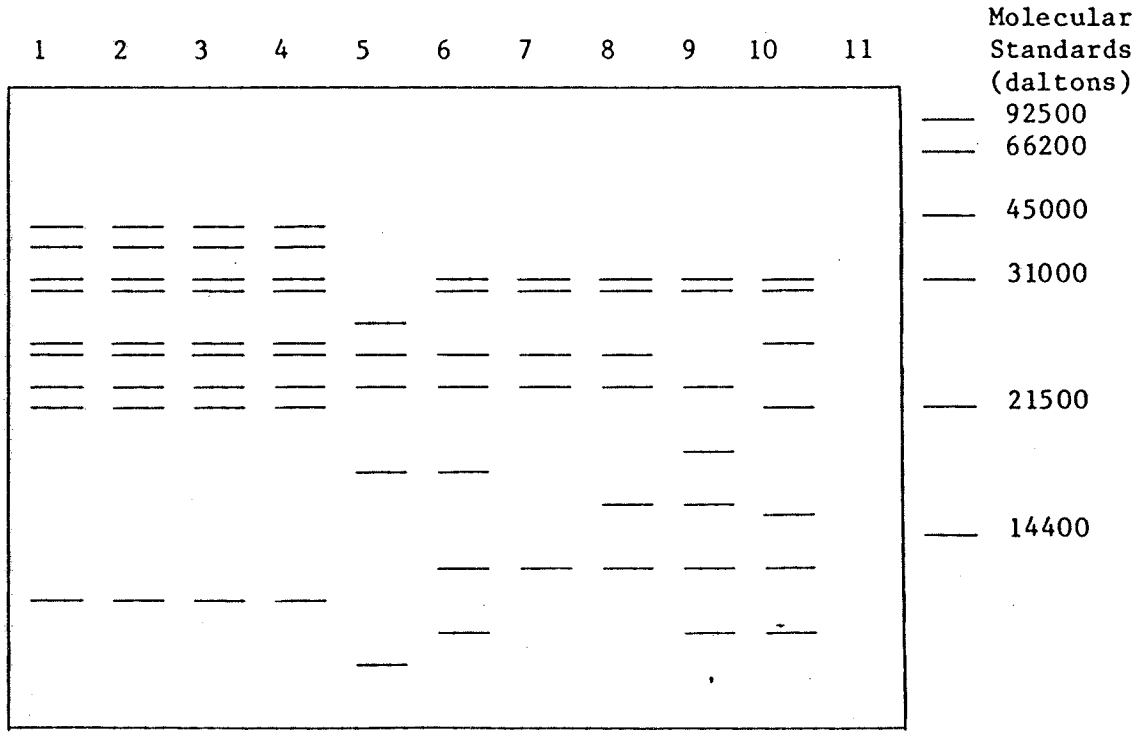
VEUILLEZ ECRIRE A L'AUTEUR POUR LES RENSEIGNEMENTS OU VEUILLEZ CONSULTER L'EXEMPLAIRE DONT POSSEDE LE DEPARTEMENT DES ARCHIVES ET DES COLLECTIONS SPECIALES, BIBLIOTHEQUE ELIZABETH DAFOE, UNIVERSITE DU MANITOBA, WINNIPEG, MANITOBA, CANADA, R3T 2N2.

Figure 35. Proteins from coupled transcription-translation of native and recombinant plasmids of H. ducreyi and N. gonorrhoeae.

A. Autoradiograph of a 15% SDS-polyacrylamide gel.



B. Line-drawing of the autoradiograph of a 15% SDS-polyacrylamide gel.



presented in Figure 35-B. The molecular weights of the proteins are presented in Table 15. Lane 11 shows the control for protein synthesis in the absence of DNA. The proteins encoded by plasmids p88557, p22209, pJB1 and pHD747 are presented in Figures 35-A and 35-B, lanes 1 through 4, respectively. Production of the proteins within these lanes utilized the entire coding capacity of the ampicillin resistance plasmids. The proteins encoded by the ampicillin transposon were identified by reference to Casadaban et al. (1981). No detectable protein was encoded by the 1.3 Mdal insertion element present within plasmids p22209 and pHD747. Two proteins of 26,300 and 22,300 daltons, were encoded by the four ampicillin resistance plasmids, as well as the mobilizing plasmid pHD147 (Fig 35-A and 35-B, lanes 1 through 5).

2. Proteins Encoded by the Mobilizing Plasmid pHD147 of H. ducreyi.

The proteins encoded by plasmid pHD147 are presented in Figure 35-A and 35-B, lane 5. The five proteins encoded by this plasmid accounted for only 9% of the potential coding capacity of this plasmid. Two of the five proteins encoded by plasmid pHD147 were also encoded by the ampicillin resistance plasmids. The positions of the regions encoding these two proteins, as well as the position of the region encoding a third protein of 16,900 daltons, were identified within specific restriction fragments of plasmid pHD147.

3. Locations, Within a Restriction Endonuclease Map, of Protein-Encoding Regions of the Mobilizing Plasmid pHD147.

ClaI and XhoII restriction fragments of plasmid pHD147 were cloned into plasmid vector pAT153. The recombinant plasmids were used in coupled transcription-translation studies. The recombinant plasmids, pATB11,

Table 15. Proteins encoded by native and recombinant plasmids of H. ducreyi and N. gonorrhoeae.

Molecular Weights of Proteins Encoded by Plasmid DNA Templates.										
Protein Origin	p88557 (dalton)	p22209 (dalton)	pJB1 (dalton)	pHD747 (dalton)	pHD147 (dalton)	pATB11 (dalton)	pATB5 (dalton)	pATC5 (dalton)	pATC5Hind (dalton)	pAT153 (dalton)
	40700	40700	40700	40700	–	–	–	–	–	–
	38000	38000	38000	38000	–	–	–	–	–	–
TnA	32000	32000	32000	32000	–	32000	32000	32000	32000	32000
TnA	30900	30900	30900	30900	–	30900	30900	30900	30900	30900
	–	–	–	–	27800	–	–	–	–	–
	27500	27500	27500	27500	–	–	–	–	–	–
	26300	26300	26300	26300	26300	26300	26300	26300	–	–
	–	–	–	–	–	–	–	–	–	26000
	22300	22300	22300	22300	22300	22300	22300	22300	22300	–
	21600	21600	21600	21600	–	–	–	–	–	–
	–	–	–	–	–	–	–	–	–	20800
	–	–	–	–	–	–	–	–	17100	–
	–	–	–	–	16900	16900	–	–	–	–
	–	–	–	–	–	–	–	16400	16400	–
	–	–	–	–	–	–	–	–	–	15300
	–	–	–	–	–	12300	12300	12300	12300	12300
	12000	12000	12000	12000	–	–	–	–	–	–
	–	–	–	–	–	10000	–	–	10000	10000
	–	–	–	–	9300	–	–	–	–	–

pATB5, pATC5 and pATC5Hind are illustrated in Figure 36-A. The proteins encoded by these recombinant plasmids are presented in Figures 35-A and 35-B, lanes 6 through 9, respectively. The proteins encoded by plasmids pHD147 and pAT153 are presented in Figure 35-A and 35-B, lane 5 and 10, respectively. By comparing the proteins encoded by the recombinant plasmids to those encoded by plasmids pHD147 and pAT153, it was possible to discern which proteins encoded by the recombinant plasmids, were encoded by the cloned fragments of plasmid pHD147. The restriction fragments of plasmid pHD147 encoding for the production of specific proteins are highlighted in the restriction map presented in Figure 36-B.

The 26,300 dalton protein was encoded by all recombinant plasmids with the exception of pATC5Hind. Therefore, the protein was encoded by a region within the 1.3 Mdal XhoII-HindIII restriction fragment. A protein of the same size was also encoded by the ampicillin resistance plasmids. As described previously, homology between the ampicillin resistance plasmids and the mobilizing plasmids resides in this same region, specifically within the 2.3 Mdal HaeII-XhoII fragment. Therefore, since this protein was encoded by both plasmid species, the region encoding for the protein was situated within the 0.8 Mdal HaeII-HindIII fragment.

A 22,300 dalton protein was encoded by both the ampicillin resistance and mobilizing plasmids. It was also produced by all the recombinant plasmids. Therefore, this protein was encoded by a region within the 1.5 Mdal HindIII-XhoII fragment (Fig. 36-B).

The location of the region of plasmid pHD147 encoding for the production of a 16,900 dalton protein was also determined. This protein was produced by recombinant plasmid pATB11 only. The inserted fragment within this recombinant plasmid was a product of partial digestion of plasmid pHD147 with XhoII restriction endonuclease. The fragment was composed of

Figure 36. Location, within restriction endonuclease maps of recombinant and native plasmids of H. ducreyi, of protein-encoding regions.

A.

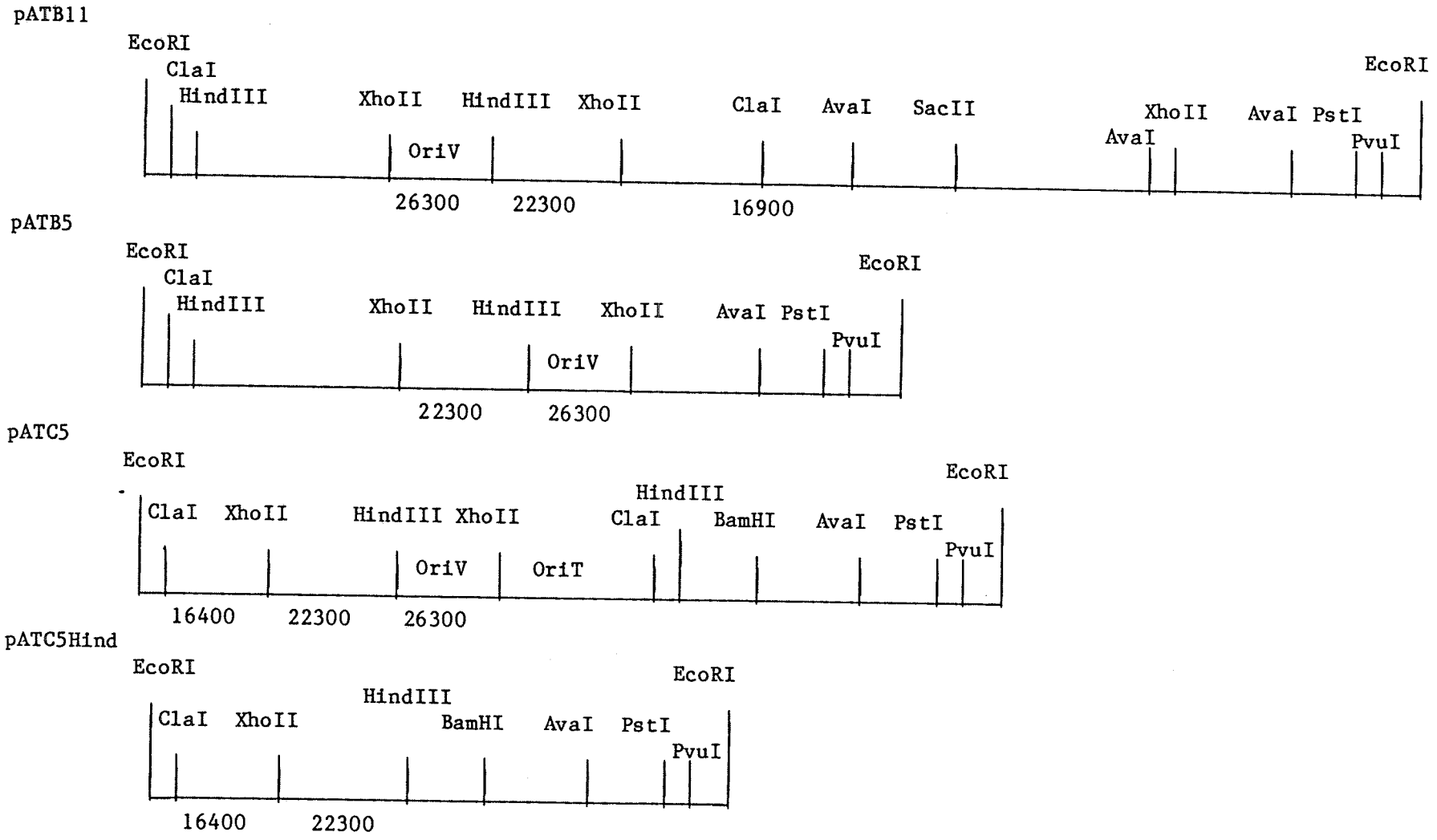
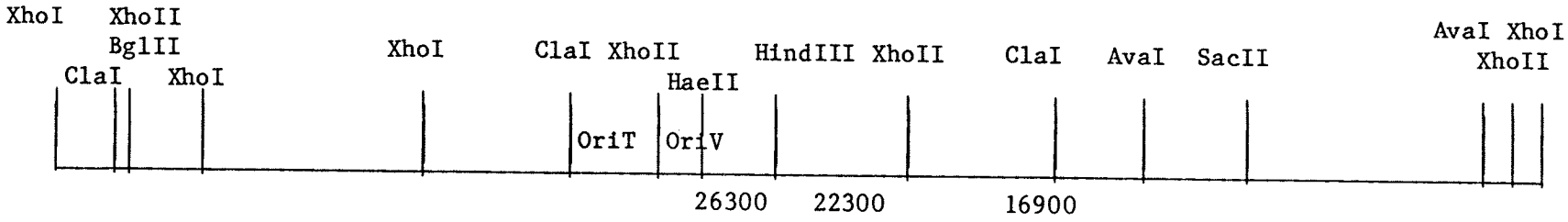


Figure 36. Location, within restriction endonuclease maps of recombinant and native plasmids of H. ducreyi of protein-encoding regions. (continued)

B.
pHD147



the 2.8 Mdal XhoII fragment and the adjacent 9.2 Mdal XhoII fragment.

A protein of 16,400 daltons was produced only by recombinant plasmids pATC5 and pATC5Hind. As illustrated in Figure 36-A, the fragment inserted within these two recombinant plasmids overlapped the fragment inserted within plasmid pATB11. For this reason, it was assumed that the 16,400 dalton protein was a truncated derivative of the 16,900 dalton protein. The promoter for this protein was situated within the 2.0 Mdal XhoII-ClaI restriction fragment. The coding sequence for this protein bridged the ClaI recognition site.

Discussion.

A. The Structural Organization of the Ampicillin Resistance Plasmids of H. ducreyi and N. gonorrhoeae.

Extensive restriction endonuclease maps were constructed as an initial step in the characterization of the ampicillin resistance plasmids of H. ducreyi and N. gonorrhoeae. As intimated by previous studies, the four plasmids are closely related with regard to physical structure (Brunton et al., 1982).

Plasmid pHD747, isolated from Haemophilus ducreyi, is the largest of the four plasmids. After a comparison of the restriction maps of the four ampicillin resistance plasmids, it appears that plasmid pHD747 could be the parental form of the plasmids pJB1, p22209 and p88557. These three plasmids can be derived from pHD747 by appropriate deletions.

The four plasmids are structurally identical with two exceptions. Plasmids pHD747 and pJB1, isolated from H. ducreyi, harbour complete ampicillin transposons. Conversely, plasmids p22209 and p88557, isolated from N. gonorrhoeae harbour only partially intact ampicillin transposons. The second variance in structure is the presence of a 1.3 Mdal insertion element within plasmids pHD747 and p22209. This insertion element is not present in plasmids pJB1 and p88557.

The ampicillin transposon within plasmids pHD747 and pJB1 is structurally intact and functional. Previous studies have demonstrated that this element can undergo transposition (Brunton et al., 1982). The transposon encodes for the production of a TEM-type beta-lactamase (Maclean et al., 1980), thus confers ampicillin resistance upon the host cell.

The ampicillin transposon present within these two plasmids has been

shown, by previous investigations, to be a Tn2-like element with regard to structure (Brunton et al., 1979; 1982; Maclean et al., 1980). Tn2 varies slightly from Tn1 and Tn3 with regard to restriction sites and has a lower frequency of transposition (Yamamoto et al., 1980). Transposition of the Tn2-like transposon within plasmids pHD747 and pJB1 was not observed during the in vitro mobilization studies of this investigation. Previously, one transposition event was observed during in vitro mobilization of plasmid pHD747 by mobilizing plasmid pHD147 (H. Deneer, M.Sc. Thesis, University of Manitoba). The ampicillin transposon transposed from plasmid pHD747 onto plasmid pHD147. Plasmid pHD147, harbouring the transposon, was isolated from a transconjugant strain. However, due to the rarity of such an event, the frequency of transposition is obviously much lower than the frequency of mobilization.

Plasmids p22209 and p88557 of N. gonorrhoeae harbour partially intact ampicillin transposons. The portion mediating resistance to ampicillin by production of a TEM-type beta-lactamase is present (Elwell et al., 1979). These transposons are unable to undergo transposition. The left-hand portion of the transposon is missing. This portion of the transposon encodes for the production of transposon resolvase and transposase proteins. Both are required for transposition (Gill et al., 1979). As well, the left-hand inverted repeat sequence is missing; a site also required for transposition (Gill et al., 1979).

The partial transposons present within the ampicillin resistance plasmids of N. gonorrhoeae are most likely derived from an intact element, as opposed to being created de novo. The left-hand portion of the transposon could have been deleted by one of two different mechanisms. Deletion may have occurred by homologous recombination, or as a result of transformation.

Deletion by homologous recombination may have occurred in the following manner. The internal resolution site of the ampicillin transposon shares extensive sequence homology with the left- and right-hand inverted repeat sequences of the element (Heffron et al., 1979). A recombination event between the resolution site and the left-hand inverted repeat would result in the formation of a partial transposon, identical to that present within plasmids p22209 and p88557.

Alternatively, deletions have occurred in plasmids following *in vitro*, transformation of a host cell (Sox et al., 1979). The partial transposons within the gonococcal ampicillin resistance plasmids may have arisen by transformation of *N. gonorrhoeae* with plasmid DNA initially harbouring an intact element.

The partial ampicillin transposon terminates at the same site within plasmids p22209 and p88557. It is highly unlikely that a deletion event resulting from transformation would occur at an identical site within the progenitor of each of the two plasmids. It is more likely that such an event occurred at the time of creation of the progenitor of plasmid p22209, since it is the larger of the two gonococcal plasmids. A plasmid identical to plasmid p88557 was subsequently derived from it.

Plasmid pHD747 of *H. ducreyi* and plasmid p22209 of *N. gonorrhoeae* harbour a unique insertion element of 1.3 Mdal. The 1.3 Mdal insertion element is bounded by inverted repeat sequences (Dickgiesser et al., 1982). The element is cryptic in that it does not encode for a selectable phenotypic trait. Transposition of the 1.3 Mdal insertion element has not been observed, yet such an event would not be easily detected, due to the small size and cryptic nature of the element.

The presence of the element within the larger of the two ampicillin resistance plasmids of *H. ducreyi* and its counterpart in *N. gonorrhoeae* is

intriguing. The element is located at an identical position within the core region of plasmids pHD747 and p22209. The common location of the element can be explained in two ways.

The site of the element within each plasmid could represent a "hot-spot" for insertion of the element by a transposition event. The element present within each of the two plasmids was acquired by separate transposition events.

Alternatively, the element was acquired by transposition at random into the progenitor of plasmid pHD747. Plasmid p22209 was subsequently derived from plasmid pHD747 by deletion of the left-hand portion of the ampicillin transposon.

The ampicillin resistance plasmids of H. ducreyi and N. gonorrhoeae form DNA-protein relaxation complexes. Upon relaxation of these complexes, a nick is introduced at a unique site in one strand of the DNA duplex. This site is the origin of transfer of the plasmid molecule.

The OriT site of each of the four ampicillin resistance plasmids is situated within the same region of each of the plasmid molecules. Thus the plasmids are organized similarly with regard to transfer function.

The OriV site of each plasmid is also located within a region common to the four plasmids and the site is in close proximity to the OriT site of each plasmid. Thus the region of each plasmid harbouring the transfer and maintenance functions is highly conserved.

The close proximity of the OriT and OriV sites has been observed for other plasmid molecules (Finnegan and Sherratt, 1982). Nordheim et al. (1980) suggested that the close proximity is necessary due to a functional relationship between the two sites. However, even if such a functional relationship did not exist, it is not surprising that the sites are located in a highly conserved region of the plasmid molecule. The replication

function, and to a lesser degree, the transfer function of a plasmid molecule are essential for survival. The clustering of these functions may represent the most advantageous arrangement to ensure stable inheritance.

B. A Structural Relationship Exists Between the Ampicillin Resistance Plasmids of H. ducreyi and N. gonorrhoeae, and a Mobilizing Plasmid of H. ducreyi.

The ampicillin resistance plasmids of H. ducreyi and N. gonorrhoeae are efficiently mobilized by a plasmid isolated from H. ducreyi. As part of an investigation to determine by what mechanism mobilization was occurring, the ampicillin resistance plasmids were probed for homology to the mobilizing plasmid pHD147. Homology was present.

Two regions, common to all four plasmids, are homologous to sequences within plasmid pHD147. The first region encompasses the OriV site of the ampicillin resistance plasmids. Since the ampicillin resistance and mobilizing plasmids are compatible, it is unlikely that the homology is due to identical maintenance functions. Plasmids with identical loci for replication, copy number control, compatibility and partitioning are not normally compatible (Broda, 1979). The homology exhibited in the region encompassing the OriV site may be due to sequences encoding a product required for a maintenance function by both species of plasmid molecules. However other maintenance functions are distinctly different.

Evidence to support the existence of different maintenance functions within the ampicillin resistance and mobilizing plasmids comes from the following observations. The ampicillin resistance plasmids are maintained at a high copy number under relaxed conditions for replication. The mobilizing plasmid pHD147 is maintained at a low copy number, under stringent conditions for replication. Thus the maintenance functions for the two

species of plasmids appear to be quite different.

A second region, common to the four ampicillin resistance plasmids, shares homology with the mobilizing plasmid. This region extends from the AvaI restriction site within the plasmid core to the left terminus of the ampicillin transposon. The function of this region of the plasmid molecule is not known, although Sox et al. (1979) demonstrated that deletions within this region of a 4.4 Mdal plasmid identical to plasmid p22209, prevented subsequent mobilization of the molecule.

The region encompassing the OriT site of the ampicillin resistance plasmids is not homologous with the mobilizing plasmid. This indicates that the transfer origins of the ampicillin resistance plasmids and mobilizing plasmids are different. Plasmids pHD747 and p22209 share more extensive homology with the mobilizing plasmid than do plasmids pJB1 and p88557. The 1.3 Mdal insertion element present within these two plasmids is also present within the mobilizing plasmid.

The presence of the 1.3 Mdal insertion element within two plasmids isolated from the common background of H. ducreyi is not surprising. However, the presence of the same insertion element within a plasmid isolated from N. gonorrhoeae warrants discussion.

The insertion element could have been acquired by plasmid pHD747 and p22209 from mobilizing plasmid pHD147 by two different mechanisms. The first mechanism is based upon acquisition of the element as a result of in cis mobilization. The pre-requisite co-integration of the ampicillin resistance and mobilizing plasmids is effected by recombination at regions of homology. Within the ampicillin resistance plasmids, the 1.3 Mdal insertion element is situated adjacent to sequences demonstrating homology with the mobilizing plasmid. Homologous recombination of the plasmids in this region, then inaccurate resolution of recombination after mobilization,

could result in acquisition of the 1.3 Mdal insertion element.

Alternatively, the presence of the 1.3 Mdal insertion element within plasmids pHD747 and p22209 can be explained in the following manner. Co-integrate formation between the ampicillin resistance and mobilizing plasmids is effected as an intermediate step in transposition of the 1.3 Mdal insertion element. Resolution of the co-integrate would result in the presence of the insertion element within the mobilizing and ampicillin resistance plasmids.

Acquisition of the element at identical sites within the two plasmids pHD747 and p22209, by inaccurate resolution of recombination following mobilization is unlikely. More plausible is the idea that the element was acquired from the mobilizing plasmid by a transposition event. The element transposed to a "hot-spot" site at identical locations within the two ampicillin resistance plasmids. An alternate explanation for the location of the element at identical sites within the two plasmids follows.

The element was acquired by the progenitor of plasmid pHD747 from plasmid pHD147 by one of the previously described mechanisms. Plasmid p22209 was subsequently derived from plasmid pHD747 by an event that deleted the lefthand portion of the transposon. Mechanisms for deletion of the left-hand portion of the ampicillin transposon have been described previously.

Thus far, it is assumed that the 1.3 Mdal insertion element originated within the mobilizing plasmid pHD147, and is subsequently acquired by plasmid pHD747, if not by both plasmids pHD747 and p22209. Acquisition of the 1.3 Mdal insertion element is a result of an *in cis* mobilization and/or transposition event. Plasmids pJB1 and p88557 do not harbour the 1.3 Mdal insertion element, yet are mobilized by plasmid pHD147. This observation would tend to support acquisition of the element by a rare transposition

event. It would also support *in trans* mobilization of the ampicillin resistance plasmids, as co-integrate formation is not required by this transfer mechanism. However, the absence of the insertion element from plasmids pJB1 and p88557 can be explained in another way. First, the assumption is made that the progenitor of plasmids pJB1 and p88557 harboured the insertion element. The manner by which it was acquired is not relevant to this argument. Upon transformation of a host cell with the progenitor plasmid, the element was deleted from it, creating plasmids identical to pJB1 and p88557. There is precedence for such an event. Sox et al. (1979) found that upon transformation of *N. gonorrhoeae* with DNA from a plasmid identical to p22209, some transformants were found to harbour a plasmid identical to p88557. The 1.3 Mdal insertion element had been deleted.

C. Mechanism of Mobilization of the Ampicillin Resistance Plasmids of *H. ducreyi* and *N. gonorrhoeae* by the Mobilizing Plasmid of *H. ducreyi*.

Kilbane and Malamy (1980) defined *in vitro* mobilization of a non-conjugative plasmid by an *in trans* mechanism in the following manner. Mobilization is dependent upon the presence of an OriT site within the non-conjugative plasmid. Diffusible gene products encoded by the mobilizing plasmid interact with functions encoded by the non-conjugative plasmid. Co-integrate formation between the non-conjugative and mobilizing plasmids does not occur. Co-transfer of the mobilizing plasmid to the recipient cell is not essential, but may occur. Mobilization fitting this description is defined as Class I.

Each of the four ampicillin resistance plasmids, pHD747, pJB1, p22209 and p88557, harbours an OriT site. As well, each of the plasmids encodes for the production of an endonuclease(s), capable, *in vitro*, of introducing

a specific nick at the OriT site, in one strand of the DNA duplex. Thus each of the four non-conjugative plasmids is capable of participating in the mobilization event by providing a functional site for the initiation of DNA transfer. When *in vitro* mobilization of these plasmids is carried out, evidence of co-integration with mobilizing plasmid pHD147 is not detected. While co-transfer of the mobilizing plasmid does occur, such co-transfer is not excluded by the definition of *in trans* mobilization. Thus, initial observations indicate that *in vitro* mobilization of the ampicillin resistance plasmids of *H. ducreyi* and *N. gonorrhoeae* occurs by an *in trans* mechanism. This is Class I mobilization as defined by Kilbane and Malamy (1980).

Three classes of *in vitro* mobilization by an *in cis* mechanism are defined by Kilbane and Malamy (1980). By definition, Class II mobilization of a non-conjugative plasmid molecule occurs by an *in cis* mechanism. Mobilization is dependent upon the recombination proficiency of the host cell to effect co-integrate formation between the mobilizing and non-conjugative plasmids.

Class III mobilization, is also an *in cis* mechanism, but co-integrate formation is not dependent upon the recombination system of the host cell. Co-integrate formation of the mobilizing and non-conjugative plasmids is mediated by recombination at regions of homology between the two plasmids.

The final mechanism of *in cis* mobilization, Class IV mobilization, is also independent of the recombination system of the host cell. The mechanism is based upon co-integration of the non-conjugative and mobilizing plasmids at regions of homology created as a result of transposition of a transposable element present within one of the plasmids.

In order to determine by what mechanism *in vitro* mobilization of the

ampicillin resistance plasmids of H. ducreyi and N. gonorrhoeae is occurring, transfer frequencies were calculated after mobilization of the four ampicillin resistance plasmids in recombination proficient and deficient backgrounds. Despite the state of the recombination system of the donors or intermediate recipients, transfer of all four plasmid is effected. The in vitro mobilization frequency, determined for each of the ampicillin resistance plasmids, is not dependent upon a proficient recombination system within the host cell. Plasmids present in transconjugant strains are unaltered in size or structure relative to those in parental strains. Mobilizing plasmid pHD147 is always co-transferred, but evidence of co-integration is not found. The characteristics of the mobilization events described above fit those of both Class I and Class III mobilization, as defined by Kilbane and Malamy (1980). Based upon these observations, in vitro mobilization of the ampicillin resistance plasmids can occur by an in trans and/or an in cis mechanism. Further analysis of the data is required to ascertain if one or both of the two mechanisms is functioning.

Independent of the recombinational proficiency of the host cells, the ampicillin resistance plasmids of H. ducreyi and N. gonorrhoeae are mobilized at reproducibly different frequencies. The differences observed in transfer frequency can be correlated with differences in physical structure, as well as to the extent of homology with the mobilizing plasmid.

Plasmid pHD747 is consistently mobilized at the highest frequency. Plasmid p22209 is consistently mobilized at the second highest frequency. Plasmids pJB1 and p88557 are mobilized at the lowest and not significantly different frequencies.

Plasmid pHD747 is the largest of the four ampicillin resistance plasmids due to the presence of both the 1.3 Mdal insertion element, and a complete ampicillin transposon. By relating the difference in mobilization

frequency to the difference in structure between plasmids pHD747 and p22209, some important observations can be made. The only physical difference between these two plasmids is the presence of the left-hand portion of the ampicillin transposon in plasmid pHD747. Therefore, the presence of this part of the transposon must be responsible for the difference observed in mobilization frequency. It is interesting to note that plasmids pHD747 and p22209 are equally homologous to the mobilizing plasmid, therefore a difference in the extent of homology is not a factor in the difference observed in transfer frequency. It appears that the functions encoded by the left-hand portion of the transposon have a significant influence upon mobilization frequency of plasmid pHD747.

However, it is not the products of the left-hand portion of the transposon alone that are responsible for the higher frequency of mobilization of plasmid pHD747. Plasmid pJB1 harbours an intact ampicillin transposon, identical to that of plasmid pHD747. Yet plasmid pJB1 is mobilized at the same low frequency as plasmid p88557. Plasmid p88557 lacks the left-hand portion of the transposon. Therefore it appears that a second factor is required, in conjunction with the left-hand portion of the transposon, to effect high frequency mobilization of plasmid pHD747. This factor is the 1.3 Mdal insertion element.

The presence of the 1.3 Mdal insertion element only, has some influence upon transfer frequency. This influence is demonstrated by the higher mobilization frequency of plasmid p22209 relative to the frequency obtained for plasmid p88557. The only physical difference between these two plasmids is the presence of the 1.3 Mdal insertion element within plasmid p22209. It should be noted that the presence of this element within plasmids p22209 is also responsible for the more extensive homology of plasmid p22209 with the mobilizing plasmid. There are three options to explain the

higher mobilization frequency of plasmid p22209.

1. The higher mobilization of plasmid p22209 is due to a product encoded by the insertion element.
2. This higher mobilization frequency of plasmid p22209 is due to the presence, within this plasmid, of more extensive homology with the mobilizing plasmid. This option would only be correct if Class III mobilization takes place.
3. The higher transfer frequency of plasmid p22209 is due to the presence of more extensive homology with the mobilizing plasmid and to the functions of a product encoded by the 1.3 Mdal insertion element.

It is not known which of the three options is correct.

The presence of both the left-hand portion of the ampicillin transposon and the 1.3 Mdal insertion element has the greatest effect, with regard to the enhancement of mobilization frequency. Plasmid pHD747 has both these components and is mobilized at the highest frequency. Since the products of the left-hand portion of the transposon alone have little or no effect upon mobilization, the enhancement in transfer of plasmid pHD747 over plasmid p22209 has to be due to an interaction between the products of the left-hand portion of the transposon and the 1.3 Mdal insertion element. The form that such interaction would take is unknown, but the following hypothesis is presented.

The left-hand portion of the ampicillin transposon encodes for the production of two proteins, transposase and transposon resolvase (Gill et al., 1979). Transposase is a high molecular weight protein of 120,000 daltons. This protein is required for transposition of the transposable element. The protein mediates recombination between the element itself, and the target molecule.

Transposon resolvase is a smaller protein of 19,000 daltons. This protein acts as a repressor of synthesis of both transposase and of itself. In addition, the protein facilitates resolution of the co-integrate formed between the host and target molecules during transposition of a transposon.

These two proteins have previously been shown to facilitate transposition of other transposons, unable to transpose themselves (Heffron et al., 1981). Since the 1.3 Mdal insertion element resembles a transposon in structure, the proteins encoded by the left-hand portion of the ampicillin transposon may facilitate recombination between the element within plasmid pHD747 and its counterpart within the mobilizing plasmid pHD147.

However, recombination of the elements may occur spontaneously without the action of the proteins. Ohtsubo et al. (1981) found that the presence of identical insertion elements within two different plasmids facilitated homologous recombination between the molecules. This recombination was independent of the recombination system of the host cell, and resolved as spontaneously as it formed.

Thus the recombination of the elements within plasmids pHD747, p22209 and pHD147 may occur. The recombination may be facilitated by the proteins encoded by the left-hand portion of the ampicillin transposon within plasmid pHD147.

The presence of the elements within both plasmids p22209 and pHD147 results in homologous recombination as well, but at a lower frequency than when assisted by the transposase and transposon resolvase proteins. Thus plasmids pHD747 and p22209 are mobilized by a Class III mechanism, but plasmid pHD747 is mobilized at a higher frequency than plasmid p22209.

The previous sections have provided evidence for mobilization by both

in trans and *in cis* mechanisms. These two mechanisms are not mutually exclusive. Both may be used during mobilization of the ampicillin resistance plasmids of *H. ducreyi* and *N. gonorrhoeae* by mobilizing plasmid pHD147.

The four ampicillin resistance plasmids should be efficiently mobilized by an *in trans* mechanism due to the presence of an OriT site, and to the production of OriT site-specific endonuclease(s). Thus Class I mobilization may be responsible for the base mobilization frequency observed for plasmids pJB1 and p88557.

Plasmids pHD747 and p22209, may in addition to Class I mobilization, undergo mobilization *in cis*. Plasmid p22209, mobilized at the second highest frequency may be capable of low frequency recombination with the mobilizing plasmid at the site of the 1.3 MdaI insertion element. Thus this plasmid undergoes predominately Class I mobilization, but is capable of undergoing Class III mobilization as well.

Plasmid pHD747 is mobilized at the highest frequency. This plasmid may be capable of higher frequency recombination with the mobilizing plasmid pHD147 at the site of the 1.3 MdaI insertion element, as a result of the function of the transposase and resolvase enzymes. Thus plasmid pHD747 undergoes not only Class I mobilization, but Class III mobilization as well.

D. Reciprocal Conjugal Transfer of the Ampicillin Resistance Plasmids of *H. ducreyi* and *N. gonorrhoeae*.

Due to the structural similarities between the ampicillin resistance plasmids of *H. ducreyi* and *N. gonorrhoeae*, an effort was made to transfer plasmids pHD747 and pHD147 from a background of *H. ducreyi* to both *N. gonorrhoeae* and *N. cinerea*, *in vitro*. Transfer did not occur.

Several reasons can be suggested to explain the failure of the plasmids to transfer, however the exact reasons are unknown. Maintenance of plasmids pHD747 within a background of Neisseria should not be a problem since the maintenance functions of this plasmid are identical to those of the native gonococcal plasmids p22209 and p88557. However, H. ducreyi and N. gonorrhoeae may fail to form a stable mating aggregate, an essential initial step for plasmid transfer. Even if such an aggregate is formed and plasmid transfer initiated, the plasmid DNA coming from a background of H. ducreyi may be degraded by the restriction endonucleases of the Neisseria species.

While transfer was not effected from a background of H. ducreyi to N. gonorrhoeae or N. cinerea, it was effected from a background of H. influenzae to N. cinerea. Ampicillin resistance plasmids pHD747, pJB1, p22209 and p88557, in addition to mobilizing plasmid pHD147, were transferred to N. cinerea. The reason why transfer to N. cinerea should be effected while a barrier exists to transfer to N. gonorrhoeae is unknown. One can speculate that the restriction endonucleases within N. cinerea are isoschizomers of those within H. influenzae strain Rd. Precedent for such a relationship exists between these genera. The restriction endonucleases NgoI and NgoII of N. gonorrhoeae are iso-schizomers of restriction endonucleases HaeII and HaeIII respectively, of H. aegyptius.

Since direct transfer of the ampicillin resistance plasmids from a background of Haemophilus to Neisseria gonorrhoeae was not successful, several different organisms were introduced as intermediates in conjugal transfer. These particular intermediates were selected for specific reasons.

As previously stated, the restriction endonucleases HaeII and HaeIII

of H. aegyptius are iso-schizomers of NgoI and NgoII respectively of N. gonorrhoeae. If restriction of incoming plasmid DNA is the barrier to transfer, then DNA entering N. gonorrhoeae from a background of H. aegyptius should be appropriately modified. However, H. aegyptius is not an effective intermediate for plasmid transfer. The failure of transfer to occur may indicate that there are other type II restriction endonucleases or other types of restriction-modification systems present within N. gonorrhoeae that do not have iso-schizomers within H. aegyptius. These enzymes degrade the incoming DNA. Alternatively, H. aegyptius may not form a stable mating aggregate with N. gonorrhoeae. Either of these two factors would prevent plasmid transfer.

The decision to use H. parainfluenzae as an intermediate in transfer was based upon an observation of Sparling et al. (1978). They were able to transfer a small ampicillin resistance plasmid from a clinical isolate of H. parainfluenzae to N. gonorrhoeae, by conjugation. However, H. parainfluenzae fails to act as an effective intermediate for plasmid transfer under the conditions of this investigation.

Finally N. cinerea was chosen as an intermediate in plasmid transfer between Haemophilus and N. gonorrhoeae. This organism has previously acted as a recipient for the ampicillin resistance and mobilizing plasmids during this investigation. As well, other researchers have effected transfer of the ampicillin resistance and mobilizing plasmids of gonococcus between N. cinerea and N. gonorrhoeae (Joan Knapp, personal communication). For these reasons, this organism was chosen to act as a transfer intermediate.

N. cinerea is capable of acting as an intermediate for transfer between Haemophilus and Neisseria gonorrhoeae. However, the efficiency of plasmid transfer is low. Despite repeated attempts, only a single gonococcal transconjugant was isolated. This transconjugant harbours plasmid

p22209. Thus conjugal transfer of plasmid DNA from a background of Haemophilus to N. gonorrhoeae is possible, but only at a very low efficiency.

To date, the genus Haemophilus appears to be the most likely source of the ampicillin resistance plasmids of N. gonorrhoeae. However, conjugal transfer of the plasmids from Haemophilus to Neisseria gonorrhoeae in this investigation is neither direct, nor efficient. For this reason, an attempt was made to determine if transfer in the reverse direction was more easily facilitated. Conjugal transfer of plasmid DNA is not effected from a background of N. gonorrhoeae to H. ducreyi, however transfer to H. influenzae is efficient. Barriers to plasmid transfer exist between these two genera, therefore it is not possible to identify the mechanism for acquisition of plasmid-mediated ampicillin resistance by N. gonorrhoeae.

Studies were carried out to determine if the barrier to efficient plasmid transfer between Haemophilus and N. gonorrhoeae is a function of the mobilizing plasmid mediating transfer. In vitro tri-parental conjugation studies were carried out to determine if transfer of the ampicillin resistance plasmids from H. influenzae to N. gonorrhoeae could be effected by the 24.5 Mdal mobilizing plasmid of N. gonorrhoeae. While this mobilizing plasmid promotes transfer of the ampicillin resistance plasmids between strains of Haemophilus influenzae, transfer is not effected from H. influenzae to N. gonorrhoeae. The barrier to conjugal transfer of the ampicillin resistance plasmids is not a function of the species of the mobilizing plasmid.

Much of the evidence accumulated to date supports the hypothesis that the ampicillin resistance plasmids originated in the genus Haemophilus. The mole % G+C ratio of the DNA of these plasmids is essentially identical

to the value calculated for the chromosomal DNA of the genus Haemophilus (De Graaff et al., 1976). Plasmids isolated from the genus are closely related in structure to the ampicillin resistance plasmids of H. ducreyi and N. gonorrhoeae (Albritton et al., 1982; Brunton et al., 1983). Plasmid-mediated ampicillin resistance in Haemophilus pre-dates the plasmid-mediated ampicillin resistance in N. gonorrhoeae (Brunton et al., 1983). Conjugal transfer of plasmid-mediated ampicillin resistance from Haemophilus to Neisseria gonorrhoeae occurs, albeit at a low frequency (Sparling et al., 1978). All these factors support the genus Haemophilus as the source of the ampicillin resistance plasmids of H. ducreyi and N. gonorrhoeae.

Due to the nature of the diseases caused by H. ducreyi and N. gonorrhoeae, a co-infection by these organisms would not be an unusual occurrence. Perhaps conjugal transfer of plasmid DNA from H. ducreyi, or from another species of Haemophilus, to N. gonorrhoeae is facilitated by environmental conditions in vivo. Indeed, even if an intermediate in transfer is required, such as N. cinerea, the presence of the organism in the uro-genital tract is not unusual. The strain of N. cinerea used in this investigation was a clinical isolate from the cervix (Joan Knapp, personal communication).

Transfer of plasmid DNA between these two genera may also occur in vivo by transformation. Transformation of N. gonorrhoeae with plasmid DNA does occur in vitro at low frequencies, however the organism is refractory to transformation with DNA isolated from a background of Haemophilus (Graves et al., 1982). The process may be more efficient in vivo. Primary isolates of N. gonorrhoeae are of the piliated colonial form. This form is essential for transformation of the organism. Therefore, N. gonorrhoeae is competent for transformation in vivo and so transformation with plasmid DNA may be much more efficient under these conditions. Transformation in vivo

may represent the principle mechanism for genetic exchange between Haemophilus and Neisseria.

Several observations made during this investigation also support the genus Haemophilus as the source of the ampicillin resistance plasmids of H. ducreyi and N. gonorrhoeae. Conjugal transfer of plasmid-mediated ampicillin resistance from a background of Haemophilus to N. gonorrhoeae occurs, but at a low frequency, and only by the intervention of an intermediate. The mobilizing plasmid of H. ducreyi shares extensive homology with the ampicillin resistance plasmids isolated from H. ducreyi and N. gonorrhoeae. Of particular interest is the presence of an insertion element within plasmids pHD147 and pHD747 of H. ducreyi and plasmid p22209 of N. gonorrhoeae. This element was not present within the 24.5 Mdal mobilizing plasmid of N. gonorrhoeae studied.

As stated previously, plasmids pJB1, p22209 and p88557 could be derived from plasmid pHD747 by appropriate deletions. While deletion events have not been observed throughout the course of this investigation, it is not difficult to envision that such deletions may occur at a low frequency. One can speculate as to the route the creation of the ampicillin resistance plasmids of H. ducreyi and N. gonorrhoeae could take. Plasmid pJB1 may have been created by transposition of an ampicillin transposon onto a cryptic plasmid resident in the genus Haemophilus. The transposon could have been carried into the genus Haemophilus by an enteric plasmid not maintained in the background of Haemophilus. Indeed, cryptic plasmids identical to the core region of plasmid pJB1, have been isolated from H. parainfluenze (Brunton et al., 1983) and H. influenzae (Albritton et al., 1982). Subsequent mobilization of this plasmid into H. ducreyi by a plasmid similar to pHD147 could occur. If, during the mobilization event, the

1.3 Mdal insertion element was acquired by the ampicillin resistance plasmid, a plasmid identical to plasmid pHD747 would be created. Should the plasmid be mobilized to H. ducreyi without acquisition of the 1.3 Mdal insertion element, then H. ducreyi would acquire a plasmid identical to plasmid pJB1.

The proposed scenario for acquisition of the ampicillin resistance plasmids by N. gonorrhoeae is more complex. Conjugal transfer of a resistance plasmid from H. parainfluenzae to N. gonorrhoeae has been observed (Sparling et al., 1978). Thus conjugal transfer between the two genera is possible. Plasmids identical to p22209 and p88557 could have been acquired by N. gonorrhoeae as a result of conjugal transfer of plasmids pHD747 and pJB1 respectively. However deletion of the left-hand portion of the ampicillin transposon within both plasmids pHD747 and pJB1 would have to occur. The occurrence of two identical deletion events is unlikely, therefore it is more likely that a plasmid identical to plasmid pHD747 was initially acquired by N. gonorrhoeae. Since N. gonorrhoeae readily undergoes autolysis releasing DNA, and is also competent for transformation in vivo, subsequent in vivo transformation of a second strain of N. gonorrhoeae with this specific plasmid could take place. Upon transformation, the left-hand portion of the ampicillin transposon may be deleted. This newly formed plasmid would be identical to plasmid p22209. Upon subsequent transformation events the 1.3 Mdal insertion element may be deleted, creating a plasmid identical to plasmid p88557. Deletion of the 1.3 Mdal insertion element by transformation has been demonstrated to occur by this route under laboratory conditions (Sox et al., 1979), however deletion of the left-hand portion of the ampicillin transposon has not been demonstrated as yet. It should be emphasized that the previous discussion is

speculation based upon experimental observation, and may or may not represent the route for acquisition of the ampicillin resistance plasmids by H. ducreyi and N. gonorrhoeae.

E. The Structural Organization of the Mobilizing Plasmid pHD147 of H. ducreyi.

As an initial step in the characterization of plasmid pHD147, a restriction endonuclease map was constructed. The plasmid does not have restriction sites for many commonly used restriction endonucleases; neither those with hexanucleotide recognition sequences, nor even those recognizing a four base pair sequence. The significance of the paucity of sites is unknown, but may represent the ability of the plasmid to be maintained in a broad range of host cells.

The map constructed for plasmid pHD147 does not resemble the map of the 24.5 Mdal mobilizing plasmid of N. gonorrhoeae (Tenover et al., 1980). Therefore these two plasmids are distinctly different, despite the fact that both have mobilizing function.

The OriV site of the mobilizing plasmid is located within a 1.3 Mdal XhoII-HindIII restriction fragment. A portion of this fragment shares homology with the ampicillin resistance plasmids of H. ducreyi and N. gonorrhoeae. However, due to the compatibility between the ampicillin resistance and mobilizing plasmids, it is unlikely that sequences specifying all of the maintenance functions are responsible for the homology.

Plasmid pHD147 is capable of replication in the absence of E. coli DNA polymerase I, a characteristic of the ampicillin resistance plasmids as well. Several plasmids of enteric origin require this enzyme for replication (Som and Tomizawa, 1982). This observation emphasizes the likelihood

that the ampicillin resistance and mobilizing plasmids originated in a genus other than E. coli.

The OriT site of the mobilizing plasmid is located within a 1.1 Mdal ClaI-XhoII restriction fragment, in close proximity to the OriV site. This region is not homologous to the ampicillin resistance plasmids. The OriT site of plasmid pHD147 appears to be structurally different from those of the ampicillin resistance plasmids. Plasmid pHD147 cannot be isolated as a DNA-protein relaxation complex, by conventional methods. While the reasons for this are unknown, other mobilizing plasmids demonstrate this characteristic as well (Johnson et al., 1981).

The maintenance and transfer functions of plasmid pHD147 are clustered within one region of the plasmid molecule. This appears to be a common feature of the plasmids characterized during this, and other investigations (Nordheim et al., 1980).

As stated previously, the 1.3 Mdal insertion element is present within mobilizing plasmid pHD147. The element is located in close proximity to the OriV and OriT sites of the molecule. Since the element appears to be an essential feature in the enhancement of mobilization frequency of ampicillin resistance plasmids pHD747 and p22209, the location of the element within the transfer region of plasmid pHD147 may be more than coincidental. The element may play a role in the mobilizing function of plasmid pHD147.

Homology between plasmid pHD147 of H. ducreyi and the 24.5 Mdal mobilizing plasmid of N. gonorrhoeae is present. Homology is not extensive and appears to be confined to the regions of each plasmid that is involved in transfer functions (Young et al., 1980). Since both plasmids are conjugative and mediate mobilization of the four ampicillin resistance plasmids, the presence of some homology is not surprising. However, these

plasmids are distinctly different molecules, indicating a diverse origin.

F. Characterization of Proteins Encoded by the Ampicillin Resistance and Mobilizing Plasmids by In Vitro Coupled Transcription-Translation of Plasmid DNA Templates.

The ampicillin resistance plasmids of H. ducreyi and N. gonorrhoeae each encode for the production of nine proteins. Since two of these proteins of 30,900 and 32,000 daltons are the beta-lactamase enzyme and its precursor, respectively (Casadaban et al., 1981), the plasmids encode for the production of eight different proteins. Seven of these proteins are encoded by the non-TnA portion of the plasmid molecules. It should be noted that production of the proteins encoded by the left-hand portion of the ampicillin transposon within plasmids pHD747 and pJB1 is under stringent control. As a result, transposase and transposon resolvase are not detected by the conditions of this procedure (Casadaban et al., 1981). The production of the seven proteins utilizes the entire coding capacity of the core region of these plasmids.

The 1.3 Mdal insertion element within plasmid pHD747 and p22209 does not encode for production of a detectable protein. This is an important observation. This finding implies that the enhanced mobilization frequencies obtained for plasmids pHD747 and p22209 may not be due to a protein encoded by the insertion element, but rather due to recombination between the elements within these plasmids and the mobilizing plasmid.

At least one of the proteins encoded by the four ampicillin resistance plasmids must be an endonuclease responsible for specifically nicking at the OriT site. Plasmid ColE1 encodes for the production of three endonuclease proteins of 60,000, 16,000, and 11,000 daltons in molecular weight

(Lovett et al., 1974b). Proteins identical in molecular weight to these proteins are not encoded by the ampicillin resistance plasmids. However, more than one of the proteins encoded by the ampicillin resistance plasmids may be involved in the nicking process.

Two proteins of identical molecular weight are encoded by the ampicillin resistance plasmids and by the mobilizing plasmid pHD147. The proteins are 26,300 and 22,300 daltons in molecular mass. Data derived from *in vitro* transcription-translation studies indicate that these proteins are encoded by homologous regions of the two plasmid species. The function of these proteins and of the five remaining proteins, unique to the ampicillin resistance plasmids, is unknown. As previously stated, at least one is an OriT site-specific endonuclease.

Plasmid pHD147 encodes for the production of five proteins. Production of these plasmids utilizes only nine percent of the potential coding capacity of this plasmid. The function of these five proteins is unknown.

Plasmid pHD147 is maintained and expresses mobilizing function in a background of *E. coli*. Therefore there is no reason to question the low number of proteins produced by the *E. coli* cell extract-based transcription-translation system. While plasmid pHD147 produces far fewer proteins than the mobilizing plasmid F (Broda, 1979), there is no reason to suspect that more are required for the plasmid's maintenance and transfer functions.

There is evidence to suggest that the mobilizing system of plasmid pHD147 is much less complex than that of the F factor or other F-like plasmids. Conjugal transfer mediated by plasmid pHD147 is facilitated when the parental cells are concentrated together on a solid surface. Mating is inefficient in broth culture. This is an indication that sex pili do not play a role in conjugation. Additionally, electron microscopy of

mating cells has never revealed the presence of sex pili (H. Deneer, M.Sc. Thesis, University of Manitoba). Rather, mating pair formation involved longitudinal alignment of mating cells. Thus the five proteins encoded by the mobilizing plasmid pHD147 may indeed be sufficient for mediation of mobilization.

At least one of the proteins is an OriT site-specific endonuclease. The function of the remaining proteins is unknown, although the 26,300, 22,300 and 16,900 dalton proteins are encoded by sequences clustered within the maintenance and transfer regions of the plasmid molecule. These proteins may be involved in either of these functions.

Summary and Conclusions.

This investigation has provided insight into the relationship between structure and function of certain plasmid molecules. It has provided additional evidence for a common ancestral origin of the ampicillin resistance plasmids of H. ducreyi and N. gonorrhoeae. Not only does this investigation demonstrate the existance of a close physical relationship between these plasmids, it also demonstrates the presence of identical transfer and maintenance functions.

This investigation has also provided insight into the structure and function of the first plasmid to express mobilization function in the genus Haemophilus. Characterization of a plasmid mediating mobilization of resistance factors amongst Haemophilus species was of value, considering the clinical significance of many of these organisms. Several conclusions have been reached.

1. The ampicillin resistance plasmids of H. ducreyi and N. gonorrhoeae are structurally identical with two exceptions. The plasmids of H. ducreyi harbour complete ampicillin transposons; the plasmids of N. gonorrhoeae retain only a portion of the transposon. The larger plasmids of H. ducreyi and N. gonorrhoeae harbour an insertion element.

2. The ampicillin resistance plasmids are functionally organized in an identical manner. The maintenance and transfer functions are located within the same regions.

3. The ampicillin resistance plasmids replicate in a unidirectional fashion and do not require E. coli DNA polymerase I for replication.

4. The ampicillin resistance plasmids share homology with a mobilizing plasmid isolated from H. ducreyi. The homologous sequences are common to all four plasmids with one exception. The insertion element within the larger plasmids of H. ducreyi and N. gonorrhoeae is also present within the mobilizing plasmid of H. ducreyi.

5. The ampicillin resistance plasmids are mobilized at reproducibly different frequencies. Mobilization frequency is correlated with physical structure and also with the extent of homology with the mobilizing plasmid.

6. Mobilization of the ampicillin resistance plasmids is not dependent upon the recombination system of the host cell.

7. Mobilization of the four ampicillin resistance plasmids occurs by an *in trans* Class I mechanism. Additionally, the larger plasmids of *H. ducreyi* and *N. gonorrhoeae* may be mobilized *in cis* by a Class III mechanism. The presence of the 1.3 Mdal insertion element within these plasmids is correlated with an enhanced frequency of mobilization.

8. The transfer regions of the ampicillin resistance plasmids and the mobilizing plasmid are different. The ampicillin resistance plasmids can be isolated as DNA-protein relaxation complexes *in vitro*. The mobilizing plasmid can not.

9. The ampicillin resistance and mobilizing plasmids encode for the production of several different proteins. However two proteins encoded by these plasmids are the same molecular weight.

10. Conjugal transfer of plasmid-mediated ampicillin resistance between the genus *Haemophilus* and *N. gonorrhoeae* takes place, but at a low frequency. Transfer is mediated by the presence of an intermediate organism.

11. The mobilizing plasmids of *H. ducreyi* and *N. gonorrhoeae* are distinctly different molecules.

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Appendices.

Appendix A

Culture Media

L Broth

Bacto-tryptone	10 g
Bacto-yeast extract	5 g
NaCl	0.5 g
1 M NaOH	2 ml
H_2O	1000 ml
Autoclave for 15 min.	
20% sterile dextrose	10 ml

GC Broth

Protease Peptone (No. 3, Difco)	15 g
K_2HPO_4	4 g
KH_2PO_4	1 g
NaCl	0.5 g
H_2O	1000 ml

Appendix B

Buffers

Cell Lysis Buffers

Triton-Sucrose Lysis Buffer

8% sucrose

0.5% Triton X-100

0.05 M EDTA

0.01 M Tris-HCl, pH 8.0

Triton Lytic Mix

10% Triton X-100 in 10 mM Tris-HCl, pH 8.0	1.0 ml
0.25 M EDTA	25.0 ml
1 M Tris-HCl, pH 8.0	5.0 ml
H_2O	69.0 ml

Tris-Sucrose Buffer

1 M Tris-HCl pH 8.0	5.0 ml
0.5 M EDTA	1.0 ml
sucrose	125 g
H_2O	494 ml

DNA Suspension Buffers

Tris-EDTA-Saline Buffer (TES)

0.03 M Tris-HCl, pH 8.0

0.05 M EDTA

0.005 M NaCl

Tris-EDTA Buffer

10 mM Tris-HCl, pH 8.0

1 mM EDTA

Restriction Endonuclease Buffers

10X Low-Salt Buffer

10 mM Tris-HCl, pH 7.5

10 mM $MgCl_2$

1 mM dithiothreitol

10X Medium-Salt Buffer

50 mM NaCl

10 mM Tris-HCl, pH 7.5

10 mM $MgCl_2$

1 mM dithiothreitol

10X High-Salt Buffer

100 mM NaCl

50 mM Tris-HCl, pH7.5

10 mM $MgCl_2$

1 mM dithiothreitol

Agarose Gel Electrophoresis Buffers

Tris-Borate Buffer

Boric Acid	22.0 g
Tris Base	43.2 g
EDTA (disodium)	3.72 g
H_2O	1000 ml

Miscellaneous Buffers

10X Ligation Buffer

0.5 M Tris-HCl, pH 7.4

0.1 M $MgCl_2$

0.1 M dithiothreitol

10 mM ATP

Stop Buffer (Nick Translation)

30 mM EDTA

3% SDS

600 ug/ml sheared Salmon sperm DNA

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis Buffers

Discontinuous Gel Buffer

1.5 M Tris-HCl, pH 8.8

Stacking Gel Buffer

0.5 m Tris-HCl, pH 6.8

Glycine Buffer

Tris Base	15 g
Glycine	72 g
10% SDS	50 ml
H_2O	5000 ml

Loading Buffer (2X)

0.08 M Tris-HCl, pH 6.8

0.1 M dithiothreitol

2% SDS

10% Glycerol

0.1 mg/ml bromophenol blue

Appendix C

Reagents

Reagents for DNA Hybridization

Standard Saline Citrate (SSC) (20X)

3 M NaCl

0.3 M Sodium Citrate

Denhardt's Solution (50X)

Ficoll 400	5 g
Polyvinylpyrolidone-360	5 g
BSA Fraction V	5 g
H_2O	500 ml

Pre-Hybridization Mix

20X SSC	30 ml
50X Denhardt's Solution	10 ml
10% SDS	5 ml
1 M EDTA	1 ml
2mg/ml sonicated denatured salmon sperm DNA	5 ml

Hybridization Mix

20X SSC	30 ml
50X Denhardt's Solution	4 ml
10% SDS	5 ml
1 M EDTA	1 ml
2 mg/ml sonicated denatured Salmon sperm DNA	5 ml

Amersham Nick Translation Kit, Solution 1

250 mM Tris-HCl, pH 7.8

25 mM $MgCl_2$

50 mM 2-mercaptoethanol

100 uM dATP, dGTP, dTTP

Amersham Nick Translation Kit, Solution 2

5 units E. coli DNA polymerase I

100 pg E. coli DNAse I

Reagents for Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

Acrylamide:Bis Stock Solution

acrylamide	30 g
bis-acrylamide	0.8 g
H_2O	100 ml

10% SDS Stock Solution

SDS	10 g
H_2O	100 ml

Stacking Gel Solution; 5% Acrylamide.

Acrylamide:Bis Stock Solution	1.67 ml
Stacking Gel Buffer, pH 6.8	2.5 ml
10% SDS Solution	0.1 ml
TEMED	0.01 ml
H_2O	5.22 ml
1.5% Ammonium Persulfate Stock	0.50 ml

Discontinuous Gel Solutions

% Acrylamide	7.5%	10.0%	12.5%	15.0%	20.0%
Acrylamide:Bis Stock Solution	7.5	10.0	12.5	15.0	20.0
Discontinuous Gel Buffer	3.75	3.75	3.75	3.75	3.75
10% SDS Stock	0.3	0.3	0.3	0.3	0.3
TEMED	0.03	0.03	0.03	0.03	0.03
H_2O	16.92	14.42	11.92	9.42	4.42
1.5% Ammonium Persulfate	1.5	1.5	1.5	1.5	1.5

Miscellaneous Reagents

DNase-Free RNase

Ribonuclease	20 mg
50 mM Sodium Acetate	20 ml

Heat at 90°C for 10 min.

Chromogenic Cephalosporin

Nitrocefin (Glaxo)	0.05 g
Dimethylsulfoxide	0.5 ml
0.1 M Phosphate Buffer, pH 7.0	9.5 ml

Agarose Gel Electrophoresis Tracking Dye

Bromophenol Blue	0.07 g
SDS	7.0 g
Glycerol	33.0 ml
H_2O	67.0 ml