A STUDY OF THE EPIDEMIOLOGY OF PLASMID ORIGIN AND DISTRIBUTION IN *HAEMOPHILUS DUCREYI*: CHARACTERIZATION OF A NOVEL 2.6 Mdal β-LACTAMASE PLASMID IN *H.DUCREYI*

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David Malcolm Ian McLean

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DAVID MALCOLM IAN McLEAN

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

MASTER OF SCIENCE

"This above all,-to thine ownself be true;

and it must follow, as the night, the day,

thou canst not then be false to any man."

William Shakespeare

This thesis is dedicated to the memory of my father

Robert C. Fiander

who impressed upon me this fundamental understanding.

I take great pleasure in acknowledging the many people whose collective efforts helped to make this thesis a reality.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Page

ABSTRACT

The rapid dissemination of plasmid mediated resistance to a number of antimicrobials has prompted studies of the epidemiology of plasmid evolution and distribution in Haemophilus ducreyi, the etiological agent of chancroid. In addition to sulfonamide, tetracycline, and chloramphenicol resistant plasmids, three ampicillin resistant plasmids have been described in this organism. The latter express a TEM-1 type β-lactamase as encoded on the transposon, Tn2. After having performed plasmid profiles of 830 H.ducreyi isolates, collected from four continents since 1978, 11 plasmids making up 14 different profiles were identified. Additionally, a number of interesting epidemiologic patterns were observed.

Of the three known β-lactamase plasmids, the 5.7 and 7.0 Megadalton (Mdal) plasmids predominate in Africa, occurring in almost 100% of isolates. A 4.9 Mdal sulfonamide resistant plasmid was also found in Africa, but in significantly fewer isolates. Multiple plasmid profiles appearing within a given year were also found in Amsterdam, suggesting multiple introductions. In North America, a single plasmid profile persisted throughout chancroid microepidemics. The 7.0 Mdal β-lactamase plasmid has not appeared in North America and this may reflect the introduction of strains harbouring the 5.7 and 3.2 Mdal β-lactamase plasmids from the Far East, South America, and Mexico. The 3.2 Mdal β-lactamase plasmid, observed in California in 1982, was found in Amsterdam isolates in the same year. Of considerable significance, a 3.2 Mdal plasmid present in all Thailand isolates was shown by hybridization studies not to encode a β-lactamase producer. Rather, the 5.7 and 7.0 Mdal plasmids, and a novel 2.6 Mdal plasmid, were ascribed this phenotype. Characterization of this novel β-lactamase producing plasmid revealed that it is non-conjugative and non-mobilizable. Transformation into a rec^+ Escherichia coli host (C600)

yielded a deletion of about 300 base pairs. This deletion was found to be common to all of the transformants. Subsequent experiments using a rec$^-$ E.coli (HB101) failed to yield transformants. Restriction endonuclease analysis of this plasmid revealed it to be unique from those β-lactamase producing plasmids previously described in H.ducreyi.

Restriction endonuclease digests and DNA hybridization studies with the parental plasmid (pDMI), the transformed deletion of this plasmid, pDMI-del, and pATI53, a deletion derivative of pBR322, indicated extensive homology shared between pAT153 and pDMI-del, and homology shared between pDMI-del and pDMI. This may suggest that homology is shared between pDMI and progenitors of the recombinant plasmid, pAT153.

INTRODUCTION

Since the description by Hammond et al (1978) of an effective culture media for Haemophilus ducreyi, studies of the organism's molecular biology have become possible. These investigations have primarily been concerned with typing the organism by outer membrane protein analysis (Odumeru et al, 1983) or with the characterization of numerous antimicrobial-resistant plasmids (Albritton et al, 1984; Anderson et al, 1984; Deneer et al, 1982; Brunton et al, 1979). Due to the epidemiological association of chancroid with human immunodeficiency virus-1 (HIV-1) and the rapidly changing incidence of antimicrobial resistance among H.ducreyi, the epidemiology of plasmid origin and distribution in this organism is of considerable importance for controlling this pathogen.

Initial studies on ampicillin resistance in H.ducreyi led to the isolation of a 5.7 Mdal β-lactamase-producing plasmid (Brunton et al, 1979) in three of 21 isolates cultured from an epidemic population in Winnipeg. Subsequent work by Maclean et al (1980) revealed that these plasmids encoded a TEM-1-type β-lactamase as found on the transposon, Tn2. A 7.0 Mdal β-lactamase plasmid was later described in this organism (Brunton et al, 1982). Both the 5.7 and 7.0 Mdal plasmids possess 100% of the transposon in contrast with the 4.4 Mdal and 3.2 Mdal plasmids of Neisseria gonorrhoeae which possess only 40% of Tn2. A third β-lactamase plasmid in H.ducreyi is 3.2 Mdal in molecular mass (Anderson et al, 1984). This plasmid is identical to the plasmid present in N.gonorrhoeae and more recently in Haemophilus influenzae (Slaney et al, 1986). Due to its extensive homology with the other H.ducreyi β-lactamase plasmids and due to the close resemblance of the restriction endonuclease map of this plasmid with the 5.7 Mdal plasmid, we assume it has evolved as a deletion of the 5.7 or 7.0 Mdal plasmid. Homology between the non-TnA regions of the small non-

conjugative ampicillin resistance plasmids of H.ducreyi, N.gonorrhoeae, and the small cryptic plasmids of Haemophilus parainfluenzae suggest that these resistance plasmids originated through a transposition event of the resistance gene to the cryptic plasmids found in H.parainfluenzae (Brunton et al, 1986a). In addition to these β-lactamase plasmids, the organism harbours a 4.9 Mdal sulfonamide resistant plasmid (Albritton et al, 1982).

Large conjugative plasmids in H.ducreyi (21.7 Mdal) and in N.gonorrhoeae (24.5 Mdal), that are capable of mobilizing these smaller resistance plasmids in intergeneric and interspecific matings, have been described (McNicol et al, 1986a). Recently, Brunton et al (1986a) has proposed that through these mobilizing plasmids, dissemination of small β-lactamase plasmids has occurred in Haemophilus spp. and Neisseria spp. Additionally, two large conjugative plasmids encoding resistance to tetracycline (30 Mdal) and to both tetracycline and chloramphenicol (34 Mdal) increase the plasmid pool in H.ducreyi.

This study was initiated in an attempt to identify the extent of plasmid evolution and distribution in H.ducreyi.

LITERATURE REVIEW

A. PLASMIDS

1. Description

By the mid-1950's, strains of bacteria expressing multiple resistance to a range of unrelated antimicrobials had been recognized. Due to the extreme improbability that such resistance genes arose through simple mutation and selection, an alternative explanation was sought. Plasmids, autonomously replicating extrachromosomal genetic elements, were found to carry various genes responsible for resistance to many different antimicrobials. These double-stranded circles of DNA ranged in size from one to over 150 Megadaltons (Mdal). Since 1500 base pairs, or one Mdal, is sufficient to code for one to two proteins, plasmids may encode many proteins conferring numerous different phenotypes that are beneficial to the bacterial host. Resistance to antibiotics, production of antibiotics, bacteriocin production, enterotoxin production, and the production of restriction and modification enzymes are among these inherited traits.

2. Incompatibility

If two closely-related plasmids are introduced into the same cell, one will become predominant and effectively eliminate the other (Broda, 1979). This is referred to as plasmid incompatibility (Olds and Primrose, 1981). Presumably the close homology between the replicative mechanisms of incompatible plasmids is responsible for a competition for essential replicative proteins. As a result, one plasmid becomes diluted out of the bacterial population. Broda (1979) adds that since dissimilar, and hence compatible, plasmids lack this homogeneity of replicative mechanism, plasmid incompatibility represents evolutionary relatedness.

3. Transition or Amplification of Plasmid DNA

It has been observed that amplification of the plasmid can occur in the presence of some antibiotics. Concurrent with this increase in the number of resistance determinants is an increase in the level of resistance to the antibiotic (Broda, 1979). Exemplifying this phenomenon are certain strains of E.coli harbouring Col-El-like plasmids. With the addition of chloramphenicol to a late log phase culture, chromosomal replication ceases due to the inhibition of requisite protein production. Plasmid replication is not affected and can result in up to 3000 copies of the plasmid in each bacterial cell (Old and Primrose, 1981). As expected, transfer to antibiotic free media results in a marked decrease in the amount of plasmid DNA. These observations demonstrate two important facts regarding plasmid amplification:

1) The imprudent use of antibiotics can act selectively for resistant pathogens. This is, therefore, a public health concern which must be addressed.

2) The capacity to enrich for plasmid DNA has beneficial applications for molecular studies.

4. Purification and Isolation

To facilitate such molecular studies, plasmids can be isolated and purified by density gradient centrifugation of cell lysates or through chemical extractions of the lysate.

Density gradient centrifugation takes advantage of the fact that when a solution of CsCl is ultracentrifuged, a gradient is established. The salt is forced to the bottom of the tube by the centrifugal force creating an area of greater density. In an attempt to restore homogeneity to the solution, the cesium will diffuse in the opposite direction thereby defining the slope of the gradient. Broda (1979) adds that the cesium salt

of DNA has a density of about 1.7 g/cm^3, but due to its far greater mass than either cesium or chloride ions, it is less likely to randomly diffuse within the gradient's density parameters (1.60 g/cm^3 at top, 1.80 g/cm^3 at bottom). Therefore, the DNA will slowly form a relatively stable narrow band. Since chromosomal and plasmid DNA possess different sedimentation coefficients in the presence of ethidium bromide dye, distinct bands will be observed. The purified plasmid DNA band can then be collected free of chromosomal DNA.

Isolation of plasmid DNA from the bacterial cell generally involves lysis of the bacterial cell wall, followed by digestion of the resulting spheroplast with a suitable detergent such as sodium dodecyl sulfate (SDS). Purification of plasmid DNA, involving the removal of protein, RNA, and chromosomal DNA, can be achieved through phenol and chloroform extractions, and RNAse (Maniatis et al, 1982).

5. Sizing

Once plasmid DNA has been extracted from the host cell, determination of the molecular weight of the molecule can be performed. This is done by electrophoresis of either purified plasmid DNA or cell lysates. With this technique, samples are loaded at one end of an agarose gel and a constant voltage is applied through the cell, with the cathode at the sample end. Since DNA molecules are negatively charged, they will migrate towards the anode. Rate of migration is dependent upon both the size of the molecule, where smaller plasmids demonstrate faster migration than larger ones, and plasmid conformation where open circular (O.C.), linear, and covalently closed circular (C.C.C.) forms of a plasmid are observed. Due to a nick in one strand of DNA, the O.C. plasmid takes longer to travel through the agarose matrix. Similarly, the linearized plasmid also is slightly more inhibited in its passage through the gel, but not to the extent of the

O.C. form. The C.C.C. conformation requires the least amount of time to pass through the agarose and therefore migrates the furthest. Upon completion of the run, the DNA will be distributed throughout the gel as thin bands which may be visualized by staining with ethidium bromide, EtBr. This dye intercalates between the bases within the DNA and fluoresces when exposed to ultraviolet (UV) light. The distances migrated are inversely proportional to the logarithms of their molecular weights. Therefore, by plotting unknown plasmid migration distances on a standard curve of known molecular weights and migration distances, sizing of closed circular or linear DNA is possible. Alternatively, electron microscopic measurement of the contour length of the plasmid, compared with a known standard, can also be employed.

B. HOMOLOGOUS RECOMBINATION

The increasing prevalence of plasmids expressing resistance to several antimicrobials (R plasmids) suggests that an understanding of the intergeneric and interspecific transfer of these molecules is of epidemiologic significance. There are three mechanisms responsible for the exchange of genetic material in prokaryotes: transformation, transduction, and conjugation. All three involve the transfer of exogenous DNA from a donor parent, and the subsequent homologous recombination of this DNA within the genome of the recipient bacterium (Fox, 1978).

1. Transformation

Transformation involves the transfer of exogenous donor DNA into a recipient cell where recombination of this genetic material occurs. In the course of transformation, the donor DNA attaches to the recipient cell at specific recognition sites on the cell surface (Bacterial Genetics, see Library). Although DNA uptake has been proven to be a Mg^{2+} requiring reac-

tion in such organisms as Bacillus subtilis and in the Pneumococcus, DNA binding to the appropriate sites on the recipient bacterium can proceed in the absence of this cation (Fox, 1978). With the addition of Mg^{2+}, induction of cell competence occurs where DNA sequence specific surface receptors are generated, making DNA binding possible. Although binding of these receptors involves double-stranded DNA, this is hydrolysed to single-stranded DNA as it enters the cells. Both homologous and heterologous DNA can bind and enter competent cells, but, as explained below, only single stranded homologous sequences demonstrate recombination. Studies illustrate that entry of DNA is dependent upon a number of factors including the competence period of the cell growth cycle which varies in duration and time of occurrence for each species. Once inside the cell, the homologous single stranded DNA integrates within the recipient genome with remarkable efficiency (Fox, 1978).

The recombinational process is controlled by the genes, recA and recB, recC, and recD, of which the latter three contribute to form the recBCD protein. The function of these proteins has been reviewed by Stahl (1987). The recA gene encodes a protein responsible for the coating of single-stranded DNA. This protein-DNA complex then invades intact double-stranded DNA causing its complementary strands to separate. The protein-DNA complex then scans the duplex DNA until a homologous sequence is reached. Here, a new duplex is formed and the previous companion chain is abandoned. At this point, the recA protein leaves. The recBCD protein precedes the recA enzyme and dissociates the duplex DNA. This provides a free chain of DNA with which the recA protein-DNA complex can integrate and recombine with a homologous region. Mutations of recA reduce recombination by about one-thousandth of the normal level, whereas mutations in the BCD gene complex are less effective, resulting in a 100x reduction.

Transformation occurs naturally in a number of organisms such as H.influenzae and N.gonorrhoeae, which are known to develop the competent state. As a means of phenotypic and genotypic variation, these organisms become transformed with DNA liberated from cells which have released their nucleic acids due to disruption by autolysis or similar mechanisms. Since these and a number of other organisms develop the competence state naturally, presumably transformation is a viable mechanism of genetic exchange in vivo. In those organisms, such as E.coli, which do not naturally develop a state of competency, treatment with $CaCl_2$ and heat-shock alters the cell surface so as to permit entrance of DNA.

2. Transduction

Another means of genetic recombination involves the transfer of bacterial DNA, either chromosomal or plasmid, through a bacteriophage vector. Bacterial DNA is mistaken for phage DNA and packaged into a phage head (Rose and Barren, 1983). This is released in phage-infected recipients with about 2-5% of encapsidated DNA possessing sufficient homology to permit recombination with the recipient genome (Fox, 1978). Since the phage is devoid of viral DNA in such events, viral replication cannot occur. Due to the infrequency of this event and the absence of reports documenting bacteriophage infection of Neisseria and/or Haemophilus, it should be assumed that, in these genera, this method of genetic recombination is rare if it occurs at all.

3. Conjugation

Bacterial mating or conjugation involves the transfer of genetic information through cell contact. The initial description in E.coli proposed the transfer of plasmid DNA from a male to a female cell. The donor male cell possesses a 60 Mdal plasmid, a fertility factor designated F^+, which codes for self-replication, transfer, and production of hollow sex

pili. A bridge is formed by these pili, permitting physical contact with the female recipient cell (F^-). Contact presumably generates a signal for the synthesis or activation of enzymes involved in DNA transfer, perhaps including the linearization of the F^+ plasmid (Broda, 1979). Presumably, the donor DNA then passes through this physical bridge in a unidirectional manner ($F^+ \rightarrow F^-$) and enters the recipient conjugant (Clark and Warren, 1979). Transferred DNA, as exemplified by the F plasmid, is episomal or capable of existing in two states. It may be incorporated into a molecule which possesses an origin of replication through a recombination event (that is, the DNA is integrated into an existing replicon), or it may exist autonomously as a plasmid. To genetically determine whether conjugal transfer has occurred, an inherited phenotype encoded by the donor genes is monitored in the recipient cell.

In addition to these conjugative plasmids which encode genes that determine the specific contact cycle, many plasmids, which do not carry such genes or whose genes have become mutated, have been observed. Such plasmids are termed nonconjugate. Within this latter class, plasmids can be categorized as either mobilizable or non-mobilizable. Clark and Warren (1979) describe mobilization as the series of metabolic events which prepare the DNA for exist from the donor conjugant. They add that non-mobilizable plasmids are mutant derivatives of those which are mobilizable. Although Novick et al (1976) consider mobilization to involve the integration and subsequent transfer of a non-mobilizable plasmid with a conjugative one, evidence involving the nonconjugative E.coli plasmid, ColE1, suggests that physical association with conjugative plasmids is not essential (Clark and Warren, 1979). Rather, mobilization is a process of donation whereby a nonconjugative mobilizable plasmid is transferred via the effective contact determined by a conjugative plasmid, without physical

association of the two plasmids (Clark and Adelberg, 1962). Mobilization is initiated through the binding of specific mobilization/relaxation proteins to the sequence responsible for mobilization, the origin of transfer (OriT). A single-stranded nick within this sequence produces a 3' terminus suitable for priming DNA synthesis and a 5' terminus covalently attached to a protein. It has been suggested that this protein acts as a pilot protein permitting the effective exist of the DNA strand from the donor and entrance into the recipient (Kornberg, 1974). Once within the recipient cell, recircularization of transferred linear DNA occurs and the onset of vegetative replication ends the process.

Both conjugative and mobilizable plasmids have been observed in the Neisseria and Haemophilus. Plasmids longer than 20 Mdal, as exemplified by the large tetracycline-resistant plasmid of H.ducreyi (30 Mdal) and the large 30 Mdal H.influenzae β-lactamase plasmids, demonstrate conjugal matings (Brunton et al, 1983). These plasmids participate in intraspecific and intergeneric transfers through their production of the requisite conjugal proteins (Albritton et al, 1984; Brunton et al, 1983; Flett et al, 1981). Although these large conjugative plasmids are often co-resident with smaller non-conjugative R-plasmids, they fail to mobilize them (Brunton et al, 1983; Flett et al, 1981). Transfer of these R plasmids is dependent upon a 21.7 Mdal mobilizing plasmid (Deneer et al, 1984), or a 24.5 Mdal mobilizing plasmid (McNicol et al, 1986a; Guiney and Ho, 1982). These mobilized plasmids include the β-lactamase producers: the 7.0 Mdal, the 5.7 Mdal, the 4.4 Mdal, the 3.2 Mdal, and the 4.9 Mdal sulfonamide-resistant plasmid.

In 1983, McNicol et al described the unique nicking site or origin of transfer (OriT) for each of these β-lactamase plasmids. Conjugal transfer is effected by the enzyme-induced nicking of one strand of the plasmid

DNA. The isolation of relaxable DNA-protein complexes for each of the four plasmids suggests that the plasmids encode the specific nicking protein necessary for their own mobilization. The mobilizing plasmid, pHD147 or pNG029, would contribute all additional requirements to the transfer process. The positive correlation between plasmid structure, homology with the conjugative plasmid and mobilization restricts the promiscuity of the transfer process (McNicol et al, 1983). Since the β-lactamase plasmids described possess homology with a 2.3 Mdal HaeII-XhoII restriction fragment of pHD147 (McNicol et al, 1986b), mobilization is possible. Transfer of plasmids to N.gonorrhoeae have been reported when a Neisseria cinerea intermediate is employed (McNicol et al, 1986a). However, H.parainfluenzae and Haemophilus aegyptius failed as mating intermediates. This suggests that genetic exchange will be more prevalent among ancestrally related segments of DNA such as those found in N.cinerea and N.gonorrhoeae, than among genetic material of more divergent origin as witnessed with Haemophilus and Neisseria DNA. These findings reaffirm the importance of conjugal matings in the dissemination of haemophilus and gonococcal plasmids, while also emphasizing the limitations of homologous recombination.

C. ORIGIN AND EVOLUTION OF ANTIBIOTIC RESISTANT PLASMIDS

The demonstration that discrete sequences of DNA on one replicon could be inserted into a separate non-homologous replicon (chromosome or plasmid) and undergo recombination was relevant to the evolution of multiresistant plasmids. This process, referred to as transposition, is responsible for the development of plasmid-mediated resistance to numerous antimicrobials including: ampicillin (Rubens et al, 1976; Datta and Kontomichalov, 1965), tetracycline (Traub and Beck, 1985; Kleckner et al, 1975), kanamycin and streptomycin (Collis and Hall, 1985) and chloramphenicol (Gottesman and

Rosner, 1975). It is therefore obvious that the evolution of multiresistance plasmids among highly divergent replicons and hence, unrelated bacteria, is a serious public health concern. Transposition studies have therefore been conducted to delineate the mode of transfer of promiscuous resistance genes.

Transposable genetic elements are discrete, mobile, segments of DNA that are common constituents of viral genomes, plasmids, and bacterial chromosomal DNA (Grindley and Reed, 1985). Within this class of genetic elements are the composite transposons (Tn) and the insertion sequence (IS) elements, both of which are defined at their termini by repeated DNA sequences (Kopecko, 1980). IS elements are generally small (800-1400 base pairs) and perform functions such as the inactivation of genes in a replicon through insertion, formation of the boundaries between two independent replicons, or the creation of deletions in a replicon through excision of the IS element. Each of these functions occurs independent of the host's recombination (rec) system (Rose and Barren, 1983). Transposons, Tn, are more complex sequences with IS elements flanking a segment of DNA and almost always being bound by inverted repeat, IR, sequences. They can carry genes responsible for any one of a number of traits such as antibiotic resistance or resistance to heavy metal ions (Kopecko, 1980). In addition, they possess sequences responsible for gene regulation and for transposition, such as the repressor and the transposase (Grindley and Reed, 1985).

The translocation of IS elements and transposons involves the selective duplication of these elements followed by transposase-induced resolution of the duplicated DNA sequence at unique IR sequences (Grindley and Reed, 1985). Through this process of replication, excision and insertion,

a mosaic of genetic combinations have become evident resulting in the development and spread of numerous resistance genes.

This phenomenon is particularly obvious in the plasmids of Haemophilus. For example, the TEM-type β-lactamase specified by the plasmids of Haemophilus is encoded on transposon A, TnA. This enzyme causes ampicillin resistance worldwide in a broad group of organisms, including Salmonella sp., Proteus sp., and E.coli (Brunton et al, 1986a). Laufs et al(1981) demonstrated that in vitro-generated H.influenzae R-plasmids and with two or three resistance determinants, expressing this TEM β-lactamase were very similar to the natural isolates. This supports the hypothesis that plasmids of H.influenzae, and presumably all plasmids could have arisen as a result of independent transposition events onto similar indigenous plasmids in different parts of the world. Heffron et al (1976) concur with these results. They observed that several of laboratory recombinant plasmids were identical to naturally occurring sulfonamide-streptomycin-ampicillin multiresistant plasmids isolated from a range of organisms. The tetracycline resistant transposons found in H.influenzae are similar to Tn10 of E.coli. The chloramphenicol acetyl transferase encoding transposon of H.influenzae is strikingly similar to the type 11 enzyme of enteric bacteria (Brunton et al, 1986a). This transposon is often found inserted in one of the inverted repeat sequences that flank the tetracycline-resistance transposon. The entire unit is transposable, which may explain why the two resistance genes are often found associated in transposon Tn1894 in haemophilus plasmids (Laufs et al, 1981). These observations indicate that transposons have been important participants in the emergence and continual evolution of antibiotic resistance in Haemophilus.

D. β-LACTAMASES

1. Introduction

Plasmid mediated resistance to penicillins and cephalosporins is conferred by the presence of β-lactamases. Which hydrolyse the amide bond in the β-lactam ring of these antibiotics, producing inactive acidic derivatives (Medeiros, 1984). Although both Gram-negative and Gram-positive organisms produce β-lactamase, the enzyme is most prevalent among the Gram-negatives. Genetic diversification has resulted in a variety of β-lactamase types in these organisms, and five classes have therefore been proposed (Medeiros, 1984): Class I consists of β-lactamases with a high rate of cephalosporin hydrolysis (cephalosporinases), Class II and IV are most active against penicillins in Proteus and Klebsiella respectively, the TEM-type β-lactamases making up Class III and a heterogeneous group of oxacillin and carbenicillin-hydrolyzing β-lactamases constitute Class V. Of the five, the latter two, the TEM and O-type enzymes, form the major classes with the TEM enzymes with TEM-1 being the most common by far (Heffron et al, 1976).

The rapid emergence of dozens of β-lactamase types in recent years (Medeiros et al, 1985; Hedges et al, 1985; Jaurin and Grundstrom, 1981; Labia et al, 1981; Matthew, 1979) has justifiably led to the speculation that transposons are responsible for this rapid genetic recombination. Medeiros (1984) suggested that an increasing number of β-lactamases are known to be encoded on transposons. Three of the TEM-type enzymes are included in this burgeoning list: TEM-1 is encoded by Tn2 and Tn3, TEM-2 by Tn1 and SHV-1 is determined by a large transposon unrelated to Tn1. To distinguish these enzymes from one another, a number of assays based on biophysical properties have been developed.

2. β-Lactamase Assays

A large number of assay techniques have been developed for the detection and characterization of β-lactamases. Below is a list of a few of the most common. Each technique has advantages and disadvantages (Sykes and Matthew, 1976), and for this reason, a combination is routinely used for accurate classification.

a) Substrate Profiles. Perhaps the most common method for the characterization of β-lactamases is to examine the substrate profile of the enzyme. This refers to the hydrolytic activity of an enzyme preparation against a number of β-lactam substrates. Although not a good parameter for identification purposes, due to the similarities of substrate profiles for different enzymes, this method does provide comparisons.

b) Analytical Isoelectric Focussing. Since the β-lactamases can be separated by isoelectric focussing (IEF), it can be deduced that they differ biophysically (Matthew, 1979). This biophysical uniqueness detected by IEF can therefore be used to classify the enzymes. With this procedure, the proteins are separated in a pH gradient produced electrophoretically in thin layers of polyacrylamide gel (Matthew et al, 1975). The proteins align themselves as thin bands at their isoelectric points (pI). It is therefore possible to differentiate enzymes which are structurally distinct but whose DNA sequence is highly homologous. Isoelectric focussing alone is not sufficient for characterization, however, since similar pI are shared among some β-lactamases.

c) DNA-DNA Hybridization. Studies have been performed using gene probes for the detection of β-lactamases (Jouvenot et al, 1987; Ouellette et al, 1986; Cooksey et al, 1985). As Pechere and Levesque (1983) predicted, DNA probes have proven useful in evaluating the distribution of different β-lactamase genes, and may eventually provide a rapid test for

hospitals and clinicians. Jouvenot et al (1987) compared the efficacy of isoelectric focussing with that of molecular hybridization and concluded that concordance was very high (93%). This technology detects the gene itself and could prove to be a rapid screening method; however, the evolution of so many new β-lactamase types necessitates an ever-increasing number of DNA probes. For this reason, the future of this technology in clinical screenings remains uncertain.

3. Gram-Negative Organisms

β-lactam antibiotic resistance in Gram-negative bacteria can result from either β-lactamase synthesis or reduced permeability of the cell envelope (Sykes and Matthew, 1976). The enzyme is specified by a chromosomal gene in nearly all Gram-negatives investigated; however, plasmid-specified activity is also reported (Matthew and Harris, 1976).

Plasmid-mediated TEM-1 β-lactamases and the genetically similar TEM-2 enzymes account for as much as 70% of resistance (Matthew, 1979). These enzymes can be differentiated by their pI; 5.4 for TEM-1 and 5.6 for TEM-2 (Matthew, 1979). TEM-1 and TEM-2 are known to be carried by transposons Tn2 and Tn1 respectively. This presumably accounts for their predominance throughout the Gram-negative organisms and even within some Gram-positives.

The first documented account of TEM-1 in strains of H.influenzae occurred in 1972. Since this time, the enzyme has risen in prevalence and host range appearing in N.gonorrhoeae isolates in 1976 and, more recently, in Neisseria meningitidis (Medeiros, 1984). In N.gonorrhoeae and H.ducreyi isolates studied to date, all of the small, mobilizable, ampicillin-resistance plasmids specify the production of a TEM-1 type enzyme.

E. PLASMID EPIDEMIOLOGY IN HAEMOPHILUS DUCREYI

1. Origin and Evolution

Since the description by Brunton et al (1979) of a 5.7 Mdal plasmid specifying ampicillin resistance in Haemophilus ducreyi, 10 additional plasmids have been identified. These plasmids range in size from 34 Mdal to less than 2 Mdal and confer resistance to ampicillin, sulfonamides, aminoglycosides, tetracycline and chloramphenicol. Three plasmids are phenotypically cryptic, the largest of which (21.7 Mdal) has been described as a mobilizing plasmid (Deneer et al, 1982).

Three ampicillin-resistant plasmids have been identified. Each encodes a TEM-1 type β-lactamase as encoded on the transposon Tn2 or TnA. The 5.7 Mdal β-lactamase plasmid was initially observed in three isolates from a chancroid epidemic in Winnipeg, Manitoba, Canada in 1979. Brunton et al (1979) compared the guanine plus cytosine (G + C) content of the plasmid with that of H.influenzae and H.ducreyi chromosomal DNA. This ratio serves as a measure of evolutionary relatedness between the plasmid and chromosomal DNA. The 41 mol% G + C content observed concurred with that for H.ducreyi and H.influenzae chromosomal DNA indicating that the plasmid may have been formed through the transposition of 100% of TnA on a rare indigenous cryptic plasmid (Brunton et al, 1979). Alternatively, the plasmid may represent an extension of the enteric plasmid pool. Restriction endonuclease analysis and molecular hybridization of this plasmid in conjunction with the 7.0 Mdal ampicillin resistant plasmid (Handsfield et al, 1981) and the 3.2 Mdal ampicillin resistant plasmid (Anderson et al, 1984) indicated that the three share common fragments and are homologous (Brunton et al, 1981; Brunton et al, 1982). Included in these studies were comparisons of the two β-lactamase plasmids in N.gonorrhoeae and the 5.4 Mdal β-lactamase plasmid of H.influenzae (Brunton et al, 1986b). These

data strongly endorse the hypothesis that the small β-lactamase plasmids of Haemophilus species and N.gonorrhoeae have arisen through the insertion of the transposable element, TnA, onto small cryptic plasmids indigenous to H.parainfluenzae. Subsequent work demonstrated that the 7.0 Mdal plasmid was identical to the larger β-lactamase plasmid in N.gonorrhoeae (4.4 Mdal) with the exception that the latter carried only 40% of the TnA transposon while the 7.0 Mdal plasmid possessed the entire TnA sequence. Likewise, with the 3.2 Mdal plasmid and the 5.7 Mdal plasmid, the 3.2 Mdal plasmid carried only 40% of the transposon while the 5.7 Mdal plasmid carried the entire TnA transposon. A 1.3 Mdal insertion element in the 7.0 Mdal plasmid was the only differentiating factor between it and the 5.7 Mdal plasmid. The 3.2 Mdal plasmid in H.ducreyi, the 3.2 Mdal plasmid in N.gonorrhoeae, and the recently described 3.2 Mdal plasmid in H.influenzae (Slaney et al, 1986) were identical. These data suggest that the ampicillin resistant plasmids originated through transposition of TnA onto a small cryptic plasmid indigenous to Haemophilus, followed by deletions of non-essential sequences. Recent data combining detailed hybridization studies and electron microscope heteroduplex analysis suggest that groups of small phenotypically cryptic replicons found in H.parainfluenzae are completely homologous to the non-TnA sequences of the 5.7 Mdal plasmid, the 3.2 Mdal plasmid and highly homologous to the 5.4 Mdal β-lactamase plasmid of H.influenzae (Brunton et al, 1986b). The complete complement of β-lactamase plasmids in H.ducreyi presumably developed through subsequent deletions or insertions, and bacterial matings.

A 4.9 Mdal sulfonamide-resistance plasmid has also been described in Haemophilus ducreyi (Albritton et al, 1982). The G + C content of this plasmid was found to be 57%, which compares well with that reported for the E.coli plasmid RSF1010 (55%). Hybridization and restriction endonuclease

analysis also demonstrated homology with RSF1010. A second 3.0 Mdal sulfonamide-resistance plasmid has recently been characterized (Slaney et al, 1987) and again demonstrates homology with RSF1010. These findings indicate that these sulfonamide-resistance plasmids originated from an enteric plasmid pool.

In 1985, Sanson-LePors presented data on the characterization of a 2.9 Mdal aminoglycoside-resistance plasmid which encodes an aminoglycoside phosphotransferase (APH). This plasmid confers resistance to streptomycin and kanamycin as does the sulfonamide-resistant plasmid of similar molecular weight (2.8 Mdal). Although these enzymes are encoded on transposons and their substrate profiles and immunological cross-reactivity suggested Tn903 to be the transposon responsible, restriction endonuclease analysis suggested that Tn903 is not implicated. Further studies need to be performed to elucidate the origin of these plasmids.

The large (30 Mdal) tetracycline-resistance plasmid in H.ducreyi, described by Albritton et al (1984), represents yet another plasmid which appears to have originated in another Haemophilus species and been transferred, presumably through one of the mechanisms previously described. Studies demonstrated common restriction endonuclease digestion patterns between this and tetracycline-resistance plasmids of H.influenzae. The extent of similarities extends beyond the boundaries of the tetracycline transposon, Tn10, suggesting relatedness between the plasmid core regions. Similarly, the large conjugative tetracycline-chloramphenicol-resistance (Tc^rCm^r) plasmid shares 70-80% of its DNA sequence with the H.influenzae Cm^r plasmid, pRI234 (Roberts et al, 1985). Restriction endonuclease analysis and molecular hybridization confirm this. The chloramphenicol resistance determinant for H.ducreyi and H.parainfluenzae was compared with the plasmid from H.influenzae. Each was found to encode a chloramphenicol

acetyltransferase (CAT) similar to the enteric type II class. Speculation as to the origin of both Tc^r and Cm^r on the same transposon includes the possibility of a single genetic event introducing this combination, or that a series of genetic rearrangements with a similar gene combination is responsible.

2. Geographic Distribution

Prior to 1978, data on the antimicrobial susceptibility of H.ducreyi was largely unavailable due to the lack of an identified technique for specimen collection and a medium for isolation. With the elucidation of these procedures (Hammond et al, 1978a; Hammond et al, 1978b) and the subsequent refinements of the selective media (Nsanze et al, 1984), numerous susceptibility studies have been performed. Hammond et al (1978c) observed that ampicillin-resistance was due to β-lactamase production in three isolates from a chancroid epidemic in Winnipeg, Manitoba, Canada. Further analysis revealed the presence of a 5.7 Mdal plasmid to be responsible for this resistance. The 5.7 Mdal plasmid has since been observed in isolates from around the world including the Netherlands, the Gambia, the United States, Mexico, Thailand and Kenya.

Following the description of the 7.0 Mdal β-lactamase plasmid, presumably originating from the Phillipines (Brunton, 1979), this plasmid was observed in Thailand, The Netherlands, and again in about 50% of isolates, from Kenya. Surprisingly, this plasmid has not been observed in North America.

The smallest of the three described β-lactamase plasmids, the 3.2 Mdal plasmid, was isolated from a strain of H.ducreyi originating from Brazil. This plasmid has since emerged in Amsterdam isolates in 1982, and was observed in 88% of isolates from an outbreak in California in the same year

(Anderson et al, 1984). The 3.2 Mdal β-lactamase plasmid has failed to emerge in Kenyan isolates.

Associated with the recent resistance of H.ducreyi to the sulfonamides was the emergence of the 4.9 Mdal plasmid (Nsanze, 1981). Observations of sulfonamide treatment failures in Kenya (Plummer et al, 1983a), coupled with data from the Netherlands (Sturm and Zanen, 1983) where this plasmid has also been observed, suggests that the sulfonamides no longer can maintain their status as an effective regimen. The plasmid has also been detected in a few isolates from the United States. In Thailand, where sulfonamide-resistance was again evident, the presence of a small 3.0 Mdal multiresistance plasmid was implicated (Slaney et al, 1987). To date, this latter plasmid has not been observed elsewhere.

Additional susceptibility studies testing the efficacy of tetracycline (Tc) for the treatment of chancroid have reported resistance in more than 90% of isolates in Kenya (Plummer et al, 1983b) and in more than 50% of isolates from the Phillipines, Singapore, and Johannesburg (Bilgeri et al, 1982; Sng et al, 1982; Fast et al, 1983). In Amsterdam, 13 of 19 strains studied were resistant to tetracycline (Sturm and Zanen, 1983). Despite such high levels of resistance, neither the 30.0 Mdal Tc^r plasmid nor the 34.0 Mdal Tc^r Cm^r plasmid are prevalent. To date, these plasmids have been observed only in a decreasing proportion of Kenyan isolates. It would appear the majority of this resistance is being mediated by a chromosomally-situated determinant as suggested by McNicol and Ronald (1984).

Responsible for the mobilization of non-conjugative plasmids during conjugal matings, the 21.7 Mdal plasmid described by Deneer et al (1982) extends its function to each of the 3.2, 5.7, and 7.0 Mdal β-lactamase plasmids and the 4.9 Mdal sulfonamide-resistant plasmid. Although the incidence of this plasmid remains low (about 3%) and it remains indigenous

to Kenyan isolates, it is responsible for the interspecific and intergeneric dissemination of haemophilus plasmids. For this reason, the mobilizing plasmid remains epidemiologically important.

MATERIALS AND METHODS

A. BACTERIAL STRAINS

Haemophilus spp. is a fastidious, facultative anaerobe requiring hemin (factor X) and/or nicotinamide adenine dinucleotide, NAD, (factor V) for growth. The bacterium is a Gram-negative coccobacilli and is oxidase positive.

Haemophilus ducreyi is hemin dependent and oxidase positive, but otherwise, is a biochemically inert bacterium, failing to ferment lactose, sucrose or glucose. The organism grows optimally under humid conditions at 34-35°C, with an environment of 5% CO_2. The yellowish-gray colonies can be pushed intact across the agar surface. Upon Gram staining, the Gram-negative coccobacilli form long, parallel chains which assume a characteristic "swirling" or "fingerprint" pattern.

Haemophilus influenzae requires both hemin and NAD supplements for growth. The organism can be serotyped on the basis of distinct capsular polysaccharides, of which there are six, a-f. Biotyping based on urease, ornithine decarboxylase, and indole production further differentiates the organism into categories I-VIII.

Escherichia coli is a Gram-negative bacillus. This facultative anaerobe can be differentiated from other *Enterobacteriaceae* on the basis of distinct biochemical reactions including the lack of oxidase production, a positive indole reaction, and the inability to metabolize citrate.

B. BACTERIAL TAXONOMY

Prior to storage or experimentation with the bacterial strains mentioned, each was taxonomically identified.

H.ducreyi was defined by Gram stain characteristics, colonial morphology, and oxidase reaction. The presence of *H.influenzae* was confirmed by

Gram reaction and colony morphology. All H.influenzae isolates had previously been serotyped, biotyped, and their X and V factor requirements had been determined. E.coli was identified by the biochemical profile obtained on API-20E analytical profile strips (Analytab Products, New York, USA).

C. CULTURE MEDIA AND GROWTH CONDITIONS

1. Solid Media

Haemophilus ducreyi and Haemophilus influenzae were grown on chocolate agar medium which consisted of GC agar base (Gibco) supplemented with 1% bovine hemoglobin (Gibco) and 1% CVA enrichments (Appendix I). Five percent fetal calf serum was also added on occasion for particularly fastidious organisms. Antibiotics were added to media at the following concentrations: ampicillin 20 to 50 ug/ml, novobiocin 5 ug/ml, streptomycin 250 to 1000 ug/ml and vancomycin 3 ug/ml.

Mueller-Hinton agar base (Gibco) was selected as the medium for Escherichia coli culture (Appendix I).

Blood agar (BA) and MacConkey's agar were also used for maintaining E.coli (Appendix I).

2. Liquid Media

Haemophilus ducreyi and H.influenzae cultures were suspended and/or grown in brain-heart infusion (BHI) broth (Gibco) supplemented with 1% nicotinamide adenine dinucleotide (NAD) (Sigma) and 1% hemin (Sigma) (Appendix II).

With E.coli, both BHI broth and Luria broth (LB) (Appendix II) were used.

3. Growth Conditions

All Haemophilus isolates were incubated at 34-35°C with 5% CO_2 under humid conditions. E.coli cultures were incubated at 37°C.

4. β-Lactamase Production

The production of beta-lactamase was determined by spotting a few drops of chromogenic cephalosporin substrate on a piece of Whatman No. 1 filter paper and manipulating the bacteria with a platinum loop into the spotted area. If the yellow substrate reacted with the bacteria, a red color appeared indicating the presence of β-lactamase.

5. Storage

All strains of H.ducreyi, H.influenzae, and E.coli were suspended in labelled dram vials of 10% skim milk containing 10% glycerol and stored at -70°C.

D. PLASMID PROFILING

1. Plasmid Screening

Although a number of procedures were attempted to isolate plasmids in H.ducreyi, the protocol which reproducibly yielded the best results was a modification of that described by Meyers et al (1976).

Cultures were grown for 18-24 hours. A partial loop (0.01 ml) of solid culture was emulsified in 10 ul of a 1% lysozyme (Sigma) solution and incubated at 37°C for 30 minutes. To this suspension, 190 ul of a 1% TE-SDS detergent solution (Appendix III) was added followed by further manipulation of the bacteria to ensure cell lysis. Tubes were incubated at 37°C for 30minutes; 12 ul of 2M Tris-HCl (pH 7.0) was added, and the contents were gently agitated. Twenty microliters of a 5M NaCl solution was added immediately and the solution was agitated, then incubated at 4°C for 30 minutes. Following this, 200 ul of 3% NaCl saturated phenol was added and tubes were inverted several times. Tubes were centrifuged for eight minutes at 4°C in a Fisher model 235B microcentrifuge. The aqueous layer (200 ul) was removed and 20 ul of 3M sodium acetate was added. Following

agitation of the tube contents, 400 ul of cold (-20°C) ethanol was added to precipitate the plasmid DNA. Tubes were held at -20°C overnight or at -70°C for 20 minutes. The plasmid DNA was pelleted by centrifugation for 10 minutes at 4°C. The supernatant was poured off and the DNA pellet was dried at 37°C. The DNA pellet was resuspended in 25 ul of TE buffer.

E. AGAROSE GEL ELECTROPHORESIS

Electrophoresis was conducted through agarose gels ranging in concentration from 0.5% to 1.2%, depending upon the extent of separation desired with lower gel concentrations providing better band separation, and higher concentrations permitting the resolution of small DNA sequences. Both vertical and horizontal gels were employed for the purposes of plasmid profiling and resolution of plasmid endonuclease digestions, respectively.

1. Vertical Gel Electrophoresis

Electrophoresis of bacterial lysates for the purposes of plasmid profiling was performed on a vertical apparatus due to the superior resolution of plasmids observed in the 34 Megadalton (Mdal) to <2 Mdal range. Lysates were therefore loaded on 0.7% agarose (SeaKem) gels and electrophoresed in a BRL model 1161 electrophoresis apparatus using Tris-borate buffer (Appendix III). This was performed for approximately three hours at 40 milliamperes (mAmp) and 100 volts using a BioRad model 500/200 power supply. The gels were then stained in a 0.1% ethidium bromide (EtBr) (Sigma) solution for 10 minutes, then destained in demineralized water overnight. The gels were photographed over an ultraviolet transilluminator (UVP) using a Polaroid MP4 land camera and Polaroid high speed type 57 film.

2. Horizontal Gel Electrophoresis

Horizontal agarose gel electrophoresis was used to separate endonuclease digested DNA fragments and to make electroelution of DNA possible. Gels ranged in concentration from 0.5% to 1.2% in Tris-borate buffer. A current of approximately 40 mAmp and 100 volts was applied for about five hours using the Aquebogue gel electrophoresis apparatus (Aquebogue Machine and Repair Shop, Aquebogue, N.Y.) and the BioRad model 500/200 power supply. When gels were electrophoresed overnight, a current of about 16 mAmp and 20 volts was used. As with vertical gels, staining in a 0.1% EtBr solution for 10 minutes followed by destaining of the gel overnight in demineralized water followed electrophoresis. Gels were photographed as previously described.

F. MOLECULAR WEIGHT DETERMINATION

1. Plasmids

Two methods were used to ascertain the molecular weights of electrophoresed plasmid DNA. The most common approach involved the co-electrophoresis of lysates from strains with plasmids of known molecular weight. Typically, two strains containing a total of five plasmids ranging from 2.6 to 21.7 Mdal in size were used. To avoid ambiguity of distinguishing linear or open circle plasmid conformations in a multiplasmid molecular weight marker, dye-buoyant-density gradient purified plasmids were also used as markers. By measuring the distance of marker plasmid migration, a standard curve could be constructed relating migration distance to molecular weights. Subsequent measurements of the migration distances of the unknown plasmids could be plotted on the standard curve to determine their molecular weights.

2. Restriction Endonuclease Fragments

When plasmid DNA is digested with restriction endonucleases, it assumes a linear form. Since linear DNA migrates at a different rate than covalently closed circular (c.c.c.) DNA of identical molecular weight, it is necessary to use linear DNA fragments for molecular weight markers. Therefore, all digest reactions were co-electrophoresed with either HindIII digested lambda phage DNA (λ) or HindIII and EcoRI digested λ DNA. Since the molecular weights of the fragments resulting from these digestions have been well documented, it is again possible to construct a standard curve by measuring migration distances. Plasmid fragments could then be sized by plotting their relative migration distances on the curve (Figure 1).

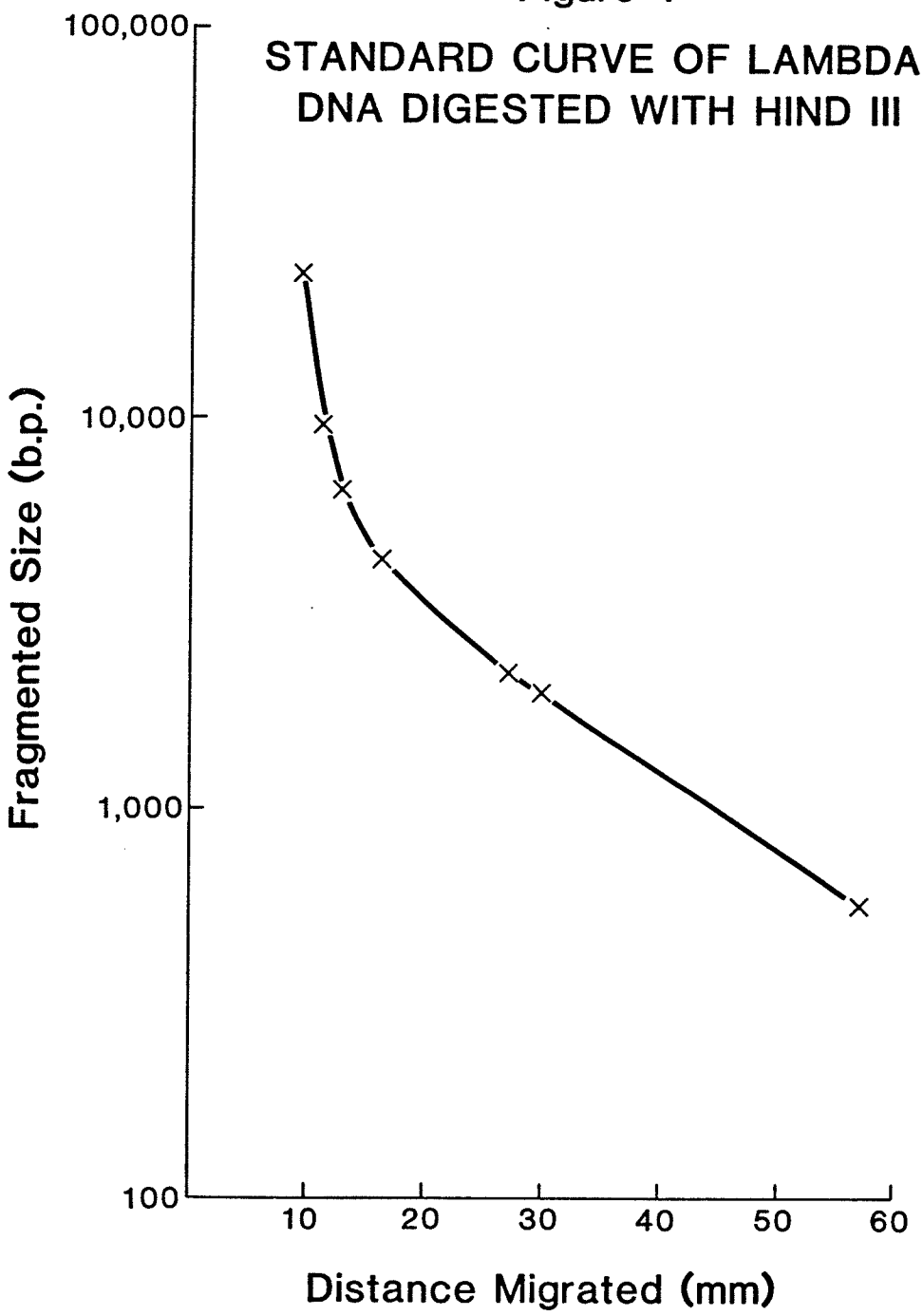
G. ISOLATION OF DNA FROM AGAROSE GELS

At times, a particular fragment of a plasmid, or a single plasmid in a multi-plasmid strain was required for more detailed study. Electroelution of this DNA was therefore performed, and this DNA was subsequently purified. This provided a means for obtaining pure DNA, otherwise unattainable, to be used for the development of probes or for plasmid mapping.

1. Electroelution

Endonuclease digested plasmid DNA or plasmid lysates were electrophoresed as described in 0.7% agarose gels in tris-borate buffer. The gel was stained in 0.1% EtBr for 10 minutes and the DNA bands were visualized over a UV transilluminator. A scalpel was used to cut a trough measuring approximately 4 mm in width at the base and sides of the required DNA band. The trough was lined on the two sides and base with nuclease free dialysis tubing and filled with Tris-EDTA buffer (Appendix III). A current of 40 mAmp was applied for five minute intervals while the progress of the DNA migration into the trough was monitored with a model UVL-28 handheld UV

Figure 1


STANDARD CURVE OF LAMBDA
DNA DIGESTED WITH HIND III
100,000
10,000
1,000
100
Fragmented Size (b.p.)
10
20
30
40
50
60
Distance Migrated (mm)

light (Ultraviolet Products Inc., California, USA). Since the DNA could not penetrate the dialysis tubing, it became concentrated in the trough. Following each five minute period, the DNA was eluted from the trough, and fresh TE buffer was added. This procedure was repeated until all of the DNA had been eluted.

2. Purification of Eluted DNA

The eluted DNA was subjected to equal volumes of tris saturated phenol in two extractions. Residual phenol, which can inhibit endonuclease activity, was then extracted with chloroform:isoamyl alcohol at a ratio of 24:1. The latter constituent acted as an anti-foaming agent. After adding a 1:10 ratio of 3M sodium acetate, an equal volume of cold isopropanol was added to precipitate the DNA and to remove the intercalated EtBr from the DNA. After holding at -20°C for 20 minutes, the reaction was centrifuged at 4°C for 10 minutes in a microcentrifuge. The DNA pellet was dried and resuspended in an appropriate volume of sterile 10 mM tris.

H. DYE-BUOYANT-DENSITY GRADIENT CENTRIFUGATION

Two similar, but nevertheless distinct, protocols were employed for the isolation of pure plasmid DNA, depending upon whether the host strain was E.coli or H.influenzae.

1. Protocol for E.coli

The organism was grown at 37°C overnight in 400 ml of antibiotic supplemented BHI broth with continuous shaking. A cell pellet was obtained by centrifuging the culture at 7000 rpm for 10 minutes at 4°C in an IEC type B20-A centrifuge (International Equipment Company, Massachusetts, USA) and type 870 rotor. The pellet was washed once in 20 ml of TES solution (Appendix III), the suspension was divided in half, centrifuged again using the same parameters, and the pellet was stored at -20°C overnight. The

pellet was thawed and suspended in 2 ml of Tris-sucrose (Appendix III), and 0.4 ml of a 5 mg/ml lysozyme solution in 0.25 M Tris (pH 8.0) was added. After one incubation period for 3-5 minutes at room temperature, 0.8 ml of a 0.5 M EDTA solution was added and the tubes were incubated at room temperature for 20-30 minutes. Then, 4.4 ml of Triton-lytic mix (Appendix III) was added, followed by a second 20-30 minute incubation at room temperature. The suspension was then centrifuged in the IEC centrifuge at 7000 rpm and 4°C for 30 minutes. To each milliliter of supernatant, 0.9 g of CsCl was added, and was allowed to completely dissolve. Two-hundred microliters of a 10 mg/ml EtBr solution in TES was then added and aliquots of the suspension were pipetted into Beckman polyallomer centrifuge tubes. The tubes were capped, topped with mineral oil, and balanced. Centrifugation occurred in a Beckman type L-8 ultracentrifuge using the Ti50 rotor for 40 hours at 40,000 rpm and at 18°C. The resulting gradient was dripped at the plasmid band using a Beckman fraction collector. The EtBr was removed by several extractions with TES/CsCl-saturated isopropanol. DNA was dialyzed in nuclease free dialysis tubing against 10 mM Tris (pH 8.0) solution overnight to remove the CsCl, yielding pure plasmid DNA.

2. Protocol for H.influenzae

The organism was grown with continuous shaking overnight in 400 ml of BHI broth supplemented with 1% hemin and 1% NAD and antibiotics. The cells were pelleted, washed once in TES, and the resulting pellet was frozen at -20°C overnight. The thawed pellet was suspended in 4 ml tris-sucrose and 0.5 ml of a 10 mg/ml lysozyme solution in 0.25 M Tris (pH 8.0) was added. After an incubation of 10 minutes at 37°C and a five minute incubation at room temperature, 0.8 ml of 0.5 M EDTA was added. The addition of 1.5 ml of Triton-lytic mix followed a 10 minute incubation at room temperature. Another 15-30 minute incubation at room temperature followed. The suspen-

sion was centrifuged for 30 minutes at 17,000 rpm and 4°C in an IEC centrifuge. Cesium chloride at a concentration of 0.9 g/ml was completely dissolved in the supernatant. To this, 200 ul of a 10 mg/ml EtBr solution in TE was added. Aliquots were prepared in Beckman polyallomer centrifuge tubes which were then capped, topped with mineral oil, and balanced. The parameters for centrifugation were as for E.coli. The plasmid band was collected, the EtBr extracted, and the DNA was dialyzed as previously described.

I. DETERMINATION OF DNA CONCENTRATION AND PURITY

Once DNA had been collected and purified either by means of electroelution or through CsCl gradient ultracentrifugation, the purity and concentration of the plasmid(s) was determined. This was done by spectrophotometric analysis of a sample of the DNA at wavelengths of 260 nm and 280 nm. The reading at 260 nm enables one to calculate the concentration of DNA in the sample since an optical density reading of one at 260 nm corresponds to approximately 50 ug DNA per milliliter of sample. Purity of the sample can be estimated by calculating the ratio between the 260 nm and 280 nm readings. A value of 1.8 or greater signifies a pure preparation. Contamination with RNA gives ratios greater than 1.9 whereas contamination by protein or phenol lowers the ratio significantly.

J. CONJUGATION

Bi- and triparental matings were performed to test plasmids for conjugal and mobilizing ability. Twenty-two donor strains of Thailand origin were used. Positive control strains originating from Kenya or Thailand and possessing one of the mobilizable β-lactamase plasmids were used concurrently. The intermediate strain was H.influenzae possessing the 21.7 Mdal

H.ducreyi mobilizing plasmid. A number of recipient organisms were employed including H.influenzae, H.ducreyi, and E.coli (Table 1).

The basic protocol was similar for all matings. Strains were suspended in BHI broth to final concentrations ranging from 10^7 to 10^8 organisms per milliliter (ml) as determined by MacFarlane standards. Both a 10:1 donor-recipient ratio and a 1:100 donor-recipient ratio were used. The intermediate strain was used consistently at a concentration of 10^8 organisms per milliliter. Triparental matings were performed using the following ratios: 1.5 ml donor (10^7 or 10^8 organisms/ml), 1.5 ml intermediate (10^8 organisms/ml), and 1.5 ml of recipient (10^8 or 10^7 organisms/ml). The appropriate volume of BHI broth was substituted for the intermediate organism in biparental matings. The mixtures were vortexed and 3 ml was aseptically pipetted into a syringe fitted with a Millipore Swinnex-25 filter unit (Millipore Corportion, Bedford, Mass.) containing a Nucleopore 4 um polycarbonate filter (Nucleopore Corporation, Pleasanton, CA). The cells were collected on the filter, which was then placed filtrate side up on a chocolate agar plate. The plates were incubated overnight under 5% Co_2 under humid conditions at about 35°C. The resulting bacterial growth on the membrane was resuspended in 1 ml of BHI broth. Ten-fold dilutions of this suspension were made and 100 ul of each was plated on selective media. Controls included placing a drop of the donor on the surface of selective media, inoculating the selective media with a drop of the intermediate suspension, and placing a drop of the recipient suspension on the selective media, as well as plating the BHI broth used. Plates were incubated for 24-48 hours under the appropriate growth conditions. Colonies which grew on the selective media were resistant to both ampicillin and the antibiotic used to select for the recipients. Any colonies which grew when

TABLE 1. Bacterial strains used in biparental and triparental conjugal matings.

	Bacterium	Strain Designation	Phenotype	Plasmid Profile
Recipients	H.influenzae	Rd^{nov}	Nov^r	-
	H.influenzae	$1\text{-}008^{rif}$	Rif^r	-
	H.ducreyi	35000^{str}	Sm^r	-
Intermediate	H.influenzae	T1002	mob	21.7
Donors	H.ducreyi	CH17	Ap^r,Km^r,Sm^r,Su^r	1.8,2.6,2.8,3.2
	H.ducreyi	CH28	Ap^r,Km^r,Sm^r,Su^r	1.8,2.6,2.8,3.2
	H.ducreyi	CH37	Ap^r,Km^r,Sm^r,Su^r	1.8,2.6,2.8,3.2
	H.ducreyi	CH39	Ap^r,Km^r,Sm^r,Su^r	1.8,2.6,2.8,3.2
	H.ducreyi	CH40	Ap^r,Km^r,Sm^r,Su^r	1.8,2.6,2.8,3.2
	H.ducreyi	CH50	Ap^r,Km^r,Sm^r,Su^r	1.8,2.6,2.8,3.2
	H.ducreyi	CH85	Ap^r,Km^r,Sm^r,Su^r	1.8,2.6,2.8,3.2
	H.ducreyi	CH90	Ap^r,Km^r,Sm^r,Su^r	1.8,2.6,2.8,3.2
	H.ducreyi	Pu1	Ap^r,Km^r,Sm^r,Su^r	1.8,2.6,2.8,3.2
	H.ducreyi	Pu2	Ap^r,Km^r,Sm^r,Su^r	1.8,2.6,2.8,3.2
	H.ducreyi	Pu6	Ap^r,Km^r,Sm^r,Su^r	1.8,2.6,2.8,3.2
	H.ducreyi	Pu12	Ap^r,Km^r,Sm^r,Su^r	1.8,2.6,2.8,3.2
	H.ducreyi	Pu13	Ap^r,Km^r,Sm^r,Su^r	1.8,2.6,2.8,3.2
	H.ducreyi	Pu16	Ap^r,Km^r,Sm^r,Su^r	1.8,2.6,2.8,3.2
	H.ducreyi	Pu18	Ap^r,Km^r,Sm^r,Su^r	1.8,2.6,2.8,3.2
	H.ducreyi	Pu20	Ap^r,Km^r,Sm^r,Su^r	1.8,2.6,2.8,3.2
	H.ducreyi	Pu23	Ap^r,Km^r,Sm^r,Su^r	1.8,2.6,2.8,3.2
	H.ducreyi	Pu29	Ap^r,Km^r,Sm^r,Su^r	1.8,2.6,2.8,3.2
	H.ducreyi	Pu33	Ap^r,Km^r,Sm^r,Su^r	1.8,2.6,2.8,3.2
	H.ducreyi	Pu35	Ap^r,Km^r,Sm^r,Su^r	1.8,2.6,2.8,3.2
	H.ducreyi	Pu37	Ap^r,Km^r,Sm^r,Su^r	1.8,2.6,2.8,3.2
	H.ducreyi	Pu45	Ap^r,Km^r,Sm^r,Su^r	1.8,2.6,2.8,3.2
	H.ducreyi	896	Ap^r	7.0
	H.ducreyi	CH48	Ap^r,Km^r,Sm^r,Su^r	2.8, 3.2, 5.7
	H.ducreyi	CH25	Ap^r,Km^r,Sm^r,Su^r	2.8, 3.2, 7.0
	H.ducreyi	038	Ap^r	5.7

streaked on fresh selective media were then tested for plasmid content. If the donor plasmid was present, then the colony was termed transconjugant.

A variation on this method involved plating a drop of the mating mixture on a CA plate, then incubating the plate overnight. A replicator was then used to transfer discrete colonies of this growth to selective media. These plates were incubated for 24-48 hours at 35°C with 5% CO_2 under humid conditions. Approximatly 10 colonies were picked and streaked again on selective media as was a loopful of colonies. Any subsequent growth was analyzed for plasmid content.

A third procedure involved plating 100 ul of the membrane growth suspension on CA selective media and inoculating 225 ml of BHI broth supplemented with hemin, NAD, and ampicillin (20 ug/ml) with the remaining 900 ul of membrane growth suspension. Broth culture was grown at 35°C with shaking for approximately seven hours. Twenty-five milliliters of broth was then centrifuged and the pellet was tested for β-lactamase production. If a positive reaction was observed, then the remaining broth was centrifuged, and the pellets wer plated on the appropriate selective media.

K. TRANSFORMATION OF BACTERIAL CELLS

Transformation using both recombination proficient (rec^+) and recombination deficient (rec^-) cells was performed (Table 2). Forty milliliters of L-broth was inoculated with 2 ml of an overnight broth culture then grown with shaking for 90 minutes or until a density of 50 Klett units was attained. The flask was then put on ice for 15 minutes and the cells were pelleted by centrifugation. Cells were washed in 30 ml of sterile 10 mM NaCl, the pellet was resuspended in 10 ml of sterile 30 mM $CaCl_2$ and then incubated on ice for 20 minutes. Cells were again pelleted, resuspended in 1 ml of 100 mM CaCl2 and dispensed into aliquots of 200 ul. From 50 to

1000 ng of exogenous DNA in a 100 ul suspension was added to the cells. Reaction mixtures were incubated on ice for one hour, then heat shocked at 42°C for two minutes. L-broth was then added to bring reaction volumes up to 3 ml. An incubation period of about two hours at 37°C with shaking permitted the transformed cells to express their new phenotypes. Ten-fold dilutions of the test reactions were then plated on the appropriate selective media. Each transformation experiment included a control for cell growth and a control for L-broth contamination.

A modification of the method described by Cohen et al (1972) was also used. One hundred ml of L-broth was inoculated with 3 ml of an overnight broth culture. The culture was grown up to early log phase by incubation at 37°C for about 90 minutes with shaking. After chilling on ice for 15 minutes, cells were pelleted by centrifugation. Resuspension of the cells in 15 ml of ice cold 0.1 M $MgCl_2$ followed. The cells were again pelleted and resuspended in 5 ml of ice cold 0.1 M $CaCl_2$ and held on ice for 20 minutes. After centrifuging the cells, each pellet was resuspended in 0.5 ml of ice cold 0.1 M $CaCl_2$ and the cells were held on ice. A range from 10 to 100 ng of DNA was added to 100 ul of cells, the transformation reactions were held on ice for 30 minutes, then heat shocked at 42°C for two minutes. The reactions were then returned to ice for 10 minutes. L-broth was added to a final volume of 1 ml and incubation for 60 minutes at 37°C allowed for expression of the plasmid-mediated antibiotic resistance. Ten plates of selective medium were each inoculated with 0.1 ml of the reaction mixture. Again, controls were employed as previously mentioned. Plates were then incubated at 37°C overnight. Any subsequent colonies were streaked on fresh selective medium, and the resulting growth was analyzed for plasmid content.

TABLE 2. Strains employed in bacterial transformations.

	Strain			
	Bacterium	Designation	Phenotype	Plasmid Profile
Exogenous DNA	H.ducreyi	CH28	Ap^r, Km^r, Sm^r, Su^r	1.8,2.6,2.8,3.2
Host Cell	E.coli	C600	rec^+	-
	E.coli	HB101	rec^-	-

L. RESTRICTION ENDONUCLEASE DIGESTIONS

For the purposes of plasmid mapping and the development of probes, purified plasmid DNA suspended in sterile 10 mM Tris was used. Varying amounts of DNA were digested, depending upon the specific experiment, with a range from 1-1000 ng being used. Restriction endonucleases were used as per the manufacturer's recommendations in conjunction with low, medium, or high salt buffers (Appendix IV). Both single and double digestions were performed to make plasmid mapping possible. Single digestions were terminated by the addition of stop buffer (Appendix IV) after three hours; double digests were terminated after the enzymes had acted sequentially for three hours each. Enzymes employed included BamHI and HindIII (Pharmacia), and PvuI, PvuII, PstI, AvaI, XhoI, SalI, and HindII (Boerhinger Mannheim).

M. IN SITU HYBRIDIZATION

1. Southern Transfer

Southern blots were prepared by the method of Maniatis et al (1982). After electrophoresis, agarose gels were stained with EtBr and photographed with a ruler on each gel from which relative distances could be measured. Excess agarose was removed and the DNA was denatured by soaking the gel in about 500 ml of 1.5 M NaCl and 0.5 M NaOH for about one hour with constant shaking. The gel was neutralized in about 500 ml of 1 M Tris-Cl (pH 8.0) and 1.5 M NaCl for one hour at room temperature with constant shaking. A wick of 3 MM Whatman paper (Whatman Ltd., Maidstone, England) was wrapped over a stack of glass plates which were placed in the middle of a large baking dish. The dish was filled with 10 x SSC (Appendix III) very near to the top of the glass support, and air bubbles in the saturated paper wick were removed by a glass rod. The gel was inverted on the 3 MM paper and a piece of 0.45 Micron nylon hybridization membrane (MSI, Fisher Scientific,

Ottawa, ON) cut slightly larger than the gel was briefly immersed in 2 x SSC then placed on top of the gel. Two pieces of Whatman 3 MM paper cut to the same dimensions as the gel were soaked in 2 x SSC then placed on top of the nitrocellulose. A stack of paper towels, cut smaller than the 3 MM paper, was then added. A glass plate with a 500 g weight topped the stack to promote the flow of SSC from the reservoir, through the gel to result in the movement of the DNA from the gel to the nylon membrane. Transfer proceeded for approximately 15 hours at which time the apparatus was disassembled and the membrane was soaked in 6 x SSC for five minutes. The membrane was allowed to dry at room temperature before being baked for four hours at 68°C.

2. Nick Translation

Purified DNA was nick-translated with α-^{32}P-dCTP using an Amersham (Illinois, USA) nick-translation kit (Appendix IV). The reaction involved using 10 ng of DNA for each milliliter of hybridization buffer. Usually, 200 ng of DNA was used. To the DNA suspension was added 4 ul of solution one, which consisted of the four deoxynucleotide triphosphates. Upon addition of the α-^{32}P-labelled 2'-deoxycytosine, 5'-triphosphate, dCTP, 2 ul of the DNA polymerase-DNaseI solution was added. The final reaction volume was 20 ul, if 200 ng of DNA was to be nick-translated. The reaction proceeded at 16°C for 90 minutes and was then stopped by the addition of 25 ul of stop buffer (Appendix V).

3. Random Priming of Probe DNA

In the event that DNA could not be obtained in a purified state, a new technique was employed involving the use of random hexanucleotides as primers for the probe DNA. As described by Feinberg and Vogelstein (1983), the probe DNA was initially rendered single stranded through boiling and cooling on ice. A mixture of all possible hexanucleotides was then added

and allowed to anneal to the single strands. The complementary strand emerged from the 3' OH end of the primer through the collective action of the Klenow enzyme, 2'-deoxynucleoside, 5'-triphosphates, and ^{32}P-labelled 2'-deoxycytosine, 5'-triphosphate. The reaction was then terminated after 30 minutes incubation at 37°C by the addition of 20 ul of stop buffer (Appendix V).

Free nucleotides which could compete with the probe in hybridization were then separated from the radioactive probe by Sephadex G-100 (Pharmacia) column chromatography. The Sephadex was heated to 42°C, degassed for 10 minutes, then packed in a 5 ml disposable pipette fitted with a three-way stopcock. The column was layered with TE buffer which also served as the eluent. The progress of the nick-translated probe through the column was monitored by a Ludlum Model 3 geiger counter (Ludlum Measurements, Texas, USA). The probe was collected and the specific activity determined using a Beckman Rackbeta Scintillation counter. The probe was then denatured by boiling for three to five mintues.

4. Hybridization

Southern membranes were prehybridized in 20 ml of an appropriate buffer (Appendix III) for 3-4 hours to prevent non-specific binding of the probe. Hybridization was performed under stringent conditions, defined as the melting temperature of DNA minus 20°C, or at 65°C. Following prehybridization, denatured probe was added to the reaction mixture and hybridization proceeded overnight at 65°C. To remove any non-specifically hybridized probe, membranes were washed twice with 250 ml of a 2 x SSC, 0.1% SDS buffer at 65°C, followed by two washes with 250 ml of a 0.1 x SSC, 0.1% SDS buffer at 65°C. Fifteen minutes was permitted for each wash with gentle agitation maintained constantly. Excess moisture was removed by placing

the membranes on a piece of Whatman 3 MM paper. The membranes were immediately sealed in bags before drying could occur and autoradiographed.

5. Autoradiography

The bagged membranes were taped into position on Kodak type T-Mat G x-ray film and placed in Kodak x-omatic cassettes with Lanex intensifying screens (Eastman Kodak Company, Rochester, N.Y.). The cassettes were held at -70°C for varying lengths of time before being developed by a Kodak X-AR-omat automatic film developer (Eastman Kodak Company, Rochester, N.Y.).

RESULTS

A. EPIDEMIOLOGY OF ORIGIN AND DISTRIBUTION OF H.DUCREYI PLASMIDS

1. Development of a Lysate Protocol for H.ducreyi

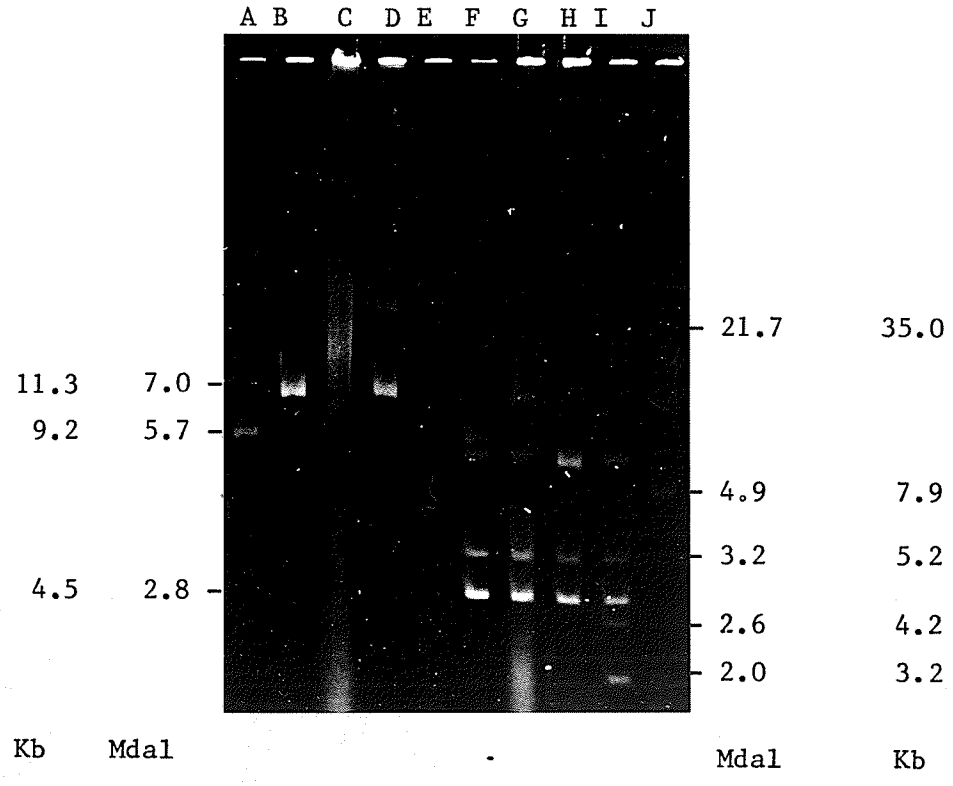
Prior to discovering a satisfactory and reproducible method for preparation of plasmid lysates, a number of modifications of the basic protocol of Meyers et al (1976) were essential. Cell lysis was attempted both with and without lysozyme, the quantity of cells used was varied and incubation parameters were altered. The system found to consistently yield interpretable results involved incubation of an 18-24 hour culture in a 1% lysozyme solution prior to detergent lysis and removal of cell debris. It was observed that removal of cell debris through the addition of a high molar salt could be facilitated by incubating at 4°C rather than at room temperature. Once the DNA had been precipitated with ethanol, samples were dried, resuspended and electrophoresed on agarose gels. Figure 2 illustrates some of the plasmid profiles in H.ducreyi which can be seen following illumination of a stained gel.

As can be seen in this figure, numerous plasmids are indigenous to H.ducreyi and contribute to a mosaic of plasmid profiles. Through analysis of a representative number of H.ducreyi isolates from around the world, it was felt that the epidemiology of plasmid origin and distribution could be understood. Isolates collected from 1976 through 1986 were therefore analyzed from four continents. In Africa, Kenya and the Gambia were represented, while Thailand isolates from Asia, Canadian and American isolates from North America and a European sample of isolates collected from The Netherlands formed the balance.

2. Plasmids Indigenous to H.ducreyi

Over 95% of the 830 isolates studied produced beta-lactamase. It was found that four small plasmids, ranging from 7.0 to 2.6 Mdal molecular

Figure 2. Agarose gel (0.7%) of electrophoresed H.ducreyi lysates illustrating several indigenous plasmids.



H.ducreyi Strain Designation:

A: HD 781
B: HD 782
C: HD 785
D. HD 786
E: HD 787
F: HD 511
G: HD 512
H: HD 516
I: HD 519
J: control strain, HD 047

weight, were responsible for encoding this enzyme. Two plasmids conferring resistance to sulfonamides were observed to be 4.9 and 2.8 Mdal in size, respectively. The latter of the two encoded multiresistance, maintaining kanamycin and streptomycin resistances as well. Also contributing to the diversity of the plasmid pool in H.ducreyi were two large conjugative plasmids responsible for tetracycline (30 Mdal) and tetracycline-chloramphenicol (34 Mdal) resistances. A 21.7 Mdal mobilizing plasmid and two small phenotypically cryptic plasmids of about 1.8 and 3.2 Mdal molecular weight were also oberved. The presence of a number of plasmids within a given isolate, and the subsequent array of profiles from isolates around the world precipitated a chronological comparison of plasmid profiles for the six countries represented.

3. Plamid Profiles of H.ducreyi Collected from 1976 to 1986

The modified lysate protocol described above was applied to 830 H.ducreyi isolates with the results shown in Table 3. Evident from these data is the fact that, among 560 African isolates, the 3.2 Mdal β-lactamase plasmid did not appear. Rather, 40% of these isolates possessed a 5.7 Mdal plasmid while 60% possessed a 7.0 Mdal plasmid. Although the 5.7 Mdal β-lactamase plasmid has emerged in outbreaks in every location represented, the 7.0 Mdal β-lactamase plasmid has yet to be found in any of the North American epidemics. Presently, it appears that the 5.7 and 7.0 Mdal plasmids are approaching equilibrium in Kenyan isolates with neither becoming predominant (Figure 3). Nevertheless, the 5.7 Mdal plasmid is more evident than the 7.0 Mdal plasmid in other areas of the world. Additionally, as evident in Figure 3, the 21.7 Mdal mobilizing plasmid found solely in Kenyan isolates, and the 4.9 Mdal sulfonamide resistance plasmids are observed infrequently and do not appear to be disseminating. The larger conjugative tetracycline and tetracycline-chloramphenicol resistance plas-

TABLE 3a. Plasmid profiles of H.ducreyi isolates collected from 1980-1986 in Africa and Thailand. Number of isolates with a given plasmid profile.

		Year of Isolation/Country of Origin								
		1980	1981	1982	1984		1985			1986
Phenotype	Profile	Kenya	Kenya	Kenya	Kenya	Thailand	Kenya	Thailand	Gambia	Kenya
Ap^r	3.2									
Su^r	4.9	1								1
Ap^r	5.7	19	31	25	44		57		5	30
Ap^r	7.0	36	64	40	56		60		2	26
Su^r, Ap^r	4.9/5.7	6	16	10	7		8			2
Su^r, Ap^r	4.9/7.0	2	1	1	1		1			1
mob, Ap^r	21.7/5.7		1	1			2			
mob, Ap^r	21.7/7.0		3	1						1
Tc^r, Ap^r	30/7.0	2	4							
Ap^r, Km^r, Sm^r, Su^r	7.0/3.2/2.8					29				
Ap^r, Km^r, Sm^r, Su^r	5.7/3.2/2.8					14		2		
mob, Su^r, Ap^r	21.7/4.9/7.0	1		1						1
Tc^r, Ap^r, Su^r	30/5.7/4.9		1							
Km^r, Sm^r, Su^r, Ap^r	3.2/2.8/2.6/1.8					47		20		
	No Plasmids	7		1	1					
	Total	74	121	79	108	90	128	22	7	62
										691

TABLE 3b. Plasmid profiles of H.ducreyi isolates collected from 1976-1985 in North America. Number of isolates with a given plasmid profile.

		Year of Isolation/Origin of Isolate							
		1976	1982		1983	1985			
Phenotype	Profile	Canada	Orange County, California	Atlanta, Georgia	WPB, Florida	Orlando, Florida	Boston, Mass.	Cleveland, Ohio	New York, New York
Ap^r	3.2		32	1					
Su^r	4.9								
Ap^r	5.7	3		2	8	2	4	1	21
Ap^r	7.0								
Su^r, Ap^r	4.9/5.7			5					
Su^r, Ap^r	4.9/7.0								
mob, Ap^r	21.7/5.7								
mob, Ap^r	21.7/7.0								
Tc^r, Ap^r	30/7.0								
Ap^r, Km^r, Sm^r, Su^r	5.7/3.2/2.8								
Ap^r, Km^r, Sm^r, Su^r	7.0/3.2/2.8								
mob, Su^r, Ap^r	21.7/4.9/7.0								
Tc^r, Ap^r, Su^r	30/5.7/4.9								
Km^r, Sm^r, Su^r, Ap^r	3.2/2.8/2.6/1.8								
	No Plasmids	18							2
	Total	21	32	8	8	2	4	1	23

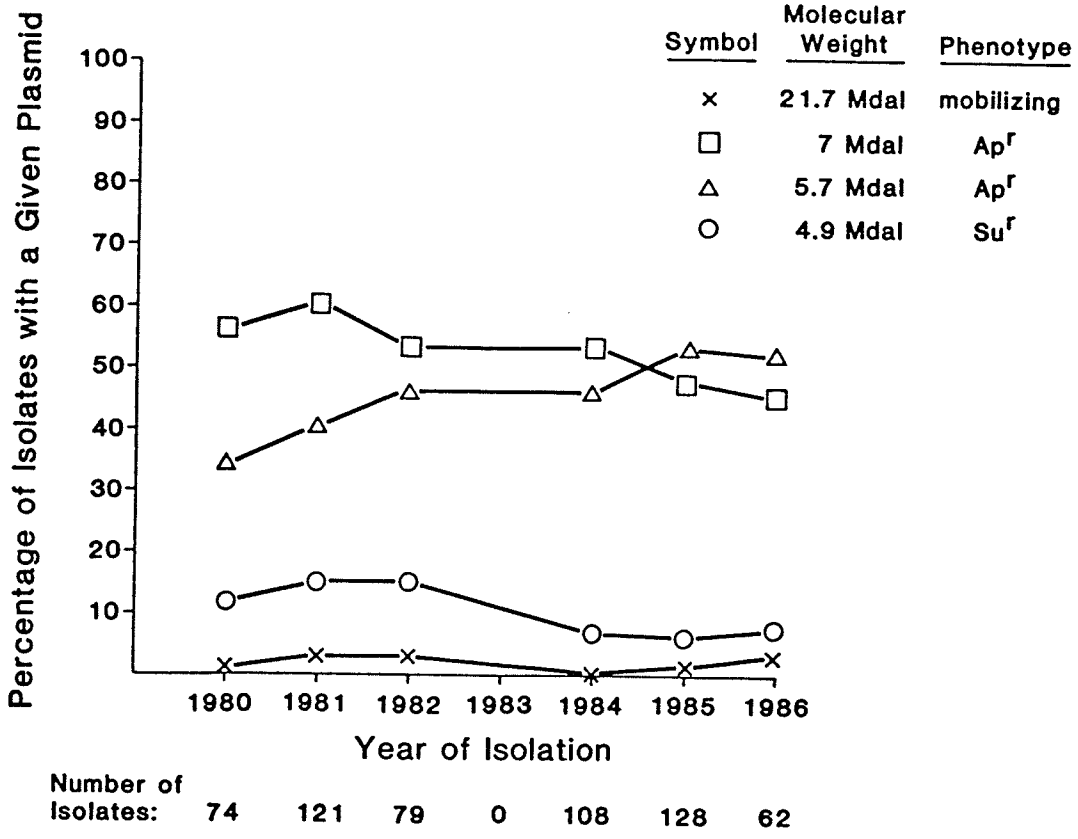
TABLE 3c. Plasmid profiles of H.ducreyi isolates collected from 1978-1985 in The Netherlands. Number of isolates with a given plasmid profile.

Phenotype	Profile	Year of Isolation 1978	1979	1980	1981	1982	1983	1984	1985
Ap^r	3.2					2			3
Su^r	4.9								2
Ap^r	5.7		1	3	2	3	3	2	
Ap^r	7.0	2	2		1	1			
Su^r,Ap^r	4.9/5.7	4		1	1				
Su^r,Ap^r	4.9/7.0	3			1	1	1		
mob,Ap^r	21.7/5.7								
mob,Ap^r	21.7/7.0								
Tc^r,Ap^r	30/7.0								
Ap^r,Km^r,Sm^r,Su^r	5.7/3.2/2.8								
Ap^r,Km^r,Sm^r,Su^r	7.0/3.2/2.8								
mob,Su^r,Ap^r	21.7/4.9/7.0								
Tc^r,Ap^r,Su^r	30/5.7/4.9								
Km^r,Sm^r,Su^r,Ap^r	3.2/2.8/2.6/1.8								
	No Plasmids			1					
	Total	9	3	5	5	7	4	2	5

40

Ap^r = ampicillin resistance
Su^r = sulfonamide resistance
Tc^r = tetracycline resistance
Km^r = kanamycin resistance
Sm^r = streptomycin resistance
mob = mobilizing plasmid

FIGURE 3. ANNUAL INCIDENCE OF FOUR Haemophilus ducreyi PLASMIDS IN KENYA



mids found in Kenyan isolates in 1980 and 1981 have not since appeared in H.ducreyi lysates. Two phenotypically cryptic plasmids of about 1.8 and 3.2 Mdal molecular weight have recently been observed, but to date, these remain confined to Thailand H.ducreyi isolates.

A final issue regarding plasmid evolution has also been illustrated in these data by the fact that a fourth β-lactamase plasmid of unique molecular weight has recently emerged along with a novel kanamycin-streptomycin-sulfonamide multiresistance plasmid in Thailand H.ducreyi isolates.

B. IDENTIFICATION OF A NOVEL 2.6 Mdal β-LACTAMASE PLASMID IN THAILAND H.DUCREYI LYSATES THROUGH DNA-DNA HYBRIDIZATION

1. Introduction

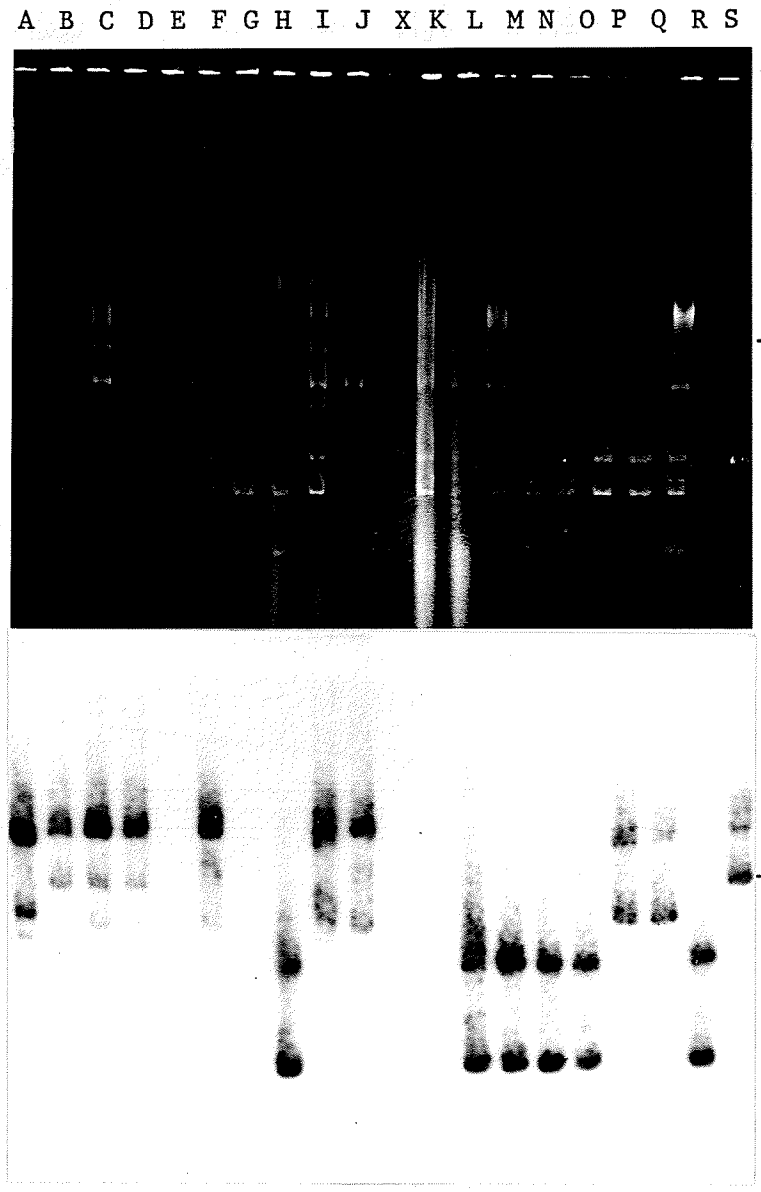
As discussed earlier, recent isolates of Thailand origin possessed four plasmids of unique molecular weights which have not been observed elsewhere. Although the 5.7 and 7.0 Mdal plasmids were found in these isolates, both of which have been previously characterized as β-lactamase producers, a number of isolates possessed neither the 5.7 nor the 7.0 Mdal plasmid. Since all of the Thailand strains were β-lactamase positive, as determined by testing on a chromogenic cephalosporin substrate, it remained to be determined which plasmids encoded this enzyme for ampicillin resistance. It was hypothesized that the 3.2, 5.7 and 7.0 Mdal plasmids, each historically identified as a β-lactamase producer, would also encode the β-lactamase gene in these isolates since all three molecular weights were represented in the plasmid profiles. To test this hypothesis, it was decided to develop a β-lactamase gene probe and test for homology to the plasmids in the Thailand isolates. Possible candidates for such a probe were therefore considered.

2. Development of a β-Lactamase Probe

To facilitate hybridization of the probe to the plasmid DNA, it was necessary to ensure that the probe encoded a β-lactamase of the same or similar type as that found on the plasmid. To this end, the 3.2 Mdal β-lactamase plasmid previously described in H.ducreyi as having a TEM-1 type β-lactamase encoded by transposon, Tn2, was used. A strain harbouring this plasmid was grown in broth and the plasmid was purified by ethidium bromide-cesium chloride gradient ultracentrifugation. Approximately 200 nanograms (ng) of this DNA was then nick-translated using α-^{32}P-dCTP. This probe was hybridized under stringent conditions to a Southern blot of Thailand H.ducreyi lysates (Figure 4). Following autoradiography of the probed membrane for 24 hours, the film was developed and depicted strong hybridization signals at various locations (Figure 5). Since a positive control on the Southern blot possessed the 7.0 Mdal β-lactamase plasmid, it was possible to identify hybridization signals with other plasmids of this molecular weight. However, hybridization with the other plasmids could not be unequivocally ascertained since there was no means to measure the relative distances between hybridization signals and plasmids on the gel. Additionally, it was discovered that previous research (McNicol et al, 1983) reported homology between non-Tn2 regions of the 3.2 Mdal plasmid and non-Tn2 regions of other plasmids in H.ducreyi. To eliminate the possibility of hybridization of the probe with non-Tn2 regions of the plasmid, a new probe was developed.

Purified 3.2 Mdal plasmid DNA was digested with Pst1 and BamH1, to yield three fragments of 1.8, 0.9 and 0.5 Mdal in size as can be seen in Figure 6. Previous studies of this plasmid had established that the β-lactamase gene was carried on the 0.5 Mdal fragment (Anderson et al, 1984; McNicol et al, 1983). This fragment was therefore electroeluted from

FIGURE 4. Agarose gel (0.7%) of Thailand H.ducreyi lysates.



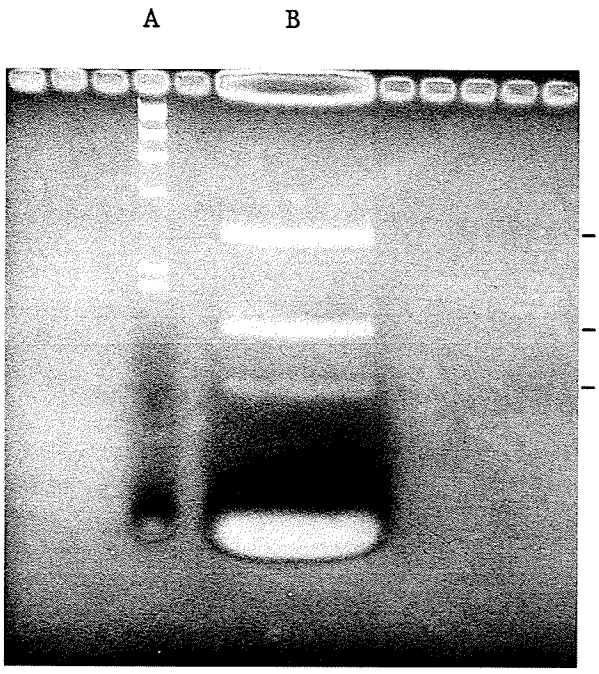
A-R: H.ducreyi isolates of Thailand origin.

X = pool of gradient purified plasmid DNA from various sources, neg. control for probe.

S: H.ducreyi of Kenyan origin, positive control for probe.

FIGURE 5. Autoradiograph of Southern blot from Figure 4.

FIGURE 6. BamHI-PstI restriction endonuclease digestion of p88557 (3.2 Mdal or 5.2 Kb).



A: Lambda DNA digested with HindIII.

B: BamHI-PstI digest of p88557 DNA.

the agarose and the DNA was purified by phenol-chloroform extraction. Following this, the DNA was nick-translated as above and used to probe a series of Southern membranes.

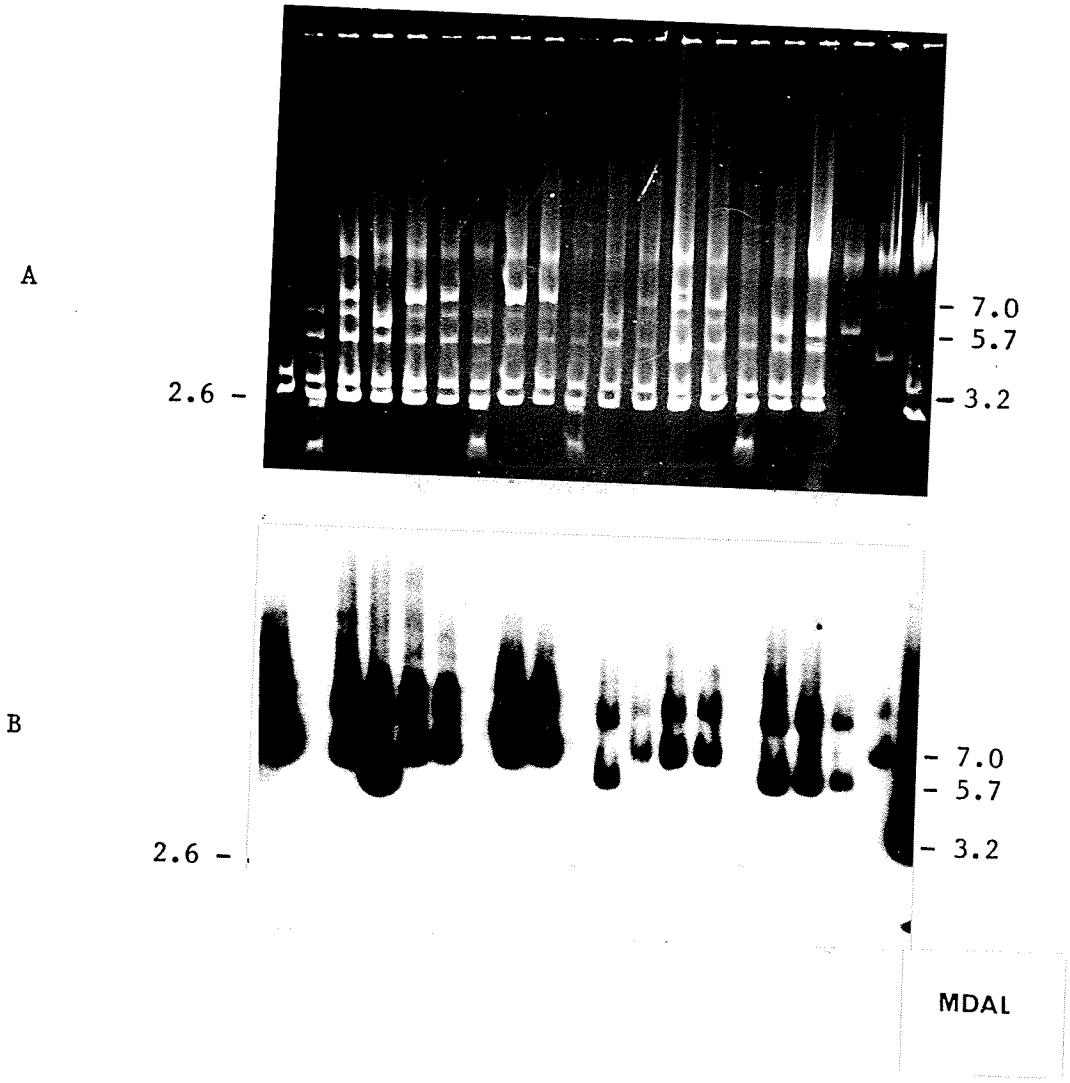
3. Southern Blotting

In an attempt to control for non-specificity of the probe and to ensure probe efficacy, three control strains were lysed and coelectrophoresed on each agarose gel. The positive controls consisted of lysates of strains containing one of the three previously characterized β-lactamase plasmids. Since two of these strains possessed additional plasmids, each of which did not encode TnA, these could be used as controls for the specificity of the β-lactamase probe. After electrophoresis of the lysates was complete, the gels were stained and then photographed with a ruler on each gel. This was done to enable an accurate comparison of plasmid migration distances with hybridization signals. Southern blotting was performed on each of six gels, representing 90 different Thailand lysates. The membranes were allowed to blot overnight, at which time they were baked and prepared for hybridization.

4. Hybridization and Autoradiography

Two membranes were placed in each bag and hybridized with boiling probe overnight at 65°C in 30 ml of hybridization buffer. The following day, the membranes were washed to remove nonspecific hybridization and then placed on x-ray film. Autoradiography was allowed to occur for 24, 48 or 72 hours before the film was developed. The results of these hybridizations (Figure 7) demonstrate that hybridization occurred with the 7.0 and 5.7 Mdal plasmids as hypothesized, but also with a smaller plasmid of 2.6 Mdal in size. This latter finding was unexpected; hybridization was expected to be with the 3.2 Mdal plasmid in the isolates. These data suggested that β-lactamase in Thailand H.ducreyi is encoded on a 7.0 Mdal

FIGURE 7. Hybridization of Thailand lysates with a TEM-1 type β-lactamase probe.



A: Agarose gel (0.7%) of electrophoresed Thailand H.ducryei lysates.

B: Hybridization results from Southern blot of figure A.

plasmid, a 5.7 Mdal plasmid, as well as on a novel 2.6 Mdal plasmid. Since such a finding had not been previously described, it was agreed that characterization of the 2.6 Mdal plasmid was a natural extension of studying plasmid evolution and distribution in H.ducreyi.

C. CONJUGAL TRANSFER OF THE NOVEL 2.6 Mdal β-LACTAMASE PLASMID

1. Introduction

To determine if the novel β-lactamase plasmid could be disseminated to other genera of bacteria, to species within the same genus, or between the same species, biparental and triparental matings were performed.

2. Intergeneric Transfer of the 2.6 Mdal Ampicillin Resistance Plasmid

Four donor strains possessing the 2.6 Mdal ampicillin resistance plasmid were grown on solid media, then suspended in broth, before being mated on a polycarbonate filter with one of two recipient E.coli strains, HB101 (rec-) or C600 (rec+). In the triparental matings, an intermediate H.influenzae strain, T1002, which possessed the 21.7 Mdal mobilizing plasmid was used, whereas the biparental matings involved only the donors and recipients. Donor strains were grown on fresh chocolate agar plates containing 20 ug/ml of ampicillin. Gram stains were performed on each organism, both prior to the experiment and afterwards if growth was apparent. As evident in Table 4, both conjugation and mobilization of the small ampicillin-resistant plasmid was not observed. Mobilization of the 7.0 Mdal ampicillin-resistance plasmid found in Kenyan strains (no. 896) did occur when using the rec^+ E.coli C600 as recipient, but was not observed when using rec^- E.coli HB101.

3. Interspecific Transfer of the 2.6 Mdal Ampicillin Resistance Plasmid

Three protocols were employed when interspecific transfers were attempted to two H.influenzae recipient strains. First, a number of

TABLE 4. Intergeneric transfer of the 2.6 Mdal ampicillin resistance plasmid.

							Conjugants			
		Mating	Growth				C600		HB101	
Strain	Organism	Function	CA	CA+amp^{20}	MH	MH+amp^{20}	x2	x3	x2	x3
CH90	H.ducreyi	Donor	+	+	-	-	-	-	-	-
CH28	H.ducreyi	Donor	+	+	-	-	-	-	-	-
Pu45	H.ducreyi	Donor	+	+	-	-	-	-	-	-
Pu13	H.ducreyi	Donor	+	+	-	-	-	-	-	-
896[1]	H.ducreyi	Donor Control	+	+	-	-	-	+	-	-
T1002	H.influenzae	Intermediate	+	-	-	-				
C600	E.coli	Recipient	+	-	+	-				
HB101	E.coli	Recipient	+	-	+	-				

\+ Denotes growth.

\- Denotes no growth.

1 896 is of Kenyan origin and possesses a 7.0 Mdal β-lactamase plasmid.

x2 Biparental mating.

x3 Triparental mating.

H.ducreyi donor isolates were mated biparentally and triparentally on polycarbonate membranes with an H.influenzae recipient, Rd^{nov}. Both a 10:1 donor-recipient ratio and a 1:100 donor-recipient ratio were attempted. A Thailand strain possessing the 7.0 Mdal ampicillin resistance plasmid (CH25) was used as a control for mating capability. As shown in Table 5, conjugation and mobilization of the small ampicillin resistance plasmid did not occur. Mobilization of the 7.0 Mdal control plasmid was possible, however.

To investigate plasmid mobility further, a second system was employed. Another H.influenzae recipient, $1\text{-}008^{rif}$, and the above donors were mixed with or without an intermediate (H.influenzae, T1002) and this mating mixture was inoculated on chocolate agar and incubated overnight. The resulting growth was transferred to selective media by replicator. Subsequent colonies were then individually streaked on selective media along with a loopful of colonies. This procedure failed to yield transconjugants (Table 5).

In a third and final attempt at mobilizing the plasmid interspecifically, triparental matings were performed with the H.influenzae recipient cell $1\text{-}008^{rif}$ and a series of seven donors, including a Kenyan strain harbouring the 5.7 Mdal ampicillin resistance plasmid, 038. Membrane growth was suspended in 1 ml of BHI broth. One hundred microliters was plated on selective media as usual, but the other 900 ul was then inoculated into 225 ml selective broth (BHI + hemin + NAD + Am^{20}) and incubated with shaking for seven hours. Since H.ducreyi cannot be grown in broth, and the presence of 20 ug/ml of ampicillin would inhibit the growth of the intermediate and recipient strains, the only possible organism that could grow under these conditions would be an H.influenzae transconjugant strain. After incubation, 25 ml of broth was pelleted by centrifugation and the

TABLE 5. Inter- and intraspecific transfer of the 2.6 Mdal ampicillin resistance plasmid.

Strain	Organism	Mating Function	Growth					Conjugants					
			CA	CA+ nov	CA+ amp	CA rif	CA str	Rd^{nov} x2	Rd^{nov} x3	$1\text{-}008^{rif}$ x2	$1\text{-}008^{rif}$ x3	35000^{str} x2	35000^{str} x3
Pu60	H.ducreyi	Donor	+	-	+			-	-				
BRH109	H.ducreyi	Donor	+	-	+			-	-				
RTAH3524	H.ducreyi	Donor	+	-	+			-	-				
CH90	H.ducreyi	Donor	+	-	+		-	-	-			-	-
Pu45	H.ducreyi	Donor	+	-	+		-	-	-				
Pu13	H.ducreyi	Donor	+	-	+		-	-	-			-	-
CH28	H.ducreyi	Donor	+	-	+		-	-	-			-	-
CH37	H.ducreyi	Donor	+		+	-				-	-		
CH39	H.ducreyi	Donor	+		+	-				-	-		
CH40	H.ducreyi	Donor	+		+	-				-	-		
CH50	H.ducreyi	Donor	+		+	-				-	-		
CH85	H.ducreyi	Donor	+		+	-				-	-		
Pu1	H.ducreyi	Donor	+		+	-				-	-		
Pu2	H.ducreyi	Donor	+		+	-				-	-		
Pu6	H.ducreyi	Donor	+		+	-				-	-		
Pu12	H.ducreyi	Donor	+		+	-				-	-		
CH25	H.ducreyi	Control 7.0	+					-	+				
038	H.ducreyi	Control 5.7	+							-	+		
T1002	H.influenzae	Intermediate	+	-	-	-	-						
Rd^{nov}	H.influenzae	Recipient	+	+	-								
1008^{rif}	H.influenzae	Recipient	+		-	+							
35000^{str}	H.ducreyi	Recipient	+		-		+						

x2 = biparental mating.

x3 = triparental mating.

nov = novobiocin 5 ug/ml.

amp = ampicillin 20 ug/ml.

rif = rifampin 25 ug/ml.

str = streptomycin 1000 ug/ml.

CA = chocolate agar.

- denotes no growth.

+ denotes growth.

pellet was tested for β-lactamase activity by chromogenic cephalosporin substrate. If pellets were β-lactamase positive, then they would be plated on selective media. All pellets tested, however, were β-lactamase negative and the cells appeared dead. It was therefore necessary to test for intraspecific mating of this plasmid.

4. Intraspecific Transfer of the 2.6 Mdal Ampicillin Resistance Plasmid

To determine if the novel ampicillin resistance plasmid was nonconjugative and nonmobilizable, a final mating experiment involving the H.ducreyi recipient strain 35000^{str} was performed.

Four H.ducreyi donor strains were used in biparental and triparental filter matings and transconjugants were selected by their resistance to 20 ug/ml of ampicillin and 1000 ug/ml of streptomycin. It should be noted that these H.ducreyi isolates were resistant to 250 ug/ml of streptomycin, but sensitive to the concentration chosen. After plating on selective media, no growth was apparent (Table 5).

D. BACTERIAL TRANSFORMATION OF E.COLI WITH THE NOVEL 2.6 Mdal β-LACTAMASE PLASMID

1. Introduction

Since this small β-lactamase plasmid could not be horizontally disseminated through bacterial mating, it was unique among the plasmids of H.ducreyi. Studies were therefore initiated to expedite molecular characterization of this plasmid, and to ascertain if the plasmid could be introduced into another host by bacterial transformation.

Prior to the transformation experiments, it was necessary to obtain the single β-lactamase plasmid. Since the plasmid in question was coresident with three others, the method of DNA electroelution appeared the only viable means for isolating one plasmid from a gradient purified fraction

containing plasmid DNA of four molecular weights. The eluted DNA was purified by phenol-chloroform extractions and used for each experiment. The 3.2 Mdal β-lactamase plasmid, previously described as having transformed E.coli (Anderson et al, 1984), was used as a positive control for all experiments.

2. Transformation of a Recombination Proficient (rec^+) E.coli, C600

An aliquot of L-broth was inoculated with an overnight suspension of E.coli C600. This strain of E.coli was identified by colony morphology, Gram stain, and API biochemical analysis, before and after the addition of exogenous DNA. In addition, β-lactamase activity was monitored and plasmid analysis was performed before and after transformation of the cells. The cells were grown to a desired concentration, cooled on ice, and then subjected to increasing molarities of salt solutions to facilitate the competency phase. The plasmid DNA was then added to the cells, and a cell control was included in which 100 ul of TES (Appendix III) replaced the plasmid DNA. All test reactions and controls were held on ice, then heat shocked at 42°C briefly. With the addition of a volume of L-broth to each test, growth of newly transformed E.coli was facilitated. Dilutions of the reaction mixtures and controls were then plated on selective media. In addition, an aliquot of the sterile L-broth, subsequently used for suspension and growth of the cells, was plated as a control for sterility. Following overnight incubation, it was observed that a single colony grew on chocolate plates containing 20 ug/ml ampicillin. This colony was streaked on fresh media and grew well after 24 hours. All indices previously described as parameters for identification proved the organism to be a β-lactamase positive E.coli bearing a plasmid of about 2.4 Mdal molecular weight. Table 6 summarizes the data from this experiment. As made evident in Figure 8, the E.coli C600 strain which was plasmid free, acquired a

TABLE 6. Summary of transformation data.

Test Reaction	Growth			
	MH_1	$MH + Ap^{20}_2$		
	Plate Dilutions	10^0	10^{-1}	10^{-2}
A	+	-	-	-
B	+	-	+	-
Cell Control (C600)	+	-	-	-
Broth Control (LB)	-	-	-	-

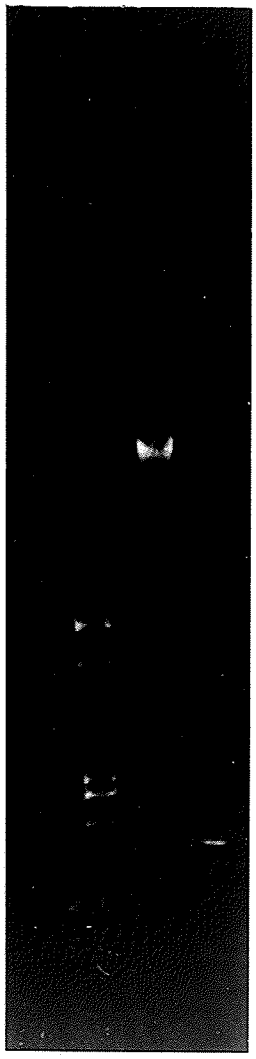
MH_1 = Mueller-Hinton agar.

Ap^{20}_2 = 20 ug/ml ampicillin.

A = C600 + 100 ul of gradient-purified plasmid DNA from a strain possessing the novel 2.6 Mdal β-lactamase plasmid.

B = C600 + 100 ul of electroeluted 2.6 Mdal DNA.

FIGURE 8. Agarose gel (0.7%) of transformant lysate depicting the transformed 2.4 Mdal plasmid, pDMI-del.



A: Thailand strain, CH28, possessing the 2.6 Mdal (4.2 Kb) plasmid, pDMI.

B: E.coli strain, C600, with no plasmids.

C: Transformed E.coli, C600, possessing a single 2.4 Mdal (3.8 Kb) plasmid, pDMI-del.

small β-lactamase plasmid through transformation with the 2.6 Mdal parental β-lactamase plasmid. The difference in size between the parental plasmid, named pDMI, and that observed in the transformant was about 0.2 Mdal as determined by molecular weight analysis on a standard curve. The plasmid observed in the transformant was therefore named pDMI-del. Subsequent experiments were performed to test the validity of these findings. Repeatedly, the deleted form of pDMI was observed. The DNA from eight such transformants was then extracted by CsCl-EtBr ultracentrifugation and the transformants were frozen for future reference.

3. Transformation of Both Recombination Proficient (rec^+) and Recombination Deficient (rec^-) E.coli

At this time, transformation experiments were repeated using a modification of the protocol of Cohen (1972). Cells were made competent by suspension in magnesium chloride, and then a known concentration of exogenous DNA was added. The cells were heat shocked and then broth was added to provide for amplification of transformed cells. The entire reaction mixture was then plated by aliquots on each of 10 plates to ensure that detection of an infrequent transformation event would be possible. Again, a cell control and broth control were employed. Additionally, HB101, an E.coli recombination deficient (rec^-) cell line was used along with C600 to ascertain if the recombination status of the cells contributed to the formation of the deletion in pDMI transformants. The recombination status of the cells was confirmed by UV exposure of a freshly streaked plate containing both organisms. Gram stains, citrate, indole, and oxidase reactions and growth characteristics on blood agar and MacConkey's agar were noted on both E.coli strains before and after transformation.

As evident in Table 7, by the failure of HB101 to grow following UV exposure, this strain is a recombination deficient organism. Identifica-

TABLE 7. Data obtained from the transformation of pDMI into rec^+ and rec^- E.coli.

Exogenous DNA	C600 (rec^+)	HB101 (rec^-)	Growth MH	Growth MH + Ap^{40}
2.6 (pDMI)			+	-
100 ng			+	-
50 ng			+	-
25 ng			+	-
10 ng			+	-
3.2 (p88557)			+	+
3.0 (pJD7)			+	+
2.4 (pDMI-del)			+	+
UV exposure (on BA plate)	+	-		
MacConkey's agar	+	+		
Blood agar	+	+		
Citrate indole	-	-		
β-lactamase	-	-		
Oxidase	-	-		
Gram stain	-ve bacilli	-ve bacilli		

+ denotes growth.

- denotes no growth.

MH = Mueller-Hinton agar.

Ap^{40} = 40 ug/ml ampicillin.

The identity of C600 and HB101 were confirmed at all stages of the procedure.

tion regimens confirmed that both isolates were, in fact, E.coli. It can be seen that transformation failed to occur in both HB101 and C600 when pDMI was used. However, successful transformation was observed when the 3.2 Mdal β-lactamase plasmid was introduced. Two additional positive controls included pDMI-del and pJD7, a small 3.0 Mdal β-lactamase plasmid indigenous to Neisseria gonorrhoeae. The latter two controls yielded numerous colonies in both hosts which, upon plasmid analysis, proved to be true transformants. It should be noted, however, that at least a 10-fold reduction in the number of colonies observed in the HB101 host cells was characteristic of each of the three controls.

To ascertain if the quantity of DNA used for transformation was responsible for the infrequency of pDMI-transformed cells, DNA concentrations of 10, 25, 50 and 100 ng were used. Again, it was found that transformation failed to occur, regardless of the amount used (Table 7).

E. CHARACTERIZATION OF THE DELETED PLASMID, pDMI-del, BY RESTRICTION ENDONUCLEASE MAPPING

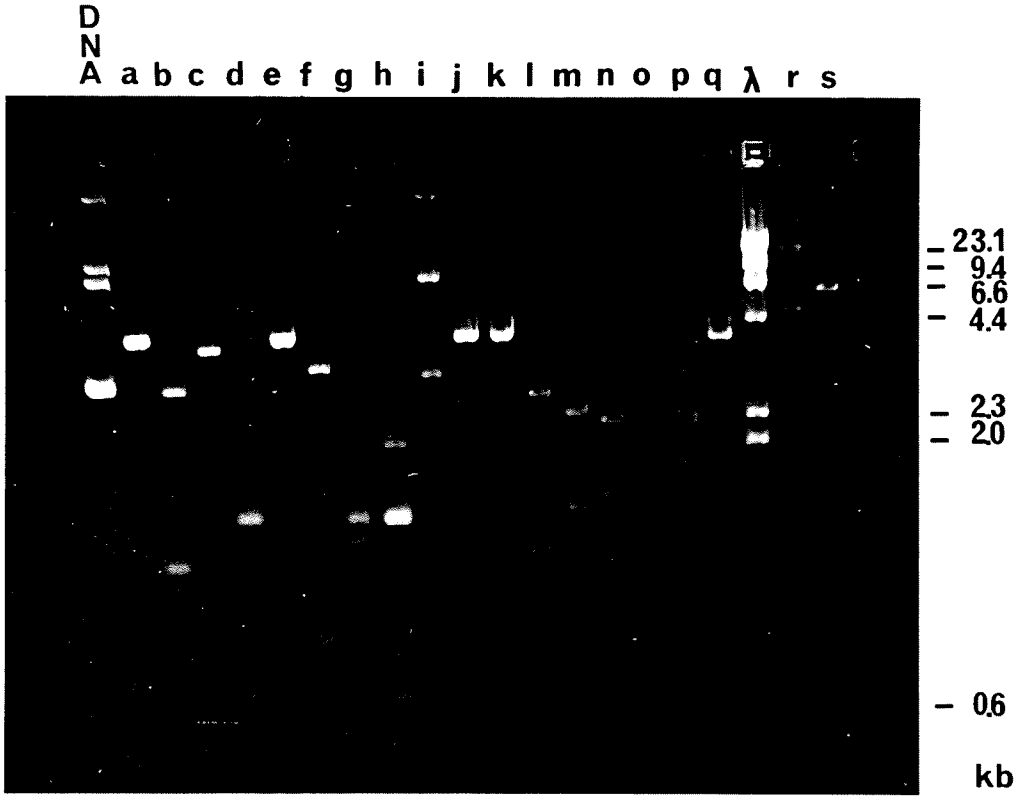
1. Introduction

With the isolation of purified plasmid DNA from eight transformants, it was felt that plasmid mapping should be pursued to help delineate the evolutionary origin of the plasmid, and to determine if the deletion in each transformant plasmid occurred at the same location.

2. Construction of a Restriction Endonuclease Map of pDMI-del

In an effort to determine the restriction endonulcease map of the deleted plasmid, restriction endonuclease digestions were performed. Use of a number of endonucleases, including BamHI, AvaI, HindII, HindIII, PstI, XhoI, PvuI, PvuII, and SalI, both in single and double digestions resulted in banding patterns as shown in Figure 9. Obvious in this photograph of

FIGURE 9. Restriction endonuclease banding patterns observed upon digestion of pDMI-del.



DNA: pDMI-del (Note: dimers and multimers are evident).

Enzymes:
a: BamHI
b: BamHI/PstI
c: BamHI/HindIII
d: BamHI/EcoRI
e: PstI
f: PstI/HindIII
g: PstI/EcoRI
h: EcoRI
i: PvuII
j: HindIII
k: AvaI
l: AvaI/BamHI
m: AvaI/PstI
n: AvaI/HindIII
o: AvaI/EcoRI
p: AvaI/PvuI
q: PvuI
λ: DNA-HindIII
r: p88557-BamHI
s: p88557-PstI

electrophoresed digestions is the presence of molecular weight markers. Lambda (λ) DNA digested with HindIII and EcoRI, as purchased from Boerhinger Mannheim Company, and λDNA digested with HindIII only, as a control for the digestion reactions, were included on each gel. By using such markers, fragment sizes could be calculated from a standard curve (Table 8) and consequently, a physical map could be constructed (Figure 10).

A panel of single and double digestions of eight transformant plasmids was performed to determine if the deletion occurred in a common site. Single digests with each of the above enzymes yielded bands of common sizes. A double digestion with AvaI and BamHI resulted in two fragments common to all eight DNA samples. It should be noted that multimeric forms of the undigested plasmid DNA were frequently observed as is evident in Figures 11a and 11b.

3. Comparison of pDMI-del With Other Plasmids

Upon successful construction of the map of pDMI-del, it was necessary to determine if this plasmid bore any similarity with any of the other small β-lactamase plasmids characterized in H.ducreyi or N.gonorrhoeae. Consequently, digests of the deleted plasmid were paired with digests of the 3.2 Mdal β-lactamase plasmid and coelectrophoresed. Figure 12 shows that the 3.2 Mdal (p88557) plasmid possesses two BamHI sites compared with one in pDMI-del, p88557 has a PvuII site which is not found in pDMI-del, and no HindIII or HindII sites are evident in the p88557 whereas they are found in pDMI-del. This comparison can be summarized in Figure 13, which illustrates some obvious dissimilarities between the plasmids. Comparison with the published map of pJD7 (Yeung and Dillon, 1985) was also performed with obvious incongruencies detected.

TABLE 8. Fragment sizes[1] of pDMI-del upon digestion with restriction endonucleases.

Enzyme	BamHI	AvaI	HindII	HindIII	PstI	PvuI	PvuII	XhoI	SalI
BamHI	2.4	0.7 1.7	0.3 1.6 0.5	0.3 2.1	0.9 1.7	0.6 1.8	2.4	2.4	0.3 2.1
AvaI	0.7 1.7	2.4	0.5 1.1 0.8	1.0 1.4	1.0 1.4	1.1 1.3	2.4	2.4	0.5 1.9
HindII	0.8 1.6	0.5 1.1 0.8	0.8 1.6	0.3 0.5 1.6	0.2 1.4 0.8	0.1 1.5 0.8	0.8 1.6	0.8 1.6	0.05* 0.75 1.6
HindIII	0.3 2.1	1.0 1.4	0.8 0.5 1.6	2.4	0.5 1.9	0.4 2.0	2.4	2.4	0.5 1.9
PstI	0.9 1.7	1.0 1.4	0.2 1.4 0.8	0.5 1.9	2.4	0.2 2.2	2.4	2.4	1.0 1.4
PvuI	0.6 1.8	1.1 1.3	0.1 1.5 0.8	0.4 2.0	0.2 2.2	2.4	2.4	2.4	0.9 1.5
PvuII	2.4	2.4	0.8 1.6	2.4	2.4	2.4	N/C	N/C	2.4
XhoI	2.4	2.4	0.8 1.6	2.4	2.4	2.4	N/C	N/C	2.4
SalI	0.3 2.1	0.5 1.9	0.05* 0.75 1.6	0.5 1.9	1.0 1.4	0.9 1.5	2.4	2.4	2.4

[1] Fragment sizes are in megadaltons and determined by plotting on a standard curve with the following molecular weight markers:

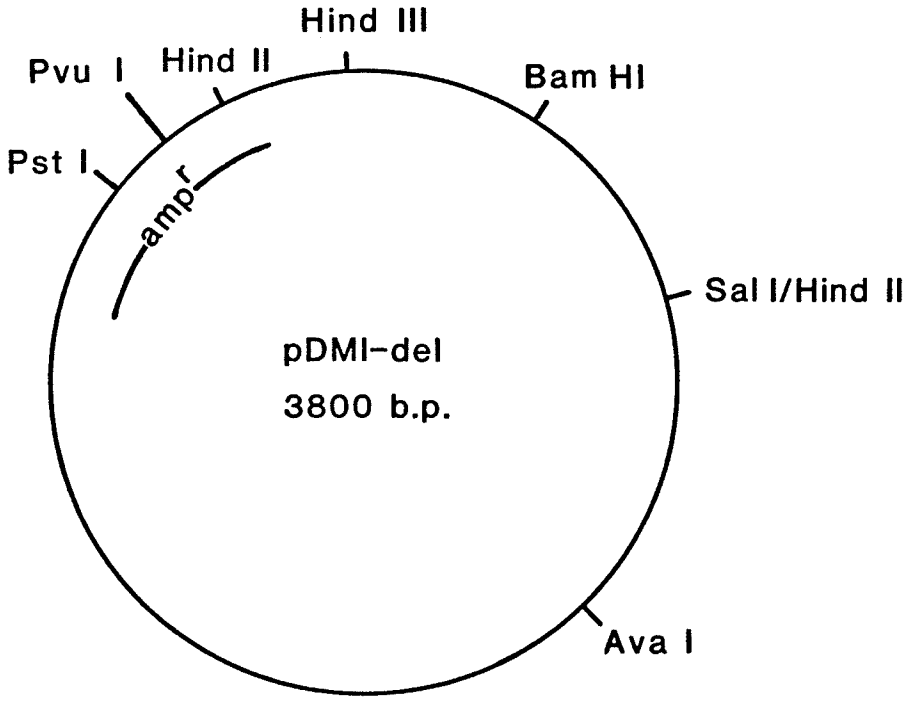
λ:HindIII + EcoRI	
13.2 Mdal	1.2 Mdal
3.2	1.0
3.1	0.8
2.7	0.6
2.2	0.5
1.3	0.3

λ:HindIII
14.3 Mdal
5.8
4.1
2.7
1.4
1.3
0.3

* Fragment size is an approximate.

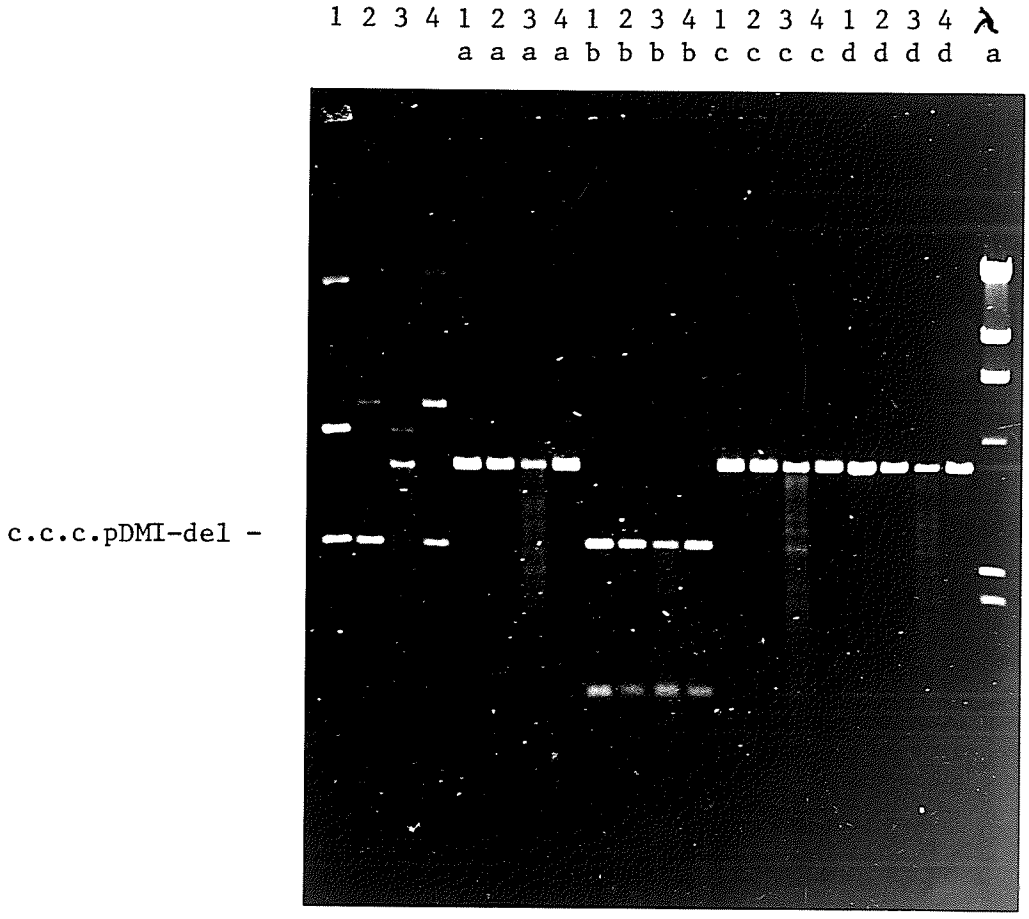
N/C = Not Cut.

Figure 10
PHYSICAL MAP OF pDMI-del.



amp^r : presumed location of the ampicillin-resistance transposon

FIGURE 11a. Restriction endonuclease mapping of pDMI-del from four distinct transformants.

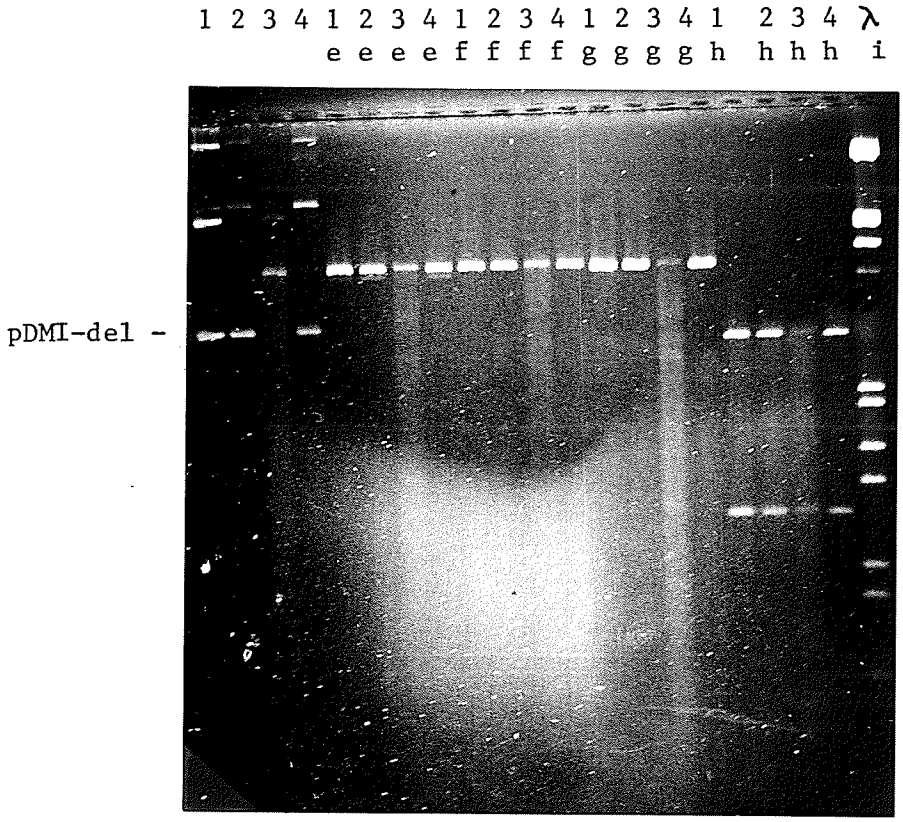


1.2% agarose

LEGEND:
1. Undigested pDMI-del-1 DNA
2. Undigested pDMI-del-2 DNA
3. Undigested pDMI-del-3 DNA
4. Undigested pDMI-del-4 DNA

ENZYMES:
a: HindIII
b: HindII
c: BamHI
d: PvuI
λ: Lambda DNA, molecular weight determinant

FIGURE 11b. Restriction endonuclease mapping of pDMI-del from four distinct transformants.

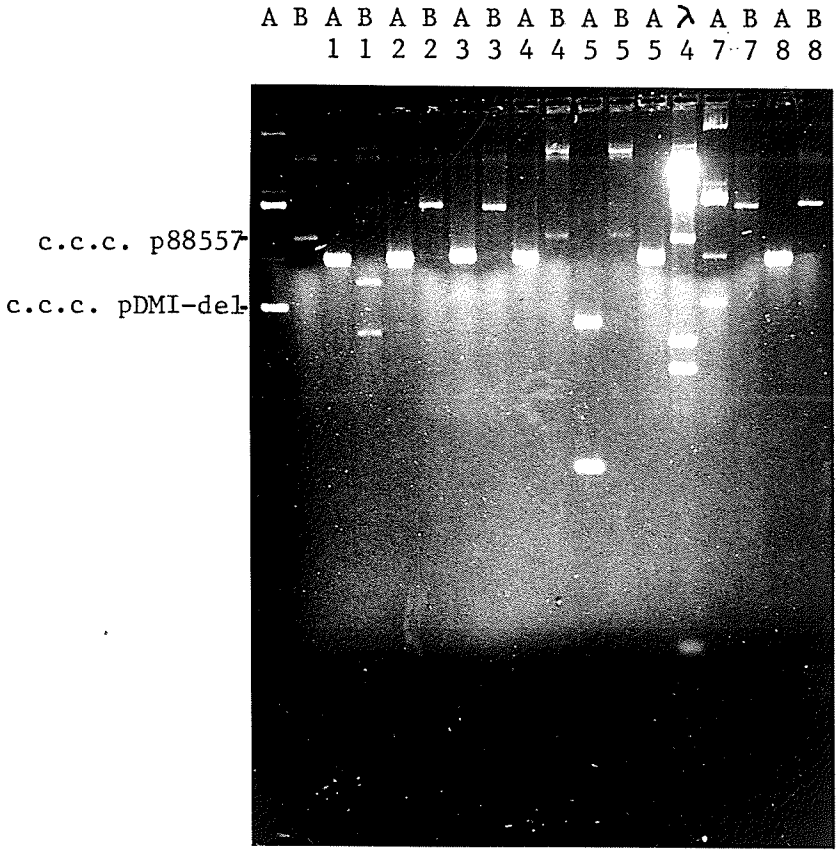


1.2% agarose

LEGEND:
1. Undigested pDMI-del-1 DNA
2. Undigested pDMI-del-2 DNA
3. Undigested pDMI-del-3 DNA
4. Undigested pDMI-del-4 DNA

ENZYMES:
e: PstI
f: SalI
g: AvaI
h: AvaI/BamHI
i: EcoRI/HindIII
λ: Lambda DNA, molecular weight determinant

FIGURE 12. A comparison of restriction endonuclease digests of pDMI-del and p88557.

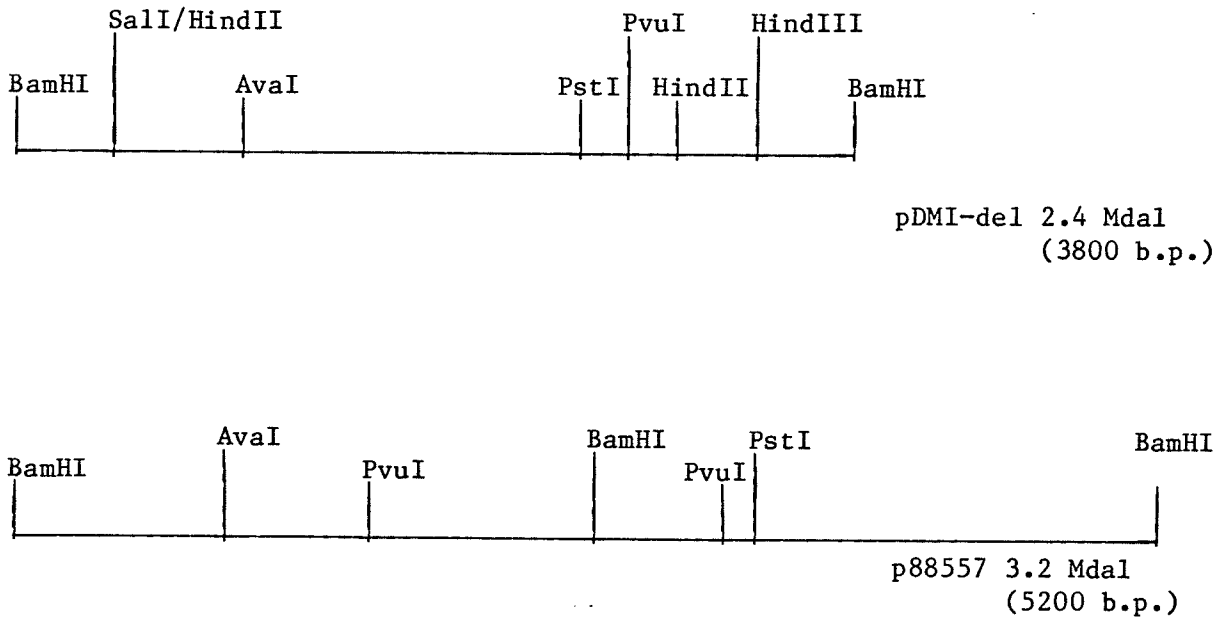


1.2% agarose

LEGEND: A: Undigested pDMI-del DNA
B: Undigested p88557 DNA

ENZYMES:
1: BamHI
2: PstI
3: AvaI
4: HindIII
5: HindII
6: SalI
7: PvuII
8: PvuI
λ: Lambda DNA, molecular weight determinant

FIGURE 13. Comparison of restriction endonuclease maps of pDMI-del and p88557.

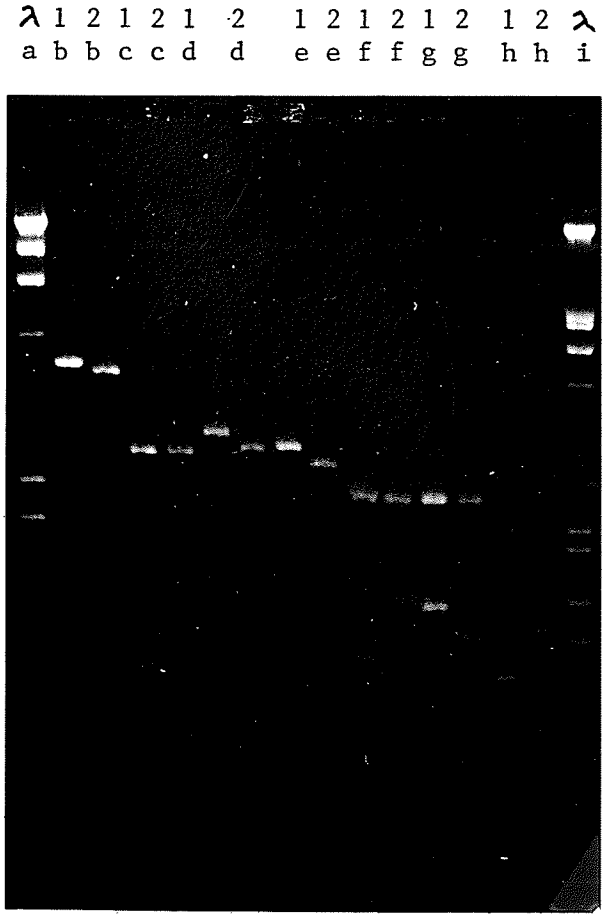


1cm = 0.2 Mdal (320 b.p.).

The restriction map of pDMI-del is very similar to that of the plasmid, pAT153, a deletion derivative of pBR322. Both plasmids are of similar size and possessed other similar characteristics such as being non-mobilizable and non-conjugative, it was necessary to determine if pDMI-del was resistant to tetracycline as is pAT153. To this end, suspensions of the E.coli transformant, C600:: pDMI-del and of C600:: pAT153 were made and plated on Mueller-Hinton agar plates with 20 ug/ml of ampicillin. Tetracycline discs (30 ug/ml) were placed on the agar surface and zone sizes were measured after 24 hours. Using the National Committee for Clinical Laboratory Standards (NCCLS, 1984) as a reference source, it was found that the 23 mm zone size surrounding C600:: pDMI-del was indicative of strong sensitivity to tetracycline. As expected, the E.coli possessing pAT153 was strongly resistant to the antibiotic disc, displaying a zone size of only 9 mm. To confirm that pDMI-del and pAT153 were, in fact, distinct plasmids, restriction endonuclease digestions were performed. The results depicted in Figure 14 illustrated pDMI-del to be about 2.4 Mdal where pAT153 was approximately 2.2 Mdal. Analysis of these data revealed many common fragments. Close inspection mapped the 200 base pair difference in the BamHI-SalI/HindII fragment of pDMI-del. This 200 base pairs, therefore, mapped within the tetracycline-resistance gene of pAT153 (Figure 15).

Due to the many similarities observed between the two plasmids, the extent of DNA homology was questioned. A hybridization experiment was performed using α^{32}P-dCTP labelled pDMI-del as a probe for the Southern blot of the gel seen in Figure 14. After 45 minutes of autoradiography, intense hybridization was observed on all fragments (Figure 16).

FIGURE 14. A comparison of the restriction endonuclease digestions observed for pDMI-del and pAT153.



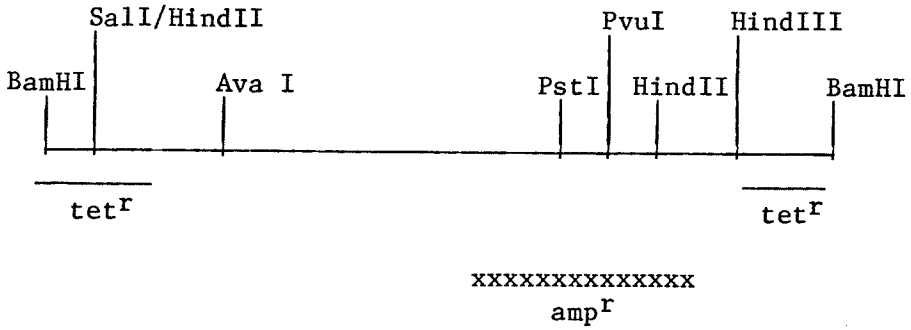
1.2% agarose

LEGEND: DNA: λ: Lambda DNA, molecular weight determinant
1: pDMI-del DNA
2: pAT153 DNA

ENZYMES:
a: HindIII
b: BamHI
c: BamHI/AvaI
d: BamHI/PvuI
e: BamHI/PstI
f: PstI/HindII
g: HindIII/AvaI
h: EcoRI/AvaI
i: HindIII/EcoRI

FIGURE 15. Comparison of the restriction endonuclease maps of pDMI-del and pAT153.

pDMI-del 2.4 Mdal
(3800 b.p.)



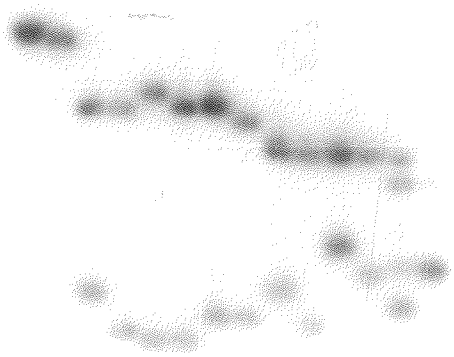
pAT153 2.2 Mdal
(3600 b.p.)

1 cm = 0.2 Mdal (320 b.p.).
tet^r = location of tetracycline resistance gene.
amp^r = location of ampicillin resistance gene.

FIGURE 16. Autoradiograph of pDMI-del and pAT153 restriction endonuclease digestions with a pDMI-del probe.

1 2 1 2 1 2 1 2 1 2 1 2 1 2
b b c c d d e e f f g g h h

linear pDMI-del
linear pAT153



45 minute exposure

LEGEND: 1: pDMI-del DNA
2: pAT153 DNA

ENZYMES: a: HindIII (for λ DNA - no hybridization observed)
b: BamHI
c: BamHI/AvaI
d: BamHI/PvuI
e: BamHI/PstI
f: PstI/HindII
g: HindIII/AvaI
h: EcoRI/AvaI

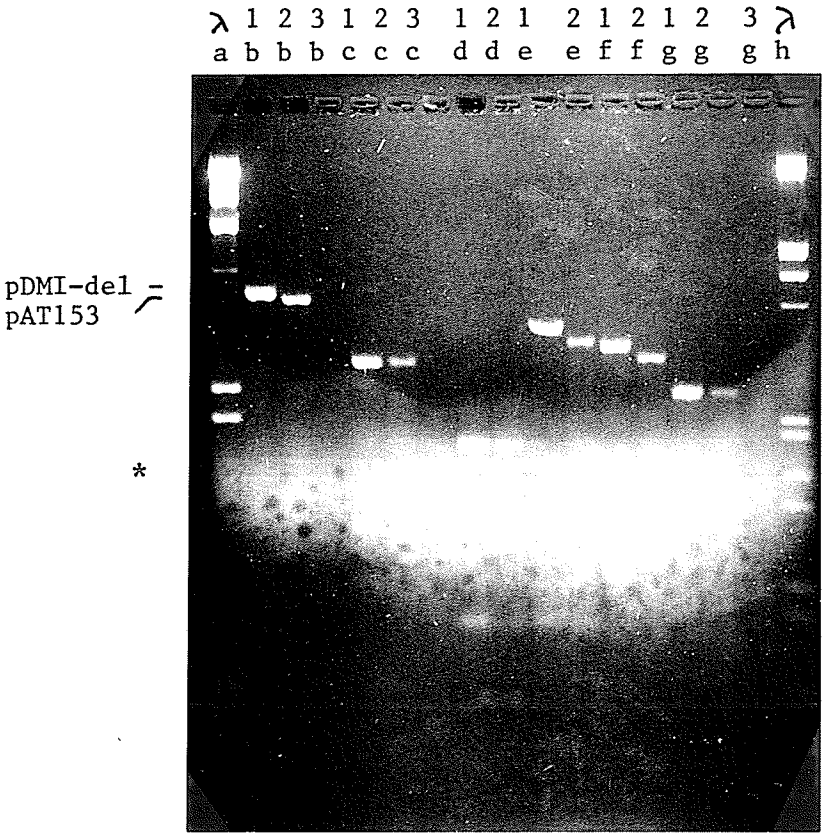
F. CHARACTERIZATION OF pDMI

1. Restriction Endonuclease Mapping and DNA-DNA Hybridization of pDMI

Difficulties were incurred with respect to purifying sufficient quantities of the plasmid for mapping. Strains harbouring the 2.6 Mdal plasmid were grown on ampicillin plates, the growth was scraped and gradients were attempted on the subsequent bacterial pellet. Following elution of the desired band, however, very little DNA remained. For this experiment, therefore, only 20 ng of pDMI was used per digest compared with the 800 ng per digest in the case of pDMI-del and pAT153. Since ethidium bromide staining failed to elucidate the banding patterns of the digested pDMI, it was proposed that probing the Southern blot of the gel would increase the sensitivity of detection, making it possible to map pDMI.

A small volume (30 ng) of pDMI DNA was labelled by the process of random priming with α^{32}P-dCTP and used to probe a Southern blot of pDMI, pDMI-del, and pAT153 digests (Figure 17). Hybridization of the probe to the pDMI fragments would aid in elucidating the physical map of the plasmid whereas homology with the other two plasmids could be detected by hybridization on these fragments. Shown in Figure 18 is the resulting autoradiogram of these hybridization experiments following three days exposure to the film. As is illustrated, weak hybridization signals can be detected corresponding to various bands from the pDMI-del digests. Hybridization with either pAT153 or pDMI fragments was not detected.

FIGURE 17. A comparison of the restriction endonuclease digestions observed for pDMI-del, pAT153, and pDMI.



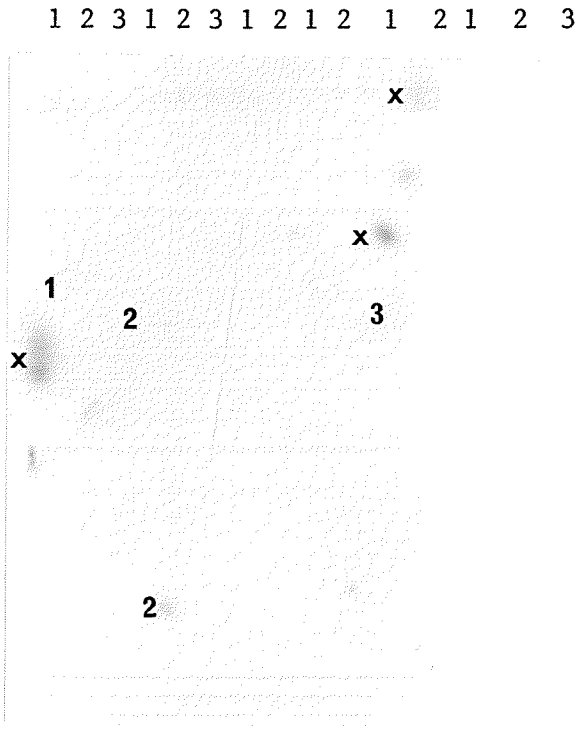
1.2% agarose

LEGEND: λ: Lambda DNA, molecular weight determinant
1: pDMI-del DNA (800 ng/lane)
2: pAT153 DNA (800 ng/lane)
3: pDMI DNA (20 ng/lane)

ENZYMES:
a: HindIII
b: BamHI
c: BamHI/AvaI
d: HindII/AvaI
e: SalI/AvaI
f: BamHI/PvuI
g: HindIII/AvaI
h: HindIII/EcoRI

* Intense fogging in the center of gel is of unknown origin.

FIGURE 18. Autoradiograph of pDMI-del, pAT153, and pDMI restriction endonuclease fragments following hybridization with a pDMI probe.



72 hour exposure

DNA:
1: pDMI-del DNA.
2: pAT153 DNA.
3: pDMI DNA.

LEGEND:
1: Hybridization detected on the linear pDMI-del.
2: Hybridization detected on the two BamHI/AvaI fragments of pDMI-del.
3: Hybridization detected on one of two BamHI/PvuI fragments of pDMI-del.
X: Non-specific hybridization.

NOTE: Unfortunately, reproduction of the hybridization data in this instance was poor. Hybridization, although detectable, was weak and some background, nonspecifically hybridized, probe was evident.

DISCUSSION

A. ANALYSIS OF DATA COLLECTED FROM PLASMID PROFILES OF H.DUCREYI FROM 1976 TO 1986

1. Distribution of β-Lactamase Plasmids

As stated previously, the 3.2 Mdal β-lactamase plasmid found in North America and Amsterdam has yet to emerge in African isolates. This observation may be explained by the fact that the plasmid presumably originated in Brazil, and since its emergence, has been geographically confined to a chain of epidemics from Brazil up into California. The presence of the plasmid in Amsterdam would suggest a North American contact was involved. Given the fact that this plasmid is mobilizable in a triparental mating and has been observed in N.gonorrhoeae, and more recently in H.influenzae isolates in Kenya, one would expect that H.ducreyi in Kenya will acquire this plasmid shortly.

Accounting for β-lactamase production in Kenya are the 5.7 Mdal and 7.0 Mdal plasmids which, as shown previously, are approaching a state of equilibrium. This trend is a divergence from that observed in the past where a 60:40 ratio of 7.0 Mdal to 5.7 Mdal plasmids was characteristic. The larger plasmid possesses a 1.3 Mdal insertion sequence which has been found to increase the frequency of mobilization over that observed for the 5.7 or 3.2 Mdal plasmids which lack this sequence (McNicol, 1983). This may indicate that the capacity to enhance dissemination is becoming less advantageous than was observed in the past due to plasmid saturatio of the bacterial host.

This pattern towards an increase in the proportion of β-lactamase-producing strains harbouring the 5.7 Mdal plasmid is borne out in other regions of the world as well. In Amsterdam, Thailand, and in most outbreaks in North America, this plasmid is responsible for β-lactamase pro-

duction. The 7.0 Mdal plasmid has not been observed in North America. These data are consistent with the above theory whereby the additional sequence has become biologically redundant and therefore selected against. Mobilization of these small resistance plasmids is dependent upon the mobilizing plasmid. However, sufficient data comparing the incidence of the 5.7, 21.7 Mdal profile with the 7.0, 21.7 Mdal profile is not available. Therefore, this theory cannot yet be substantiated. Alternatively, this trend may simply reflect a geographic bias whereby North American source contacts have had no association with strains originating in the Philippines where this plasmid was first isolated. A prospective evaluation of the relative incidences of these plasmids in the next few years should elucidate this pattern.

Additionally, a novel 2.6 Mdal β-lactamase plasmid has recently emerged in Thailand H.ducreyi isolates. To date, this plasmid species remains confined to Southeast Asia but accounts for β-lactamase production in 52% of isolates. This would suggest that since the recent introduction of this plasmid, it has spread quite effectively, becoming the dominant β-lactamase producer in Thailand H.ducreyi.

2. Prevalence of Sulfonamide, Tetracycline and Chloramphenicol Plasmid-Mediated Resistance

Further analysis of the plasmid profiles of H.ducreyi suggests that the 4.9 Mdal sulfonamide-resistant plasmid is observed infrequently. About 10% of Kenyan isolates from 1984 through 1986 harboured this R-factor. As alluded to earlier, there has emerged a 2.8 Mdal sulfonamide-streptomycin-kanamycin multiresistant plasmid in Thailand isolates. As with the novel β-lactamase plasmid, this 2.8 Mdal plasmid has only been isolated from Thailand H.ducreyi isolates to date. However, the plasmid was demonstrated in each of 90 Thailand isolates examined, suggesting that it carries out

its functions very efficiently or that it has a cryptic function required by the host. Contrasting with this ubiquitous replicon are the large conjugative tetracycline and tetracycline-chloramphenicol plasmids found in Kenya. Neither has been observed in a lysate since 1981. Since resistance to these antibiotics has been demonstrated even in the absence of a visible plasmid, it is presumed that these large plasmids incorporate well into the chromosome or that the resistance in these isolates is chromosomally-mediated (Albritton et al, 1984; McNicol and Ronald, 1984).

3. Prevalence of Mobilizing Among the Small R-Plasmids of H.ducreyi

The 21.7 Mdal mobilizing plasmid is relatively rare, appearing in about 2-3% of Kenyan isolates. Despite its infrequent occurrence, this plasmid remains an important precursor to plasmid spread in H.ducreyi and related organisms such as H.influenzae (McNicol et al, 1983) and N.gonorrhoeae (McNicol et al, 1986a). The fact that nearly 100% of H.ducreyi isolates from Kenya possess one of two β-lactamase plasmids, and that the 3.2 Mdal β-lactamase plasmid has recently been observed for the first time in Kenyan H.influenzae, suggests that plasmid dissemination through mobilization is still an ongoing and dynamic process in Haemophilus and Neisseria.

4. Multiplicity of Plasmid Profiles as an Index of Bacterial Strains

An informative feature of H.ducreyi infection was also implied by the data illustrated in Table 3. It was evident that single strain epidemics characterized by a common plasmid profile are found in recent North American outbreaks whereas in Thailand, The Netherlands and Kenya, multiple strains with diverse profiles are continuously endemic. These data would seem to support the assumption that in the case of the North American epidemic, a single strain carried by one (or few) source contact(s) is introduced into a population and spread. Plasmid profiles in most North

American epidemics illustrate a homologous population of a single plasmid. Contrasting this is the situation in Europe, Africa and Asia where a number of different profiles, usually consisting of many plasmids, are observed within a given epidemic. In such outbreaks, it appears that a number of source contacts, each harbouring a different strain as suggested by the heterogeneity of plasmid profiles, are responsible for introducing and spreading the disease. Presumably, locations where the disease is endemic, such as Amsterdam, Kenya and Thailand, prove permissive environments for recurring infection with different strains of the bacterium. As a result, plasmid exchange has occurred resulting in profiles consisting of two or more plasmids. An extreme demonstration of such promiscuous genetic exchange has recently emerged in Thailand and must be addressed.

B. RECOGNITION OF A 2.6 Mdal β-LACTAMASE PLASMID INDIGENOUS TO THAILAND H.DUCREYI

1. Molecular Hybridization of p88557 with Thailand Plasmids

With the observance of a number of novel plasmids in Thailand isolates, it became necessary to determine which were responsible for β-lactamase production. To this end, the 3.2 Mdal β-lactamase plasmid, p88557, which encodes a TEM-1 type enzyme on Tn2, was nick-translated with α-^{32}P-dCTP and used to probe a Southern membrane of electrophoresed lysates. Autoradiography revealed intense hybridization at several points. Since a strain possessing the 7.0 Mdal plasmid had been included on the gel, it was possible to ascertain hybridization of the probe to 7.0 Mdal β-lactamase plasmids. It should be noted that, as expected, hybridization also occurred with the open circular form of the electrophoresed plasmids. Hybridization was noted below the 7.0 Mdal plasmid in some strains, presumably indicative of the 5.7 Mdal β-lactamase plasmid. Strong signals were

evident below these as well. Unfortunately, however, hybridization with these other plasmids could not be assessed due to the lack of an appropriate panel of positive controls at various molecular weights, and/or a means of comparative measurement between the gel and hybridization signals. Additionally, contributing to the ambiguity of these results was the fact that previous authors (Brunton et al, 1982; Brunton et al, 1981) have proven homology among the non-TnA regions of all the β-lactamase plasmids of N.gonorrhoeae and H.ducreyi. Therefore, the homology observed may, in fact, have been between non-TnA encoding regions. For this reason, it could not be conclusively stated that hybridization was with β-lactamase encoding plasmids.

2. Homology Shared Between the BamHI/PstI Fragment of p88557 with pDMI

To circumvent these problems, three positive controls were included for each hybridization, and a ruler was placed on each gel before being photographed so as to enable relative measurements between plasmids on the gel and the hybridization signals. A new β-lactamase probe was also developed specific for the TEM-1 type β-lactamase gene of TnA or Tn2. This consisted of the BamHI/PstI fragment of p88557. Since this 0.5 Mdal sequence encodes a portion of the β-lactamase protein (Yeung et al, 1985), it was believed to be a more specific indicator of β-lactamase-producing plasmids than the complete plasmid probe. The fact that plasmids of 3.2, 5.7 and 7.0 Mdal had been previously described as β-lactamase producers in H.ducreyi led us to hypothesize that each of these three plasmids, observed in Thailand isolates, would carry the gene for this enzyme. The hybridization experiment reaffirmed this presumption.

Autoradiography revealed that β-lactamase was encoded on 7.0 and 5.7 Mdal plasmids, but not on the 3.2 Mdal plasmid. Rather, in those isolates not carrying either the 7.0 or 5.7 Mdal plasmids, a weak hybridization sig-

nal was observed on a 2.6 Mdal plasmid. These data suggested that, contrary to a widely held belief, a 3.2 Mdal plasmid in H.ducreyi isolates is not conclusive evidence of β-lactamase production. To date, the 3.2 Mdal plasmid in Thailand remains phenotypically cryptic. The obvious disparities in the intensity of hybridization signals remains unexplained. Perhaps this is due to a lesser degree of homology expressed between the TEM-1 probe and the 2.6 Mdal plasmid. This may be explained by the fact that the new plasmid encodes a different but similar type of β-lactamase. This could be elucidated better through hybridization with a panel of β-lactamase probes, and isoelectric focussing; studies which incidentally are in progress. Alternatively, the difference in hybridization signals may be due to the residual cross-contamination of fragments associated with electroelution. The fraction DNA used for the β-lactamase probe inevitably had small amounts of the other two fragments as well. Upon hybridization then, all of the fragments would hybridize with the 5.7 and 7.0 Mdal plasmids, but only the β-lactamase fragment would hybridize with pDMI. This would therefore account for the differences in hybridization signals.

C. CONJUGAL TRANSFER OF THE 2.6 Mdal PLASMID, pDMI

1. Intergeneric Transfer

With the isolation of a new plasmid of novel molecular weight, it became necessary to determine if the plasmid could be spread by conjugal matings. An intergeneric mating was attempted using four donor strains and two E.coli isolates as recipients, one recombination proficient (rec^+) recipient strain, C600, and one rec^- recipient, HB101. One would expect that if genetic recombination through conjugation were to occur, then the likelihood of observing transconjugants in C600 would be 100-1000 times that for the rec^- HB101 (Stahl, 1987). The results suggested that the 2.6

Mdal donor plasmid failed to be mobilized into either recipient. One positive control carrying the 7.0 Mdal β-lactamase plasmid from a Thailand isolate was successfully mobilized into C600, but was not evident in HB101. Although one would expect the ratio of C600 transconjugants to be much higher than that observed for HB101, one would still expect transconjugants in the latter. The absence here may reflect a discrepancy in the mating ratios from that employed in the control, since McFarland standards were used for determining the mating concentrations.

2. Interspecific Transfer

Although a number of different protocol were used to mobilize the plasmid into either of two H.influenzae recipients, successful transfer was not observed. Again, the 7.0 Mdal plasmid maintained in a Thailand donor control strain was transferred into H.influenzae (Rd^{nov}) in a triparental mating. The 5.7 Mdal plasmid of Kenyan origin was also mobilized.

3. Intraspecific Transfer

In a final effort to transfer the 2.6 Mdal β-lactamase plasmid through bacterial conjugation, four H.ducreyi donors were mated with an H.ducreyi recipient either with or without an intermediate H.influenzae strain; mobilization was not observed.

These data indicate that the 2.6 Mdal β-lactamase plasmid in H.ducreyi is both nonconjugative and nonmobilizable. This may indicate that the sequence essential for plasmid mobilization, the origin of transfer, or OriT, is missing. Consequently, relaxation proteins prove useless in effecting plasmid mobilization since their target site is absent. It is of interest to note that pAT153 is not mobilizable either due to a deletion of the OriT site. The mechanism by which pDMI will spread therefore remains to be elucidated.

D. TRANSFORMATION OF E.COLI WITH pDMI

1. Recognition of a Deletion-Derivative of pDMI in Recombination Proficient E.coli, C600

In an attempt to better understand the biology of this novel plasmid, transformation experiments were conducted. Initially, the plasmid DNA was electroeluted and purified before proceeding with the transformation protocol. It was perceived that if transformation was possible, it would be more evident in a rec^+ background since homologous recombination occurs at a greater frequency here, and for this reason, the plasmid-free E.coli C600 was used. The results of these experiments revealed a transformed E.coli C600 strain bearing a single plasmid species of about 2.4 Mdal in size, slightly smaller than the parental plasmid, 2.6 Mdal. Identical findings were reported when the experiment was repeated. Although transformation-induced deletions had not been observed in our laboratory with the other H.ducreyi β-lactamase plasmids, similar deletions with these plasmids have been reported elsewhere (Totten et al, 1982). Sox et al (1979) have also described deletions of the gonococcal plasmids when transformations into N.gonorrhoeae were attempted. To explain this phenomenon, he conjectured that endonucleases converted the exogenous DNA to the linear form during uptake, and recircularization resulted in deleted plasmids. Alternatively, he suggested that the linear DNA may have recombined with the chromosome. Whether either hypothesis could be substantiated for the case of pDMI-transformed E.coli C600 remains to be determined.

2. Cotransformation of E.coli C600 (rec^+) and HB101 (rec^-) with pDMI

To determine if the deletions in pDMI observed with C600 were induced by the recombination system of this strain, transformation was attempted with HB101 as well. This strain of E.coli is isogenic to C600, but has been mutated to provide a rec^- background. When the experiments were

repeated using both recipient strains, transformants were not detected in either strain. Three positive controls were included on this panel of tests: pJD7, a 3.0 Mdal β-lactamase plasmid indigenous to N.gonorrhoeae (Yeung, 1986); pDMI-del, the deleted form of pDMI; and p88557, the 3.2 Mdal β-lactamase plasmid. Interestingly, each of these successfully transformed both C600 and HB101. As expected, colony counts on the C600 plates were at least ten times greater than those on the HB101 plates. The fact that pDMI failed to transform both strains in this experiment, while previous experiments involving C600 were successful (albeit with very few colonies observed), suggests that transformation of an E.coli host with pDMI is an infrequent event, which when successful, appears to be dependent upon the integrity of the host's recombination system.

E. RESTRICTION ENDONUCLEASE MAPPING AND MOLECULAR HYBRIDIZATION IN THE CHARACTERIZATION OF pDMI AND pDMI-del

1. Mapping of the Plasmid Deletion in Eight Transformants

Given that transformation of C600 by the 2.6 Mdal plasmid is presumably a rec dependent event and results in a deletion of about 0.2 Mdal, it was questioned as to whether or not the deletion would occur at the same site on the plasmid in all transformed colonies. To determine if the transformants were homogeneous with respect to the deletion site, plasmid DNA extracts were prepared from eight transformants. Restriction endonuclease digestions were performed on each DNA sample using either one enzyme or two in concert. Electrophoresis of the samples revealed the banding pattern for each enzyme reaction to be identical. Although these data do not prove unequivocally that the deletion is from a common site, they do provide evidence in support of this theory. The deleted fragment may be inserted in the host chromosome. This hypothesis could be tested by

probing the chromosome of a transformant with pDMI, or more specifically, with the 0.2 Mdal fragment of pDMI not found on pDMI-del.

2. Comparison of pDMI-del with p88557, pJD7 and pAT153 by Restriction Endonuclease Analysis and Molecular Hybridization

Successful construction of the physical map for pDMI-del by restriction endonuclease digestions has made it possible to compare the plasmid with others to ascertain if any similarities existed. This was done in the hopes of identifying the evolutionary origin of pDMI. To this end, digests of pDMI-del were coelectrophoresed with either p88557 or pAT153 and banding patterns were examined. Since the map of pJD7 is known (Yeung et al, 1986), comparison with pDMI-del was expedited. No homologies were apparent between pDMI-del and either p88557 or pJD7. In contrast, the maps of pDMI-del and pAT153 were remarkably similar. In addition, both plasmids fail to be mobilized in triparental matings. Although it was confirmed that pAT153 is strongly resistant to both ampicillin and tetracycline, whereas antibiotic disc diffusion assay revealed pDMI-del to be sensitive to tetracycline, examination of their respective physical maps revealed the two plasmid maps to be identical with the exception that pDMI-del possesses an additional 200 b.p. of sequence in the BamHI-SalI/HindII fragment. Since the tetracycline gene maps in this location on pAT153, it is tempting to speculate that pDMI-del is identical to pAT153 with the exception that it has a 200 b.p. insertion sequence in this fragment thereby inactivating the tetracycline gene.

In an attempt to examine the extent of homology between pDMI-del and pAT153, DNA-DNA hybridization studies were performed on digests of both plasmids. After only 40 minutes of autoradiography, intense hybridization was observed with all fragments including fragments known to carry non-transposon sequences. Since these hybridizations were conducted under

stringent conditions, it is safe to assume that at least 85% of the sequences are homologous. This would indicate that pDMI-del and the progenitors of pAT153, a recombinant plasmid, originated from a common evolutionary source, or that pAT153 has become introduced into H.ducreyi.

3. Comparison of pDMI with pDMI-del and pAT153

Given the results obtained from hybridization of pDMI-del and pAT153, comparisons with the parental plasmid, pDMI, were essential. Due to the problems associated with acquiring sufficient quantities of this DNA in purified form for restriction digests or molecular hybridization, it was necessary to reduce the amount of DNA used. Twenty ng of pDMI was employed in each restriction endonuclease digest, compared with 800 ng for pAT153 and pDMI-del. Hybridization was performed under stringent conditions using 30 ng of α ^{32}P-dCTP labelled pDMI as a probe. After 72 hours of autoradiography, weak hybridization was observed with various pDMI-del fragments, but no signal was detected for either pDMI digests or pAT153 digests. Since the specific activity of the probe was low at the initiation of hybridization, it was not surprising that detection of pDMI DNA was not possible. However, this does not account for the lack of a hybridization signal on the pAT153 fragments. Demonstrable hybridization on these fragments would be expected since previous data showed marked homology between pDMI-del and pAT153, and this experiment detected homology between pDMI and pDMI-del. After examining the agarose gel of the respective digests, it became apparent that the amount of pDMI-del DNA per digestion exceeded that for pAT153. This may explain why hybridization is not apparent, since the signals for pDMI-del are weak in themselves. Nevertheless, the fact remains that pDMI has been shown to be homologous to pDMI-del which has been proven to be highly homologous to pAT153. It may be that pDMI and pAT153 share sequence homology. If pDMI and pAT153 are proven to be homo-

logous, then pAT153 is a deletion derivative of pBR322, a chimeric construct (Balbas et al, 1986), presents two potential hypotheses as to the emergence of pDMI in H.ducreyi:

1. The plasmid, pDMI, in Thailand H.ducreyi has evolved through a number of recombination events which, by chance, have mimicked those in vitro manipulations producing pBR322, or
2. The plasmid, pBR322 or pAT153, has become introduced into the human host, possibly recombining in an enteric organism such as E.coli. This organism has, in turn, passed the plasmid to Haemophilus presumably by conjugation. Subsequently, a HaeII fragment possessing the OriT has been lost, and a 200 b.p. insertion sequence is inserted in the BamHI-SalI/HindII fragment thereby inactivating the tetracycline resistance gene.

Although both theories appear unlikely, it appears that pDMI presumably possesses a few "hot spots" for insertion and removal of DNA sequence; one 200 b.p. element is present in the tetracycline gene, another of twice the size accounts for the deletion, pDMI-del, observed in transformation, and perhaps a third which encodes the OriT site for conjugative transfer.

Regardless of the mechanisms that have prevailed in the evolution of pDMI, it remains that another novel and very successful β-lactamase plasmid has arisen in H.ducreyi. Because of the very profound public health implications of a naturally-occurring plasmid that bears striking physical and genetic homology to a recombinant plasmid, further studies need to be undertaken to elucidate the precise origins of pDMI.

CONCLUSIONS

Having analyzed plasmid profiles of 830 H.ducreyi isolates, collected from 1976-1986, from four continents, a number of trends were observed. In addition, a novel 2.6 Mdal β-lactamase plasmid, which has emerged in Thailand, was characterized. From these data, the following can be concluded:

1. The 3.2 Mdal β-lactamase plasmid has not appeared in Kenyan H.ducreyi despite the existence of the identical plasmid in N.gonorrhoeae and H.influenzae isolates found here.
2. The 5.7 Mdal and 7.0 Mdal β-lactamase plasmids have approached an equilibrium in Kenyan isolates with neither becoming predominant. This may reflect the biological redundancy inherent to the 1.3 Mdal insertion sequence of the 7.0 Mdal plasmid.
3. The 7.0 Mdal β-lactamase plasmid has not yet appeared in North America.
4. The 21.7 Mdal mobilizing plasmid and the 4.9 Mdal sulfonamide-resistant plasmid are observed infrequently and do not appear to be disseminating.
5. Tetracycline-resistance plasmids, 30 Mdal, and the tetracycline-chloramphenicol-resistance plasmids, 34 Mdal, have not been observed in H.ducreyi isolates since 1981.
6. Single strain epidemics characterized by a common plasmid are found in recent North American outbreaks. This contrasts with that found in Thailand, The Netherlands, and Kenya where multiple strains with diverse profiles are continuously endemic.

7. Plasmid stability in H.ducreyi has been witnessed throughout the course of numerous outbreaks in North America, suggesting that the introduction of new plasmids or modifications of existing ones is an infrequent event.

8. Evidence in support of plasmid evolution in H.ducreyi was found in the recent emergence of a fourth and novel 2.6 Mdal β-lactamase plasmid, pDMI, and a 2.8 Mdal kanamycin-streptomycin-sulfonamide multiresistance plasmid, both from Thailand.

9. The novel plasmid, pDMI, is observed in 52% of isolates examined from Thailand thereby proving it is efficiently maintained and spread.

10. This plasmid has been found to be both nonconjugative and non-mobilizable in intergeneric, interspecific, and intraspecific conjugal matings.

11. The plasmid can transform a recombination proficient E.coli strain C600 with the resulting transformant bearing a plasmid deleted by about 400 b.p., pDMI-del. Transformation of the recombination deficient E.coli HB101 was not possible with pDMI.

12. Characterization of pDMI-del revealed it to be dissimilar to the previously described β-lactamase plasmids of H.ducreyi as well as to pJD7, a small β-lactamase plasmid found in N.gonorrhoeae. Extensive homology was found between fragments of pDMI-del.

13. Although pAT153 carries a tetracycline-resistance gene and pDMI-del was sensitive to this antibiotic, it was observed that the 200 b.p. size difference in the plasmid maps in the area of this gene. This provides some latitude for speculation that perhaps insertion of a 200 b.p. sequence in this fragment prohibits the expression of this gene.

14. Comparison of pDMI and pDMI-del by molecular hybridization was suggestive of homology between pDMI and pDMI-del.

15. The plasmid, pDMI, represents a very successful, newly evolved R-factor whose origin, in light of the evidence provided, must be determined.

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APPENDIX I

SOLID MEDIA

Chocolate Agar

GC agar base (Gibco)	36.0 g
Bovine haemoglobin	10.0 g
CVA enrichment (Atlas)	10.0 ml
Distilled water	1000.0 ml
Sterile fetal calf serum (optional)	50.0 ml

Mueller-Hinton Agar

Mueller-Hinton agar base (Gibco)	38.0 g
Distilled water	1000.0 ml

Blood Agar

Heart infusion agar base	40.0 g
5% sheep blood	50.0 ml
Distilled water	1000.0 ml

MacConkey Agar

MacConkey agar base without sodium chloride	47.0 g
Distilled water	1000.0 ml

APPENDIX II

LIQUID MEDIA

Brain-Heart Infusion Broth

Brain-heart infusion broth (Gibco)	38.0 g
Distilled water	1000.0 ml
For the growth of *H.influenzae* or to suspend *H.ducreyi* growth, the following ingredients were added:	
Nicotinamide adenine dinucleotide or NAD (Sigma) to 1%	10.0 ml
Hemin (Sigma) to 1%	10.0 ml

Luria Broth (pH 7.5)

Bacto-tryptone (Difco)	1.0 g
Yeast extract (Gibco)	0.5 g
Sodium chloride	1.0 g
IM $MgSO_4$	0.1 ml
0.1 M $CaCl_2$	0.1 ml
40% glucose	3.0 ml
Distilled water	100.0 ml

APPENDIX III

Tris-Borate Buffer (pH 8.0-8.5)

Boric acid (Sigma)	110.0 g
Trizma base (Sigma)	216.0 g
Disodium EDTA (Sigma)	18.6 g
Distilled water	20.0 L

Tris-EDTA Buffer, TE (pH 8.0)

1 M Tris	5.0 ml
0.25 M EDTA	8.0 ml
Distilled water	87.0 ml

Tris-EDTA-Salt, TES (pH 8.0)

Trizma base	14.536 g
EDTA	5.84 g
Sodium chloride	11.68 g
Distilled water	4.0 L

Tris-EDTA-Sodium Dodecyl Sulfate, TE-SDS (pH 12.45)

TE buffer	15.0 ml
Sodium dodecyl sulfate (SDS)	0.15 g

Tris-Sucrose Buffer (pH 8.0)

1 M Tris	5.0 ml
0.5 M Edta	1.0 ml
Sucrose	125.0 g
Distilled water	494.0 ml

APPENDIX III (Continued)

Triton-Lytic Mix

10% Triton X-100	1.0 ml
0.25 M EDTA	25.0 ml
1 M Tris, pH 8.0	5.0 ml
Distilled water	69.0 ml

20 X SSC (pH 7.0)

Sodium citrate	88.2 g
Sodium chloride	175.3 g

Dissolve the above in 800 ml of distilled water, adjust pH to 7.0, and increase volume to 1000 ml with distilled water.

50 X Denhardt's Solution

Ficoll 400 (Pharmacia)	5.0 g
Polyvinyl pyrolidone MW 360,000	5.0 g
BSA fraction V (Sigma)	5.0 g
Distilled water	(to) 500.0 ml

Prehybridization/Hybridization Buffer

6X SSC (20x SSC)	30.0 ml (20x SSC)
0.5% SDS (10% SDS)	5.0 ml (10% SDS)
5X Denhardt's solution	10.0 ml (50x Denhardt's)
100 ug/ml denatured salmon sperm DNA	1.0 ml (10 mg/ml)
Distilled water	54.0 ml

APPENDIX IV

RESTRICTION ENDONUCLEASE BUFFERS

Low Salt Buffer

10 mM Tris.Cl, pH 7.5

10 mM $MgCl_2$

1 mM dithiothreitol (DTT)

Medium Salt Buffer

50 mM NaCl

10 mM Tris.Cl, pH 7.5

10 mM $MgCl_2$

1 mM DTT

High Salt Buffer

100 mM NaCl

50 mM Tris.Cl, pH 7.5

10 mM $MgCl_2$

1 mM DTT

Restriction Endonuclease Stop Buffer

Bromophenol blue	70.0 mg
Sodium dodecyl sulfate	7.0 g
Glycerol	33.0 ml
Distilled water	67.0 ml

APPENDIX V

Nick Translation Kit (Amersham)

Nucleotide/Buffer Solution (1)

100 uM dATP

100 uM dGTP

100 uM dTTP

Above nucleotides are suspended in a concentrated buffer solution containing Tris.Cl, pH 7.8, magnesium chloride and 2-mercaptoethanol.

Enzyme Solution (2)

Each 10 ul aliquot contains:

5 units DNA polymerase 1

100 pg DNase 1

These enzymes are suspended in a buffer solution containing Tris.Cl, pH 7.5, magnesium chloride, glycerol and bovine serum albumin.

Nick Translation Stop Buffer

30 mM EDTA

3% SDS

600 ug/ml yeast tRNA

APPENDIX VI

Conversion table of plasmid molecular weight (Mdal) and length (Kb) for the plasmids indigenous to H.ducreyi.

Molecular Weight (Megadaltons, Mdal)	Length (Kilobase Pairs, Kbp)
34 Mdal	55 Kbp
30 Mdal	48.5 Kbp
21.7 Mdal	35.0 Kbp
7.0 Mdal	11.3 Kbp
5.7 Mdal	9.2 Kbp
4.9 Mdal	7.9 Kbp
3.2 Mdal	5.2 Kbp
2.8 Mdal	4.5 Kbp
2.6 Mdal	4.2 Kbp
<2.0 Mdal	3.2 Kbp